EFFICIENCY OF ELICITORS ON INDUCED RESISTANCE AGAINST CASSAVA ROOT ROT DISEASE

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A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy in Crop Science

Suranaree University of Technology

Academic Year 2020

ประสิทธิภาพของอิลิซิเตอร์ในการชักนำความต้านทานต่อโรคหัวเน่ามันสำปะหลัง

นายชานนทร์ แสงจันทร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาพืชศาสตร์
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2563

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ชานนทร์ แสงจันทร์ : ประสิทธิภาพของอิลิซิเตอร์ในการชักนำความต้านทานต่อโรคหัวเน่ามันสำปะหลัง (EFFICIENCY OF ELICITORS ON INDUCED RESISTANCE AGAINST CASSAVA ROOT ROT DISEASE) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.ณัฐธิญา เบือนสันเทียะ, 131 หน้า.

โรคหัวเน่ามันสำปะหลัง (CRRD) เกิดจากเชื้อราสาเหตุโรคหนึ่งหรือหลายสกุล เชื้อราฟูซาเรียมถือเป็นหนึ่งสาเหตุสำคัญที่ทำให้เกิดโรคหัวเน่ามันสำปะหลังในประเทศไทย ซึ่งลักษณะของเชื้อที่แสดงอาการรุนแรงสามารถทำให้เกิดอาการได้หลายแบบ เช่น เน่าแห้ง เน่าเละ และเน่าดำ โดยเฉพาะอย่างยิ่งในฤดูฝน อาการเหล่านี้สามารถทำลายผลผลิตมันสำปะหลังได้มากถึง 80-100% ดังนั้น วัตถุประสงค์ของการศึกษาในครั้งนี้คือ (1) เพื่อประเมินประสิทธิภาพของสูตรสำเร็จกรดซาลิไซลิกในการกระตุ้นความต้านทานต่อโรคหัวเน่าฟูซาเรียมในมันสำปะหลัง และ (2) เพื่อศึกษากลไกของการกระตุ้นความต้านทานต่อโรคหัวเน่ามันสำปะหลังภายหลังการฉีดพ่นด้วยตัวกระตุ้นสูตรสำเร็จ Zacha (สารออกฤทธิ์คือกรดซาลิไซลิก 6%) ที่ความเข้มข้น 500 ppm และสูตร JN2-007 (ชีวภัณฑ์เชื้อแบคทีเรียบาซิลลัส ซับทิลิส) สามารถลดการเจริญเติบโตของเส้นใยเชื้อรา *Fusarium solani* ได้ นอกจากนี้การสะสมของไฮโดรเจนเปอร์ออกไซด์ และกิจกรรมของเอนไซม์บางชนิดที่เกี่ยวข้องกับกลไกการป้องกันตัวเองของพืช (เปอร์ออกซิเดส, โพลีฟีนอลออกซิเดส และคาตาเลส) พบว่าการกระตุ้นด้วย Zacha ที่ความเข้มข้น 500 ppm แสดงให้เห็นถึงการแสดงออกที่เพิ่มขึ้นในพืชมันสำปะหลัง 24 ชั่วโมงหลังการปลูกเชื้อสาเหตุโรคเมื่อเทียบกับพืชที่ไม่ติดเชื้อ มันสำปะหลังที่ถูกฉีดพ่นด้วยสารกระตุ้น Zacha11 และปลูกเชื้อด้วยสารแขวนลอยเชื้อราฟูซาเรียมมีกิจกรรมของ β-1,3-glucanase เพิ่มขึ้นเล็กน้อยที่ 24 ชั่วโมงหลังการปลูกเชื้อสาเหตุโรค (15.62 μg min^{-1} mg^{-1} protein) เมื่อเทียบกับกรรมวิธีควบคุม สำหรับกิจกรรมของไคติเนสก็มีการเพิ่มขึ้นเช่นเดียวกับกิจกรรม β-1,3-glucanase ในทำนองเดียวกันการสะสมของกรดซาลิไซลิกจะเพิ่มขึ้นใน 24 ชั่วโมงหลังการปลูกเชื้อสาเหตุโรค คือ 69.95 μg g^{-1} fresh weight ยิ่งไปกว่านั้นมันสำปะหลังที่ผ่านการกระตุ้นด้วย Zacha11 แสดงให้เห็นถึงการเปลี่ยนแปลงขององค์ประกอบทางชีวเคมี เช่น ลิกนินและเพคตินในผนังเซลล์มันสำปะหลัง จากนั้นต้นมันสำปะหลังที่ได้รับการกระตุ้นด้วย Zacha11 ให้ความสูงของลำต้น ความยาวราก และจำนวนรากสูงสุด คือ 11.67 เซนติเมตร, 18.91 เซนติเมตร และ 49.50 ตามลำดับ เมื่อเปรียบเทียบกับกรรมวิธีการควบคุม นอกจากนี้ยังสามารถลดโรคหัวเน่าฟูซาเรียมได้อีกด้วย จากการศึกษาผลของเชื้อสาเหตุโรคที่เกิดขึ้นตามธรรมชาติในมันสำปะหลัง (พันธุ์ระยอง 72 และพันธุ์ CRM-89) ที่ถูกฉีดพ่นนั้น พบว่าสารกระตุ้นสามารถยับยั้งโรคหัวเน่าได้ทั้งสองสายพันธุ์ รวมถึงโรคใบไหม้จากเชื้อแบคทีเรีย นอกจากนี้ยังช่วยลดความรุนแรงของโรค

แอนแทรคโนสและจุดใบสีน้ำตาลที่พบในสภาพไร่ได้ สุดท้ายการกระตุ้นด้วย Zacha11 ยังมีประสิทธิภาพสูงสุดในการส่งเสริมการเจริญเติบโตและเพิ่มผลผลิตมันสำปะหลังในทั้งสองพื้นที่ ดังนั้น จึงเป็นไปได้ว่าอิลิซิเตอร์สามารถใช้ลดความรุนแรงของโรคหัวเน่าฟูซาเรียมได้ โดยการกระตุ้นสัญญาณที่เป็นสื่อกลางในการตอบสนองต่อการป้องกันตัวเองในมันสำปะหลัง

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา ชาคริยา แสงจันทร์

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา

CHANON SAENGCHAN : EFFICIENCY OF ELICITORS ON INDUCED RESISTANCE AGAINST CASSAVA ROOT ROT DISEASE. THESIS ADVISOR : ASST. PROF. NATTHIYA BUENSANTEAI, Ph.D., 131 PP.

SALICYLIC ACID/ELICITORS/INDUCED RESISTANCE/FUSARIUM ROOT ROT DISEASE/CASSAVA

Cassava root rot disease (CRRD) is caused by one or several fungal genera. Fusarium species are an important component of the fungal complex that causes root rot disease in Thailand. Severe infection can cause a range of symptoms from dry rot, soft rot and black rot, especially during the rainy season. These symptoms can potentially destroy as much as 80-100% of cassava production. Therefore, the aims of this study were (1) to evaluate the efficacy of salicylic acid formulation for inducing resistance against Fusarium root rot in cassava plants and (2) to study the mechanism of induced resistance in cassava plants against the CRRD after being treated with the elicitors. Zacha elicitor formulations (the active ingredient was 6% salicylic acid) at a concentration of 500 ppm and JN2-007 (*Bacillus subtilis* bioproduct elicitor) could reduce mycelial growth of *Fusarium solani*. Also, the accumulation of hydrogen peroxide and activity of some enzymes related to plant defense mechanisms (peroxidase (PO), polyphenol oxidase (PPO) and catalase (CAT)) revealed that the 500 ppm Zacha treatment showed an increased regulation in cassava plants at 24 hours after inoculation (HAI) compared to that of the noninfected plants. Cassavas sprayed with Zacha11 elicitor and inoculated with Fusarium suspension had a slight increase of β-1,3-glucanase activity at 24 HAI (15.62 μg min^{-1} mg^{-1} protein) compared to that of the negative control. For chitinase activity, the increase was similar to that of the

β-1,3-glucanase. Likewise, the accumulations of salicylic acid increased at 24 hours after inoculation of 69.95 μg g^{-1} fresh weight. Moreover, cassava treated with Zacha11 showed the biochemical components of lignin and pectin in the cassava cell wall. Then, the cassava plants treated with Zacha11 gave the maximum stem height, root length and number of roots by 11.67 cm, 18.91 cm, and 49.50, respectively compared to the negative control. In addition, cassava plant treatment can reduce Fusarium root rot disease. The results of naturally occurring diseases on treated cassava varieties (Rayong 72 and CRM-89) revealed that elicitors could suppress root rot disease in both cultivars as well as bacterial leaf blight. They also reduced the severity of anthracnose and brown leaf spot found in the field. Furthermore, Zacha11 showed the highest efficiency in enhancing cassava growth-promotion and increasing the yield of cassava in both locations. Thus, it is possible that elicitors could be used to reduce Fusarium root rot disease severity by inducing signals mediating defense responses in cassava.

School of Crop Production Technology Student's Signature Chanon Saengchan

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ACKNOWLEDGEMENTS

This research could not have been accomplished without the backing of the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission. The partial funding supported by the Research and Researcher for Industries (RRi), the Thailand Research Funds for Ph.D. program number PHD58I0070 and Bioactive Agro-Industry Co., Ltd.

I would like to express my heartfelt gratitude to my advisor, Asst. Prof. Dr. Natthiya Buensanteai who critically reviewed the work and for her tireless guidance, encouragement, and assistance in making this successful dissertation. My co-advisor, Dr. Piyaporn Phansak, for providing me his profound knowledge and experiences shared, support, understanding, trust and appreciation.

Special thanks to Dr. Sopone Wongkaew and Dr. Toan Le Thanh, for their kindness, instructions, and assistance. My sincere thanks go to all my fellow members of Plant Pathology Laboratory for their useful input and kind help. I would also like to thank you very much for Dr. Kanjana Thumanu, Dr. Supatcharee Siriwong, for managing beam times, instruments, consultation and facilities on FTIR spectroscopy analysis at The Synchrotron Light Research Institute (Public Organization), Thailand.

Finally, I take this opportunity to express my heartfelt thanks and gratitude to all those people who have helped immensely in the successful completion of my thesis.

