การตอบสนองระดับเซลล์ระยะเริ่มต้นและระยะยาวในเซลล์เฮเทอโรโทรฟแขวนลอย
ของ *Chenopodium rubrum* L. ต่อไคโทซาน

นายอารัติ สนทนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพฤกษศาสตร์ ภาควิชาพฤกษศาสตร์
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2558

EARLY AND LONG-TERM CELLULAR RESPONSES IN HETEROTROPH CELL SUSPENSION OF *Chenopodium rubrum* L. TO CHITOSAN

Mr. Arrat Sontana

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Botany
Department of Botany
Faculty of Science
Chulalongkorn University
Academic Year 2015

Thesis Title	EARLY AND LONG-TERM CELLULAR RESPONSES IN HETEROTROPH CELL SUSPENSION OF *Chenopodium rubrum* L. TO CHITOSAN
By	Mr. Arrat Sontana
Field of Study	Botany
Thesis Advisor	Assistant Professor Anchalee Chaidee, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Science
(Associate Professor Polkit Sangvanich, Ph.D.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Tosak Seelanan, Ph.D.)

.....Thesis Advisor
(Assistant Professor Anchalee Chaidee, Ph.D.)

.....Examiner
(Assistant Professor Kanogwan Seraypheap, Ph.D.)

.....External Examiner
(Chatchawal Wongchai, Ph.D.)

อารัติ สนทนา : การตอบสนองระดับเซลล์ระยะเริ่มต้นและระยะยาวในเซลล์เฮเทอโรโทรฟแขวนลอยของ *Chenopodium rubrum* L. ต่อไคโทซาน (EARLY AND LONG-TERM CELLULAR RESPONSES IN HETEROTROPH CELL SUSPENSION OF *Chenopodium rubrum* L. TO CHITOSAN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. อัญชลี ใจดี, 69 หน้า.

การศึกษาก่อนหน้า พบการเพิ่มปริมาณซิลิกาบอดีส์ในกล้วยไม้หวายสกุล *Dendrobium* ทำให้เห็นความเป็นไปได้ว่าไคโทซานอาจมีผลเพิ่มการดูดซิลิกอน ซึ่งเป็นธาตุเสริมประโยชน์ของพืช เพื่อพิสูจน์ประเด็นดังกล่าว การตอบสนองของพืชในระดับเซลล์ต่อไคโทซานเป็นสิ่งจำเป็น ดังนั้น จึงมีการพัฒนาเซลล์แขวนลอย *Chenopodium rubrum* L. ชนิดเฮเทอโรโทรฟขึ้นจากแคลลัส จากนั้นจึงมีการตรวจวัดการตอบสนองระยะเริ่มต้นของเซลล์แขวนลอยต่อไคโทซาน (O-80) 3 ความเข้มข้น พบภาวะเป็นด่างภายนอกเซลล์แบบชั่วคราวเกิดขึ้นโดยขึ้นกับปริมาณความเข้มข้นที่ใช้ ทั้งนี้ ความเป็นกรดด่างเพิ่มขึ้น 0.30 หน่วย ที่ไคโทซานระดับ 5 และ 10 พีพีเอ็ม และ 0.55 หน่วย ที่ 100 พีพีเอ็ม อีกทั้งกรดแอซีติก (ตัวทำละลายควบคุม) ชักนำให้เกิดภาวะเป็นกรดภายนอกเซลล์ ในขณะที่โปรตีนภายนอกและการตายของเซลล์ไม่เปลี่ยนแปลงเมื่อได้รับไคโทซานระดับ 5 และ 10 พีพีเอ็ม แต่เพิ่มขึ้นอย่างมากที่ไคโทซานระดับ 100 พีพีเอ็ม จากผลของค่าความเป็นกรดด่างภายนอกและความมีชีวิตของเซลล์ จึงได้นำไคโทซานที่ระดับ 10 พีพีเอ็ม มาใช้ในการตรวจวัดการตอบสนองระยะยาว (60 วัน) ในสภาวะที่ให้และไม่ให้ซิลิกอนเพิ่มเติม หลังจาก 12 วันพบว่า เซลล์ทำให้อาหารเพาะเลี้ยงมีสภาวะเป็นกรดที่ค่า 4.0 แม้ไม่ได้รับไคโทซานและกรดแอซีติก นอกจากนี้ยังพบว่า ปริมาณซิลิกอนภายนอกเซลล์ลดลงถึง 70% โดยที่เพิ่มขึ้นอย่างมากถึง 30% ภายในเซลล์ของทุกชุดการทดลอง ในระหว่างการเพาะเลี้ยงเซลล์เป็นเวลา 60 วัน พบปริมาณซิลิกอนภายในเซลล์มีค่าอยู่ระหว่าง 0.15 ถึง 0.22 มิลลิกรัมต่อกรัมน้ำหนักสด ในทุกชุดการทดลอง โดยพบเซลล์ตายมากขึ้นในสภาวะที่มีซิลิกอนสูง ทั้งนี้ แสดงถึงความเป็นไปได้ของความเป็นพิษจากซิลิกอน จากทั้งหมดที่กล่าวมาแสดงให้เห็นว่า ไคโทซานไม่ได้เพิ่มการดูดซึมซิลิกอนในสภาวะที่ศึกษา และพบผลที่น่าสนใจคือ การดูดซึมซิลิกอนเกิดขึ้นได้ในเซลล์ชนิดนี้เมื่อมีซิลิกอนในรูปที่นำไปใช้ได้ บทบาทการเสริมประโยชน์ของซิลิกอนนี้มีส่วนเกี่ยวข้องกับการลำเลียงโปรตอนและภาวะการขาดอาหารของเซลล์ซึ่งจะได้อภิปรายในรายละเอียดต่อไป

ภาควิชา พฤกษศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา พฤกษศาสตร์ ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

5572237123 : MAJOR BOTANY

KEYWORDS: CHENOPODIUM RUBRUM, CHITOSAN, EXTRACELLULAR ALKALINIZATION, SILICON UPTAKE, VITALITY

ARRAT SONTANA: EARLY AND LONG-TERM CELLULAR RESPONSES IN HETEROTROPH CELL SUSPENSION OF *Chenopodium rubrum* L. TO CHITOSAN. ADVISOR: ASST. PROF. ANCHALEE CHAIDEE, Ph.D., 69 pp.

Previous finding of chitosan-induced increase of silica bodies in *Dendrobium* orchid hints that chitosan may enhance silicon uptake, a beneficial element for plants. To prove that, plant responses to chitosan at cellular level are prerequisite. Thus, cell suspension culture has been established from callus culture of heterotroph *Chenopodium rubrum* L. Subsequently, early responses of cell suspension to three different concentrations of chitosan (O-80) were determined. Transient extracellular alkalinization occurred on a concentration-dependent manner. An increase of 0.30 pH units was detected with 5 and 10 ppm chitosan and 0.55 pH units with 100 ppm. In addition, acetic acid (solvent control) induced extracellular acidification. While extracellular protein release and cell mortality was not changed by 5 and 10 ppm chitosan, it was highly increased by 100 ppm. Based on extracellular pH and vitality, chitosan at 10 ppm was applied in 60-day long-term treatment with and without silicon supplementation. After 12 days, cells acidified the medium to pH 4.0, regardless of chitosan and acetic acid. Furthermore, extracellular silicon was decreased about 70% while intracellular silicon was increased 30% similarly in all treatments. During 60 days of cultivation, intracellular silicon varied between 0.15 to 0.22 mg g^{-1}FW in all treatments similarly. It was also observed that cell mortality was slightly higher under high silicon condition, suggesting possible silicon toxicity. In total, the results suggest that chitosan does not enhance silicon uptake under investigated condition. Interestingly, *C. rubrum* cells increase silicon uptake when soluble silicon is available. Beneficial role of silicon in association with H^+-mediated transport and cell starvation was discussed therein.

Department: Botany Student's Signature .....

Field of Study: Botany Advisor's Signature .....

Academic Year: 2015

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my advisor, Assistant Professor Dr. Anchalee Chaidee for the continuous support of my master's degree study and related research, for her patience, motivation, and immense knowledge. Her guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my master's degree study.

My sincere thanks also go to Professor Dr. Wolfgang Pfeiffer for the kind gift of Chenopodium rubrum callus culture.

Besides my advisor, I would like to thank the rest of my thesis committee: Assistant Professor Dr. Tosak Seelanan, Assistant Professor Dr. Kanogwan Seraypheap and Dr. Chatchawal Wongchai for their insightful comments and encouragement, also for the hard question which incented me to wide my research from various perspectives.

This research was partially supported by the grant for new scholar (co-funded by TRF and CHE, MRG5280200) and teaching assistant fellowships.

I thank to all my friends in Center of Excellence in Environment and Plant Physiology for the stimulating discussions, the sleepless nights we were working together before deadlines, and all the fun we have had in the last four years.

Last but not least, I would like to thank my family: my parents and my brothers and sisters, for supporting me spiritually throughout writing this thesis and my life in general.

CONTENTS

Page

Page

Page

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Abbreviations or symbols	Term
2,4-D	2,4-dichlorophenoxyacetic acid
ATP	adenosine triphosphate
^{o}C	degree Celsius
EDTA	ethylenediaminetetraacetic acid
et al.	et. alli (Latin), and others
FW	fresh weight
g	gravity force
g	gram
g/L	gram per liter
H^{+}	hydrogen ion or proton
HEPES-Tris	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-Tris
kDa	kilodalton
kinetin	kinetin-6-furfurylaminepurine
MS	Murashige and Skoog (1962) medium
M_W	molecular weight
m	meter
mg	milligram
ml	milliliter
mM	millimolar
mm	millimeter
N	normality
nm	nanometer
O-80	oligomeric (O; M_W approximately 20 kDa) chitosan with a degree of deacetylation (DD) of 80 %

LIST OF ABBREVIATIONS (cont.)

Abbreviations or symbols	Term
pH	negative logarithm of concentration of hydrogen ions
P_i	inorganic phosphate
ppm	part per million
rpm	round per minute
s	second
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
µg	microgram
µl	microliter
µM	micromolar
µmol	micromole
%	percentage
Δ	delta

CHAPTER I

INTRODUCTION

RATIONALES, THEORIES OR ASSUMPTIONS

Chitosan is known as an elicitor of plant defense genes and inhibits fungal growth and development. This molecule has potential in agriculture with regard to controlling plant diseases. In addition, chitosan treatments are able to improve plant production under normal condition. In *Dendrobium* orchid, chitosan (O-80) increased floral production, size of chloroplast and number of silica bodies in bundle sheathed cells. These responses corresponded to chloroplast regulatory gene reduction. At cellular level, chitosan triggers some signal transduction events, such as extracellular alkalinization and membrane depolarization. Likewise, the activity of proton pumping is inhibited, modifying the uptake of various ions and nutrients. Due to chitosan-induced silica bodies accumulation in *Dendrobium*, it raises a question whether chitosan enhances the uptake of silicon, a beneficial element that enhances stress tolerance of various plants. Therefore, this study is aimed to investigate early and long-term cellular responses to chitosan, O-80, in term of membrane functions and silicon uptake using heterotroph *Chenopodium rubrum* cell suspension as a model. By these approaches, putative responses to chitosan in *C. rubrum* will enhance our understanding in cellular mechanisms regulating plant growth and development to overwhelm environmental stress.

RATIONALES

Chitosan is a non-toxic biodegradable natural polymer which has antiviral, antibacterial and antifungal properties (Abdelbasset et al., 2010; Ben-Shalom et al., 2003; Limpanavech et al., 2008). Moreover, chitosan is characterized as a potent elicitor of plant resistance against fungal pathogens (Abdelbasset et al., 2010; Iriti et al., 2006). As a consequence, chitosan is often used in plant disease control as a powerful elicitor rather than a direct antifungal or toxic agent (Bell et al., 1998; Eikemo et al., 2003). Effects of chitosan highly depend on properties such as

concentration applied, molecular weight, degree of deacetylation, pH, viscosity and types of solvent (Abdelbasset et al., 2010; Iriti & Faoro, 2009; Rabea et al., 2003).

Amborabé et al. (2008) reported that chitosan was able to trigger quick and transient depolarization of *Mimosa pudica* motor cell membranes in a dose-dependent manner. They showed additionally that the concentration of 100 μg ml^{-1} is a cytotoxicity level. Ultra-structural studies by Benhamou (1992) showed that chitosan induced a series of morphological and structural modification. This was linked to the polycationic properties of chitosan, causing changes in membrane permeability and cytoplasmic aggregation. The site of action of this polysaccharide was found to be the plasma membrane H^+-ATPase, as shown by the inhibition of the proton pumping and the catalytic activity of enzyme (Amborabé et al., 2008). Moreover, the modification of extracellular pH by inhibitors and activators of H^+-ATPase could also change the expression of defense genes (Grabe et al., 2000). Chitosan was also shown to alter other H^+-mediated processes, i.e. the uptake of certain carbohydrate and amino acids (Amborabé et al., 2008).

At whole plant level, chitosan treatments are able to improve plant production under normal condition. In *Dendrobium* orchid, chitosan (O-80) increased floral production, size of chloroplast and number of silica bodies in bundle sheathed cells (Limpanavech et al., 2008). These findings were corresponded with chloroplast regulatory gene (*Ycf2*) reduction.

Silicon is the second most abundant element in soil and is known as plant beneficial element. In some species, silicon-deprived plants are often weaker structurally than silicon-replete plants, leading to abnormality of growth, development and reproduction (Ma, 2004; Ma & Yamaji, 2006; Mitani & Ma, 2005). Silicon is effective in preventing lodging in rice by increasing the thickness of the culm wall and size of the vascular bundles (Shimoyama, 1958), thereby enhancing the strength of the stem. Moreover, silicon can alleviate various kinds of abiotic stresses, such as salt and drought stress, metal toxicity, high radiation, nutrient imbalance, high temperature and freezing (Currie & Perry, 2007; Epstein, 1999; Ma, 2004). Under salinity, putative mechanism of silicon action is the activation of H^+-ATPase and H^+-PPase, increasing K^+ influx and Na^+ efflux in barley roots (Liang et al.,

2005a). Likewise, silicon enhances resistance of plants to pests and pathogenic infection, therefore plants need to accumulate large amounts of silicon as well (Tamai & Ma, 2003). However, plant species differ greatly in silicon accumulation, ranging from 0.1% to 10% of dry mass (Epstein, 1994). It has been reported that shoot Si contents were 7.3, 2.3, and 0.2% in rice, cucumber, and tomato plants, respectively (Mitani & Ma, 2005). Plants are able to control silicon transport and its polymerization in the cells. Interestingly, silicon uptake by rice roots is mediated by both passive pathway and via energy-driven transporters (Ma et al., 2006; Van Bockhaven et al., 2013).

Due to chitosan-induced silica bodies accumulation in *Dendrobium* (Limpanavech et al., 2008), it raises a question whether chitosan enhances the uptake of silicon. To answer this, responses to chitosan in term of membrane function, i.e. proton flux and pumping, and silicon uptake should be investigated at cellular level. *Chenopodium rubrum* L. cell suspension has been used successfully to characterize membrane transporters and functions (Bentrup et al., 1986; Chaidee & Pfeiffer, 2006; Kranewitter et al., 1999). By using auto- and heterotroph cells, Chaidee et al. (2008) and Wongchai et al. (2012a) have found cell-specific responses of proton flux and actin cytoskeleton to abiotic stresses.

Responses of plant cells to chitosan highly depend on cell type. Previously, Wongchai et al. (2013) have found that chitosan induces drastic repellent activity of *Tenebrio molitor* larvae in heterotroph *C. rubrum* cells but not in autotroph. This showed a cell-specific response in *Chenopodium* cells to chitosan. Consequently, to enlighten about role of chitosan on nutrient uptake, chitosan response should be specifically investigated in various cell types. Therefore, autotroph and heterotroph cells of *C. rubrum* L. performing cell-specific responses under abiotic stress are a promising plant cell model. However, they have different culturing conditions. To attain similar range of fresh weight, heterotroph cells require shorter time of cultivation than autotroph cells, i.e., 10 days are required for heterotrophs to achieve 24% packed cell volume but 6 weeks for autotrophs. Unfortunately, there is no information yet about optimal concentration and exposure time of chitosan that modify nutrient uptake. Thus, it has advantage to develop experiment conditions

using heterotroph cells as a plant material. Approaches obtained with heterotroph cells can be applied to investigate cellular responses in other cell types, including autotroph cells, in future research.

Totally, heterotroph *C. rubrum* cells will be used to investigate membrane functions and silicon uptake in short- and long-term responses to chitosan (O-80) in this study. Besides, growth and viability of heterotroph cells exposed to chitosan and silicon will be examined. Early and long-term responses to chitosan and silicon in this cell type will enhance our understanding in cellular mechanisms involving with plant nutrient uptake, growth and development.

EXPECTED BENEFICIAL OUTCOME(S) FROM THE THESIS

Roles of chitosan on silicon uptake will be well described in heterotroph plant cell type. These approaches can be applied to investigate cellular responses in other cell types, such as autotroph cells, in future research.

CHAPTER II
LITERATURE REVIEW

2.1 Chitin and chitosan: Chemical structures

Chitin (polymer of β-1,4-glycan-linked N-acetyl-D-glucosamine) is the second most numerous natural polysaccharide after cellulose (Gooday, 1990). Chitin has the main backbone similar to cellulose but comprises of an acetamide group, instead of a hydroxyl group, at the C-2 position (Ramírez et al., 2010). It is a main component found in the cell wall of fungi and exoskeleton of animals, such as insects, crabs and shrimps (Gohel et al., 2006). Chitosan is the N-deacetylated derivative of chitin and can be prepared by deacetylation of chitin using alkaline solution (Austin et al., 1981; Tsigos et al., 2000). So, it is a linear polysaccharide chain of β-(1,4)-2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose (Figure 2.1).

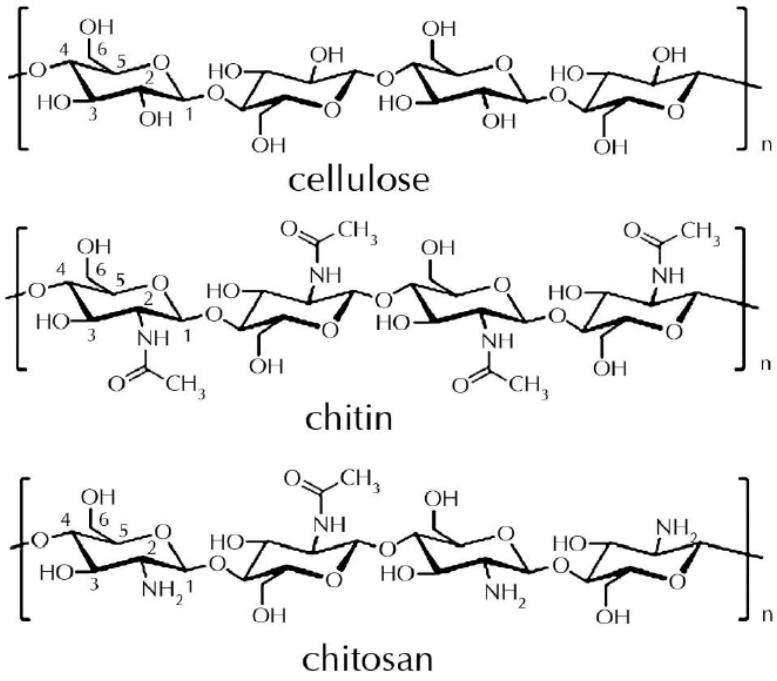

Figure 2.1 Chemical structures of cellulose, chitin and chitosan (Abdelbasset et al., 2010).