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
ABA	=	Abscisic acid
AA	=	Ascorbic acid
ASM	=	Acibenzolar-S- methyl
apx	=	Ascorbate peroxidase
BA		Benzoic acid
BLS	=	Brown leaf spot
CAD	=	Cassava anthracnose disease
CAT	=	Catalase
CFU mL^{-1}	=	Colony forming units per milliliter
CBB	=	Cassava bacterial blight
CLA	=	Carnation leaf agar
cm	=	Centimeter
CRD	=	Completely randomized design
CRRD	=	Cassava root rot disease
CV	=	Coefficient of Variation
DAI	=	Days after inoculation
DAP	=	Days after planting
DRC	=	Democratic Republic of Congo
DMRT	=	Duncan's Multiple Range Test

LIST OF ABBREVIATIONS (Continued)

DAPFS	=	Days after putting fungal slices
DS	=	Disease severity
ET	=	Ethylene
EU	=	European Union
H	=	Hour
HAI	=	Hours after inoculation
HR	=	Hypersensitive response
H_2O_2	=	Hydrogen peroxide
ISR	=	Induced systemic resistance
IAA	=	Indole-3-acetic acid
JA	=	Jasmonic acid
KI	=	Potassium iodide
mg L^{-1}	=	Milligram per Liter
µL	=	Microliter (s)
µg	=	Microgram
g	=	Gram
µm	=	Micrometer (s)
min	=	Minute (s)
mL	=	Milliliter (s)
mm	=	Millimeter
mM	=	Millimolar

LIST OF ABBREVIATIONS (Continued)

MAP	=	Months after planting
nm	=	Nanometer
O_2^-	=	Superoxide radical
OCT	=	Optical Coherence Tomography
OA	=	Oxalic acid
OH	=	Hydroxyl radical
pal	=	Phenylalanine ammonia lyase
PCA	=	Principal Component Analysis
PDP	=	Potassium dihydrogen phosphate
PGPR	=	Plant growth promoting rhizobacteria
PR proteins	=	Pathogenesis-related proteins
PDA	=	Potato dextrose agar
PDB	=	Potato dextrose broth
ppo	=	Polyphenol oxidase
ppm	=	Part Per Million
PO or POX	=	Peroxidase
ROS	=	Reactive oxygen species
RCBD	=	Randomized complete block design
rpm	=	Round per minute
SA	=	Salicylic acid
SAR	=	Systemic acquired resistance

LIST OF ABBREVIATIONS (Continued)

SE	=	Standard error
SR-FTIR	=	Synchrotron based - Fourier transform infrared microspectroscopy
t/ha	=	Ton per Hectare
TCA	=	Trichloroacetic acid
Xam	=	*Xanthomonas axonopodis* pv. *manihotis*

CHAPTER I

INTRODUCTION

1.1 Background of the selected topic

Cassava (*Manihot esculenta* Crantz) has been an important economic crop in Thailand and has been used mainly as animal feed, used in the various industrial raw materials, production of ethanol (Charaensatapon et al., 2014; Duchanee, 2015; FAO, 2002; Sangpueak et al., 2018) and as a food crop in Southeast-Asia and Africa (Ubalua and Oti, 2007). Because of initiatives to derive some or all of their food raw material from cassava and food demand on the substantial way, Thailand has operation started to substitute modified starch from imported corn with cassava starch nationally produced. Therefore, the planting acreage has been increased because of responding to consumer demand and the increasing consumption. In Thailand, the growing area of cassava has approximately 1.29 million hectares and changing cultivation practices and tends to increase which affect disease severity and pathogen diversity in cassava (Charaensatapon et al., 2014; Duchanee, 2015; Sangpueak et al., 2018). As a consequence, cassava yield has been reduced for approximately 20-100% by the diseases (Charaensatapon et al., 2014; Duchanee, 2015). Among them, cassava root rot disease (CRRD) is caused by the fungal complex can reduce as much as 80% of annual yield in Thailand (Charaensatapon et al., 2014; Machado et al., 2014b; Duchanee, 2015). A virulent infection of CRRD symptoms can range from dry rot, soft rot and black rot (Machado et al., 2012), especially during the rainy seasons

(Machado et al., 2014b; Duchanee, 2015). Since 2011, with the widespread cultivation of fast-growing, higher-yield, and disease susceptible varieties CMR89, there have been many outbreaks of the CRRD in the Northeast and East of Thailand such as Nakhon Ratchasima, Chaiyapoom, Burirum, Chonburi and Rayong (Charaensatapon et al., 2014; Duchanee; 2015). In Thailand, the CRRD has been reported to cause heavy yield loss worldwide during the past ten years (Hillocks and Wydra, 2002; Banito et al., 2010; Duchanee; 2015). In Thailand, Duchanee (2015) reported that CRRD was caused by at least 5 pathogens including *Lasiodiplodia theobromae*, *Neoscytalidium hyalinum*, *Sclerotium rolfsii*, *Fusarium* spp., and *Phytophthora* sp., with various degrees of losses (Bua and Okello, 2011). The CRRD is caused by soil-borne pathogens that also have been reported to be found in Africa and South America (annual yield loss of cassava up to more than 80%) (Lozano et al., 1981; Onyeka et al., 2005; Salami and Akintokun, 2008; Banito et al., 2010).

In current years, conventional practices for cassava disease control in Thailand have depended on cultural approaches (Duchanee; 2015; Buensanteai et al., 2009). Recently, an alternative disease management by an application of resistance elicitors which induce plant innate immunity (Onyeka et al., 2005; Buensanteai et al., 2010) and defense mechanism has been tried for the controlling of CRRD (Durrant and Dong, 2004). The inducers such as salicylic acid (SA) and *Bacillus* sp. have been widely managed in research experiment (Hukkanen et al., 2007; War et al., 2011), enhance growth in various plants and commercially to control plant pathogens (except cassava plants) (Cartea et al., 2011). SA and Bacillus have shown their efficacy in the induction of disease resistance in several economic crops (Eschen-Lippold et al., 2010) (cassava, chili, grapevine, potato, rice, pepper, soybean, vegetable soybean, cucumber, and tomato) (Park et al., 2005; Buensanteai et al,

2009; Sharma and Sohal, 2010; Prakongkha et al., 2013a; 2013b; Gharbi et al. 2017; Le Thanh et al. 2017; Thumanu et al., 2017). Zainuddin et al. (2017) reported that reactive oxygen species product can be induced by SA in defense mechanism of cassava plants against physiological disorders. In addition, B. subtilis can induce resistance against root rot disease of yam. Some examples of such pathogens include *Aspergillus* sp., *Botryodiplodia* sp., *Penicillium* sp. (Okigbo, 2005). *Bacillus subtilis* strain CaSUT007 (the plant growth promoting rhizobacterium; PGPR), has been reported to induce disease resistance and enhance growth in many crops (Buensanteai et al., 2009). Various plant growth enhancement by CaSUT007 is mediated in part by biochemical molecules and the excretion of phytohormones (Thumanu et al., 2015). Recently, there have been reports that biotic elicitors could effectively control plant diseases when applied together with abiotic elicitors (Walters et al., 2005; Zehra et al., 2017).

Although there have been proving of effectiveness on using many elicitors of resistance for preventing and managing plant diseases, none of them have been tested on the CRRD. Therefore, this research was conducted to evaluate the efficiency of resistance elicitors as SA and *Bacillus* sp. for the controlling of CRRD. Their mechanism in inducing CRRD resistance in cassava plants was also characterized.

1.2 Research objectives of the study

1.2.1 To evaluate the efficacy of salicylic acid formulation for induced resistance against *Fusarium* root rot in cassava plants (Chapter III)

Investigate the effect of the formulated salicylic acid and a *bacillus* bioproduct for inducing resistance on H_2O_2 content, peroxidase, polyphenol oxidase, and catalase activities against Fusarium root rot disease in cassava.

1.2.2 To study induced resistance mechanisms in cassava against the CRRD after being treated with the elicitors (Chapter IV and V)

First, effect of SA prototype formulations inhibited the growth of *Fusarium solani* was surveyed. Then, monitoring of biochemical changes associated with plant innate immunity accumulation of chitinase, ß-1,3-glucanase activity and indigenous SA analysis by using a spectrophotometer and their characterization using SR-FTIR microspectroscopy.

Second, evaluate the efficacy of exogenous SA and a *bacillus* bioproduct in controlling cassava root rot disease under both greenhouse and field conditions.

1.3 Research hypothesis

1.3.1 Resistance elicitors as SA and *bacillus* bioproduct could trigger systemic resistance in cassava plants against CRRD when properly applied.

1.3.2 A resistance mechanism in cassava plants could be directly related to biochemical defence.

1.4 Scope of the study

This research studied the efficacy of exogenous SA (Zacha) and *Bacillus* prototype formulation (JN2-007 elicitors) using different application methods, rate and timing to induce resistance against root rot disease in cassava cv. Rayong 72 compare with accession number CMR89. Biochemical changes in the cassava associated with plant innate immunity were monitored using traditional methods (cassava indigenous salicylic acid, phenolic compound analysis) and FT-IR microspectroscopy after the

plants were treated with Zacha and JN2-007 elicitors and then challenge inoculated with *Fusarium* sp. The productivity of the treated cassava was also evaluated.

1.5 Expected results of the study

1.5.1 Benefits expected for academia

1.5.1.1 Understanding the efficacy of elicitor prototypes as Zacha or JN2-007 in inducing resistance in cassava plants against the CRRD.

1.5.1.2 Understanding the resistance mechanisms in cassava plants after being induced by the elicitor prototypes.

1.5.2 Benefits expected for the development of prototype and application by the public

1.5.2.1 Implementing formulation of the elicitors to be used for controlling CRRD in cassava plants.

1.5.2.2 Encouraging the use of resistance elicitors in subsistent cassava farms.

1.5.2.3 Providing an alternative method for controlling the CRRD and reducing the use of chemical fungicides.

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CHAPTER II

LITERATURE REVIEW

2.1 Origins, current status and morphological characteristics of cassava plants

2.1.1 Origin and current status of cassava

Cassava, *Manihot esculenta* Crantz, is a perennial economic crop in the family of Euphorbiaceae (spurge family) originated in South America but now grown in tropical and sub-tropical areas worldwide for the edible starchy roots (Alves, 2002; Hillocks, 2002). The cassava is a major industrial and food source in the developing world, in equatorial regions including Africa, South America, and Oceania (Veltkamp and De Bruijin, 1996; Allem et al., 2002; Henry and Hershey, 2002).

Cassava belongs to the class Dicotyledonea, family Euphorbiaceae, tribe Manihoteae, genera *Manihot* Tournefort and species *Manihot esculenta* Crantz (Alves, 2002). Archaeological evidence suggests that it was cultivated in Peru 4,000 years ago and in Mexico 2,000 years ago. It was introduced to West Africa in the 16th century, and has become a major food crop. In 2010, total global production of cassava was 228 million metric tons, harvested from 18.4 million hectares, with Nigeria, Thailand, and Brazil constituting the largest amounts (Courteau, 2012). In optimal conditions, cassava plants may yield up to 68 tons per hectare in a year, but typical yields are 10 tons/hectare. In addition, cassava is often intercropped with maize, vegetables,

legumes, cocoa, and coffee (FAO, 2015). Cassava is considered one of the most important economic crops in Thailand (Treesilvattanakul, 2016). Most of the harvested roots are processed into pellets and dry chips for export as into starch, as well as animal feed, both for export (80% of the production is exported) and use domestic (FAO, 2008; Chaisinboon and Chontanawat, 2011; Jakrawatana et al., 2015; Monineath, 2016; Newby, 2016). In addition to being both a food crop and an industrial crop, cassava has recently been considered as an energy crop, being utilized for the production of bioethanol. The cassava yields have been significantly varied with cassava cultivars and their growing locations that higher root yields can be obtained by well-managed farm practices including land preparation, preparation of planting materials, time of planting, planting method, fertilization, irrigation, intercropping and weed control (Howeler, 2000a and 2000b). However, major production problems are limited genetic diversity of the crop, soil erosion, declining soil fertility, pests and diseases. Because of the market increasing demand, cassava planting area and growing cycles have also been increased resulting in the accumulation of cassava diseases over the years. In Thailand, cassava has been found to be infected by cassava bacterial blight (CBB), brown leaf spot (BLS), anthracnose (CAD) and root rot (CRRD) (Fokunang et al., 2001; Charaensatapon et al., 2014).

2.1.2 Morphological characteristics of cassava

Cassava is grown in tropical and sub-tropical areas worldwide for the edible starchy tubers, which are a major food source in equatorial regions including South America, Oceania, Africa and the developing world (Veltkamp and De Bruijin, 1996). Also known as manioc, and tapioca, the dried root is the source of tapioca. The cassava shrub may grow 9 feet high, with leaves deeply divided into 3-7 lobes. The

shrub is often grown as an annual and propagated from stem cuttings after tubers have been harvested. The fruit is small, roughly 1/2 inch in diameter, but root tubers of cultivated varieties can be 5-10 cm in diameter and 15-30 cm long (Alves, 2002). Fresh leaves and roots may have cyanide compounds including hydrocyanic acid and cyanogenic glucoside at levels that may be toxic but properly treated, the cyanide content is negligible. Sweet cultivars contain less of these compounds than bitter cultivars although flavor is an imperfect indicator but are often preferred by farmers for their pest-repellent properties.

The cassava plant is characterized by the following parts (Figure 2.1):

Stem- The stem about 4 meters long and woody with thick bark. The old part of the stem bears evident scars of fallen first leaves. The system of stem branching is controlled by environmental and genetic factors. The branching may start at any time of plant growth, producing 3 new branches and after a certain time, these produce 3 more new branches each. The level of branching depends on the variety of cassava (CIAT, 1981; IITA, 1990).

Leaves- Cassava leaves are divided into 5 to 7 lobes and have a long petiole. The leaves have a spiral insertion on the cassava stem. Also, petiole and leaf colors depend on the genotype.

Roots- The roots are the most important organ in cassava and the main storage. The cassava root is not a tuberous stem, but a true root that cannot be used for vegetative propagation (Alves, 2002). The mature cassava storage root has four distinct tissues: central vascular xylem bundle, parenchyma, peel (cortex) and bark (periderm) (IITA, 1990). The periderm (3% of total weight) is a thin layer of cells and, as growth progresses; the outermost portions usually slough off. The peel layer, which

is comprised of sclerenchyma, cortical parenchyma and phloem, constitutes 11-20% of root weight. The parenchyma, which is the edible portion of the fresh root, comprises approximately 85% of total weight, consisting of xylem vessels radially distributed in a matrix of starch-containing cells (Wheatley and Chuzel, 1993). Shape and root size depend on environmental and cultivar conditions. Variability in size between cultivars is greater than that found in other root crops (Wheatley and Chuzel, 1993).

Fruit- The fruit is a result of cross-pollination. It is a globular capsule, trilocular, 1 to 1.5 cm in diameter with six straight longitudinal aristae (CIAT, 1981). Each locule contains a single carunculate seed (Alves, 2002).

Inflorescences- Cassava is a monoecious species with panicle inflorescences at the reproductive branching points, female flowers in the base and male flowers at the top of the inflorescence (Alves, 2002).

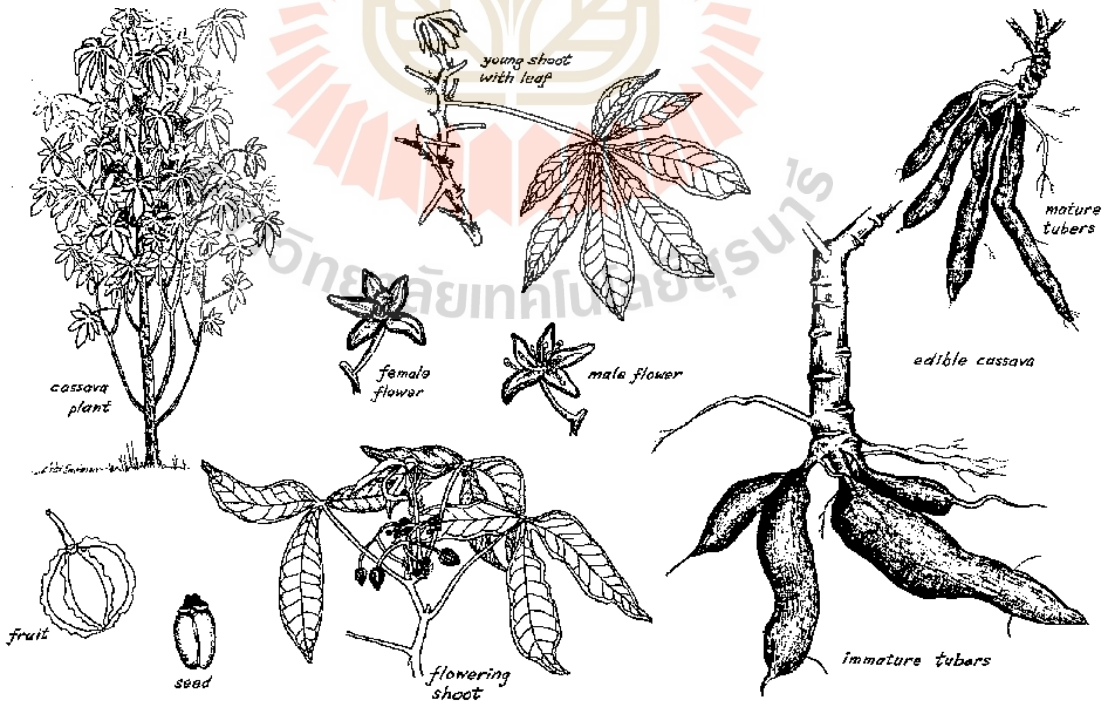

Figure 2.1 Botanical characteristic of cassava. Source: http://www.nzdl.org/cgi-bin/library?e=d-00000-00---off-0hdl--00-0----0-10-0---0---0direct-10---4-------0-0l--11-en-50---20-about---00-0-1-00-0--4----0-0-11-10-0utfZz-8-10&cl=CL1.14&d=HASH0173082659b1eafc593ba653.3.2.2&gc=1

2.2 Important diseases of cassava

Manihot esculenta is attacked by more than 34 pathogens (Banito et al., 2010), causing various degrees of losses (Msikita et al., 2005; Salami and Akintokun, 2008).

2.2.1 Cassava bacterial blight (CBB), caused by bacterial *Xanthomonas axonopodis* pv. *manihotis (Xam),* is a destructive disease in Africa and South America that affects both planting material and yield leading to seed yield reductions. The disease has a wide spectrum of symptoms including stem necrosis, die-back, angular leaf spot and blight, gum exudation, and wilt (Figure 2.2) (Maraite, 1993).

Figure 2.2 Cassava bacterial blight disease symptoms, caused by *X. axonopodis* pv. *manihotis* (Xam) (Taylor et al., 2017)

2.2.2 Brown leaf spot (BLS) is caused by fungus *C. henningsii*, *Passalora henningsii*, and *Mycosphaerella henningsii*. BLS disease appears as small spots with greyish-brown to dark brown borders on the upper leaf surface. The greyish-brown spots form between leaf veins so the veins limit their shape and size. The center of the brown spots may fall out, leaving a hole in the leaf (Figure 2.3). The disease can greatly reduce yields (Karyeija, 2012).

Figure 2.3 Brown leaf spot (BLS), caused by *C. henningsii, Passalora henningsii*, and *Mycosphaerella henningsii* infection, is one of the most important fungal diseases of cassava (Pei et al., 2014)

2.2.3 Cassava anthracnose disease (CAD) caused by the fungus *Colletotrichum gloeosporioides* f. sp. *manihotis* is one of the major economic diseases in Thailand. CAD is characterized by cankers on stems, branches and fruits, leaf spots and tip dieback symptoms. The appearance of the disease depends on the cassava varieties and the infected plant. Cassava production on an industrial scale is grown on large fields or plantations. The leaves develop and spread, forming a close canopy, which creates very

humid condition favorable for CAD development and spread in the field (Figure 2.4) (Muimba, 1982; Yeo et al., 2017).

Figure 2.4 Symptoms of severe infection caused by *C. gloesporioides* f. sp. *manihotis* (Yeo et al., 2017)

2.2.4 Cassava root rot disease (CRRD) is gradually becoming important in the major cassava producing regions of the country with high yield losses. High incidence and severity have been regularly reported by farmers and Agriculture Extension Agents in Thailand. Complete crop failure due to root rots has been observed in farms in different regions of the country (Buensanteai and Athinuwat, 2012; Charaensatapon et al., 2014; Athipunyakom et al., 2020). Because the disease is under this study, its details will be presented in depth in the next section.

2.3 Cassava root rot disease (CRRD)

2.3.1 Discovery and distribution

CRRD is caused by various kinds of fungi living in the soil. They occur mainly in forest fallow land that has been recently cleared and in soils that do not drain properly. If a cassava plant is suspected to have root rot disease, it should be confirmed by uprooting it and examining the roots for the damage symptoms. Root rot diseases kill both storage roots and feeder of cassava. The storage roots may develop light brown coloration and unusually swell that the roots may give out a bad smell as they rot. Moreover, can be seen if the roots crack in the soil or they are cut open. (Msikita et al., 2000).

2.3.2 Cassava root rot symptoms and damages

Root rots can occur on young or old cassava plants and can be caused by one or several fungal genera. Disease incidence usually is higher during the rainy season, but some pathogens, e.g., *Macrophomina phaseolina*, are more common during the dry season (Msikita et al., 2005). The main symptom of rot disease is a breakdown in tissues of the mature tuberous roots, usually associated with a foul odor and changes in color, which may be useful in distinguishing the pathogens involved. For example, storage tissues of tuberous roots primarily infected by a *Fusarium* species often are pink or yellow, whereas tissues infected with *Botryodiplodia theobromae* often is a dark blue or grayish black (Akinyele and Ikotun, 1989). Infected young feeder roots lose vitality and may desiccate, whereas infected shoots wilt either partially or completely and may lodge. In some cases, fungal structures such as mycelia, sclerotia, rhizomorphs, or pycnidia are seen attached to the infected plant parts (Onyeka, 2002). Root rots generally are classified into dry or soft rots depending on symptom

expression and on their prevalence during either the dry season or the wet season (Lozano and Nolt, 1994; Msikita et al., 1997). Soft rot is common if the soil is wet and/or temperatures lower (Theberge, 1985), and in heavy, poorly drained soils with high organic matter content. Hillocks and Wydra (2002) listed the organisms known to cause root rot or to be involved in the root rot complex. In Africa, reports of specific studies on CRRD have been published from the Democratic Republic of Congo (DRC) (Makambila, 1994; Mwangi et al., 2004), Nigeria (Msikita et al., 1997, 1998; Onyeka, 2002; Msikita et al., 2005), Cameroon (Messiga et al., 2004), and Togo (Boher et al., 1997). Ranajit (2006) revealed that, it was not possible to determine which genus was most important economically, but there was a general agreement that *Fusarium* species were an important component of the fungal complex that caused CRRD everywhere cassava was cultivated in the farmer field (Figure 2.5).