2.2 Plant cellular responses to chitosan

Chitosan is known as an elicitor that activates defense genes against pathogen in plants (Doares et al., 1995). In agriculture, chitosan can be applied to enhance growth and flowering of various crop plants (Limpanavech et al., 2008; Ohta et al., 2004; Ohta et al., 1999), both under normal and stress conditions. By which mechanisms chitosan brings about positive effects, more investigation is required.

Whole plant responses to chitosan include stomatal closing in order to prevent pathogen infection and maintain water status (Lee et al., 1999). Chitosan also increased chloroplast size, number of silica bodies and floral production in orchid (Limpanavech et al., 2008) and enhanced growth of *Eustuma grandiflorum* (Ohta et al., 2004; Ohta et al., 1999).

Early events induced by chitosan were influxes of H^+ and Ca^{2+}, transient extracellular alkalinization and membrane depolarization (Amborabé et al., 2008; Kauss, 1985; Zuppini et al., 2003). Likewise, reactive oxygen species formation and oxidative burst were induced by chitosan (Lin et al., 2005; Wendt dos Santos et al., 2008; Yang et al., 2009). These responses were detected after minutes of exposure time. It was also suggested that molecular target of chitosan was the plasma membrane H^+-ATPase (Amborabé et al., 2008). Proton pumping in membrane vesicles of *Mimosa pudica* pulvinar cells was inhibited more than 50% by 25 ppm chitosan (Amborabé et al., 2008). By measuring the uptake of amino acids and carbohydrates, chitosan-inhibited proton pumping was thought to be a cause of decreased nutrient uptake (Amborabé et al., 2008). Furthermore, cytoplasmic increase in H^+ and Ca^{2+} are parts of signal transduction cascade leading to acquired tolerant gene expression (Amborabé et al., 2008; Kauss, 1985; Zuppini et al., 2003). In addition, extracellular alkalinization is advantageous in prevention of pathogenic microorganism infection (Abdelbasset et al., 2010).

Plant responses to chitosan highly depend on physicochemical properties of chitosan, mainly the degree of deacetylation and molecular size (Abdelbasset et al., 2010). Concentration and exposure time are also important factors (Amborabé et al., 2008). For examples, effects of chitosan with 85% deacetylation degree (%DD)

depended on molecular size (Zuppini et al., 2003). That is, H_2O_2 production and cell death occurred when applied to *Phaseolus vulgaris* leaves with 6 kDa and 76 kDa but not with 322 kDa of chitosan. However, callose deposition in cell wall was induced by all molecular sizes (Iriti & Faoro, 2009). Chitosan at low concentration (25 μg ml^{-1}) with 85% DD induced a transient increase in cytosolic Ca^{2+} concentration within 5 min in suspension cultured soybean cells (Zuppini et al., 2003). When the concentration was increasing, it possibly led to plasma membrane disturbance and subsequent cell death (Zuppini et al., 2003).

Partially deacetylated chitosan (%DD) is the most efficient signals to increase of H_2O_2 production in *Araucaria angustifolia* suspension cells (Wendt dos Santos et al., 2008). Most active inducing was 31-40% DD and 9-22% DD of polymeric and oligomeric chitosan, respectively (Wendt dos Santos et al., 2008). However, fully N-acetylated chitosan is more effective than partially N-acetylated chitosan parsley cell suspension cultures (Abdelbasset et al., 2010). Effectiveness of polymeric and oligomeric chitosan are diverse. For example, polymeric chitosan is more effective than oligomeric chitosan in inducing callose formation of suspension cultured cells of *Catharanthus roseus* (Kauss et al., 1989). Likewise, most repellent activity of *Tenebrio molitor* larvae was found with 70% DD of polymeric chitosan (P-70) in autotroph suspension cells (Wongchai et al., 2013). However, much more effects of chitosan oligomers than polymers were also found in some studies (Abdelbasset et al., 2010; Kauss et al., 1989). The latter findings indicate chitosan function via the interaction between amino groups and numerous negative charges of plasma membrane (Abdelbasset et al., 2010; Kauss et al., 1989).

2.3 How do plants sense chitosan?: Chitin receptors

Chitosan is an amino polysaccharide that the molecule is too large to pass through the cell. It has amino groups containing positive charges that can interact with the plasma membrane (Abdelbasset et al., 2010; Kauss et al., 1989). Recently, there have been many reports about plasma membrane chitin receptor (Kaku et al., 2006), receptor-like kinase (Wan et al., 2008) and chitin-elicitor binding protein

(Sánchez-Vallet et al., 2014). Perception of various elicitors, such as chitin and partially deacetylated chitosan, trigger gene expression and defense response in plant cells (Petutschnig et al., 2010; Wan et al., 2008). In *Arabidopsis* and rice, chitin-elicitor receptor kinase-1 (CERK1) is required for direct chitin binding, leading to cellular signal transduction (Chen et al., 2010; Reddy et al., 1999). Additionally, rice does not only demand CERK1 but also chitin-elicitor binding protein (CEBiP) to form a sandwich-type dimerization receptor-ligand complex (Hayafune et al., 2014; Sánchez-Vallet et al., 2014). Subsequently, many downstream target proteins would be interacted leading to a variety of immune responses. For example, production of reactive oxygen species (ROS), callose deposition, lignification, pathogenesis-related (PR) proteins (chitinase & glucanase), accumulation of phytoalexins, and activation of mitogen-activated protein kinase (MAPK) cascades (Akamatsu et al., 2015; Jonak et al., 1999; Yamaguchi et al., 2015).

2.4 Silicon: beneficial element to alleviate many stresses

Silicon is a second most abundant element on the earth's crust and beneficial to many plant species. Several plants can take up silicon and become more tolerant to biotic and abiotic stresses (Epstein, 1999; Ma, 2004; Shimoyama, 1958). Silicon has both structural and biochemical functions in alleviating various environmental constraints (Currie & Perry, 2007). When silicon is taken up, it polymerizes as amorphous silica that can strengthen cell wall structures (Ma & Yamaji, 2006), preventing plants from lodging (Shimoyama, 1958) and herbivore attack (Tamai & Ma, 2003). Besides, silicon actively induces various physiological responses acquired under stress conditions. For example, silicon activates H^+-ATPase and H^+-PPase activities, leading to high proton motif force necessary for H^+-driven transporters (Liang et al., 2005a). Silicon activates various antioxidant enzymes that prevent plants from suffering by oxidative stress (Liang et al., 2003).

2.5 Plant available form of silicon and silicic acid solubility

Though silicon is abundant in the soil, it can be interacted with aluminum ions and other compounds being insoluble form as aluminosilicate complexes that plants cannot take up (Currie & Perry, 2007; Mitani & Ma, 2005). The easiest form of silicon that plants are able to take up is a monomer orthosilicic acid [$Si(OH)_4$; $Si(OH)_3O^-$]. It composes of silicon tetrahedrally coordinated with four hydroxyl groups and is a weak acid (Iler, 1979). When the concentration of silicic acid excesses of 100-200 mg kg^{-1} (1-2 %), polymerization will occur to form various structural motifs, such as globular, fibrous and sheet-like structures, approaching a particle size of about 1-3 nm as shown in Figure 2.2. Also, they have a surface negatively charged and can interact with the cell wall. Plant silica particle form in various types and localize in many areas. For examples, dumb bell shaped silica bodies and bulliform cells located in rice leaves (Currie & Perry, 2007).

The solubility of silicon in the soil is affected by many factors. Firstly, when pH in the soil is acidic (pH 5.5-6.0), aluminum solubility will be increased (Voleti et al., 2009). Exchangeable aluminum then reacts with silicic acid, leading to plant silicic acid availability reduction (Epstein, 1994). It has been reported that the solubility of silicon will be increased when pH is above 9.0 (Govett, 1961). However, adjusting soil pH by liming does not increase the solubility and plant uptake (Jones & Handreck, 1967). This is also caused by some effects of soil type. Secondly, water content in the soil has impact on silicon solubility. In other word, when the soil is wet, water can induce silicon solubilization. With dry soil, silicon mostly binds to other ions and molecules (Jones & Handreck, 1967). Lastly, some organic compounds may prevent silicon polymerization and decrease exchangeable aluminum (Voleti et al., 2009). Interestingly, it was found that the presence of soil organic matter can increase the release of silicic acid into soil solution (Voleti et al., 2009).

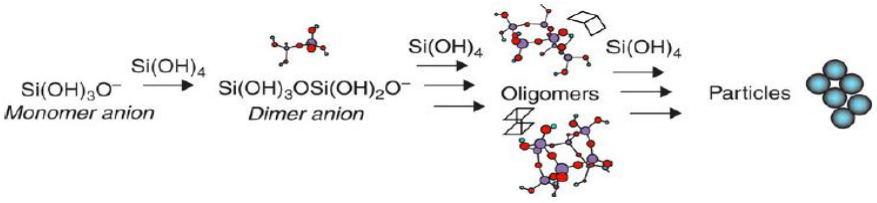

Figure 2.2 The monomeric silicic acid polymerized into larger silica molecules in various forms such as dimers, oligomers and particles (Currie & Perry, 2007).

2.6 Silicon transporters and uptake pathways

Silicon transporters have been characterized in rice and diatom. Up to now there are at least two different silicon transporter families, namely, low affinity silicon transporters, Lsi, found in rice (Ma & Yamaji, 2006) and diatom silicon transporters called SIT (Hildebrand et al., 1998).

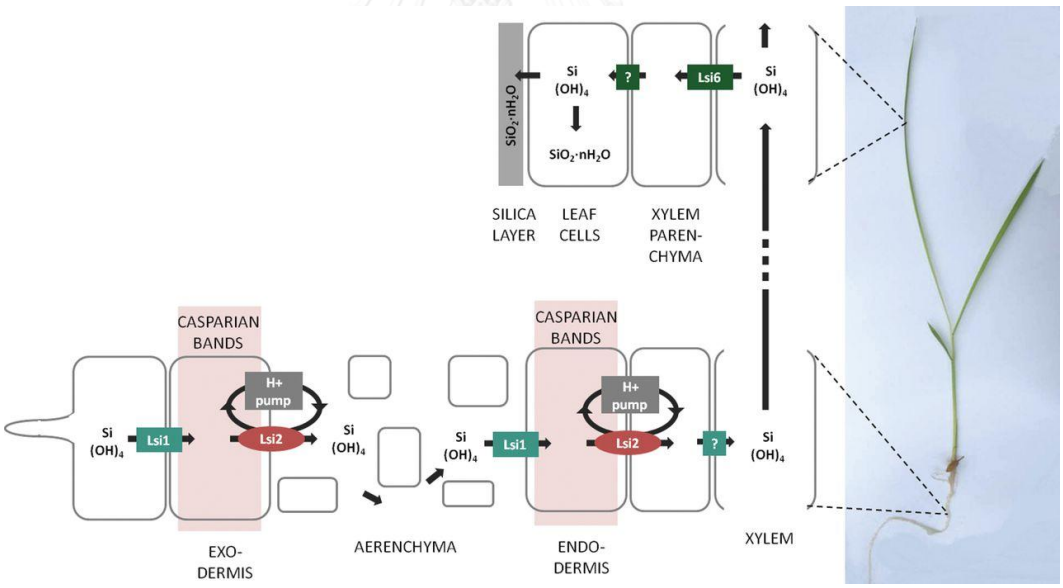

Figure 2.3 The uptake of silicon in rice seedling from roots to leaves (Van Bockhaven et al., 2013).

In rice, silicon can accumulate up to 10% of dry weight (Mitani & Ma, 2005). Two different types of transporters: the silicon permeable channel (Lsi1, Lsi2) and the efflux transporters (Lsi6), involved in silicon uptake processes (Yamaji et al., 2012). From root epidermal cells, silicic acid is transported through exodermal cells passively via Lsi1 (localized at the distal side of plasma membrane of exo- and endodermal cells) and actively via Lsi2 (localized at the proximal side of plasma membrane of exo- and endodermal cells). In the aerenchymal cells, silicic acid moves apoplastically until it reaches the endodermis where the Lsi1 and Lsi2 transporters load silicic acid into the stele. An undefined transporter loads the silicic acid in the xylem. Via the xylem, the silicic acid arrives in the shoots, where the Lsi6 transporter unloads the silicic acid into the xylem parenchyma cells. An undefined protein transports the silicic acid in the leaf cells where it is polymerised either as silica in the cell or as a sub-cuticular silica layer outside the cell (Van Bockhaven et al., 2013). Besides rice, these transporters function in silicon accumulation of silicon accumulating organisms, such as maize (Mitani et al., 2009), barley (Yamaji et al., 2012), wheat (Ma et al., 2011), pumpkin (Mitani et al., 2011), horsetail (Grégoire et al., 2012) and marine diatom (Hildebrand et al., 1998).

2.7 *C. rubrum* cells: a model for plant cellular studies

Chenopodium rubrum L. cell suspension culture established from hypocotyl fragments (HÜsemann & Barz, 1977; Weigel, 1993) composes of two cell types, auto- and heterotroph cells having different life span. Autotroph chloroplast-containing cells require two months of cultivation under continuous light and CO_2 supplementation. In contrast, fast growing heterotroph cells demand sugar as a carbon source and hormone supplementation during two-week interval (Weigel, 1993). Furthermore, continuous light is required for betalain accumulation in the vacuole of heterotroph cells (Berlin et al., 1986). More than three decades, both auto- and heterotroph cells have been used as a plant cell model for determining membrane function and cellular responses to environmental stimuli. For instance, vacuolar H^+-ATPase and H^+-PPase were isolated and characterized in both cell types

(Bentrup et al., 1986; Bille et al., 1992; Kranewitter et al., 1999). In addition, heat shock-induced proton flux with actin reorganisation has been shown to be cell type-specific (Chaidee et al., 2008). While *C. rubrum* heterotroph cells formed actin ring and increased proton uptake in response to heat shock, autotroph cells did not (Chaidee et al., 2008). Furthermore, salt stress and metabolite sensing has been described based on differential proton fluxes in both cell types (Wongchai et al., 2012b). Interestingly, by monitoring the repelling of *Tenebrio molitor* L. larvae, sensing to chitosan and abiotic stresses was shown to be cell type-specific (Wongchai et al., 2013). This implies that some putative effects hidden when investigating at whole plant level can be uncover using cell culture system.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

All chemicals and reagents used through this study were molecular biology or analytical grade purchased from different companies (Table 3.1).

Table 3.1 List of chemicals and reagents

Chemicals and reagents	Company	Country
adenosine triphosphate (ATP)	Fluka	Switzerland
ammonium heptamolybdate	Merck	Germany
ascorbic acid	Fisher Scientific	Germany
Biorad dye	Bio-Rad	USA
dithiothreitol (DTT)	Bio-Rad	USA
EDTA (Ethylenediaminetetraacetic acid)	Fluka	Switzerland
EGTA (Ethylene glycol tetraacetic acid)	Fisher Scientific	Germany
Evan's blue dye	Sigma-Aldrich	USA
glacial acetic acid	Carlo Erba	France
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Sigma-Aldrich	USA
kinetin	Sigma-Aldrich	USA
methanol	Carlo Erba	France
neutral red dye	Sigma-Aldrich	USA
sodium bisulfite	Sigma-Aldrich	USA
sodium dodecyl sulfate (SDS)	Bio-Rad	USA
sodium hydroxide	Carlo Erba	France
sodium orthovanadate	Sigma-Aldrich	USA
sodium sulfate	Merck	Germany
sodium trisilicate	Sigma-Aldrich	USA
sulfuric acid	Fisher Scientific	Germany
tartaric acid	Merck	Germany
Tris (2-Amino-2-hydroxymethyl-propane-1,3-diol)	Fisher Scientific	Germany

3.2 Plant material

3.2.1 Establishment of *Chenopodium rubrum* cell suspension

Heterotroph, betalain-containing callus of *Chenopodium rubrum* L. (Harms et al., 1977) was cultured in Murashige and Skoog liquid medium (Murashige & Skoog, 1962), as shown in Appendix (Table 1), with regular sub-cultivation for 6 months (Berlin et al., 1986). Briefly, cell suspensions were cultured in a 125-ml glass Erlenmeyer flask at 22°C on a shaker (110 rpm) with 24 hours irradiance of 70 μmol m^{-2} s^{-1}. Sub-cultivation was done every 14 days. Then, established cell suspension was observed under light-transmitted and fluorescence microscopes equipped with a digital camera. Growth curve was determined by the packed cell volume (% PCV). Cell suspensions (9±1 days old) having PCV of 24.0 ± 0.5 % were used in all experiments, otherwise indicated.

3.3 Chitosan and silicon treatments

Oligomeric (O; M_W approximately 20 kDa) chitosan with a degree of deacetylation (DD) of 80 % (denominated O-80), prepared from crab shells and solubilized in 1N acetic acid as depicted in Limpanavech et al. (2008) was used in this study. Final concentration of 5, 10 and 100 ppm chitosan, with corresponded concentration of acetic acid as a solvent control, was used in experiments. Corresponded volume of water was applied in control treatment.

Silicon stock solution of 50 mM was freshly prepared by sodium trisilicate (18% Na_2O, 60% SiO_2). Final concentration of 0.5 mM silicic acid was treated in all experiments of silicon supplementation.

3.4 Extracellular pH measurement

Cell suspension (20 ml) was transferred into a 125-ml graduated polycarbonate (PC) flask (Thermoscientific, USA) and pre-equilibrated for 60 minutes under the same condition as growth condition (Wongchai et al., 2012b). Then, cell suspension was applied with chitosan and silicon. Extracellular pH in the medium

was measured at 0, 10, 20, 30, 45, 60, 90, 120, 150, and 180 minutes using a pH meter equipped with a microelectrode (Fisher Scientific, Germany).

3.5 Extracellular protein and mortality assays

C. rubrum cell suspension (20 ml) in a 125-ml graduated PC flask was pre-equilibrated as previously described. After exposure to chitosan for 0, 12, 24, 48, 96 and 144 hours, an aliquot (1 ml) of cell suspension was centrifuged (313×g, 5 minutes, 25°C). After centrifugation, supernatant was collected and protein content was measured using Bradford assay at 595 nm with bovine serum albumin (BSA) as standard (Bradford, 1976). Cells were tested for cell viability using Evan's blue staining method. Firstly, cells were incubated with 125 µl of 1% (w/v) aqueous Evan's blue for 5 minutes. Then, cells were drained and rinsed with distilled water for 5 times to remove untrapped dye using centrifugation as described above. After washing, cells added with 1 ml of 1% (w/v) aqueous sodium dodecyl sulfate (SDS) in 50% methanol were incubated in a water bath (60°C, 30 minutes) to release trapped dye. After centrifugation, supernatant was taken and the optical density was evaluated spectrometrically at 600 nm (Baker & Mock, 1994), as shown in Appendix (Figure 1). The optical density of heat-killed *C. rubrum* cells (0.24 g FW) was set to 100 % cell mortality.