Figure 2.5 Symptoms and damages of CRRD in cassava plants

2.3.3 Morphology and physiology of *Fusarium solani*

Colony appearance was white to pale cream with sparse to cottony mycelia with concentric rings pattern on the upper surfaces and white to pale yellow on the lower surfaces (Figure 2.6a and b). White to cream sporodochia on CLA were observed (Figure 2.6c). Macroconidia were inequilaterally fusoid in shape with 3-5 septa, and 27.6-41.3×3.5-5.9 µm in size (Figure 2.6d). Microconidia were oval to ellipsoid with 0-1 septa, and 8.1-15.2×3.3-4.3 µm (Figure 2.6e). Microconidia were abundant in the form of false heads borne on long monophialides (Figure 2.6f, g and h). Chlamydospores measuring 7.4~11.2 µm were also produced on CLA (Figure 2.6i).

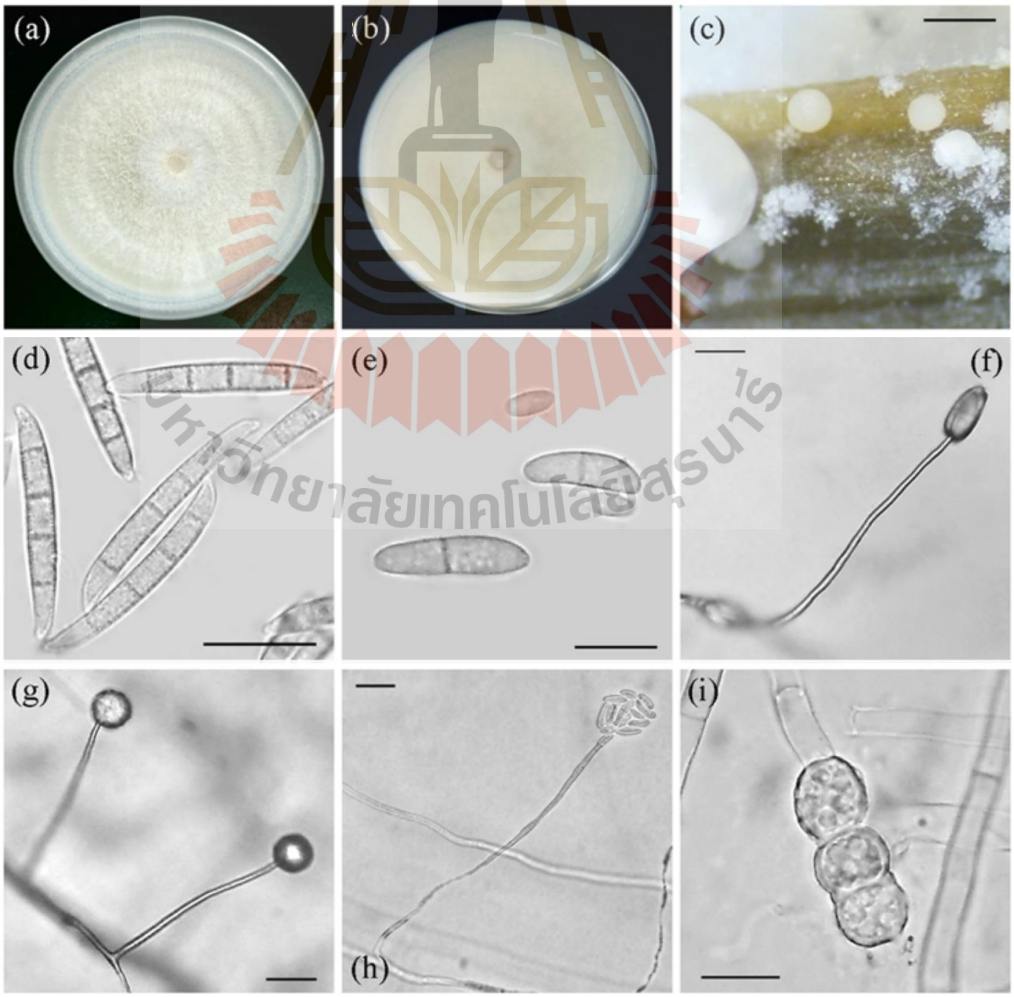

Figure 2.6 Morphological characteristics of *Fusarium solani*. (a,b) Colony's upper surface and lower surfaces of one-week old colony on CLA. (c) Sporodochia formed. (d) Macroconidia. (e) Microconidia. (f,g,h) Microconidia were abundant in the form of false heads borne on long monophialides. (i) Chlamydospores. (Kee et al., 2020).

2.3.4 Modes of pathogen infection

The *F. solani* is also a ubiquitous fungus widely distributed in the environment. Both intercellular and intracellular penetration of the root was observed, predominantly in the root hair zone (Ma et al., 2013). Hyphal growth along the depressions and anticlinal wall junctions resulted in the ingress of hyphae between adjoining epidermal cells. Alternatively, direct penetration of epidermal cells took place soon after conidial germination. The subsequent passage of the fungus through the root cortex also followed both inter- and intracellular routes. The ability of *F. solani* hyphae to penetrate the cotton root epidermis by either mechanism has been documented previously (Rodriguez-Galvez and Mendgen 1995).

Soilborne plant diseases are those caused by infection of pathogens in soil via the roots. *F. solani* and *F. oxysporum* are representative of soilborne pathogens. It inhabits the soil for a long time in the form of chlamydospores, penetrates the roots, extends in the tissues, colonizes and metastasizes in xylem vessels, and causes systemic yellowing, wilting, and death in plants (Beckman, 1987).

2.4 Control of CRRD

The important sources of CRRD are soils and cassava root and stem debris contaminated with the fungi. The fungi enter cassava plants through wounds caused by pests or farming tools or by piercing the roots by themselves. Farm tillage tools used in cassava farms with the disease should be cleaned after use to prevent the fungi on them from spreading to other areas. Similarly, cassava plant debris in farms with the disease serve as sources of root rot fungi and should be destroyed by burning (Eria, 2008). Management of CRRD there are several methods such as cultural methods, disease-resistant cultivars, biological controls, induced resistance, and using chemicals (concentrate on reducing subsequent development of the pathogen on cassava).

2.4.1 Chemical control

Cassava is one of the most cultivated food crops and is planted in backyards even in the cities in the South Africa and as such the use of chemicals in the control of cassava diseases is not recommended. CRRD is difficult to manage because the disease is caused by a complex of soil-borne fungi/oomycete species, which makes chemical control infeasible due to environmental damage and its high treatment costs for producers. In Thailand, plant pathologists of the Department of Agriculture found that the CRRD was caused by *Phythopthora meadii* and *Phythopthora* sp., then recommended the stem dipping before planting and foliar sprays with fosetyl aluminium (50 g/ 20 L water, 4 applications at 1-month intervals) as a control measure (Chatchai, 2014).

2.4.2 Cultural methods

Well-drained sandy loam soils are good for cassava cultivation. Farmers must avoid areas that are prone to flooding and water-logging. Waterlogged soils promote

root rot disease. Farms should not be located close to rivers and streams. Such areas are likely to be flooded at some time in the year. Choose lands that have no history of the root rot disease and other major cassava diseases. Look out for the presence of root rot causing mushrooms and avoid the site (Moses et al., 2007). In cases where the land has a history of root rots, good disease management was practiced to ensure good yields. Farmers must make sure the land is fertile. Fertile lands produce healthy plants that are not easily attacked by diseases. Improve soil fertility with farmyard manure. Destroy all plant debris including rotten roots and stems bearing fruiting bodies immediately after harvest to destroy spores of pathogenic fungi. This act reduces disease severity in the next season. Disease severity on farms could be reduced through destruction of debris that carry spores into the next planting season. Farm tools such as cutlasses, hoes and ploughs used on fields with a root rot history must be cleaned immediately after using (Moses et al., 2007). Continuous cropping of cassava on the same piece of land leads to the build-up of pathogen population increasing severity year after year. Rotate cassava with grains or cereals periodically (every 3 years) to help reduce the effects of root rot disease (Lozano, 1992).

2.4.3 Disease resistant cultivars

Improved cassava varieties with resistance or tolerance to certain important diseases and pests (that are also high yielding) have been developed and released by a number of agricultural institutions. Farmers must always choose disease resistant or tolerant varieties, if available, as planting materials (Moses et al., 2007). In Thailand, resistant cultivars recommended for the controlling of cassava diseases are Rayong 9 and Rayong 72 (Chatchai, 2014).

2.4.4 Biological control

CRR disease severity is directly related to yield losses caused and necessitates the development of ecology-conscious and cost-effective strategies. Biocontrol agents have been used on root rot disease of cassava such as Bacillus spp (*B. polymyxa, B. subtilus*), *Pseudomonas fluorescens* (Vasudevan et al., 2002; Velusamy and Gnanamanickam, 2003; Mukherjee et al., 2012), *P. aeruginosa, Trichoderma virens, Stenotrophomonas maltophilia, Burkholderia glumae, Streptomyces mutabilis* (Janse, 2005). Hridya et al. (2012) reports preliminary results on the efficacy of biocontrol agents, biofertilizers on root rot, harvest index and nutrient uptake, yield of cassava at two NPK rates. According to Silva et al. (2016), the antagonist capacity of *T. aureoviride* URM 5158 against CRRD caused by *F. solani* showed 88.91% of the highest inhibition of pathogen growth. All isolates showed chitinase activity, but *Trichoderma aureoviride* URM 5158 produced the highest amount of chitinase. *T. hamatum* URM 6656 and *T. aureoviride* URM 5158 were further selected to be applied *in vivo*. The two *Trichoderma* strains reduced 64 and 60% of the disease severity in the shoot and 82 and 84% in the root. Cassava plants infected with *Trichoderma* showed the highest peroxidase and ascorbate peroxidase productions. The *Trichoderma* also showed competitive antagonist capability *in vitro*, highest chitinase production and reduced the severity of CRRD in shoot and root of cassava plants. In addition, the application of the selected antagonists led to maximum enzyme activities of ROSs group in cassava plants.

2.4.5 Induced resistance

Over the last few years, resistance stimulants have been widely studied for preventing plant diseases stand on the induced resistance concept. The induced

resistance wherein the plant immune system against a wide range of plant pathogens (Heil, 2002; Mandal et al., 2009; Graham and Myers, 2011). Two important plant defense mechanisms comprise systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is also dependent on salicylic acid signaling and the systemic expression of pathogenesis related protein genes (Sticher et al., 1997; Hammerschmidt, 1999; Heil and Silva Bueno, 2007). ISR is induced by certain strains of plant growth promoting rhizobacteria (PGPR) (Van Loon et al., 1998; Buensanteai et al., 2009 and 2010). Unlike SAR, ISR is not associated with local necrotic lesion formation. ISR also differs in that it depends on perception of ethylene and jasmonic acid, and is not associated with expression of PR genes.

2.5 Mechanism of defense resistance

Economic crops have generated a complex immune and defense system against plant pathogens infection. These plant/crop defense mechanisms rely on their ability to recognize pathogen effector, carry out plant respond defensively, and signal transduction via several pathways involving various genes and their products.

The induced defence mechanisms have two forms of systemic defenses including 1) systemic acquired resistance (SAR) and 2) induced systemic resistance (ISR). The SAR requires salicylic acid (SA) as a signal molecule and is associated with the production, defense enzymes and pathogenesis-related (PR) proteins (Buensanteai et al., 2010). The ISR relies on ethylene (ET) and jasmonic acid (JA) as signaling factors (Walters et al., 2007). The elicitors could be both synthetic or natural compounds and microorganisms. Abiotic elicitors comprise SA, vitamin B1, acibenzolar-S- methyl (ASM), benzoic acid (BA), chitosan, riboflavin, oxalic acid

(OA), benzo (1,2,3)-thiadiazole-7-carbothionic acid, and ascorbic acid (AA) have been widely evaluated against various plant diseases (Perazzoli et al., 2008; Eschen-Lippold et al., 2010; Prakongkha et al., 2013a; 2013b). Moreover, biotic elicitors such as PGPR have been frequently assessed to be effective in inducing increase resistance and plant growth against several diseases (Walter et al., 2007; Buensanteai et al., 2009; Graham and Myers, 2011; Park et al., 2013). Induced resistance mechanisms of elicitors have been announced that callose deposition, cytosolic H^+ and Ca^{2+}, hypersensitive response (HR), oxidative burst, activation of mitogen activated protein kinase, accumulation of jasmonate and salicylic acid, synthesis of defense enzyme, PR-proteins and phytoalexins stimulation related with plant defense response against pathogen infection (Sana et al., 2010; Iriti et al., 2011)

2.6 Some characteristics of elicitors

Elicitors have low molecular weight and synthesized as such or released from polymeric precursors during infection and very stable molecules that induce an immune defence response in plants. Furthermore, elicitors can trigger morphological and physiological response and phytoalexin accumulation (Holopainen et al., 2009; Mejia-teniente et al., 2010).

2.6.1 Salicylic acid

Salicylic acid (SA) or 2-hydroxy benzoic acid is a plant hormone that not only mediates plant defense responses against biotic and abiotic stresses through morphological, physiological and biochemical mechanisms. It is a natural product of phenylpropanoid metabolism. Chemically, SA belongs to an extremely diverse group of plant phenolics, that possess an aromatic ring with a hydroxyl group or its

functional derivatives. SA has direct involvement in plant growth, thermogenesis, flower induction and uptake of ions. It affects ethylene biosynthesis, stomatal movement and also reverses the effects of ABA on leaf abscission. Enhancement of the level of chlorophyll and carotenoid pigments, photosynthetic rate and modifying the activity of some important enzymes are other roles assigned to SA (Figure 2.7) (Hayat et al., 2007; War et al., 2011; Zhang et al., 2017).

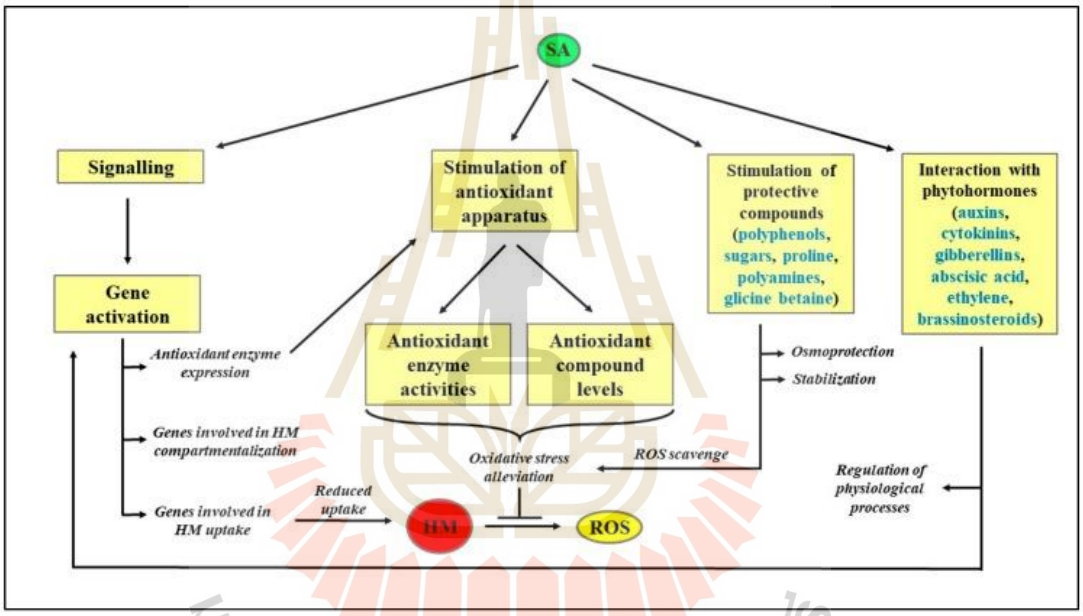

Figure 2.7 Schematic representation showing the role of SA application (Sharma et al., 2020)

Abdel-Monaim (2013) revealed that soaking faba bean seeds in chemical inducers individually or in combination and biocontrol agent significantly increased survival plants either and reduced root rot, damping-off under greenhouse or field conditions. Moreover, these treatments increased protein content, plant growth, and yield components in seeds during both growing seasons under field conditions. The

combination between chemical inducers and biocontrol agents were better than used individually especially SA + *B. megaterium* and SA + *T. viride*. The effects of microelements, antioxidants, and bioagents on *Fusarium oxysporum, F. solani*, and *Macrophomina phaseolina*, the causal pathogens of root rot and wilt diseases in roselle, were examined under field conditions (Hassan, 2014). El-Mohamedy (2014), effect of some chemical inducers such as potassium salts, salicylic acid and sorbic acid on control of root rot pathogens and their impact on growth, quantity and quality parameters of tomato cv. Super Strain B were investigated. All the tested chemical inducers significantly reduced severity of root rots under greenhouse and field conditions. Potassium salts based-treatments, followed by salicylic acid, were the most effective in decreasing incidence of root rots.

2.6.2 *Bacillus* sp.

Bacillus sp. is well documented for enhanced growth promotion and induced systemic resistance (Kloepper et al., 2004; Ryu et al., 2004). Furthermore, it directly affecting plant growth and development through plant growth regulator, *Bacillus* sp. could physiological systems to promote growth enhancement trigger plant biochemical and colonize roots (Figure 2.8). It could enhance growth in several economic crops such as soybean, vegetable, soybean, corn, rice, Chinese kale, cauliflower and cassava (Prathuangwong et al., 2005a; 2005b; 2005c; Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2011), this process is mediated in part by the excretion of phytohormones such as extracellular proteins, lipopeptides, auxin and indole-3-acetic acid (IAA) (Buensanteai et al., 2008). And induced systemic resistance (ISR) was a pattern of induced resistance associated with defense related enzymes of increased β-1,3-glucanase and peroxidase activity levels in plants and the

accumulation of phenolic content. Moreover, when *Bacillus*-treated seeds, then the seedling primed with disease inoculation, it was able to activate both rapid salicylic acid (SA) and delayed jasmonic acid/ ethylene (JA/ET) dependent pathways of induced systemic resistance in plants with high and low production levels, respectively. Song (2014) suggested that the *Bacillus* species have good potential as a microbial agent for the biocontrol of the ginseng root rot caused by *Fusarium incarnatum*. Root dip treatment with *Bacillus subtilis* formulations showed a considerable increase in root length with *B. subtilis* (33 cm) and chlorothalonil (28.5 cm) when compared to untreated control (15 cm). Growth promotion was better with root dip application while disease control was achieved better with seed application. A 66% and 84% reduction in incitation of disease was noticed with soil and seed application methods (Narasimhan and Shivakumar, 2016).

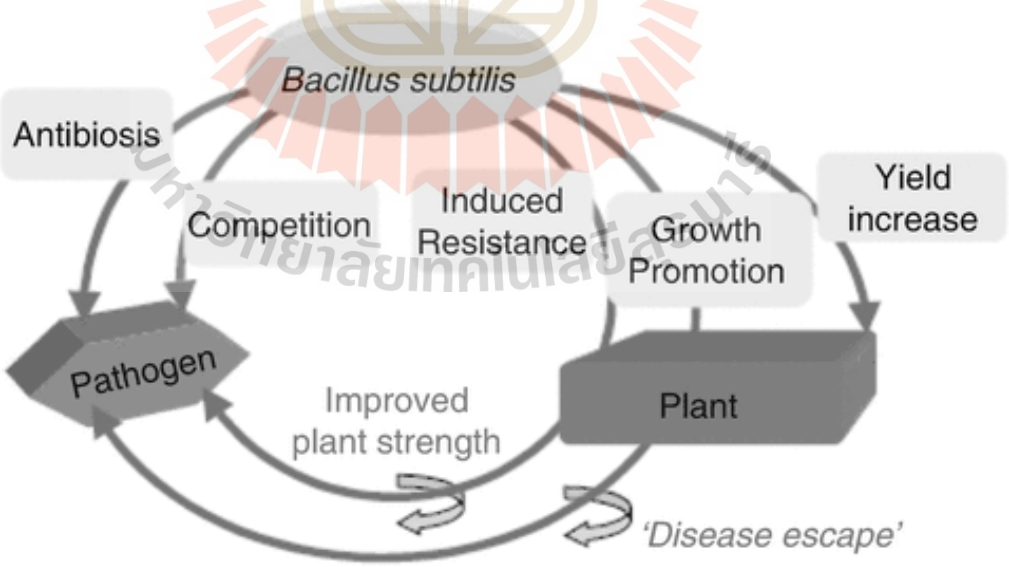

Figure 2.8 Modes of action of *Bacillus subtilis* promoting plant growth (Böhme et al., 2016)

2.7 Conclusion of literature review

The induction of systemic resistance against root rot disease was carried in many kinds of plants. The mechanisms of these resistance elicitors have been revealed that hypersensitive response (HR), an accumulation of JA and SA, phytoalexins, oxidative burst, callose deposition, synthesis of defense enzymes and PR proteins are plant defense responses against pathogen infection and invasion (Iriti et al., 2011; Kombrink and Schmelzer, 2001). Stimulants could be both microorganisms and also natural bioactive compounds or synthetic. Natural compounds elicitors including SA, benzoic acid, chitosan, 2,6-dichloroisonicotinic acid, ascorbic acid, potassium dihydrogen phosphate, oxalic acid, sodium saccharin dihydrate or BIT, riboflavin, vitamin B1, kinetin and BTH have been widely evaluated against various plant pathogens (Prakongkha et al., 2013a, 2013b; Slaughter et al., 2008). Moreover, biotic elicitors such as plant growth-promoting rhizobacteria (PGPR) presented by preventing roots from invasion by pathogens, building up resistance against several diseases (Walter et al., 2007; Graham and Myers, 2011), and enhancing growth (Eschen-Lippold et al., 2010). Its application has been registered in several countries and various crops (De Meyer et al., 1999).

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CHAPTER III

Induction of systemic resistance against Fusarium root rot disease in *Manihot esculenta*

ABSTRACT

The study aims to evaluate the effect of Zacha elicitor formulations (a formulated salicylic acid) and a bacillus bioproduct for inducing resistance on H_2O_2 content, peroxidase, polyphenol oxidase, and catalase activities against *Fusarium* root rot disease in cassava. The results showed that Zacha elicitor formulations and bacillus bioproduct reduced mycelial growth of *F. solani*. Furthermore, Zacha11 treatment at a concentration of 500 ppm could induced H_2O_2 contents and activity of some enzymes related to plant defense mechanisms were highest at 24 hours and decreased at 48 hours after pathogen inoculation in cassava plants compared with the uninfected plants. The biochemical response of plant defense mechanism pertaining to H_2O_2 in plants and enzyme activities of PO, PPO and CAT were also observed due to SAR induction. So, these results suggest the formulated salicylic acid played an important role as a plant defense elicitor, leading to reduced cassava root rot disease.