3.6 Silicon determination

C. rubrum cell suspension (20 ml) in a 125-ml PC flask was pre-equilibrated as previously delineated. Specifically, after exposure to chitosan and silicon for 0, 12, 24, 48, 96 and 144 hours, media were collected by centrifugation (313×g, 5 minutes, 25°C) and stored at -20°C. After centrifugation, cells were weighted and freshly extracted by autoclave-induced digestion method with some modification (Elliott & Snyder, 1991). Briefly, samples (4.8 g FW) were mixed with 3 ml of 15% (w/v) NaOH in a 50 ml plastic tube and autoclaved for 20 minutes at 121°C. Then, extracts were filtered and adjusted volume to 15 ml with distilled water. The extracts containing 1% (w/v) NaOH was analyzed for intracellular silicon content in the same day, to

avoid an error caused by self-polymerization of soluble silicon. For silicon content analysis, the extracts and media were determined by colorimetric molybdenum blue method, at 650 nm (Dai et al., 2005). Samples (0.6 ml) were then mixed with 3.3 ml of 20% (v/v) acetic acid and 1 ml of ammonium molybdate solution (5.4 g l^{-1}, pH 7.0). After incubation for 5 minutes, 0.5 ml of 20% (w/v) tartaric acid and 0.1 ml of reducing agent were added and thoroughly mixed altogether. Mixture solution was then incubated for 30 minutes before measuring. Reducing agent was freshly prepared by mixing 25 ml of solution A (2 g Na_2SO_4, 0.4 g 1-amino-2-naphthol-4-sulfonic acid) with 200 ml of solution B (12.5% (w/v) $NaHSO_3$), and adjusted the final volume to 250 ml with distilled water. For calibration, a standard curve of known silicon concentration was used (Figure 2, Appendix).

3.7 Preparation of membrane vesicles

Membrane vesicle of *C. rubrum* cells was prepared according to previously described method (Pfeiffer & Hager, 1993) with minor modifications. Briefly, nine-day old *C. rubrum* cells (6.3 g FW) were washed and cool homogenized with freshly prepared homogenization buffer, containing 50 mM Hepes-Tris (pH 7.2), 0.5 mM EGTA, 5.0 mM EDTA, 2.0 mM dithiothreitol (DTT) and 5% (w/w) sucrose, using a 2-ml potter. Subsequently, the homogenate was centrifuged to precipitate nuclei, mitochondria, starch grains, tissue pieces and cell wall fragments (firstly at 1,000×g for 10 minutes and lastly at 9,000×g for 10 minutes). After that, supernatant (approx. 10 ml) was gently balanced and centrifuged by ultracentrifugation (200,000×g, 1 hour). Pellet was washed and re-suspended with re-suspension buffer containing 10 mM HEPES-Tris (pH 7.2) and 5% (w/w) sucrose. All preparation steps were performed at 4°C. Then, membrane vesicle samples were kept on ice for further assay.

3.8 Procedure of H^+-ATPase activities

Membrane vesicle samples (0.118 μg protein μl^{-1}) were analyzed for vanadate-sensitive plasma membrane H^+-ATPase activity (Ames, 1966; Pfeiffer & Hager, 1993). The activity of H^+-ATPase was valued from the release of P_i from ATP in 0.3 ml

reaction volume. The assay medium containing 10 mM HEPES-Tris (pH 7.2), 100 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM ATP, sodium-orthovanadate (2.5 mM vanadate) and membrane vesicle (20 μl). After 1 hour, the reaction was stopped by adding 0.7 ml of freshly prepared stop solution: 1 part of 10% ascorbic acid and 6 parts of 0.42% ammonium heptamolybdate in 1 N sulfuric acid. Color development was allowed for 30 minutes. Then, blue reduced phospho-molybdate complex was measured spectrophotometrically at 820 nm (Ames, 1966). The enzymatic assay was performed with and without chitosan and acetic acid at different concentrations. Standard phosphate was used for calibration.

3.9 Scanning electron microscopy (SEM)

After cultivation of 9-day old *C. rubrum* cell with silicon (0.5 mM) and additives (10 ppm chitosan and acetic acid) for 12 days, cells were collected after 5-minute sedimentation, fixed, and dehydrated as standard protocol for scanning electron microscopy (SEM). In brief, *C. rubrum* cells (0.48 g FW) were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer solution (pH 7.3) at 4°C overnight. Fixed specimens were washed twice in the same buffer without fixative. Then, samples were dehydrated using graded ethanol series, 30, 50 and 70% (v/v), for 10 minutes each. Subsequently, the specimens were mounted on copper stubs and coated with gold. Then, they were illustrated by a scanning electron microscope (JSM-6400) at acceleration voltage of 20 kV. For control, 9-day old *C. rubrum* cell suspension cultivated without silicon supplementation was used.

3.10 Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA) following by Duncan's multiple range test (DMRT) with 4 replicates. *P*-values ≤ 0.05 were considered as significant.

CHAPTER IV

RESULTS

4.1 Establishment of *Chenopodium rubrum* cell suspension

Cell suspension cultures of *C. rubrum* were obtained from the friable reddish callus which was maintained by a regular sub-cultivation (Figure 4.1A). Calli separated to small clumps were transferred into MS liquid medium supplemented with 0.15 μM 2,4-D and 1.2 μM kinetin and cultivated on a shaker rotated continuously at 22°C (110 rpm). After sub-cultivating every 14 days for 6 months, suspension cultures appeared as fine, small reddish clusters (Figure 4.1B). Attainable cell suspension composed of microscopic cell colonies (≥3 cells) and macroscopic cell aggregates with diameter of about 0.13 mm (Figure 4.1C). The shape of individual cell varied, it was observed as round, oval or square. A cell with round shape had average diameter of about 44 μm. A young cell had numerous betalain-containing small vacuoles that subsequently fused into a large vacuole when mature (Figure 4.1C). The micrograph in figure 4.1D showed fluorescein diacetate (FDA)-stained live cells of 9 day-old cell suspension. Based on packed cell volume (PCV), cell suspension performed exponential growth during the cultivation period (Figure 4.1C). At the beginning of sub-cultivation (0-4 day), there was a lag phase in which cell division was low. During 7-14 days, the rate of cell growth was highest, as shown by a rapid increase of PCV (an exponential or logarithmic growth phase). Then, the growth rate of cells was reduced after 14 days of cultivation. Therefore, 8-10 day-old cell suspensions having PCV of 24.0 ± 0.5% were used in all experiments, except indicated otherwise (Figure 4.1E).

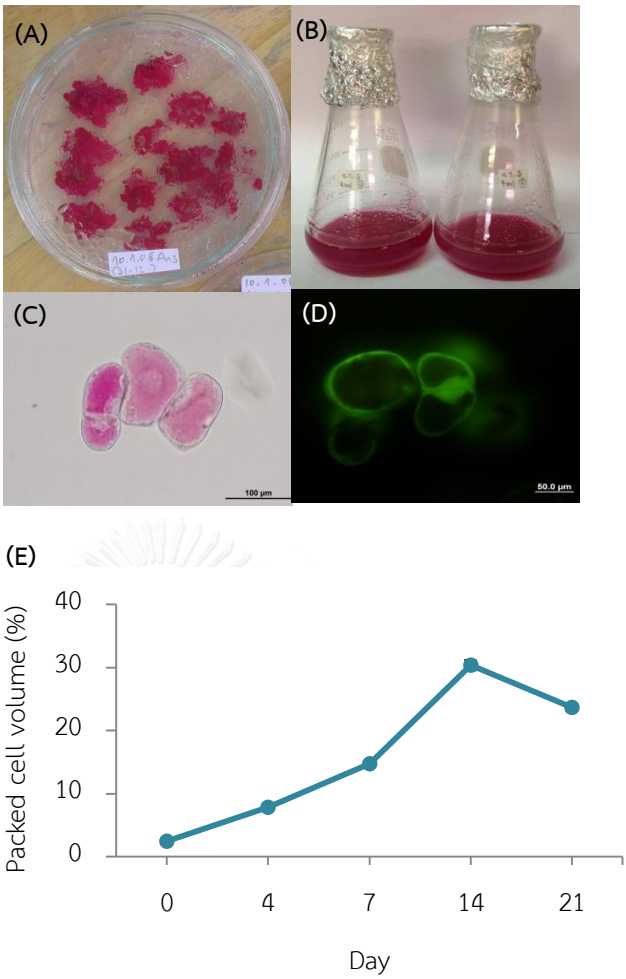

Figure 4.1 Heterotroph cells of *Chenopodium rubrum* L. The apparent friable, soft and reddish callus cultures (A). Cell suspension cultures obtained after routine long-term sub-cultivation (B). Unstained (C) and fluorescein diacetate stained (D) cells in the logarithmic growth phase. Time-courses of packed cell volume, mean±SE (E).

4.2 Short-term responses to high concentration of chitosan with silicon supplementation

4.2.1 Extracellular pH

Chenopodium rubrum cell suspension (9±1 day-old) was cultivated with 0.5 mM silicon supplementation, with and without additives, for 144 hours. The additives were 100 ppm O-80 and acetic acid. Then, extracellular pH was continuously monitored within first 3 hours (Figure 4.2A). Initially, extracellular pH of control cells was 4.52 ± 0.01 pH units and varied between 4.43 to 4.52 along the experimental period. For solvent control, extracellular pH of cells treated with acetic acid was significantly reduced to 4.11 ± 0.01 pH units. Moreover, the pH value was gradually reduced after exposure for 10 minutes. Acetic acid affected cells by inducing maximal extracellular acidification after 30 minutes, causing the pH change of 0.5 units. Then, the pH was slightly increased to control level after 90 minutes. After that, the culture medium was dramatically alkalinized, as shown by the ΔpH of 1.34 units. In contrast, extracellular pH of cells treated with chitosan was maintained at 4.11 during 30 minutes of treatment. Then, the pH value was increased about 0.55 units after exposure for 60 minutes. When compared to acetic acid treatment, therefore, chitosan inhibited extracellular acidification induced by acetic acid. Remarkably, the effect of chitosan and acetic acid was antagonized at the end of experiment.

4.2.2 Cell mortality and extracellular protein

Mortality and extracellular protein of *C. rubrum* cells were determined during 144 hours of cultivation (Figure 4.2B and 4.2C, respectively). At beginning, the mortality of cells treated with acetic acid as a solvent control was 14.1 ± 0.19% similar to water control. At the end of measurement, the mortality slightly increased to 26.9 ± 0.1% with no difference from water control (Figure 4.2B). In contrast, the mortality of cells treated with 100 ppm chitosan was abruptly increased to 43.7 ± 3.09% at the beginning of treatment. High mortality induced by chitosan was evident over the exposure time. For extracellular protein, the content was 0.01 μg g^{-1}FW

similarly in all treatments (Figure 4.2C). With acetic acid treatment, extracellular protein was 0.007 μg g^{-1}FW, which was significantly lower than water control and chitosan during 24-96 hours (Figure 4.2C). However, the value of 0.01 μg g^{-1}FW at the end of measurement was similar to water control. In contrast, extracellular protein of cells treated with chitosan was similar to water control during 96 hours. At the end of experiment, extracellular protein with chitosan treatment was 0.02 μg $μl^{-1}$, which was significantly different from acetic acid and water controls (Figure 4.2C). The increase of extracellular protein indicates that the plasma membranes might leak by hundred ppm chitosan. Taken together, hundred ppm chitosan was a cytotoxicity level for *C. rubrum* cell suspensions.

4.2.3 Extracellular silicon

Silicon (0.5 mM, equivalent to 0.125 mg g^{-1}FW) was added to pre-equilibrated *C. rubrum* cell suspension together with additives, which were 100 ppm acetic acid and chitosan. For control, equivalent volume of water was added. Then, silicon concentration in culture medium was examined during 144 hours (Figure 4.2D). For water control, extracellular silicon content was about 0.05 mg g^{-1} FW during 96 hours. This concentration was about 40% of added silicon concentration. Moreover, extracellular silicon was slightly reduced to 0.03 mg g^{-1} FW after 144 hours. In contrast, extracellular silicon in the presence of acetic acid and chitosan was significantly higher than water control at the beginning of treatment. With acetic acid and chitosan, extracellular silicon was 0.07 and 0.06 mg g^{-1} FW, respectively. In addition, extracellular silicon was high at this level during 144 hours of treatment. Also, this level were about 56% of external silicon added. However, it was clearly seen that hundred ppm chitosan and acetic acid inhibited cellular silicon uptake when compared to water control. Nevertheless, chitosan lowered medium silicon concentration about 17-18% after 48 and 96 hours, when compared to acetic acid treatment.

In total, *C. rubrum* cell responses to high concentration of chitosan with silicon supplementation were time-dependent. Chitosan effects included transient extracellular alkalinization and the increase of cell mortality and protein release (Figure 4.2A-C). By measuring extracellular silicon content, it showed that chitosan reversed the inhibitory effect of acetic acid on silicon uptake after 48-96 hours (Figure 4.2D). However, the effect of chitosan was invisible when cell grew older (at 144 hours, Figure 4.2D). Based on cell mortality, therefore, chitosan at 100 ppm was not optimum for long-term experiment. Thus, cellular responses to lower concentration of chitosan, i.e., 5 and 10 ppm, were examined subsequently.

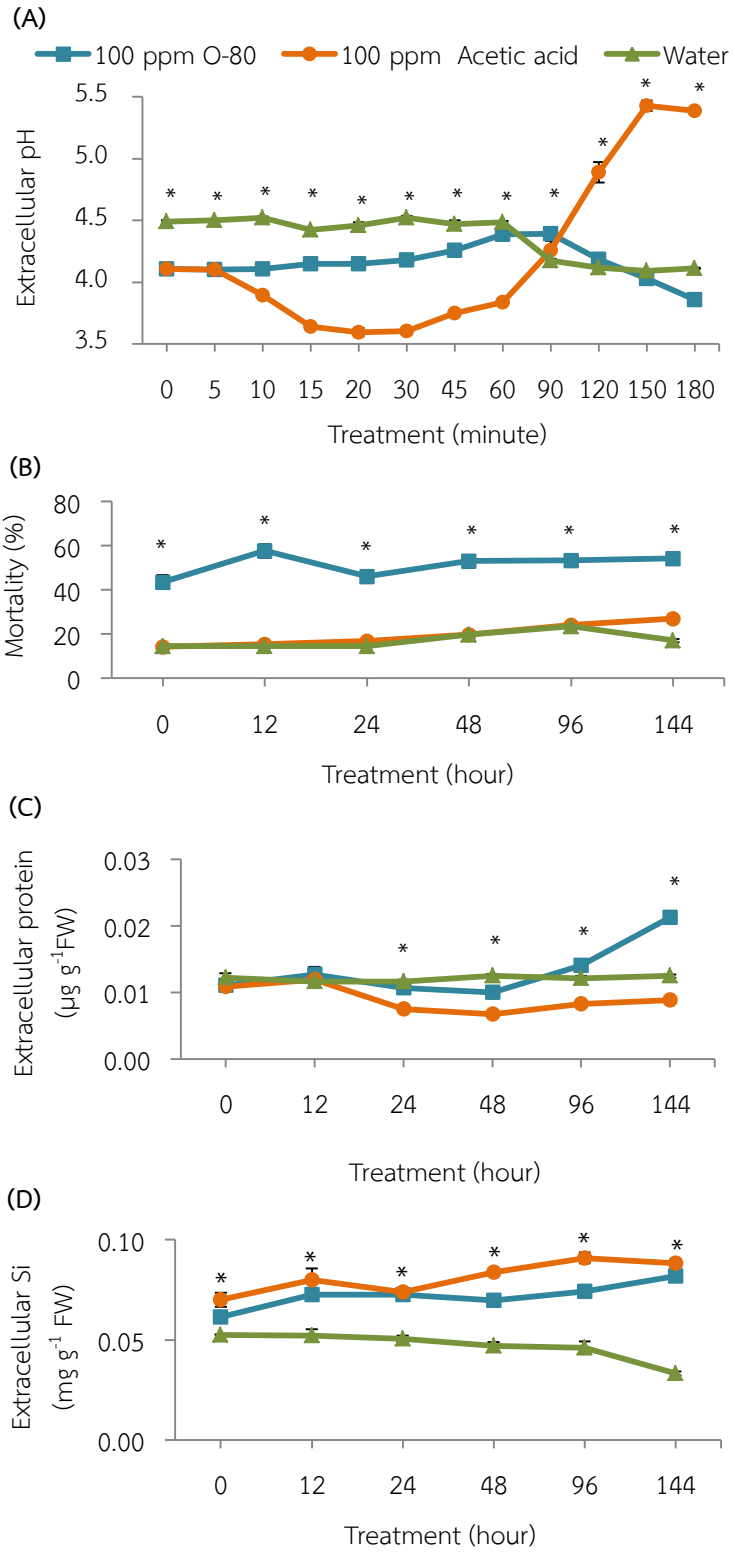

Figure 4.2 Effects of short-term treatment of chitosan (O-80) on extracellular pH (A), mortality (B), extracellular protein (C) and extracellular silicon (D) in heterotroph cell suspension of *Chenopodium rubrum* L. 100 ppm O-80 (squares), 100 ppm acetic acid as a solvent control (circles) and water control (triangles). Data shows means ± SE (n = 4). Significant results are marked with stars (*p<0.05).

4.3 Short-term responses to low concentrations of chitosan with silicon supplementation

4.3.1 Extracellular pH

Accordingly, extracellular pH was continuously explored after chitosan treatment at 5 and 10 ppm (Figure 4.3A and 4.3B, respectively). At 5 ppm concentration, extracellular pH of cells treated with acetic acid was 4.20 ± 0.02 pH units, which was slightly lower than control level (4.45 ± 0.01 pH units). In contrast, extracellular pH of cells treated with chitosan was 4.40 ± 0.05 pH units, rather similar to control (Figure 4.3A). Therefore, acetic acid induced a slight acidification (ΔpH of 0.3 units) of the medium immediately after addition. The extracellular acidification induced by acetic acid was constantly visible during 60 minutes of treatment. As compared to the solvent control, 5 ppm chitosan inhibited the acetic acid effect on extracellular pH during 30 minutes of treatment. Subsequently, the effect of chitosan on pH disappeared, as shown by a similar pH as the solvent control at 60 minutes. Then, *C. rubrum* cells treated with acetic acid and chitosan for 60 minutes similarly performed extracellular acidification with the ΔpH of 0.3 units, when compared to water control. The solvent-induced extracellular acidification was clearly visible throughout the experimental period.