3.1 Introduction

Cassava (*Manihot esculenta* Crantz) has been an important economic crop in Thailand. The increasing demand for food has led to an expansion of cassava cultivation in many countries (FAO, 2002; Ubalua and Oti, 2007; FAO, 2015). Cassava planting area in Thailand was approximately 1.29 million hectares and recognized as a major cassava producer, annual yielding at approximately 31.2 million tons. Thailand has begun to substitute modified starch produced from imported corn with modified starch produced from local cassava (Charaensatapon et al., 2014; Duchanee, 2015; Sangpueak et al., 2018). Nevertheless, cassava production is extremely reduced due to many other diseases and pests attack cassava. Among them, cassava root rot disease (CRRD) is the most important soil-borne disease and the major diseases affecting worldwide (Harman et al., 1981; Duchanee, 2015; Athipunyakom et al., 2019). Especially, the damage of *Fusarium* spp. could reduce the yield up to 81% (Theberge, 1985; Lozano and Nolt, 1994).

The elicitors such as salicylic acid (SA) and *Bacillus* sp. have been extensively studied in a research experiment and commercially trial product to promote growth and control phytopathogens in various crops (Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2012; War et al., 2011). SA could induce the level of reactive oxygen species, enzyme activities and PR-proteins in defense mechanism of cassava plants against physiological disorders and biochemical mechanisms (Buensanteai et al., 2012). So, this study aims at evaluating the effect of exogenous SA and Bacillus prototype formulation for inducing resistance against cassava root rot disease.

3.2 Materials and methods

3.2.1 CRRD causal agents and culture conditions

Fusarium solani isolate SHRD1 was obtained from stock culture (stored in potato dextrose broth (PDB) with glycerol (10%) at -80°C) of Plant Molecular Biology Laboratory, Suranaree University of Technology, Thailand. The culture was transferred from PDB onto potato dextrose agar (PDA) plates for 7 days at room temperature. The fungal culture on PDA was used throughout the research (slightly modified from Buensanteai, 2009; Duchanee, 2015; Sangpueak et al., 2018) . *Fusarium* spores suspension was obtained by filtered through layers with sterile distilled water was adjusted to 10^6 spores mL^{-1} by a hemocytometer, added 2 drops/100 mL of drop spores suspension of Tween-20 before used.

3.2.2 Preparation of abiotic and biotic elicitors

3.2.2.1 Abiotic elicitor preparation of exogenous SA (Zacha)

The exogenous SA (Zacha), 15 salicylic acid elicitor prototype formulations, the products of Bioactive Agro Industry Co., Ltd were used in the experiment. Zacha elicitors were prepared by dissolving them in sterile distilled water and adjusted to 0, 100, 200, 500, 1,000, 2,000 and 60,000 ppm (the active ingredient of Zacha elicitors were 6% salicylic acid)

3.2.2.2 *Bacillus* prototype bioformulations (JN2-007 elicitor) and culture conditions

The JN2-007 elicitor is a product prototype developed by Nikaji et al., 2015 from the Plant Molecular Biology Laboratory, Suranaree University of Technology, Thailand. The cultures were re-suspended in sterile distilled water and

adjusted to $1x10^6$ cfu mL^{-1}, $1x10^7$ cfu mL^{-1} and $1x10^8$ cfu mL^{-1} concentration (Buensanteai et al., 2009; Nikaji et al., 2015).

3.2.3 Evaluation of physical and biological properties of elicitors

3.2.3.1 Quality changes of elicitor formulations during storage

Storage quality and shelf-life of the elicitors were monitored regularly. Discoloration (by using RHS color chart), sedimentation, pH, liquid separation and contamination of each elicitors were checked every 3 months, using the methods slightly modified from Simpson (2011) and Kandeepan et al. (2013).

3.2.3.2 Phytotoxicity of Zacha elicitors

Cassava leaves were surfaces cleaning with soap and water, drying on Whatman™ filter paper. In the design of this experiment, a completely randomized design (CRD) with four replications. Three detached cassava leaves were placed in each moist box and then a drop of 50 µl of a Zacha at the concentrations of 0, 100, 200, 500, 1,000, 2,000 or 60,000 ppm was pipetted onto surface of each leaf. Subsequently, the boxes were placed in a room temperature for 48 h. The toxicity level showed by the leaves were recorded at 2 days after being treated with elicitors (Yodyotee 2010). The experiment was conducted three times.

3.2.4 Effect of Zacha and JN2-007 elicitors on CRRD causal agents in vitro

This design is used for experiments analysis of data with a split-plot in CRD with four replications to evaluate the inhibition effect of Zacha and JN2-007 elicitors on the *F. solani*. Zacha at concentrations of 0, 100, 200 or 500 ppm; and JN2-007 at concentration of $1x10^6$, $1x10^7$ and $1x10^8$ cfu mL^{-1} were added into PDA petri plates. A cork borer with diameter of 5 mm was used to drill agar blocks of the causal fungi and settle center of the elicitor-amended petri-plate. All culture plates were kept at 28±2

^{o}C for 7 days. PDA plates without Zacha and JN2-007 elicitors were used as the negative control and chemical fungicides including: carbendazim (500 ug mL^{-1}), mancozeb (2,000 ug mL^{-1}) and prochloraz (200 ppm) were used to inhibit growth of the fungi as a positive control. The mycelial growth was measured in diameter (mm) around the discs (Sobowale et al., 2005; Bandyopadhyay, 2006; Wongcharoen, 2013). Observations on width of mycelial growth of the tested pathogens were recorded and percentage of inhibition was calculated according to the formula (Vincent, 1927; Maurya et al., 2014) as follows:

$$\text{Percent inhibition (I)} = C\text{-}T/C \times 100$$

Where, C = mycelial growth of the pathogen in negative control

T = mycelial growth of the pathogen in elicitor amended culture plate

The experiment was repeated twice, with three replicates per treatment.

3.2.5 Efficacy of the elicitors in inducing resistance against Fusarium root rot disease under greenhouse conditions

The experiment was conducted in a randomized complete block design (RCB) with four replications. Cassava stalks of accession number CMR89 were surface-disinfected with NaOCl (1%). After that, washing with distilled water and let dry for 5 min at room temperature. Then, cassava stalks were soaked for 10 minutes before planting with elicitors and chemical fungicide (positive controls) that were selected in the previous experiment compare with water are negative controls. After planting, the cassava plants were inoculated with 1 x 10^{6} conidia mL^{-1} of *Fusarium* suspension, such suspension was estimated for the root rot general symptoms after 60 days and mixing

infested soil (100 mL/pot and plants) (Oliveira et al., 2013). Subsequently, at 14, 21, and 28 days after planting (DAP), cassava plants were sprayed with each elicitor. After that cassava plants were monitored for biochemical changes associated with plant defense accumulation of H_2O_2 content and enzyme activities (peroxidase, polyphenol oxidase, and catalase). The experiment was conducted three times, with a measurement of H_2O_2 content and enzyme activities for each time.

3.2.6 Measurement of hydrogen peroxide (H_2O_2) content

Cassava roots were collected at 0, 12, 24 and 48 hours after inoculation (HAI). Briefly, 0.5 g of the root samples were immersed in ice and ground with a sterile mortar and pestle to a suspension then homogenized with 2 mL ice-cold 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 3,500 g for 30 min at 4°C. Finally, 0.3 mL of the supernatant was dispensed into a new Eppendorf tube and 0.3 mL 10 mM potassium phosphate buffer (pH 7.0), 0.6 mL of 1 M potassium iodide (KI) was added and the absorbance read at 390 nm (Velikova et al., 2000). Hydrogen peroxide content was calculated from a standard curve and the concentration was expressed as μmol/g FW (Njenga et al., 2017).

3.2.7 Extraction and determination of enzyme activities

3.2.7.1 Estimation of total soluble proteins

Cassava roots were collected at 0, 12, 24 and 48 HAI. The plant tissues (0.5 g) was ground in a cold mortar with a pestle using liquid nitrogen that homogenized in 3 mL of 0.1 M sodium phosphate buffer (pH 6.5) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenate was centrifuged at 5000 rpm for 15 minutes at 4°C. The supernatant was used as the enzyme extract for the assay of peroxidase, polyphenol oxidase, and catalase activities. The protein content

was examined using the standard Bradford protein assay (Bradford, 1976). Crude protein extract was extracted by the method as described by Buensanteai et al., (2009); Prakongkha et al., (2013); Zur et al., (2013); Nair and Umamaheswaran, (2016).

3.2.7.2 Peroxidase activity (PO)

The reaction mixture consisting of 0.05 M pyrogallol (1.5 mL) and enzyme extract (0.1 mL) was taken in a 96-well plate. To initiate the reaction, 1% H_2O_2 (0.5 mL) was added. The change in absorbance was recorded at 420 nm at 1 min intervals for 3 min from zeros of incubation a room temperature. The boiled enzyme served as blank. The enzyme activity was expressed as an units g^{-1} FW min^{-1}. The activity of peroxidase was determined as described by Hammerschmidt et al., (1982); Abou-Zeid et al., (2018).

3.2.7.3 Polyphenol oxidase (PPO)

A mixture consisting of 0.1 M sodium phosphate (pH 7.0) (1.5 mL) and enzyme extract (0.1 mL) was taken in a cuvette. Then, 0.01M catechol (0.2 mL) was added to initiate the reaction. The change in absorbance was recorded at 495 nm at 1 min intervals for three min and the results were expressed as an units g^{-1} FW min^{-1} (Mayer et al., 1965; Abou-Zeid et al., 2018).

3.2.7.4 Catalase (CAT) activity

Activity was assayed in the root samples that collected from inoculated and healthy cassava plants (slightly modified by Beers and Sizer 1952; Njenga et al., 2017). Reaction mixture consisting of 0.1 M phosphate buffer (pH 7.0) (0.5 mL), enzyme extract (0.2 mL), and 1% H_2O_2 (0.1 mL) was incubated at 28±1°C. At the start of the enzyme reaction, the absorbance of the mixture was set zero at 230 nm in a spectrophotometer, and changes in the absorbance were recorded at 1 min intervals for

3 min. Values of Catalase activity was expressed as μmol of H_2O_2 oxidized mg^{-1} of protein min^{-1}.

3.2.8 Statistical analysis

Statistical analyses were performed using analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) for multiple comparisons, the finding is considered statistically significant (p-value ≤ 0.05). SPSS software (version 16 for window) was used for statistical analysis.

3.3 Results

3.3.1 Evaluation of physical and biological properties of elicitors

The color properties of elicitor formulations during storage at 9 months showed that Zacha1-5 and Zacha11-15 were Yellow group (13B) while elicitor formulation (Zacha6-10) was Non-color group by using RHS color chart (Figure 3.1). Also, it was not found the sedimentation, liquid separation, and contamination of all of Zacha's elicitor formulations. Meanwhile, its were monitored pH level revealed that the pH scale ranges from 1.71 to 1.78 (Table 3.1). Phytotoxicity test of Zacha by detached cassava leaves showed that a concentration range of 0-2,000 ppm, not observed for burn lesions but the high concentration (60,000 ppm) of Zacha's elicitor made brown burn (Figure 3.2).