At 10 ppm, the effect of acetic acid on extracellular pH was different from water control after 5 minutes (Figure 4.3B). Acetic acid caused a maximum level of acidification, reached the ΔpH of 0.3 units after 10 minutes. Then, the pH was stable at 4.30 until 60 minutes of treatment. On the other hand, extracellular pH of cells treated with chitosan was preserved at 4.52 during 45 minutes similar to water control. This indicates that chitosan inhibits extracellular acidification induced by acetic acid. Nonetheless, the inhibitory effect of 10 ppm chitosan was transient like 5 ppm (Figure 4.3A-B). That is, extracellular pH was reduced to a similar level of solvent control after 60 minutes until the end of measurement. Based on extracellular pH change, 10 ppm chitosan triggered a cellular response more than 5 ppm.

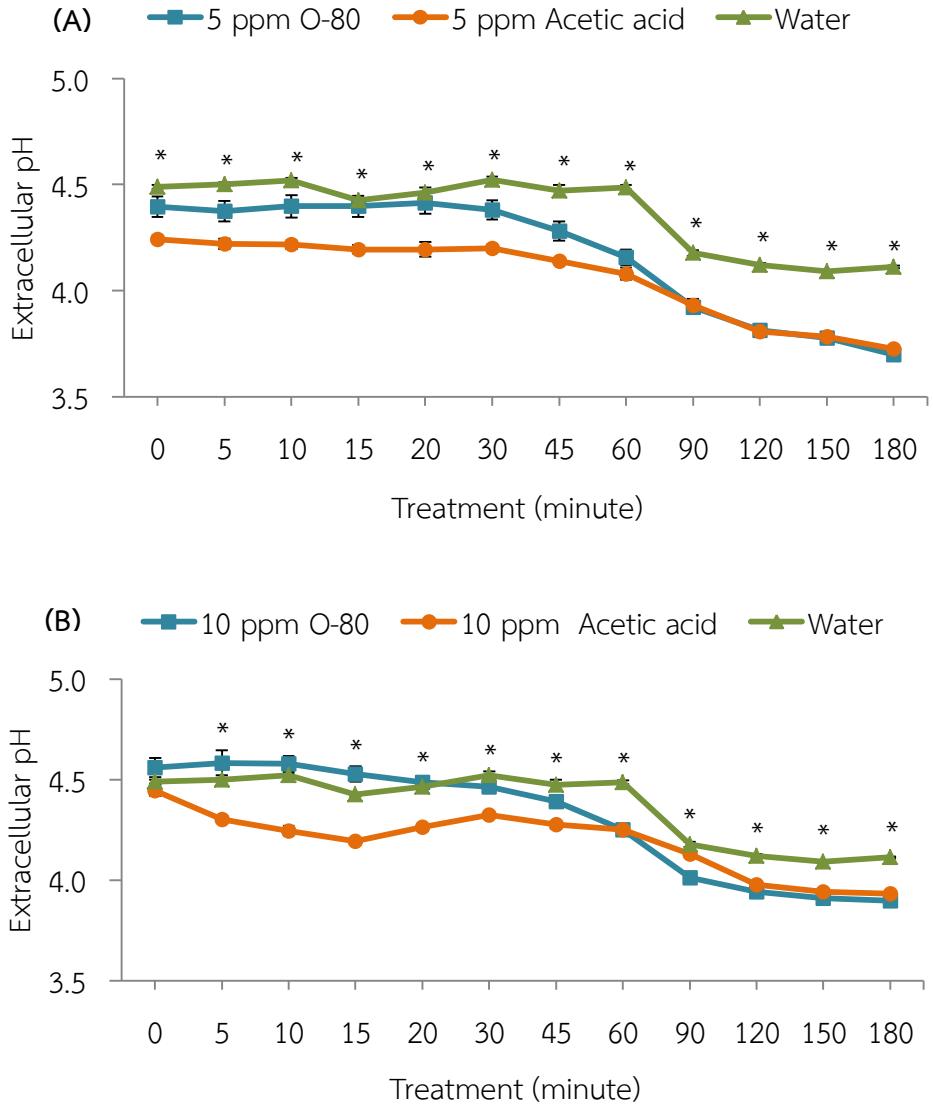

Figure 4.3 Effects of chitosan, 5 ppm (A) and 10 ppm (B) O-80, on extracellular pH of *C. rubrum* cell suspension. Cells (9 ± 1 day-old) were treated with water (triangles), O-80 (squares) and acetic acid (circles). Data show means ± SE (n = 4). Significant results are marked with stars (*p<0.05).

4.3.2 Cell mortality

The evaluation of cell mortality was determined after exposure to chitosan at 5 and 10 ppm (Figure 4.4A and 4.4B, respectively). It was found that both concentrations of chitosan had no effect on cell death when compared to acetic acid (solvent control). That is, the mortality of cells treated with chitosan was approximately 24% similar to the solvent control (Figure 4.4A-B). Nonetheless, acetic acid treatment caused a slight increase of cell mortality during 24 hours when compared to water control. Subsequently, the mortality of control cells increased at 48 and 96 hours. So, there was no difference of cell mortality in all treatments at this cultivation period. However, increased cell mortality caused by acetic acid was detected after 144 hours of cultivation. From results, it showed that chitosan at these concentrations was not harmful to cells during 144 hours of treatment, but its solvent.

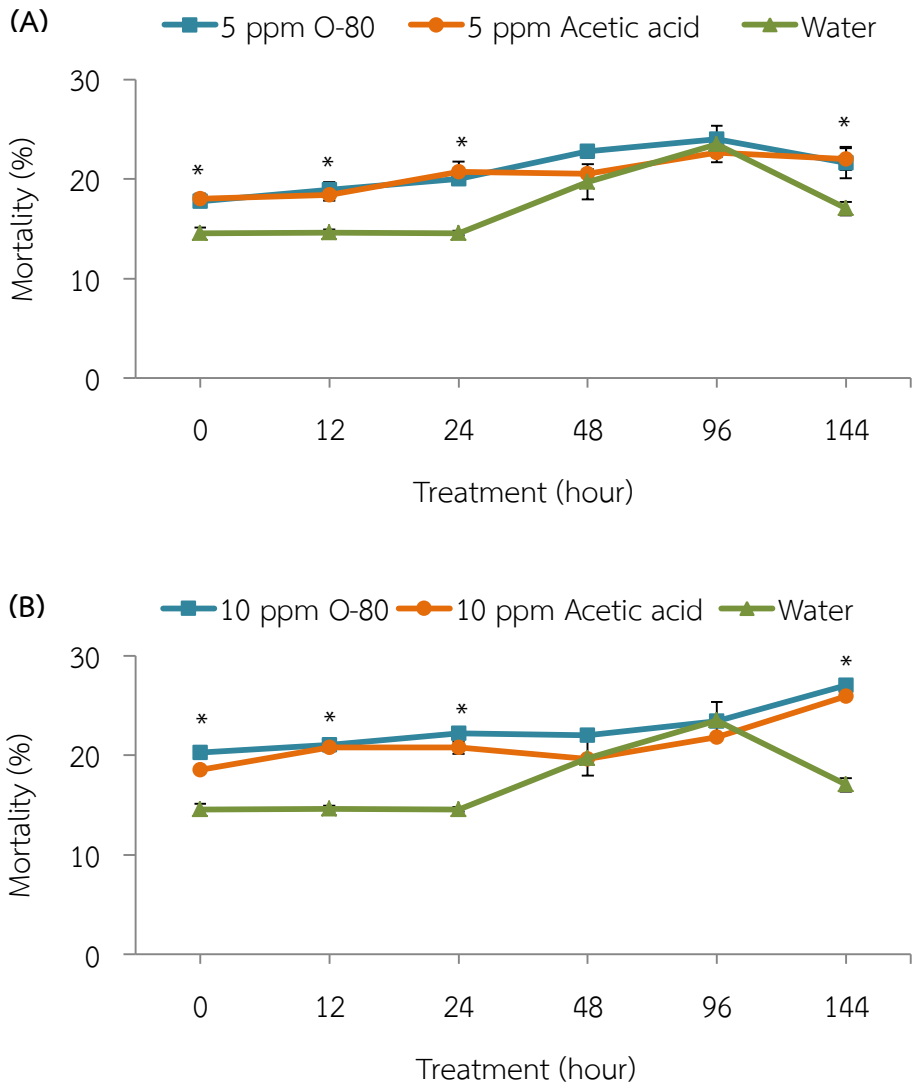

Figure 4.4 Effects of chitosan, 5 ppm (A) and 10 ppm (B) O-80, on the mortality of heterotroph *C. rubrum* cells. Water control (triangles), O-80 (squares) and acetic acid (circles). Data show means ± SE (n = 4). Significant results are marked with stars (*p<0.05).

4.3.3 Extracellular protein

Extracellular protein of *C. rubrum* cells was determined after exposure to chitosan at 5 and 10 ppm (Figure 4.5A and 4.5B, respectively). Both concentrations of chitosan had no effect on protein release. That is, extracellular protein content was 0.01 µg g^{-1}FW similarly in all treatments (Figure 4.5A-B).

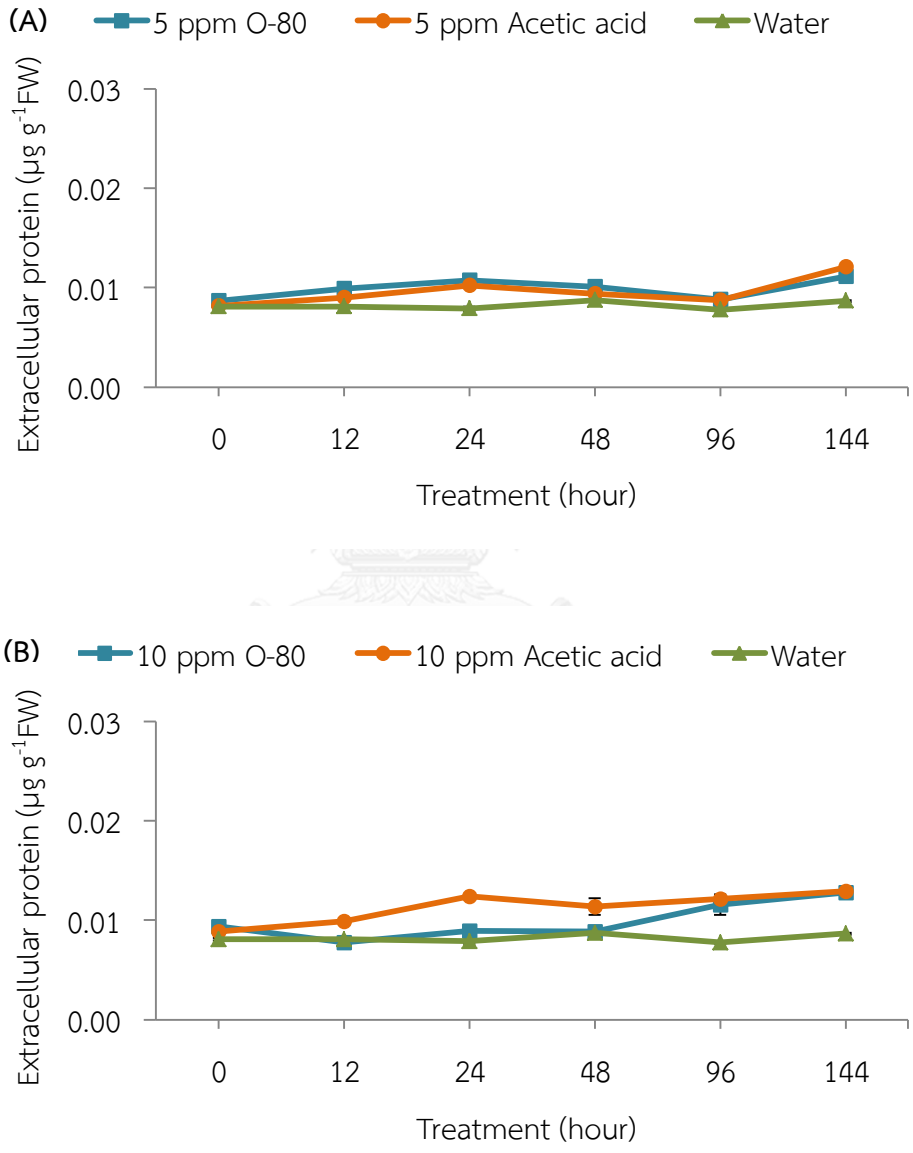

Figure 4.5 Effects of chitosan, 5 ppm (A) and 10 ppm (B) O-80, on the extracellular protein of heterotroph *C. rubrum* cells. Water control (triangles), O-80 (squares) and acetic acid (circles). Data show means ± SE (n = 4). There was no significant difference (P>0.05).

4.3.4 Extracellular silicon

The concentration of silicon in culture medium was valued after exposure to chitosan at 5 and 10 ppm during 144 hours (Figure 4.6A and 4.6B, respectively). With both chitosan concentrations, extracellular silicon varied between 0.053 and 0.058 mg g^{-1} FW along the experimental period. (Figure 4.6A-B). Therefore, there was no difference in extracellular silicon content in all treatments. That is, chitosan at these concentrations had no effect on silicon uptake in short-term experiments.

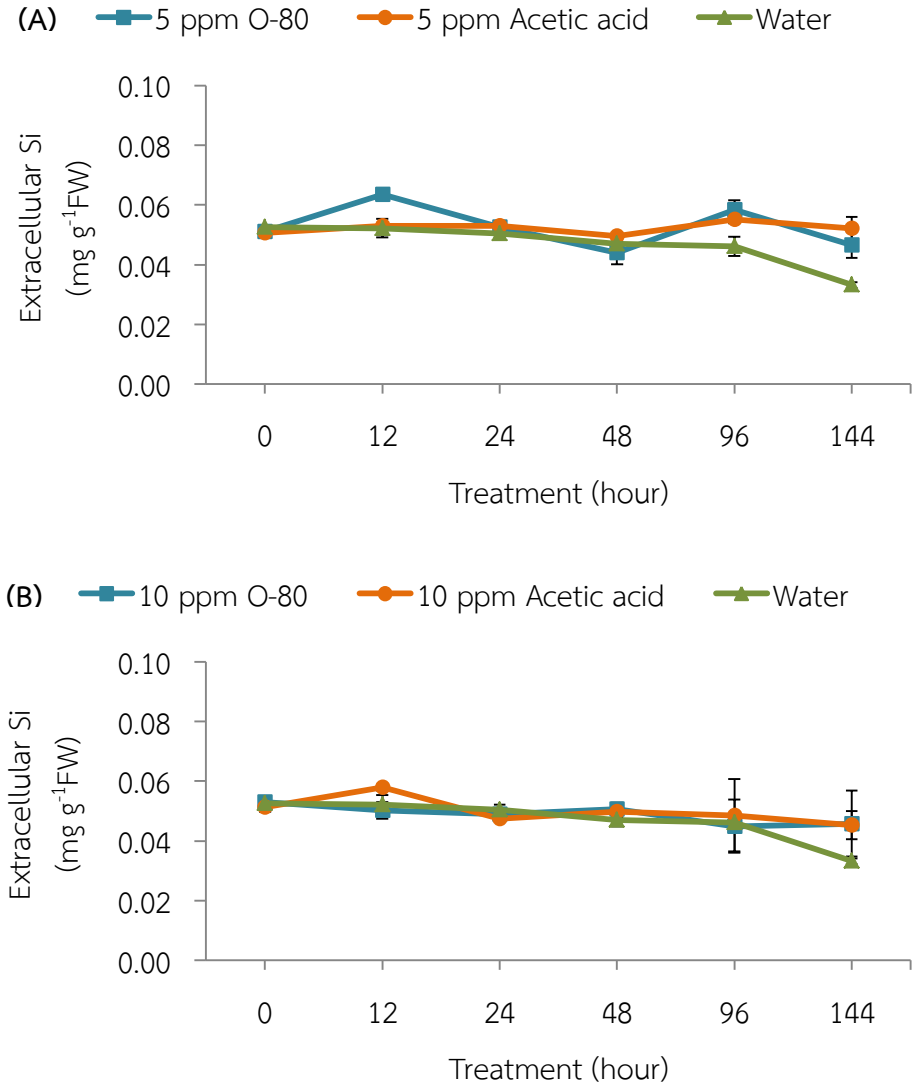

Figure 4.6 Effects of chitosan, 5 ppm (A) and 10 ppm (B) O-80, on the extracellular silicon of heterotroph *C. rubrum* cells. Water control (triangles), O-80 (squares) and acetic acid (circles). Data show means ± SE (n = 4). There was no significant difference (P>0.05).

4.4 Long-term chitosan treatment with silicon supplementation

Based on chitosan short-term responses, ten ppm O-80 chitosan was selected for investigating chitosan role on silicon uptake in long-term experiment. During 60 days of cultivation with silicon supplementation, cell suspensions were sub-cultivated every 12 days. Immediately before sub-cultivation, extra- and intracellular parameters were determined as follows (Figure 4.7-4.12).

4.4.1 Extracellular pH

With 0.5 mM silicon supplementation, time-course pH response after chitosan (10 ppm) treatment was determined during 60 days (Figure 4.7). Initially, extracellular pH of cells treated with chitosan, acetic acid and water was 4.50 pH units. Then, extracellular pH was similarly reduced to 3.86 – 4.04 pH units in all treatments after 12 days. The medium pH was maintained in acidic range until the end of experiment in all treatments. For control cells, extracellular pH was 4.04 ± 0.02 units. It was found that cells treated with acetic acid and chitosan responded similarly, except after 24 days of treatment. There, chitosan-treated cells had significantly higher extracellular pH (ΔpH = 0.1) than acetic acid and water controls. However, effects of chitosan on extracellular pH was seen only at this exposure time. Furthermore, acetic acid acidified the medium pH (ΔpH = 0.2) after 48 days. Nonetheless, the effect of acetic acid on extracellular pH was vanished when cultivated cells for 60 days.

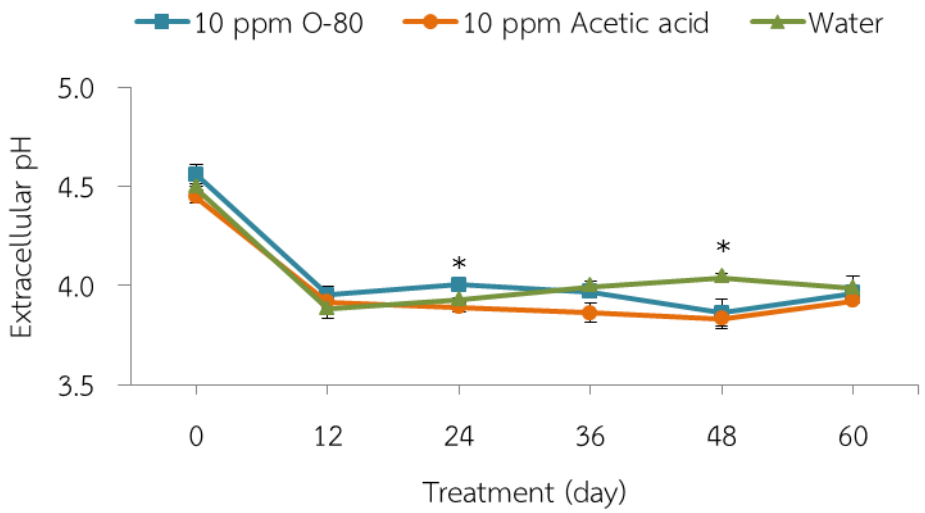

Figure 4.7 Effect of long-term treatment with chitosan (10 ppm) on extracellular pH *C. rubrum* cells. Acetic acid (circles), water control (triangles) and O-80 (squares). Data show means ± SE (n = 4). Significant results are marked with stars (*p<0.05).

4.4.2 Cell mortality and extracellular protein

An appraisement of percentage cell death and extracellular protein was valued after treatment with 10 ppm O-80 up to 60 days (Figure 4.8A and 4.8B, respectively). Control cell mortality was approximately 22.4-33.2% during 60 days of cultivation with supplemented silicon (Figure 4.8A). Surprisingly, the mortality of *C. rubrum* cells treated with acetic acid for 36 days was significantly lower than control (Figure 4.8A). However, the effect of acetic acid was visible only at this timing. For chitosan treatment, there was no significant effect on cell mortality during 60 days of cultivation (Figure 4.8A). For extracellular protein, treatment with acetic acid and chitosan had no significant effect on protein release (Figure 4.8B). That is, extracellular protein was constant at 0.01 μg g^{-1}FW similar to control during 60 days of experiment (Figure 4.8B).

In total, the presence of chitosan and acetic acid in culture medium during 60 days of cultivation did not affect cell viability and protein release of *C. rubrum* cells.

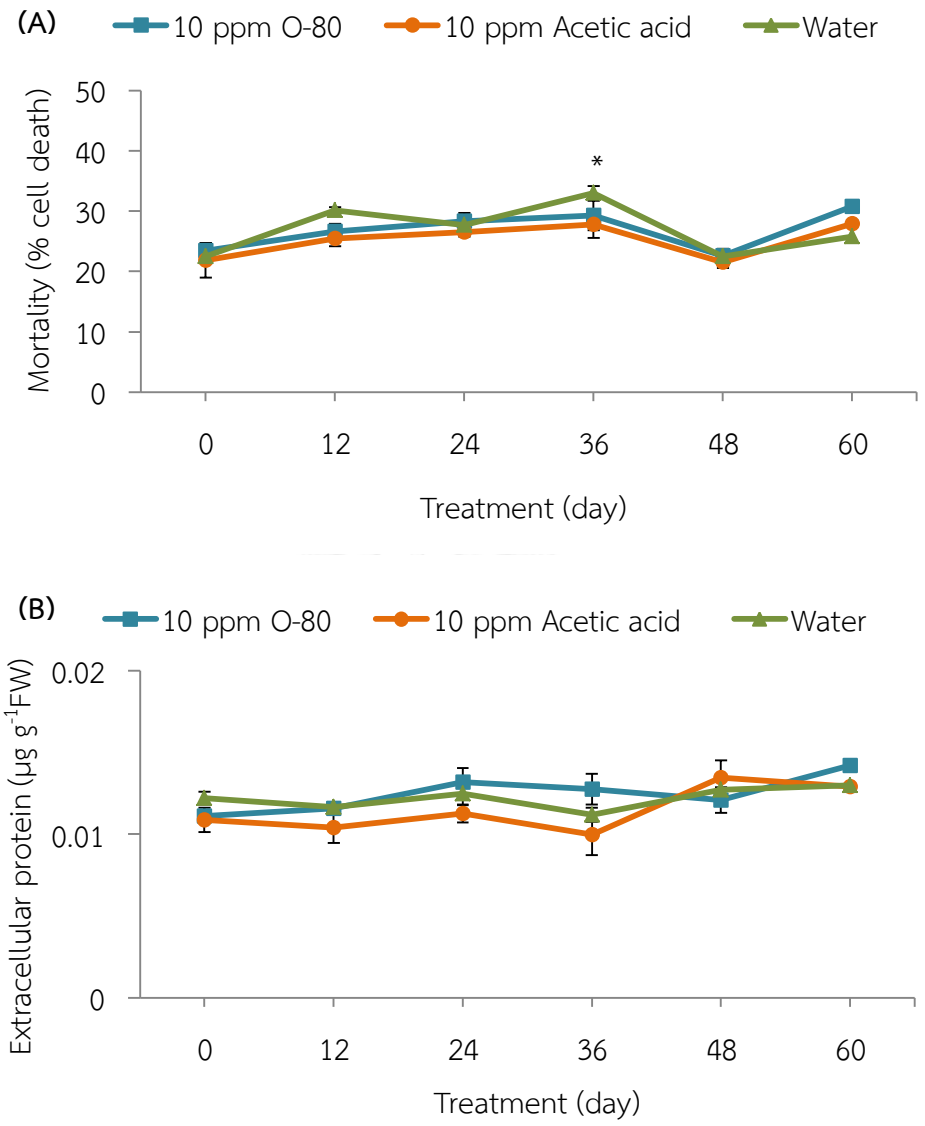

Figure 4.8 Effects of chitosan (O-80), 10 ppm, on the mortality (A) and extracellular protein (B) of heterotroph *C. rubrum* cells. O-80 (squares), water control (triangles) and acetic acid (circles). Data show means ± SE (n = 4). Significant results are marked with stars (*p<0.05).

4.4.3 Extracellular silicon

To monitor chitosan effect on silicon uptake, the concentration of silicic acid in the culture medium was estimated every 12 days (Figure 4.9). Initially, extracellular silicon in all treatments was 0.059-0.063 mg g^{-1}FW (15.2-17.0 mg L^{-1}). After treatment for 12 days, medium silicon concentration was decreased about 70% similarly in all treatments. Also, silicon concentration was this low during 48 days of cultivation. Interestingly, extracellular silicon after acetic acid treatment was significantly higher than control after 60 days. In addition, extracellular silicon was significantly lower with chitosan application than with acetic acid. By relating to initial concentration, therefore, acetic acid and chitosan caused 19.5% and 45.8% reduction of medium silicon. Nonetheless, the reduction of extracellular silicon was highest (-64.4%) without additives. Based on extracellular silicon, it is likely that silicon uptake can be induced by high silicon availability, not by acetic acid and chitosan treatment. Furthermore, acetic acid inhibits silicon uptake and chitosan antagonizes the effect of acetic acid under prolonged cultivation.

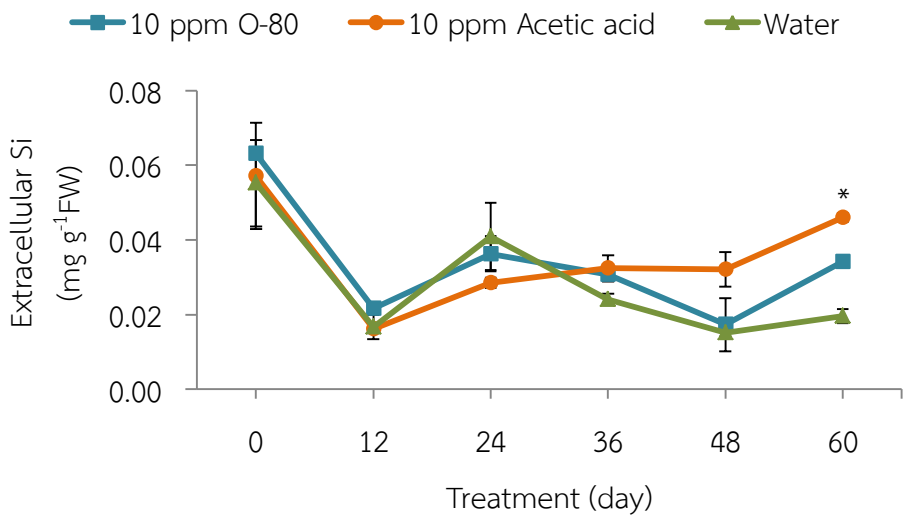

Figure 4.9 Effects of long-term treatment of chitosan (10 ppm) on silicon uptake of heterotroph *C. rubrum* cells. O-80 (squares), water control (triangles) and acetic acid (circles). Data show means ± SE (n = 4). Significant results are marked with stars (*p<0.05).

4.4.4 Intracellular silicon

Besides medium silicon, intracellular silicon content was evaluated after exposure to 10 ppm chitosan throughout 60 days (Figure 4.10). Initially, intracellular silicon of 12 day-old *C. rubrum* cells was approximately 0.15 mg g^{-1}FW. Silicon contents in cells treated with water, chitosan and acetic acid were similarly increased (49, 58 and 62%, respectively) after 12 days of treatment. During 60 days of cultivation, cellular silicon in all treatments was similar at all exposure times, the contents fluctuated between 0.15 to 0.22 mg g^{-1}FW. In total, the findings denoted that cells treated with chitosan, acetic acid and water performed similar cellular silicon uptake. Therefore, there was no effect of chitosan on silicon uptake in long-term treatment.

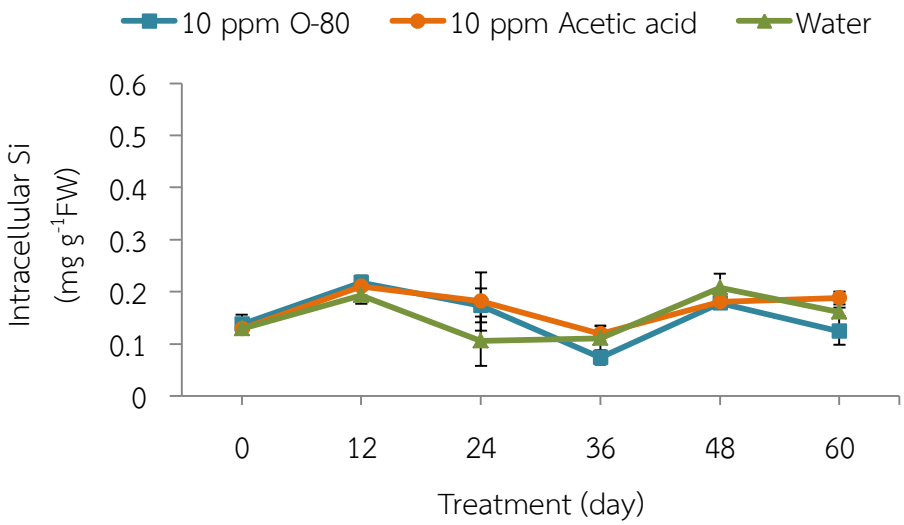

Figure 4.10 Effects of long-term treatment of chitosan (10 ppm) on silicon uptake of heterotroph *C. rubrum* cells. O-80 (squares), water control (triangles) and acetic acid (circles). Data show means ± SE (n = 4). There was no significant difference (P>0.05).

4.4.5 Percentage of extra- and intracellular silicon of heterotroph *C. rubrum* cell suspension

To clarify the data, extra- and intracellular silicon were presented in percent of total silicon contents in 20 ml cell suspension (Figure 4.11A-C). At the beginning, extracellular silicon was about 40% while intracellular silicon was 60% similarly in all treatments (Figure 4.11A-C). After 12 days of cultivation, extracellular silicon was reduced to 10% whereas intracellular silicon was increased to 90% in all treatments similarly (Figure 4.11A-C). After 24 and 36 days of cultivation, extracellular silicon was approximately 30-40% while intracellular silicon was dropped to 60-70%, regardless of additives. After 48 days, however, it was found that silicon presented 90% in the cells and 10% outside the cells. After 60 days, silicon contents were found 80% internally and 20% externally. Obviously, changes in percentage of silicon contents were similar in all treatments throughout 60 days of cultivation. Therefore, this indicated that there was no effect of chitosan on cellular silicon uptake in heterotroph *C. rubrum* cells, when applied in long-term.

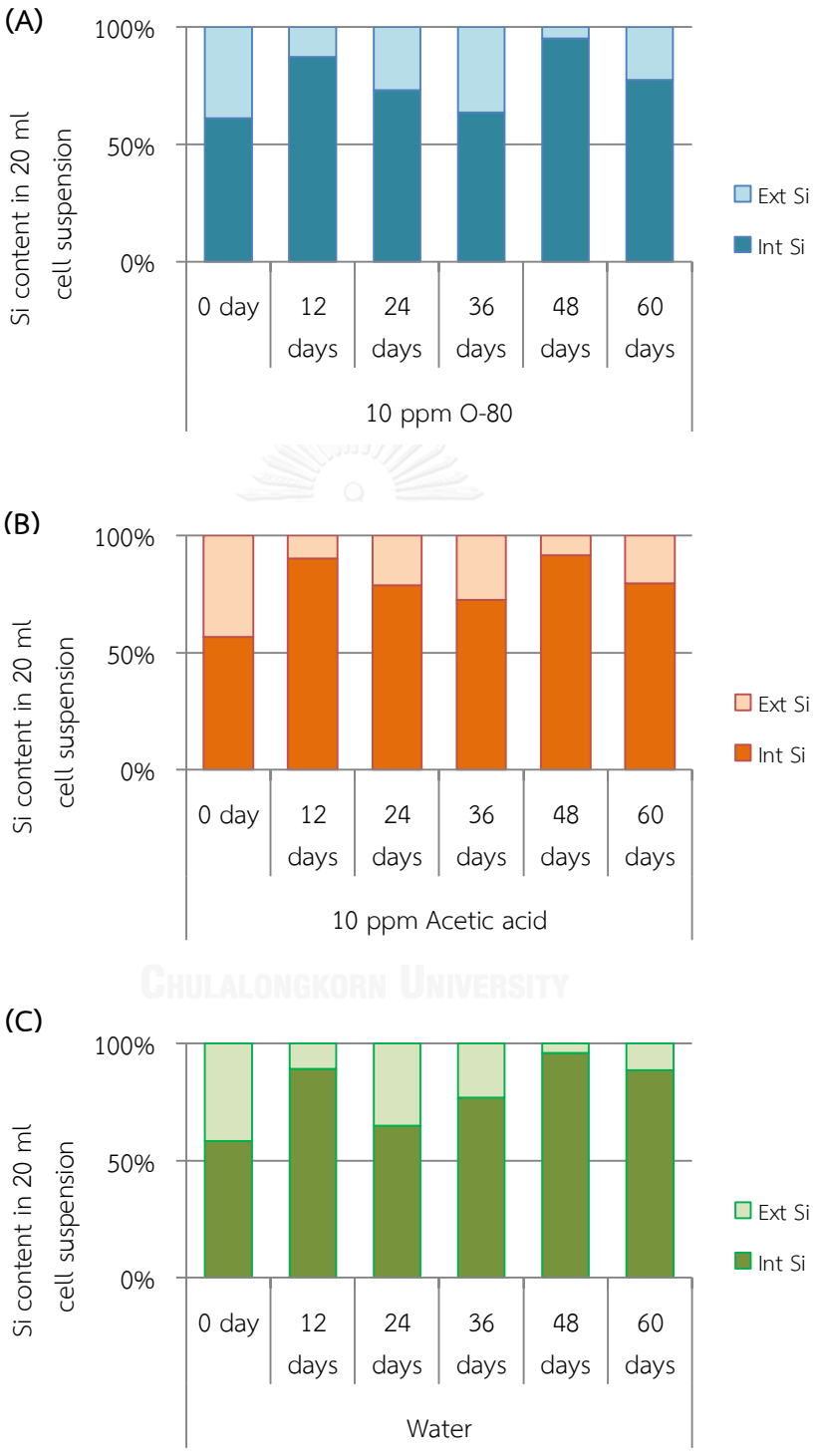

Figure 4.11 Total percentage of extra- and intracellular silicon of *C. rubrum* cells. 10 ppm O-80 (A), 10 ppm acetic acid (B) and water control (C).

4.4.6 Scanning electron microscopy (SEM) images

After 12 days of cultivation with additives and silicon supplementation, cell morphology was illustrated by SEM (Figure 4.12A-C). The morphology of cells cultivated without silicon and additives was also observed (Figure 4.12D). When compared to cells cultured without silicon and additives, it appeared that high silicon in the medium did not modify cell morphology (Figure 4.12A and 4.12D). Likewise, cell treated with water, chitosan, and acetic acid retained in their normal globular shape and appeared as cell clumps without visible difference of cell surface (Figure 4.12A-D). To note, some wrinkles on cell surface was caused by disqualified dehydration. Jointly, there was no effect of silicon and chitosan on the morphology of cells.

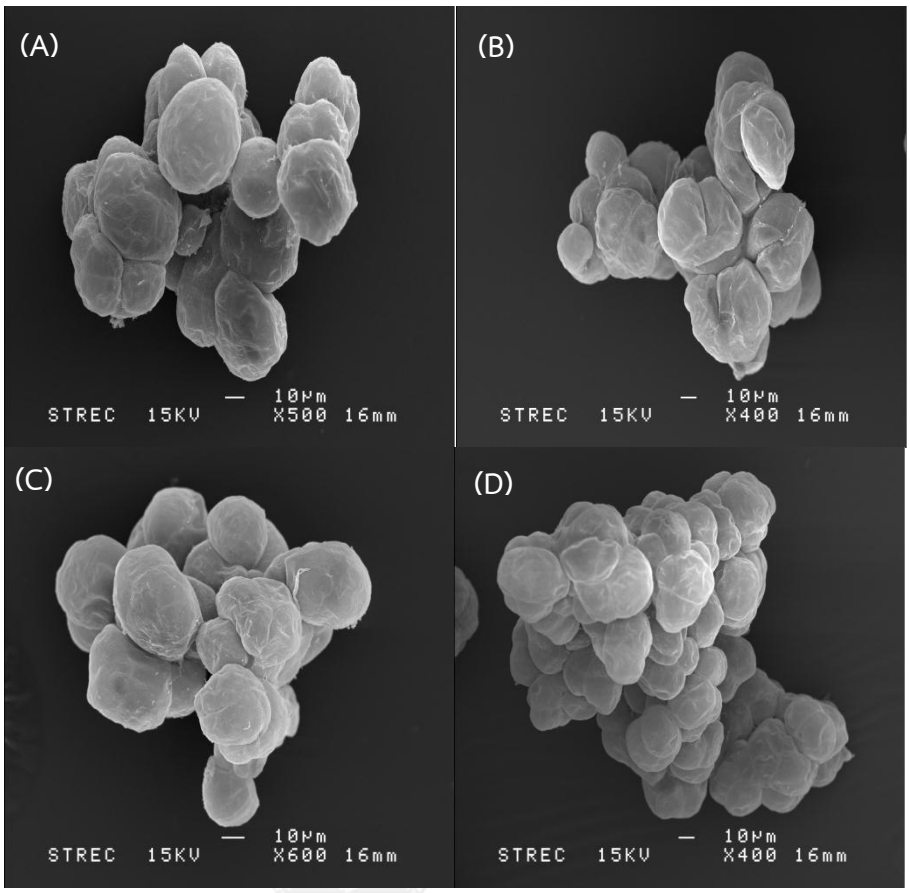

Figure 4.12 Scanning electron microscopy images of *C. rubrum* cells after long-term treatment of chitosan (10 ppm) and silicon (0.5 mM). Cells cultured with high silicon were treated with water (A), O-80 (B) and acetic acid (C) for 12 days. Cell culture (12 days old) without silicon supplementation (D).