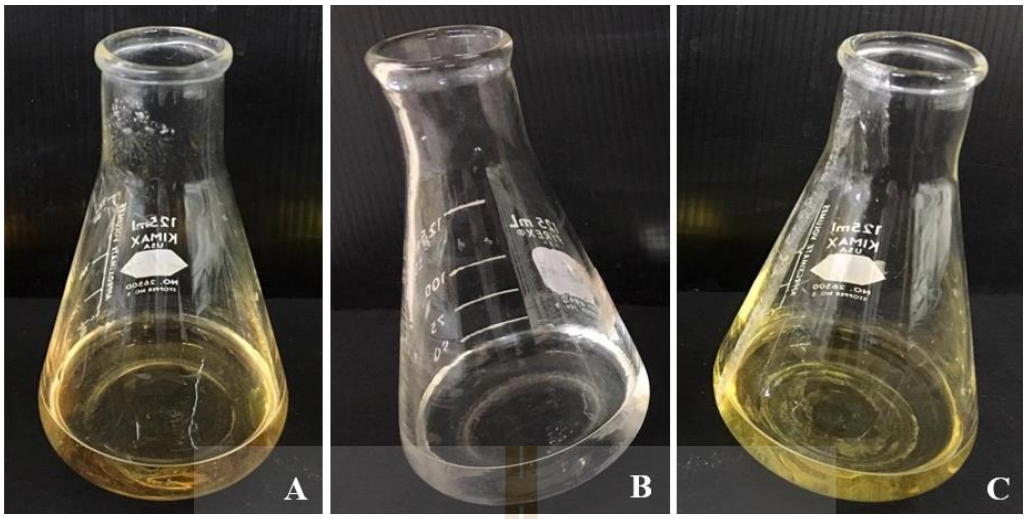

Figure 3.1 The color properties of elicitor formulations during storage (at 9 months) A) Zacha1-5 B) Zacha6-10 and C) Zacha11-15.

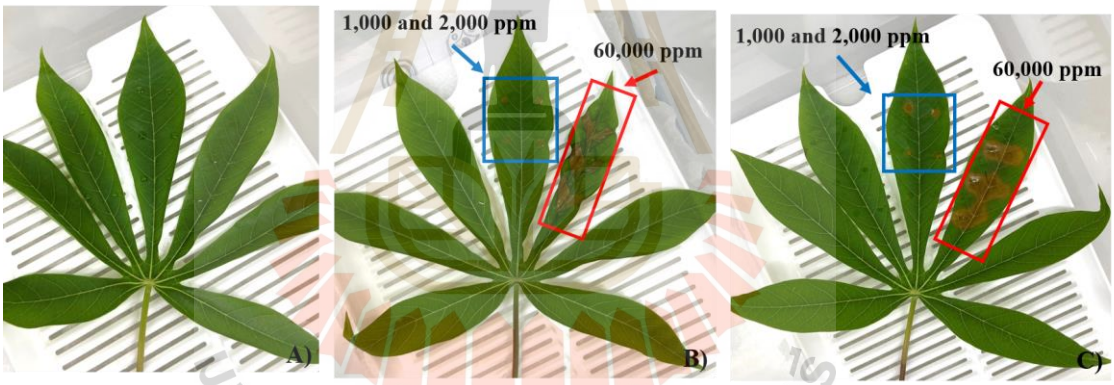

Figure 3.2 Evaluation phytotoxicity of Zacha elicitors on detached cassava leaves A) 0 day after elicitor application, B, C) Zacha11 and Zacha15 at 2 days after elicitor application.

Table 3.1 Quality changes of the 15 elicitor formulations of Zacha elicitor at 3 and 6 months

No. of elicitors	Color	Sedimentation	pH		Liquid separation	Contamination
			3 months	6 months		
Zacha1	Yellow (13B)	×	1.72	1.78	×	×
Zacha2	Yellow (13B)	×	1.79	1.80	×	×
Zacha3	Yellow (13B)	×	1.71	1.68	×	×
Zacha4	Yellow (13B)	×	1.71	1.73	×	×
Zacha5	Yellow (13B)	×	2.05	1.73	×	×
Zacha6	Non-color	×	1.81	1.78	×	×
Zacha7	Non-color	×	1.72	2.08	×	×
Zacha8	Non-color	×	1.67	1.93	×	×
Zacha9	Non-color	×	2.12	1.78	×	×
Zacha10	Non-color	×	1.99	1.80	×	×
Zacha11	Yellow (13B)	×	1.71	1.68	×	×
Zacha12	Yellow (13B)	×	1.71	1.73	×	×
Zacha13	Yellow (13B)	×	2.05	1.83	×	×
Zacha14	Yellow (13B)	×	1.71	1.88	×	×
Zacha15	Yellow (13B)	×	1.72	2.08	×	×

3.3.2 Evaluation of elicitors on inhibition of *F. solani* mycelial growth

The effect of elicitors on mycelial growth inhibition revealed that Zacha11 and Zacha15 at a concentration of 500 ppm resulted in a significant reduction in mycelial growth of *F. solani* (Table 3.2) at 12.11% and 11.83%, respectively. In addition, the mycelial growth inhibition for JN2-007 elicitor on different concentrations evaluated showed high reduction in mycelial growth of *F. solani* but no statistically significant difference. However, the chemical fungicides had better mycelial growth inhibition than other treatments. Mancozeb (2,000 ug mL^{-1}) had the highest inhibition effect on the radial mycelial growth (75.50%).

Table 3.2 Percent inhibition mycelial growth of *F. solani* in elicitors amended culture plate

Treatments	Concentration	Mycelial growth inhibition (%)[1/]	
		Day 5	Day 7
Zacha1	100 ppm	9.93 ef	1.68 hi
	200 ppm	9.93 ef	1.68 hi
	500 ppm	15.10 d	1.68 hi
Zacha2	100 ppm	11.35 def	2.22 hi
	200 ppm	8.35 ef	2.22 hi
	500 ppm	9.85 ef	2.22 hi
Zacha3	100 ppm	11.28 def	2.22 hi
	200 ppm	10.68 def	2.22 hi
	500 ppm	6.93 ef	2.22 hi
Zacha4	100 ppm	11.28 def	2.22 hi
	200 ppm	9.85 ef	2.22 hi
	500 ppm	10.68 def	2.22 hi
Zacha5	100 ppm	8.35 ef	2.22 hi
	200 ppm	10.60 def	2.22 hi
	500 ppm	9.85 ef	1.75 hi
Zacha6	100 ppm	11.43 def	1.75 hi
	200 ppm	11.43 def	1.75 hi
	500 ppm	9.85 ef	1.75 hi
Zacha7	100 ppm	11.35 def	1.75 hi
	200 ppm	9.85 ef	1.75 hi
	500 ppm	11.28 def	1.75 hi
Zacha8	100 ppm	9.93 ef	1.75 hi
	200 ppm	9.93 ef	1.68 hi
	500 ppm	9.85 ef	1.68 hi
Zacha9	100 ppm	9.78 ef	1.68 hi
	200 ppm	9.93 ef	1.68 hi
	500 ppm	9.93 ef	1.68 hi
Zacha10	100 ppm	9.85 ef	1.68 hi
	200 ppm	9.78 ef	1.68 hi
	500 ppm	9.93 ef	1.68 hi
Zacha11	100 ppm	12.03 def	9.47 defg
	200 ppm	11.35 def	9.95 defg
	500 ppm	15.18 d	12.11 d
Zacha12	100 ppm	12.78 de	10.12 defg
	200 ppm	9.93 ef	7.40 defg
	500 ppm	12.70 de	7.60 defg
Zacha13	100 ppm	9.85 ef	8.55 defg

Treatments	Concentration	Mycelial growth inhibition (%)[1/]	
		Day 5	Day 7
Zacha14	200 ppm	9.18 ef	6.72 fgh
	500 ppm	9.85 ef	6.78 efgh
	100 ppm	11.35 def	9.30 defg
Zacha15	200 ppm	12.78 de	6.18 gh
	500 ppm	11.43 def	7.90 defg
	100 ppm	12.85 de	11.74 def
JN2007	200 ppm	12.78 de	10.12 defg
	500 ppm	15.18 d	11.83 de
	$1x10^6$ cfu/ml	66.93 b	57.73 c
	$1x10^7$ cfu/ml	63.34 b	55.55 c
	$1x10^8$ cfu/ml	63.42 b	55.55 c
Carbendazim	500 ug/ml	66.85 b	68.78 b
Mancozeb	2,000 ug/ml	71.35 a	75.50 a
Prochloraz	200 ppm	55.03 c	55.45 c
Control		2.00 g	0.00 i
F-test		**	**
CV (%)		17.30	28.00

[1/] Mean ± SE (standard error) followed by the same letter do not different significantly according to DMRT at $P \leq 0.05$

3.3.3 Effect of treated cassava roots with elicitors in inducing resistance on H_2O_2 content and enzyme activities

H_2O_2 content in all treatments increased till 24 HAI and the highest value was observed in Zacha11 at 1.73 μmol/g FW, and then decreased at 48 HAI (Figure 3.3). All treatments showed an increased level of peroxidase activity (PO) after pathogen inoculation. The highest PO activity of 2.54 units g^{-1} FW min^{-1} was observed in Zacha11 followed by Zacha15 (2.20 units g^{-1} FW min^{-1}) at 24 HAI (Figure 3.4). Results in Figure 3.5 revealed that the polyphenol oxidase activity was increased in Zacha11 treatment that roots infected with *F. solani* strain SHRD1 at 24 HAI followed by Zacha15 treatment were 8.54- and 7.20-units g^{-1} FW min^{-1}, respectively, showing significant (p=0.05) difference compared to the uninfected control. Likewise, the activity of CAT was increased in challenged inoculation with *Fusarium* root rot and

less in healthy plants. The highest CAT activity in Zacha11 treatment at 24 HAI was 5.54 μmol mg^{-1} of protein min^{-1} (Figure 3.6).

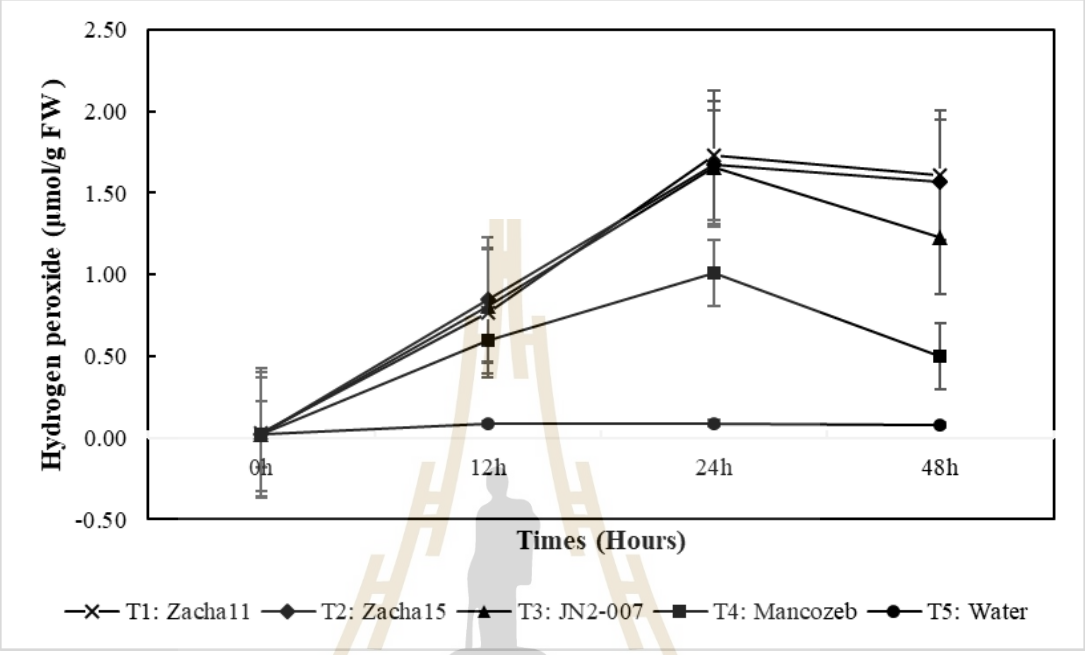

Figure 3.3 Hydrogen peroxide content infected by *F. solani* strain SHRD1; T1 Zacha11 at a concentration of 500 ppm, T2 Zacha15 at a concentration of 500 ppm, T3 JN2-007 at concentration of $1x10^6$ cfu mL^{-1}, T4 Mancozeb (2,000 ug mL^{-1}) and T5 Water (control).