4.5 Long-term chitosan treatment without silicon supplementation

Due to the finding that high silicon availability induced cellular silicon uptake regardless of chitosan (Figure 4.7-4.12), cellular responses to chitosan and silicon uptake were investigated under low silicon condition. This additional experiment was carried out to prove that cellular silicon uptake of heterotroph *C. rubrum* cells is activated by high soluble silicon in culture medium.

4.5.1 Extracellular pH

Time-course pH response to chitosan (10 ppm) without silicon supply was evaluated throughout 60 days (Figure 4.13). At the beginning, extracellular pH of cells treated with water, acetic acid and chitosan was 4.50 pH units. After 12 days, extracellular pH was similarly dropped to 3.75 – 4.03 pH units in all treatments. The medium pH in all treatments was preserved in acidic range throughout experimental period. The drop of extracellular pH after 12 days of cultivation was similar between chitosan, acetic acid, and water control. Likewise, extracellular pH was in acidic range in all treatments after cultivation for 24-60 days. The findings indicate that there is no effect of acetic acid and chitosan on extracellular pH under low silicon condition. In addition, there may be some latent culturing conditions, such as low nutrients, that can activate extracellular acidification of heterotroph *C. rubrum* cells.

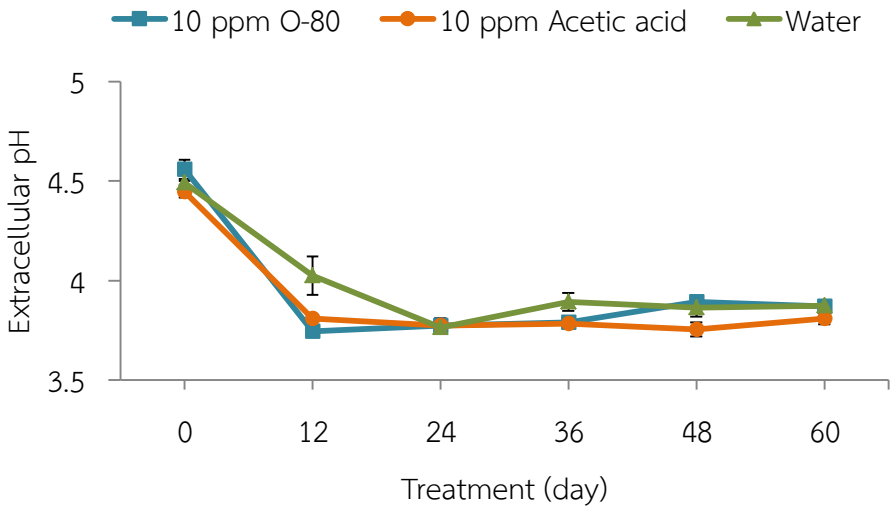

Figure 4.13 Effect of long-term treatment with chitosan (10 ppm) on extracellular pH of *C. rubrum* cells under low silicon condition. Acetic acid (circles), water control (triangles) and O-80 (squares). Data show means ± SE (n = 4). There was no significant difference (P>0.05).

4.5.2 Cell mortality and extracellular protein

Percentage cell death and extracellular protein was determined after treatment with 10 ppm O-80 up to 60 days (Figure 4.14A and 4.14B, respectively). At the beginning, the mortality of cells was approximately 10.6% in all treatments (Figure 4.14A). Cell mortality slightly increased to 20.3% when cultivation period was extended. However, there was no significant effect of chitosan and acetic acid on cell mortality during 60 days of cultivation (Figure 4.14A). For extracellular protein measurement, treatment with chitosan and acetic acid had no significant effect on protein release (Figure 4.14B). That is, extracellular protein was invariable at 0.01 µg g^{-1}FW similar to water control along 60 days of experimental period (Figure 4.14B).

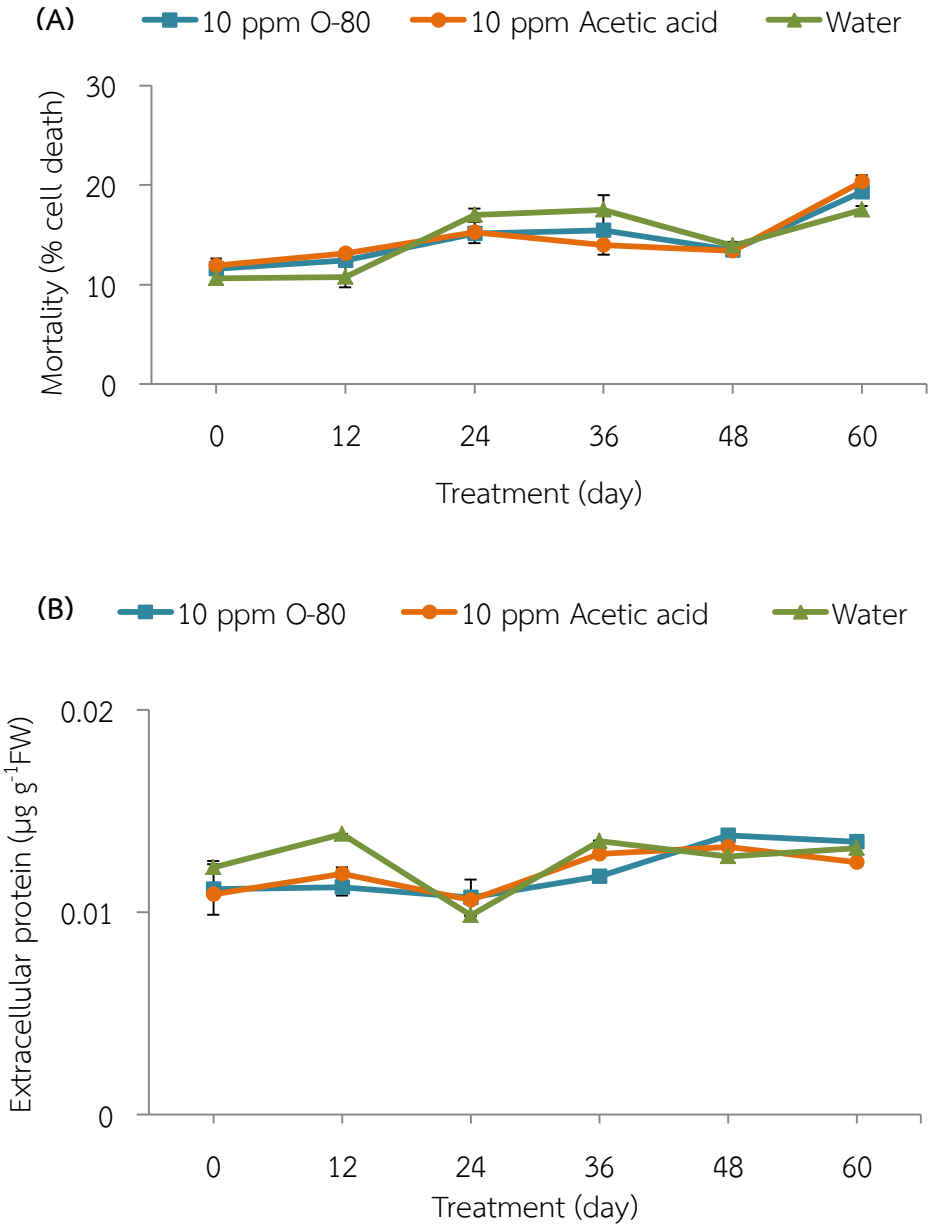

Figure 4.14 Effects of chitosan (O-80), 10 ppm, on the mortality (A) and extracellular protein (B) of heterotroph *C. rubrum* cells under low silicon condition. O-80 (squares), water control (triangles) and acetic acid (circles). Data show means ± SE (n = 4). There was no significant difference ($P>0.05$).

4.5.3 Extracellular silicon

Without silicon supplementation, the concentration of silicic acid in culture medium was 0.005 mg g^{-1}FW (1.29 mg L^{-1}) (Figure 4.15). During cultivation for 60 days, extracellular silicon in all treatments was maintained at 0.005-0.006 mg g^{-1}FW (1.29-1.55 mg L^{-1}) similarly.

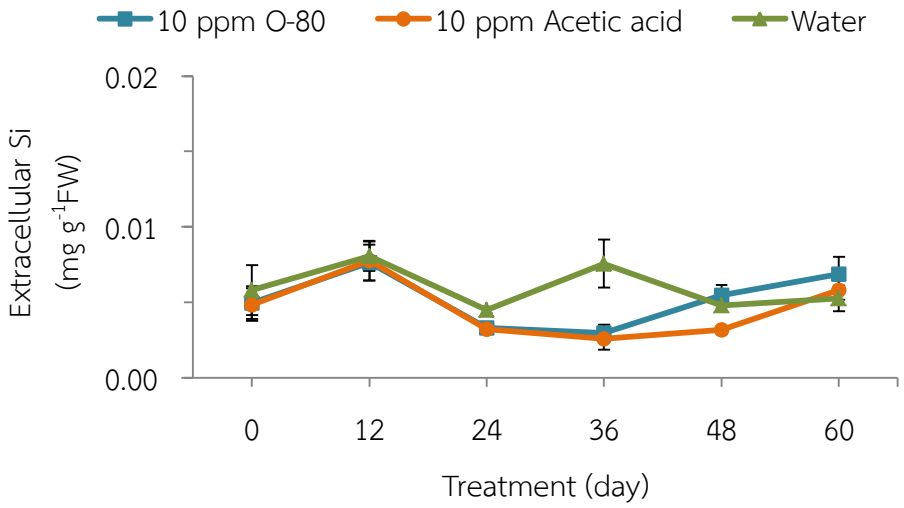

Figure 4.15 Effects of long-term treatment of chitosan (10 ppm) on extracellular silicon of heterotroph *C. rubrum* cells under low silicon condition. O-80 (squares), water control (triangles) and acetic acid (circles). Data show means ± SE (n = 4). There was no significant difference (P>0.05).

4.5.4 Intracellular silicon

Intracellular silicon content was evaluated after exposure to 10 ppm chitosan for 60 days (Figure 4.16). Silicon content in cells treated with water, chitosan and acetic acid was about 0.05 mg g^{-1} FW similarly. The content remained at this level in all treatments throughout 60 days of cultivation. Therefore, there was no increase in silicon uptake when cells were treated in long-term with chitosan under low silicon condition. Moreover, there was no accumulation of cellular silicon under low silicon in culture medium.

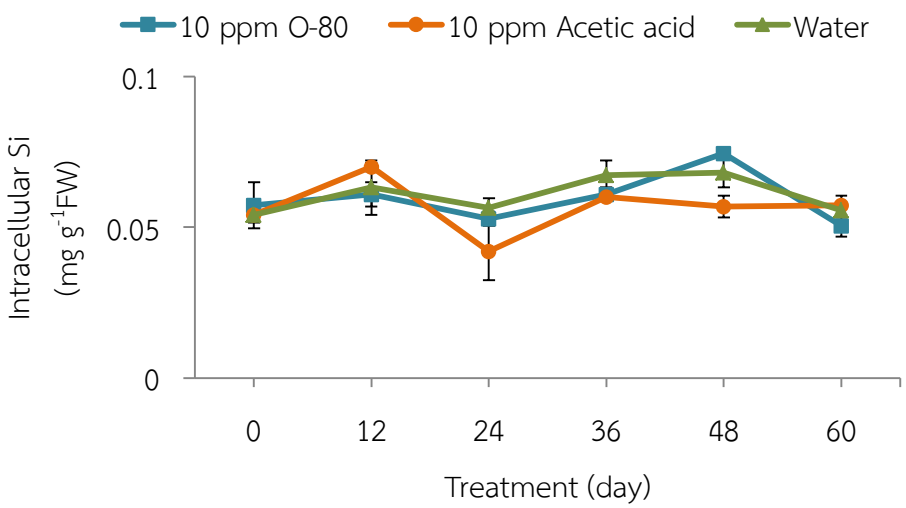

Figure 4.16 Effects of long-term treatment of chitosan (10 ppm) on intracellular silicon of heterotroph *C. rubrum* cells under low silicon condition. O-80 (squares), water control (triangles) and acetic acid (circles). Data show means ± SE (n = 4). There was no significant difference (P>0.05).

4.6 Effect of chitosan on plasma membrane H^+-ATPase activity

Membrane vesicles prepared from 12 day-old heterotroph *C. rubrum* suspension cells were measured for the activity of plasma membrane H^+-ATPase, in the presence and absence of chitosan and acetic acid (Table 4.1). The results showed that H^+-ATPase activity of cells treated with 10 ppm acetic acid was similar to control. Interestingly, the inhibitory effect was shown by 100 ppm acetic acid (47% reduction). Nevertheless, 10 and 100 ppm chitosan had H^+-ATPase activity similar to water control. When compared to its solvent control, antagonistic effect on H^+-ATPase of chitosan to acetic acid was visible at 100 ppm concentration. Though the data were from one replication, this observation corresponded with extracellular pH (Figure 4.2A) and encouraged further investigation on molecular target of chitosan and acetic acid in plant cells.

Table 4.1 Effect of chitosan and acetic acid on activity of plasma membrane H^+-ATPase on membrane vesicles prepared from heterotroph *C. rubrum* suspension cells. Protein content was 2.863 mg g^{-1} FW. Data are from one replication.

Additives	Vanadate-sensitive ATPase activity (nmol Pi mg $prot^{-1}$ min^{-1})	Relative activity (%)
Control	623.7	100
10 ppm acetic acid	616.9	99
10 ppm chitosan	615.8	99
100 ppm acetic acid	333.5	53
100 ppm chitosan	579.7	93

CHAPTER V
DISCUSSION

5.1 Short-term response to chitosan and acetic acid with silicon supplementation

Plant cells can perceive a variety of micro-organism substances such as elicitors, bioactive signal peptides and various compounds associated with pathogen defense (Darvill & Albersheim, 1984; Ebel & Cosio, 1994). The perception leads to the blocking of H^+-ATPase pumping, as shown by the reduction of proton efflux through apoplast, i.e., a transient extracellular alkalinization, detected in the culture medium of tomato (*Lycopersicon esculentum*) suspension-cultured cells stimulated by chitin fragments (Felix et al., 1998; Schaller & Oecking, 1999). In this study, results showed that extracellular pH response to chitosan depended on concentration and exposure time (Figure 4.2-4.3). In addition, there was an effect of acetic acid, which is a solvent of chitosan, on extracellular pH change. Acetic acid caused extracellular acidification immediately after 10 minutes on a concentration-dependent manner (Figure 4.2A, 4.3A-B). Interestingly, acetic acid at high concentration (100 ppm) induced biphasic changes of extracellular pH, that is, firstly extracellular acidification before 90 minutes and extracellular alkalinization after 90 minutes of exposure time (Figure 4.2A). Extracellular acidification can occur via some mechanisms as follows: (i) the increase of H^+-ATPase activity which requires ATP supply or (ii) the release of proton ions from some organic acids present in the medium. Likewise, extracellular alkalinization can be due to (i) the inhibition of H^+-ATPase or (ii) the reduction of free proton ions by means of buffering compounds in the medium. For acetic acid, it is a weak acid that release free proton ions into the medium (Felle et al., 1986). Thus, the reduction of extracellular pH at the beginning of treatment (water control: 4.52 pH units, acetic acid: 4.11 pH units) was observed (Figure 4.2A). Subsequently, acetic acid-induced cellular response by mediating extracellular acidification was obvious with increasing exposure time (Figure 4.2A). Previously, it has been reported that acetic acid (5 ppm) can passively diffuse into the cells and induce a transient membrane hyperpolarization of corn coleoptile cells (Felle et al., 1986). This leads to

cytosolic acidification, proton pumping stimulation and induction of elongation growth, well known as wall-acidification theory (Felle et al., 1986). However, long time exposure to high concentration of acetic acid may not benefit the cells, as shown by abrupt extracellular alkalinization occurring after 90 minutes of treatment (Figure 4.2A). This finding was corresponded with acetic acid inhibition of H^+-ATPase (Table 4.1). Lately, programmed cell death induction when treated with high concentration of acetic acid (in mM range) was evident in yeast and algae (Zuo et al., 2012).

Because acetic acid caused extracellular acidification, extracellular pH maintained at a similar level of water control implied chitosan-induced extracellular alkalinization (Figure 4.2A, 4.3A-B). The antagonistic effect on extracellular pH to acetic acid was obviously shown by high concentration (100 ppm) of chitosan (Figure 4.2A). The result of extracellular pH indirectly shows the inhibitory effect of chitosan on H^+-ATPase activity. Chitosan was found to increase extracellular pH and inhibit the plasma membrane H^+-ATPase of *Mimosa pudica* in a concentration-dependent manner (Amborabé et al., 2008). The inhibition of H^+-ATPase activity by chitosan was corresponded with its inhibitory effect on the uptake of amino acid and sugars (Amborabé et al., 2008).

Besides pH and H^+-ATPase, it was reported that chitosan induced hypersensitive response in *Catharanthus roseus* tissues as determined by H_2O_2 synthesis (Kauss et al., 1989). Likewise, chitosan can trigger octadecanoid pathway which induces the increase of jasmonic acid level and lipoxygenase activity (Rakwal et al., 2002). Moreover, chitosan can activate phenylpropanoid pathway, i.e., the increase of phenylalanine ammonia lyase activity, leading to the accumulation of various phenolic compounds (Kohle et al., 1984; Reddy et al., 1999; Rossard et al., 2006). The oxidative burst (Rossard et al., 2006) and production of H_2O_2 to toxicity level may lead to cell death (Kauss & Jeblick, 1996). Evidently, electrolyte leakage and extracellular protein was increased by chitosan (Young et al., 1982). It was also found that cell organization could be destroyed by high concentration of chitosan (Kohle et al., 1984). Here, the abrupt increase of cell mortality was caused by 100 ppm chitosan (Figure 4.2B). A cause is likely due to detergent-like chitosan properties

(Iriti & Faoro, 2009; Rossard et al., 2006). Polycationic properties of chitosan changed membrane permeability of suspension-cultured *Glycine max* and *Phaseolus vulgaris* cells (Young et al., 1982). This resulted in large pore formation at the plasma membrane. In this study, chitosan effect on cell mortality and protein release was dose-dependent (Figure 4.2B-C, 4.4, 4.5). Extracellular protein increase was found after 144 hours only with 100 ppm chitosan (Figure 4.2C, 4.5A-B). There was an evidence of cytotoxic effects induced by high concentration (200-1,000 ppm) of chitosan (Amborabé et al., 2008). Deformation of the plasma membrane was observed with 200 ppm chitosan (Amborabé et al., 2008).

By following changes of extracellular silicon, roles of chitosan and its solvent on silicon uptake may be unveiled. From results (Figure 4.2D, 4.6A-B), extracellular silicon was similar to water control when cells exposed to low concentration of chitosan and acetic acid. However, chitosan and acetic acid at high concentration caused significantly higher extracellular silicon than water control throughout 144 hours of treatment. Nonetheless, measured concentration of medium silicon was approximately 0.05-0.07 mg g^{-1}FW, which was about 50-60% of externally applied silicon concentration (0.125 mg g^{-1}FW, 0.5 mM). In other word, there was some amount of silicon disappeared or dissoluble, and was not detected by the applied method. Therefore, it may be that the presence of acetic acid enhances the sensitivity of silicon assay. Alternatively, acetic acid may affect the solubility of silicon in the medium.

Interestingly, the drop of extracellular silicon of control cells after 144 hours, from 0.05 to 0.03 mg g^{-1}FW, evidenced silicon uptake enhancement in *Chenopodium rubrum* cells (Figure 4.2D). It is likely that when cells get old, and/or under reduced nutrient condition, cells may enhance the uptake of silicon. Furthermore, it was found that chitosan caused more reduction of extracellular silicon than acetic acid solvent control only after 48 and 96 hours (Figure 4.2D), suggesting its beneficial role on silicon uptake. After 144 hours, chitosan role on silicon uptake may be invisible by some other factors, such as cell age and nutrition. Although silicon is not determined as an essential element, it seems that silicon is beneficial for plants (Epstein, 1999). Many studies reported that silicon can alleviate abiotic and biotic stresses such as

metal toxicity, salt stress, flooding and pathogen attack (Abe & Watanabe, 1992; Epstein, 1999; Liang et al., 2003; Neumann & zur Nieden, 2001) in order to enhance photosynthetic ability and plant growth (McNaughton & Tarrants, 1983; McNaughton et al., 1985). Silicon functions both biochemically and physically (Currie & Perry, 2007; Liang et al., 2005a). Externally applied silicon activated the activity of H^+-ATPase and H^+-PPase (Liang et al., 2005a). It also induced antioxidant mechanism and maintained photosynthetic apparatus (Liang et al., 2003; Liang, 1998). In addition, silicon deposited in cell walls as solid amorphous silica (Currie & Perry, 2007), having mechanical strength that can strengthen cell wall structures (Epstein, 1999; Perry & Keeling-Tucker, 2000; Sangster et al., 2001). Thus, it is possible that *C. rubrum* suspension cells activate silicon uptake when culturing condition is not favorable.

5.2 Long-term response to chitosan and acetic acid with and without silicon supplementation

In both high and low silicon conditions, extracellular pH in all treatments dropped from 4.5 to 4.0 after 12 days (Figure 4.7 and 4.13). Throughout 60 days, extracellular pH was maintained at this pH range similarly in all treatments and regardless of medium silicon concentration. Therefore, the similarity implies that the acidification of culture medium is not due to high availability of silicon. Extracellular acidification may occur by cellular release of some organic acids that acidify culture medium (Felle, 2001). However, most likely cause is the activation of H^+-ATPase to benefit cells in nutrient uptake (Felle, 2001). Moreover, extracellular acidification may be involved in changing plant cellular activity for growth stimulation (Iivonen & Vapaavuori, 2002). Because the sub-cultivation was done every 12 days, low nutrient condition might take place and induce extracellular acidification process. This suggestion is in agreement with previous report that extracellular acidification was a sensing mechanism found in potassium deficiency (Wang & Wu, 2013).

Furthermore, effect of chitosan and acetic acid on extracellular pH was visible only under high silicon condition (Figure 4.7 and 4.13). There, chitosan-induced extracellular alkalinization was evident after 24 days whereas acetic acid-induced

extracellular acidification was after 48 days (Figure 4.7). Though there has been no clear explanation for this finding, it is obvious that high silicon availability is involved. In other word, silicon may influence H^+-mediated processes of plant cells, including metabolite and elicitor sensing. As described before, applied silicon modifies some cellular mechanisms, such as proton transport and antioxidant enzyme activation (Liang et al., 2003; Liang et al., 2005a).

In addition to extracellular pH response, it was found that the mortality of control cells under low silicon condition was lower than under high silicon condition (Figure 4.8A and Figure 4.14A). There, control cells under low silicon (day 0) had cell mortality of 10% (Figure 4.14A) but it was 20% under high silicon condition (Figure 4.8A). Furthermore, it was found that acetic acid reduced cell mortality (after 36-day exposure time) only under high silicon condition (Figure 4.8A). At this exposure time, control cells had 25% mortality (Figure 4.8A) when intracellular silicon content was 0.11 mg g^{-1}FW (Figure 4.10). Though the effect was observed once, it gives a clue of ameliorative effect of acetic acid on silicon toxicity in this cell type. There has been no previous report about silicon toxicity in plant cells (Epstein, 1994; Epstein, 1999). The result presented in this study (Figure 4.8A) was an evidence of silicon toxicity, at least in heterotroph *C. rubrum* cell. Nonetheless, there was no detectable effect on membrane integrity based on extracellular protein measurement (Figure 4.8B and 4.14B).

After 12 days of cultivation with high silicon, extracellular silicon was reduced from 0.06 mg g^{-1} FW to 0.02 mg g^{-1} FW similarly in all treatments (Figure 4.9). The results indicated that chitosan and acetic acid had no effect on silicon uptake after 12 days of treatment. Because the reduction of extracellular silicon was found only under high silicon condition, it seems that high available silicon induced cellular silicon uptake in this cell type. There is a report that silicon can activate H^+-ATPase activity of tonoplast vesicles from roots of salt-stressed barley (*Hordeum vulgare* L.) (Liang et al., 2005a). By assuming some similarity between tonoplast H^+-ATPase and plasma membrane H^+-ATPase, silicon may activate the plasma membrane H^+-ATPase activity, leading to increased proton motif force to drive some other H^+-mediated transporters (Taiz & Zeiger, 2016). Because of being beneficial element for plants, the

increase of silicon uptake will be advantaged for cells. As shown by extracellular silicon (Figure 4.9), *C. rubrum* cell suspension enhanced the uptake when silicon was highly available. Therefore, the accumulation of silicon in *C. rubrum* cells was induced by high silicon condition, not by chitosan and acetic acid. After 60 days, the inhibition of silicon uptake by acetic acid and chitosan was found, as shown by higher extracellular silicon than water control (Figure 4.9). With acetic acid, extracellular silicon was 0.05 mg g^{-1} FW while it was 0.03 mg g^{-1} FW in the presence of chitosan. This suggested antagonistic effect of chitosan to acetic acid on silicon uptake in addition to extracellular pH. There was a report also about acetic acid-antagonized low molecular weight chitosan sensor found in *C. rubrum* cells (Wongchai et al., 2013). Likewise, response of extracellular pH to acetic acid was linked to nutritional condition in heterotroph *C. rubrum* cell suspension, but not in autotroph cells (Wongchai et al., 2012b). Based on principle component analysis of proton flux, the authors suggested that cell starvation before sub-cultivation may activate a short chain monocarboxylic transporter (Wongchai et al., 2012b).

Like extracellular silicon, intracellular silicon was found to increase significantly after 12 days of treatment (Figure 4.10). Nevertheless, the association of chitosan and acetic acid with silicon uptake was not visible by intracellular silicon (Figure 4.10, after 60 days). This may be partially due to some other factors that reduce the sensitivity of silicon assay, such as sodium hydroxide used in autoclave-induced digestion (Dai et al., 2005). In this study, silicon accumulated in *C. rubrum* cells varied between 0.15 to 0.22 mg g^{-1}FW. These levels in *C. rubrum* cells were in a similar level as in tomato plant, which is silicon non-accumulating species (Ma, 2004; Mitani & Ma, 2005). There has been reports that silicon uptake and transport in plant differ greatly in plant species (Epstein, 1994; Epstein, 1999; Jones & Handreck, 1967). Naturally, graminaceous plants will take up silicon much more than other plant species (Liang et al., 2003; Ma, 2004; Ma & Yamaji, 2006). This is because of energy-mediated transport in a silicon-accumulating plant, such as rice (Epstein, 1999; Ma et al., 2006). In contrast, dicotyledonous plants passively absorb silicon, leading to less tissue silicon content (Liang et al., 2003; Ma, 2004; Ma & Yamaji, 2006). These species are cucumber, melon, soybean and strawberry (Ma, 2004; Mitani & Ma, 2005). In

addition, some dicots: for example legumes, bean, and tomato, prefer silicon exclusion to uptake (Liang et al., 2003; Ma, 2004; Ma & Yamaji, 2006).

Silicon uptake by rice roots take place by specific transporter-mediated processes (Jones & Handreck, 1967; Ma et al., 2006). Recently, the characterization of silicon uptake and transporter using rice mutants was presented (Ma et al., 2006; Ma et al., 2011; Tamai & Ma, 2003). These studies showed that low-affinity silicon transporter contained Cys residues in its molecular structure (Tamai & Ma, 2003). Rice *Lsi1* gene, which was mainly expressed in roots, encoded a membrane protein which is structurally similar to water channel (aquaporin) protein (Ma et al., 2006). This Lsi1 protein locating on the plasma membrane is suggested to function also in silicon non-accumulating species (Liang et al., 2005b). In addition, silicon concentration of cucumber was lower in xylem sap than external solution, suggesting passively-mediated xylem loading in cucumber which was different from actively process found in rice (Liang et al., 2005b; Mitani & Ma, 2005). Moreover, silicon distribution depends on plant species and external silicon concentrations (Liang et al., 2003; Ma et al., 2011).

Hence, it is obvious that the increase of silicon uptake in *C. rubrum* cells is enhanced by silicon supplemented in the culture medium. Previous report in rice suspension culture also showed that accumulated silicon can strengthen cell wall structures (He et al., 2013). Therefore, this present study provides an evidence supporting beneficial role of silicon even in silicon non-accumulating species, like *C. rubrum*. Unfortunately, beneficial role of chitosan and acetic acid on silicon uptake is not obvious in this cell type under investigated condition.

CHAPTER VI

CONCLUSION

6.1 Short-term response to chitosan and acetic acid with silicon supplementation

Heterotroph *Chenopodium rubrum* cells responded to chitosan by mediating transient extracellular alkalinization on a concentration-dependent manner like previous findings in other plant species. However, *C. rubrum* cells also responded to chitosan solvent, acetic acid. At low concentrations (5 and 10 ppm), acetic acid caused transient extracellular acidification during 3 hours of treatment. At 100 ppm, acetic acid induced biphasic changes of extracellular pH, i.e, firstly extracellular acidification before 90 minutes and alkalinization after 90 minutes of experimental period. In latter case, chitosan strongly inversed acetic acid-induced extracellular alkalinization, suggesting that (i) chitosan activates the H^+-ATPase or (ii) cells release some organic acids that acidify the medium. At high concentration (100 ppm), chitosan caused abrupt cell death, following with extracellular protein release. At low concentration (5 and 10 ppm), there was no change of cell mortality and extracellular protein caused by chitosan. For acetic acid (5 and 10 ppm), there was no effect on cell mortality and protein release, except after 144 hours of exposure time. There, the mortality of cells added with acetic acid was significantly lower than that of control cells. Therefore, low concentration of acetic acid is favorable to cells at old age, or under starvation condition. For silicon uptake, chitosan at high concentration reduced more extracellular silicon than acetic acid after 48 and 96 hours while at low concentration did not. This may suggest its beneficial role on silicon uptake. Interestingly, extracellular silicon of control cells trended to reduce after 144 hours, indicating the increase in silicon uptake under nutrient limitation and/or cell aging. This implies silicon as plant beneficial element.

6.2 Long-term response to chitosan and acetic acid with and without silicon supplementation

When cultivating *C. rubrum* cell suspension in long-term with and without silicon supplementation, extracellular pH reduced to pH 4.0 after 12 days and maintained at this pH value throughout 60 days, regardless of silicon content in culture medium. There was no effect of acetic acid and chitosan at this exposure time. However, acetic acid (10 ppm)-induced extracellular acidification was visible after 48 days whereas chitosan-induced extracellular alkalinization was observed after 24 days under high silicon condition. Under low silicon condition, there was no effect of chitosan and acetic acid on changes of extracellular pH throughout 60 days of cultivation. Besides extracellular pH, high medium silicon seems to increase cell mortality, when compared to low silicon condition. In addition, beneficial effect of acetic acid on cell viability was observed when medium silicon was high. Therefore, these findings suggest an involvement of silicon with acetic acid and chitosan cellular responses, such as proton transport and cell viability. For silicon uptake, chitosan and acetic acid had no effect also in long-term treatment. Extracellular silicon was decreased about 70% while intracellular silicon was increased 30% similarly in all treatments. During cultivation with silicon supplementation for 60 days, *C. rubrum* cells possessed intracellular silicon about 0.15-0.22 mg g^{-1}FW. Therefore, *C. rubrum* cells increase silicon uptake when soluble silicon is highly available, not by an influence of chitosan, at least under investigated condition. The finding of increased silicon uptake in *C. rubrum* cells supports beneficial role of silicon even in a silicon non-accumulating species.

REFERENCES

Abdelbasset, E., Lorne, R., Ismali, E. and Fouad, D. 2010. Chitosan in plant protection. **Marine Drugs** 8: 968-987.

Abe, K. and Watanabe, Y. 1992. Determination of silicate in seawater by inductively coupled plasma atomic emission spectrometry. **Journal of Oceanography** 48: 283-292.

Akamatsu, A., Uno, K., Kato, M., Wong, H.L., Shimamoto, K. and Kawano, Y. 2015. New insights into the dimerization of small GTPase Rac/ROP guanine nucleotide exchange factors in rice. **Plant Signaling & Behavior** 10: 1-7.

Amborabé, B., Bonmort, J., Fleurat, L. and Roblin, G. 2008. Early events induced by chitosan on plant cells. **Journal of Experimental Botany** 59: 2317-2324.

Ames, B. 1966. Assays of inorganic phosphates, total phosphate and phosphatases **Methods in Enzymology** 8: 115-118.

Austin, P.R., Brine, C.J., Castle, J.E. and Zikakis, J.P. 1981. Chitin: new facets of research. **Science** 212: 749–753.

Baker, C. and Mock, N. 1994. An improved method for monitoring cell death in cell suspension and leaf disc assays using Evan's blue. **Plant Cell, Tissue and Organ Culture** 39: 7–12.

Bell, A., Hubbard, J., Liu, L., Davis, R. and Subbarao, K. 1998. Effects of chitin and chitosan on the incidence and severity of *Fusarium* yellows in celery. **Plant Diseases** 82: 322–328.

Ben-Shalom, N., Ardi, R., Pinto, R., Aki, C. and Fallik, E. 2003. Controlling gray mould caused by *Botrytis cinerea* in cucumber plants by means of chitosan. **Crop Protection** 22: 285–290.

Benhamou, N. 1992. Ultra structural detection of β-1,3-glucans in tobacco root tissues infected by *Phytophthoraparasitica* var. *nicotianae* using a gold-complexed tobacco β-1,3-glucanase. **Physiology and Molecular Plant Pathology** 41: 351-357.

Bentrup, F., Gogarten, B., Hoffmann, B., Gogarten, J. and Baumann, C. 1986. ATP-dependent acidification and tonoplast hyperpolarization in isolated vacuoles from green suspension cells of *Chenopodium rubrum*. **Natural Academic Science USA** 83: 2431–2433.

Berlin, J., Sieg, S., Strack, D., Bokern, M. and Harms, H. 1986. Production of betalains by suspension cultures of *Chenopodium rubrum*. **Plant Cell, Tissue and Organ Culture** 5: 163–174.

Bille, J., Weiser, T. and Bentrup, F.W. 1992. The lysolipid sphingosine modulates pyrophosphatase activity in tonoplast vesicles and isolated vacuoles from a heterotrophic cell suspension culture of *Chenopodium rubrum*. **Plant Physiology** 84: 250–254.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. **Analytical Biochemistry** 72: 248-254.

Chaidee, A., Foissner, I. and Pfeiffer, W. 2008. Cell-specific association of heat shock-induced proton flux with actin ring formation in *Chenopodium* cells: comparison of auto- and heterotroph cultures. **Protoplasma** 234: 33–50.

Chaidee, A. and Pfeiffer, W. 2006. Parameters for cellular viability and membrane function in *Chenopodium* cells show a specific response of extracellular pH to heat shock with extreme Q_{10}. **Plant Biology** 8: 42–51.

Chen, L., Hamada, S., Fujiwara, M., Zhu, T., Thao, N.P., Wong, H.L., Krishna, P., Ueda, T., Kaku, H. and Shibuya, N. 2010. The Hop/Sti1-Hsp90 chaperone complex facilitates the maturation and transport of a PAMP receptor in rice innate immunity. **Cell Host Microbe** 7: 185–196.

Currie, H. and Perry, C. 2007. Silica in plants: Biological, biochemical and chemical studies. **Annals of Botany** 100: 1383-1389.

Dai, W.-M., Zhang, K., Duan, B., Sun, C., Zheng, K., Cai, R. and Zhuang, J. 2005. Rapid determination of silicon content in rice. **Rice Science** 12: 145-147.

Darvill, A.G. and Albersheim, P. 1984. Phytoalexins and their elicitors: a defense against microbial infection of plants. **Annual Review of Plant Physiology** 35: 243–275.

Doares, S.H., Syrovets, T., Weiler, E.W. and Ryan, C.A. 1995. Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. **Proceedings of the National Academy of Sciences of the United States of America** 92: 4095–4098.

Ebel, J. and Cosio, E.G. 1994. Elicitors of plant defense responses. **International Review of Cytology** 148: 1–36.

Eikemo, H., Stensvond, A. and Tronsmo, A. 2003. Induced resistance as a possible means to control disease of strawberry caused by *Phytophthora* spp. **Plant Diseases** 87: 345–350.

Elliott, C. and Snyder, G. 1991. Autoclave-induced digestion for the colorimetric determination of silicon in rice. **Journal of Agricultural and Food Chemistry** 39: 1118-1119.

Epstein, E. 1994. The anomaly of silicon in plant biology. **Proceedings of the National Academy of Sciences of the United States of America** 91: 11-17.

Epstein, E. 1999. Silicon. **Annual Review of Plant Physiology and Plant Molecular Biology** 50: 641-664.

Felix, G., Baureithel, K. and Boller, T. 1998. Desensitization of the perception system for chitin fragments in tomato cells. **Plant Physiology** 117: 643–650.

Felle, H., Brummer, B., Bertl, A. and Parish, R.W. 1986. Indole-3-acetic acid and fusicoccin cause cytosolic acidification of corn coleoptile cells. **Proceedings of the National Academy of Sciences of the United States of America** 83: 8992-8995

Felle, H.H. 2001. pH: Signal and Messenger in Plant Cells. **Plant Biology** 3: 577-591.

Gohel, V., Singh, A., Vimal, M., Ashwini, P. and Chhatpar, H.S. 2006. Bioprospecting and antifungal potential of chitinolytic microorganisms. **Journal of Biotechnology** 5: 54–72.

Gooday, G.W. 1990. The ecology of chitin degradation. **Advanced Microbe Ecology** 11: 387–419.

Govett, G. 1961. Critical factors in the colorimetric determination of silica. **Analytica Chimica Acta** 25: 69-80.

Grabe, M., Wang, H. and Oster, G. 2000. The mechanochemistry of V-ATPase proton pumps. **Biophysical Journal** 78: 2798–2813.

Grégoire, C., Rémus-Borel, W., Vivancos, J., Labbé, C., Belzile, F. and Bélanger, R.R. 2012. Discovery of a multigene family of aquaporin silicon transporters in the primitive plant *Equisetum arvense*. **The Plant Journal** 3: 115-121.

Harms, H., Dehnen, W. and Monch, W. 1977. Bezo(a)pyrene metabolites formed by plant cells. **Zeitschrift für Naturforschung** 32: 321–326.

Hayafune, M., Rita, B., Marchetti, R., Alba, S., Miyu, K., Yoshitake, D., Sakiko, A., Flavia, S., Alessia, R., Ken, T., Antonio, M., Hanae, K. and Shibuya, N. 2014. Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. **Proceedings of the National Academy of Sciences of the United States of America** 10: 404-413

He, C., Wang, L., Liu, J., Liu, X., Li, X., Ma, J., Lin, Y. and Xu, F. 2013. Evidence for 'silicon' within the cell walls of suspension-cultured rice cells. **New Phytologist** 200: 700–709.

Hildebrand, M., Dahlin, K. and Volcani, B.E. 1998. Characterization of a silicon transporter gene family in *Cylindrotheca fusiformis*: Sequences, expression analysis, and identification of homologs in other diatoms. **Molecular and General Genetics** 260: 480–486.

HÜsemann, W. and Barz, W. 1977. Photoautotrophic growth and photosynthesis in cell suspension cultures of *Chenopodium rubrum*. **Plant Physiology** 40: 70-81.

Iivonen, S. and Vapaavuori, E. 2002. Seasonal variation in nitrogen net uptake and root plasma membrane H^+-ATPase activity of Scots pine seedlings as affected by nutrient availability. **Tree Physiology** 22: 1–10.

Iler, R. 1979. **The chemistry of silica**. New York, John Wiley & Sons.

Iriti, M. and Faoro, F. 2009. Chitosan as a MAMP, searching for a PRR. **Plant Signaling and Behavior** 4: 66-68.

Iriti, M., Sironi, M., Gomarasca, S., Casazza, A., Soave, C. and Faoro, F. 2006. Cell death-mediated antiviral effect of chitosan in tobacco. **Plant Physiology and Biochemistry** 44: 893-900.

Jonak, C., Ligterink, W. and Hirt, H. 1999. MAP kinases in plant signal transduction. **Cellular and Molecular Life Sciences** 55: 204–213.

Jones, L. and Handreck, K.A. 1967. Silica in soils, plants and animals. **Advances in Agronomy** 19: 107–149.

Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E. and Shibuya, N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. **Proceedings of the National Academy of Sciences of the United States of America** 103: 11086-11091

Kauss, H. 1985. Callose biosynthesis as a Ca^{2+}-regulated process and possible relations to the induction of other metabolic changes. **Journal of Cell Science** 2: 89-103.

Kauss, H. and Jeblick, W. 1996. Influence of salicylic acid on the induction of competence for H_2O_2 elicitation: Comparison of ergosterol with other elicitors. **Plant Physiology** 111: 755–763.

Kauss, H., Jeblick, W. and Domard, A. 1989. The degrees of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*. **Planta** 178: 385-392.

Kohle, H., Young, D.H. and Kauss, H. 1984. Physiological changes in suspension-cultured soybean cells elicited by treatment with chitosan. **Plant Science Letters** 33: 221–230.

Kranewitter, W., Gehwolf, R., Nagl, M., Pfeiffer, W. and Bentrup, F. 1999. Heterogeneity of the vacuolar pyrophosphatase protein from *Chenopodium rubrum*. **Protoplasma** 209: 68-76.

Lee, S., Choi, H., Suh, S., Doo, I.S., Oh, K.Y., Choi, E.J., Taylor, A.T.S., Low, P.S. and Lee, Y. 1999. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato

and *Commelina communis*. **Plant Physiology and Biochemistry** 121: 147–152.

Liang, Y., Chen, Q., Liu, Q., Zhang, W.H. and Ding, R.X. 2003. Exogenous silicon (Si) increases antioxidant enzyme activity and reduces lipid peroxidation in roots of salt-stressed barley (*Hordeum vulgare* L.). **Journal of Plant Physiology** 160: 1157-1164.

Liang, Y., Zhang, W., Chen, Q. and Ding, R. 2005a. Effects of silicon on H^+-ATPase and H^+-PPase activity, fatty acid composition and fluidity of tonoplast vesicles from roots of salt-stressed barley (*Hordeum vulgare* L.). **Environmental and Experimental Botany** 53: 29–37.

Liang, Y.C. 1998. Effects of Si on leaf ultrastructure, chlorophyll content and photosynthetic activity in barley under salt stress. **Pedosphere** 8: 289–296.

Liang, Y.C., Si, J. and Romheld, V. 2005b. Silicon uptake and transport is an active process in *Cucumis sativus* **New Phytologist** 167: 797-804.

Limpanavech, P., Chaiyasuta, S., Vongpromek, R., Pichyangkura, R., Khunwasi, C., Chadchawan, S., Lotrakul, P., Bunjongrat, R., Chaidee, A. and Bangyeekhun, T. 2008. Chitosan effects on floral production, gene expression and anatomical changes in the *Dendrobium* orchid. **Scientia Horticulturae** 116: 65-72.

Lin, W., Hu, X., Zhang, W., Rogers, W.J. and Cai, W. 2005. Hydrogen peroxide mediates defence responses induced by chitosan of different molecular weights in rice. **Journal of Plant Physiology** 162: 937-944.

Ma, J. 2004. Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. **Soil Science and Plant Nutrition** 50: 11-18.

Ma, J., Tamai, K., Yamaji, N., Konishi, S., Katsuhara, M., Ishiguro, M., Murata, Y. and Yano, M. 2006. A silicon transporter in rice. **Nature** 440: 688-691.

Ma, J. and Yamaji, N. 2006. Silicon uptake and accumulation in higher plants. **Trends in Plant Science** 11: 392–397.

Ma, J.F., Yamaji, N. and Mitani-Ueno, N. 2011. Transport of silicon from roots to panicles in plants. **Proceedings of the Japan Academy, Series B, Physical and Biological Sciences** 87: 377–385.

McNaughton, S.J. and Tarrants, J.L. 1983. Grass leaf silicification: Natural selection for an inducible defense against herbivores. **Proceedings of the National Academy of Sciences of the United States of America** 80: 790-791.

McNaughton, S.J., Tarrants, J.L., McNaughton, M.M. and Davis, R.H. 1985. Silica as a defense against herbivory and a growth promoter in African grasses. **Ecology** 66: 528-535.

Mitani, N., Chiba, Y., Yamaji, N. and Ma, J.F. 2009. Identification and characterization of maize and barley Lsi2-like silicon efflux transporters reveals a distinct silicon uptake system from that in rice. **The Plant Cell** 21: 2133–2142.

Mitani, N. and Ma, J. 2005. Uptake system of silicon in different plant species. **Journal of Experimental Botany** 56: 1255-1261.

Mitani, N., Yamaji, N., Ago, Y., Iwasaki, K. and Ma, J.F. 2011. Isolation and functional characterization of an influx silicon transporter in two pumpkin cultivars contrasting in silicon accumulation. **The Plant Journal** 66: 231–240.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Physiologia Plantarum** 15: 473-497.

Neumann, D. and zur Nieden, U. 2001. Silicon and heavy metal tolerance of higher plants. **Phytochemistry** 56: 685-692.

Ohta, K., Morishita, S., Suda, K., Kobayashi, N. and Hosoki, T. 2004. Effects of chitosan soil mixture treatment in the seedling stage on the growth and flowering of several ornamental plants. **Journal of the Japanese Society for Horticultural Science** 73: 66-68.

Ohta, K., Tanguchi, A., Konishi, N. and Hosoki, T. 1999. Chitosan treatment affects plant growth and flower quality in *Eustuma grandiflorum*. **Horticultural Science** 34: 233-234.

Perry, C.C. and Keeling-Tucker, T. 2000. Biosilicification: the role of the organic matrix in structure control. **Journal of Biological Inorganic Chemistry** 5: 537-550.

Petutschnig, E.K., Alexandra, M.E., Liliya, S., Ulrike, L. and Volker, L. 2010. The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. **Journal of Biological Chemistry** 285: 28902-28911.

Pfeiffer, W. and Hager, A. 1993. A Ca^{2+}-ATPase and a Mg^{2+}/H^{+}- antiporter are present on tonoplast membranes from roots of *Zea Mays* **Planta** 191: 377-385.

Rabea, E., Badawy, M., Stevens, C., Smagghe, G. and Steurbaut, W. 2003. Chitosan as antimicrobial agent: Applications and mode of action. **Biomacromolecules** 4: 1457-1465.

Rakwal, R., Tamogani, S., Agrawal, G.K. and Iwahashi, H. 2002. Octadecanoid signalling component 'burst' in rice (*Oryza sativa* L.) seedlings leaves upon wounding by cut and treatment with fungal elicitor chitosan. **Biochemical and Biophysical Research Communications** 295: 1041–1045.

Ramírez, M.Á., Rodriguez, A.T., Alfonso, L. and Peniche, C. 2010. Chitin and its derivatives as biopolymers with potential agricultural applications. **Biotechnology and Applied Biochemistry** 27: 270–276.

Reddy, M.V., Arul, J., Angers, P. and Couture, L. 1999. Chitosan treatment of wheat seeds induces resistance to *Fusarium graminearun* and improves seed quality. **Journal of Agricultural and Food Chemistry** 47: 1208–1216.

Rossard, S., Luini, E., Pérault, J.M., Bonmort, J. and Roblin, G. 2006. Early changes in membrane permeability, production of oxidative burst and modification of PAL activity induced by ergosterol in cotyledons of *Mimosa pudica*. **Journal of Experimental Botany** 57: 1245–1252.

Sánchez-Vallet, A., Jeroen, R.M. and Thomma, B. 2014. The battle for chitin recognition in plant-microbe interactions. **FEMS Microbiology Reviews** 10: 1-13.

Sangster, A.G., Hodson, M.J. and Tubb, H.J. 2001. Silicon deposition in higher plants. **Studies in Plant Science** 8: 85-113.

Schaller, A. and Oecking, C. 1999. Modulation of plasma membrane H^{+}-ATPase activity differentially activates wound and pathogen defense responses in tomato plants. **The Plant Cell** 11: 263–272.

Shimoyama, S. 1958. Effect of calcium silicate application to rice plants on the alleviation of lodging and damage from strong gales: Studies in the improvement of ultimate yields of crops by the application of silicate materials. **Japanese Association for the Advancement of Science** 2: 57-99.

Taiz, L. and Zeiger, E. 2016. **Plant Physiology**. Fifth ed. USA, Sinauer Associates.

Tamai, K. and Ma, J. 2003. Characterization of silicon uptake by rice roots. **New Phytologist** 158: 431-436.

Tsigos, I., Martinou, A., Kafetzopoulos, D. and Bouriotis, V. 2000. Chitin deacetylases: New, versatile tools in biotechnology. **Trends in Biotechnology** 18: 305–312.

Van Bockhaven, J., De Vleesschauwer, D. and Höfte, M. 2013. Towards establishing broad-spectrum disease resistance in plants: Silicon leads the way. **Journal of Experimental Botany** 64 1281-1293.

Voleti, S.R., Babu, S.M. and Ranganathan, S. 2009. Biocompatible small molecules that enhance silica solubilization under ambient conditions: Chemical profile of such complexes, possible mechanism for enhancement, and their effect on the growth and protection from pests in the rice plant (*Oryza sativa* L.). **Phosphorus Sulfur and Silicon and the Related Elements Journal** 184: 1975–1990.

Wan, J., Zhang, X.C., Neece, D., Ramonell, K.M., Clough, S., Kim, S.-Y., Stacey, M.G. and Stacey, G. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. **The Plant Cell** 20: 471–481.

Wang, Y. and Wu, W.H. 2013. Potassium transport and signaling in higher plants. **Annual Review of Plant Biology** 64: 451–476.

Weigel, B. 1993. Zellsuspensionskulturen aus explantaten von *Chenopodium rubrum* L.: Etablierung und charakterisierung mit speziellen untersuchungen zu cytogenetischem status, membranproteinen und transportaktivität, Vol. Ph.D. Thesis. Gießen.

Wendt dos Santos, A.L., El gueddari, N.E., Trombotto, S. and Moerschbacher, B.M. 2008. Partially acetylated chitosan oligo- and polymers induce an oxidative burst in suspension cultured cells of the gymnosperm *Araucaria angustifolia*. **Biomacromolecules** 9: 3411-3415.

Wongchai, C., Chaidee, A. and Pfeiffer, W. 2013. Enhancement and quantification of repellent activity in *Chenopodium* cells. **Arthropod-Plant Interactions** 7: 69–82.

Wongchai, C., Chaidee, A. and Pfeiffer, W. 2012b. Multivariate analyses of salt stress and metabolite sensing in auto- and heterotroph *Chenopodium* cell suspensions. **Plant Biology** 14: 129–141.

Yamaguchi, K., Yamada, K. and Kawasaki, T. 2015. Receptor-like cytoplasmic kinases are pivotal components in pattern recognition receptor-mediated signaling in plant immunity. **Plant Signaling & Behavior** 10: 1-4.

Yamaji, N., Chiba, Y., Mitani-Ueno, N. and Ma, J.F. 2012. Functional characterization of a silicon transporter gene implicated in silicon distribution in barley. **Plant Physiology** 160: 1491–1497

Yang, F., Hu, J., Li, J., Wu, X. and Qian, Y. 2009. Chitosan enhances leaf membrane stability and antioxidant enzyme activities in apple seedlings under drought stress. **Plant Growth Regulation** 58: 131–136.

Young, D.Y., Kohle, H. and Kauss, H. 1982. Effect of chitosan on membrane permeability of suspension-cultured *Glycine max* and *Phaseolus vulgaris* cells. **Plant Physiology** 70: 1449–1454.

Zuo, Z., Zhu, Y., Bai, Y. and Wang, Y. 2012. Acetic acid-induced programmed cell death and release of volatile organic compounds in *Chlamydomonas reinhardtii*. **Plant Physiology and Biochemistry** 51: 175-184.

Zuppini, A., Baldan, B., Millioni, R., Favaron, F., Navazio, L. and Mariani, P. 2003. Chitosan induces Ca^{2+}-mediated programmed cell death in soybean cells. **New Phytologist** 161 557-568.

APPENDIX

Table 1. Heterotroph - *Chenopodium rubrum* medium (Murashige & Skoog, 1962)

Stock solution	Chemicals	Company	g L^{-1}
Macronutrient (10X)	NH_4NO_3	Fluka, Switzerland	16.50
	KNO_3	Merck, Germany	19.00
	$MgSO_4•7H_2O$	BDH, England	3.70
	$CaCl_2•2H_2O$	BDH, England	4.40
	KH_2PO_4	Fluka, Switzerland	1.70
Micronutrient (100X)	H_3BO_3	Merck, Germany	0.62
	$MnSO_4•H_2O$	BDH, England	1.69
	$ZnSO_4•7H_2O$	Merck, Germany	0.86
	KI	Merck, Germany	0.083
	$Na_2MoO_4•2H_2O$	Merck, Germany	0.025
	$CoCl_2•6H_2O$	Carlo Erba, France	0.0025
	$CuSO_4•5H_2O$	Merck, Germany	0.0025
Vitamins & Amino acids (100X)	Glycine	Cleveland, U.S.A.	0.20
	Nicotinic acid	Fluka, Switzerland	0.05
	Thiamine HCL	Sigma, U.S.A.	0.01
	Pyridoxine HCL	Fluka, Switzerland	0.05
Iron – EDTA (100X)	$Na_2•EDTA•2H_2O$	Merck, Germany	3.73
	$FeSO_4•7H_2O$	Carlo Erba, France	2.78

1. A standard curve of known Evan's blue concentration

Linear regression equation for the determination of the Evan's blue dye was acquired as: y = 84.511x, R^2 = 0.9989 Where, y is OD value and x is Evan's blue dye in the solution used for setting up standard curve.

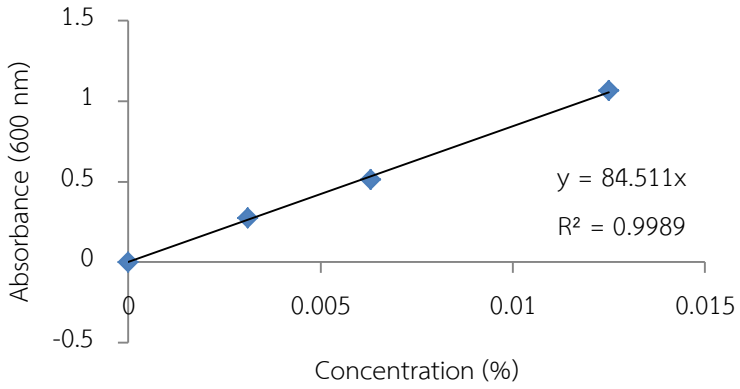

Figure 1. Standard curve of determination of Evan's blue concentration in *C. rubrum* cells.

2. A standard curve of known silicon concentration

Linear regression equation for the determination of the silicon content was acquired as: y = 0.0082x, R^2 = 0.9998 Where, y is OD value and x is silicon content in the solution used for setting up standard curve.

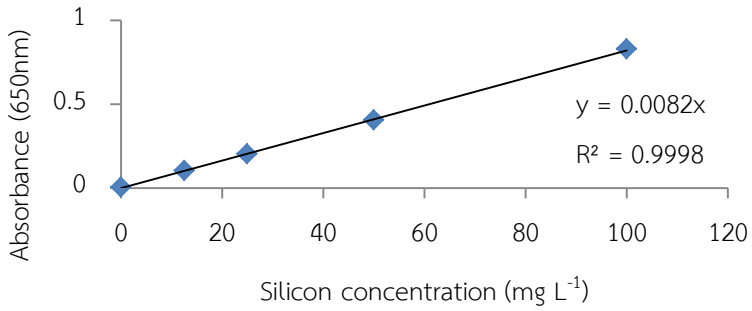

Figure 2. Standard curve of determination of silicon concentration in *C. rubrum* cells.

VITA

Mr. Arrat Sontana was born on January 22, 1989 in Khon Kaen province, Thailand. After finishing high school from Sarakhampittayakhom school in 2007, he enrolled for Bachelor's degree in Science (Biology) at the Department of Biology, Faculty of Science, Khon Kaen University and graduated in 2010. He continued his study in Master degree in Science (Botany) at the Department of Botany, Faculty of Science, Chulalongkorn University.

AWARD

ORAL PRESENTATION

The honorable mention award in the 10th Botanical Conference of Thailand in topic "Effects of chitosan on extracellular pH, vitality and silicon uptake in heterotroph cell suspension of Chenopodium rubrum L."

PUBLICATION

Sontana, A. and Chaidee, A. 2016. Effects of chitosan on extracellular pH, vitality and silicon uptake in heterotroph cell suspension of Chenopodium rubrum L. Proceedings in: The 10th Botanical Conference of Thailand.

Sontana, A., Wangsomnuk, P.P., Pongdontri, P., Wangsomnuk, P., Srivong, T., Ruttawat, B., Jogloy, S. and Patanothai, A. 2016. Protein pattern analysis from germinating Helianthus tuberosus L. seeds. Proceedings in: 17th KKU's Agricultural conference.