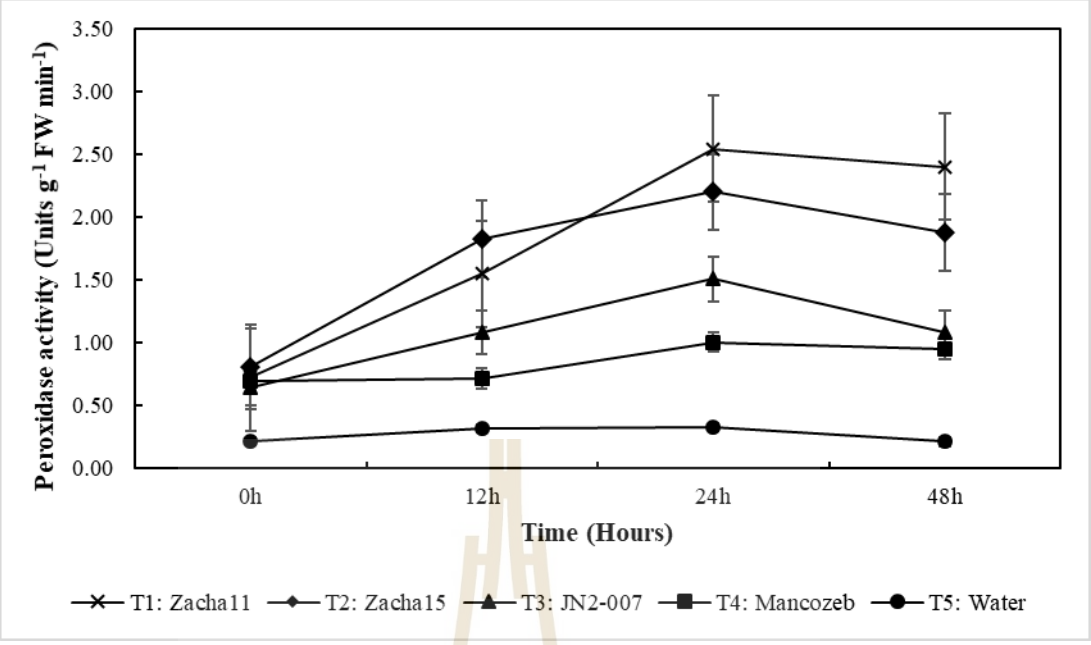

Figure 3.4 PO activity of cassava roots after treated with Zacha elicitor formulations at 0, 12, 24 and 48 HAI and challenged inoculation with Fusarium root rot; T1 Zacha11 at a concentration of 500 ppm, T2 Zacha15 at a concentration of 500 ppm, T3 JN2-007 at concentration of $1x10^6$ cfu mL^{-1}, T4 Mancozeb (2,000 ug mL^{-1}) and T5 Water (control).

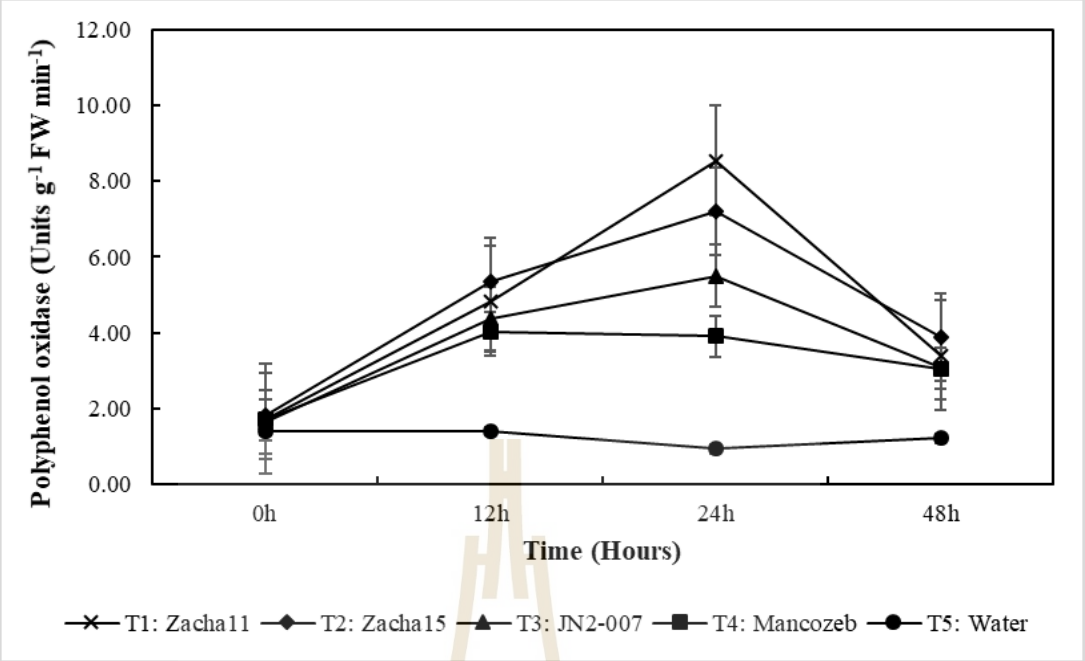

Figure 3.5 PPO activity of cassava roots after treated with salicylic formulation at 0, 12, 24 and 48 HAI and challenged inoculation with Fusarium root rot; T1 Zacha11 at a concentration of 500 ppm, T2 Zacha15 at a concentration of 500 ppm, T3 JN2-007 at concentration of $1x10^6$ cfu/mL, T4 Mancozeb (2,000 ug/mL) and T5 Water (control).

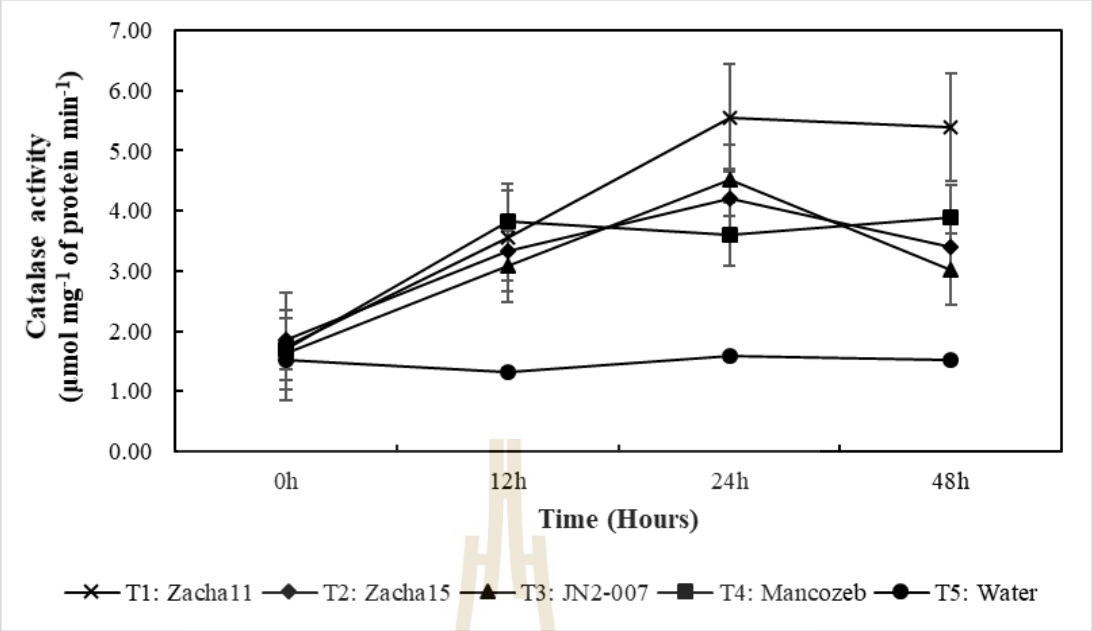

Figure 3.6 CAT activity of cassava roots after treated with salicylic formulation at 0, 12, 24 and 48 HAI and challenged inoculation with Fusarium root rot; T1 Zacha11 at a concentration of 500 ppm, T2 Zacha15 at a concentration of 500 ppm, T3 JN2-007 at concentration of $1x10^6$ cfu mL^{-1}, T4 Mancozeb (2,000 ug mL^{-1}) and T5 Water (control).

3.4 Discussion

To our knowledge, this is the first report on the *in vitro* mycelial growth inhibition of SA against *F. solani* causing root rot in cassava was observed. SA reduced the mycelial growth of *F. solani* isolate SHRD1 at concentrations 500 ppm as compared to the control. Similar researches of Kendra and Hadwiger (1984) also indicated that the mycelial growth of *F. solani* with different crops was inhibited at concentrations of 12 and 18 mg mL^{-1}, respectively. El-Mohamedy et al., 2014 found that sprays with SA at 100 mM for 48 h before root inoculation showed a reduction of

disease severity and root rot incidence caused by *F. solani,* and *S. rolfsii*. Kumar and Bains (2018) reported that in most cases, a higher concentration than 0.5 mM of salicylic acid decreased the mycelial growth of *F. mangiferae*. The finding was in accordance with those of Jendoubi et al. (2015) who reported that the different concentrations of SA (100, 200 and 300 μM) had significantly inhibited *F. oxysporum* f.sp. *lycopercisi* and *F. oxysporum* f.sp. *radicis-lycopercisi* growth compared to the control. Also, SA at a minimum concentration of 2.5 mM could control for other pathogens such as *F. oxysporum, R. solani, Pythium* sp., *Phytophthora, R. stolonifer, M. phaseolinae* and *S. rolfsii* (Panahirad et al., 2012; El-Mohamedy et al., 2013). Moreover, in some reports, SA acts as a toxic chemical and inhibited the mycelial growth of other fungi. This strengthens the hypothesis that the signal transduction pathways activated by SA, their expression of systemic acquired resistance, rather than inhibiting the fungus directly (Metraux et al. 2002; Fragnière et al., 2011; Qi et al., 2012). Hydrogen peroxide acts as a local signal molecule for the induction of protective plant defense mechanisms (Quan et al., 2008). In this study, the H_2O_2 content in elicitors treated plants was higher compared to the non-treated plants, and the one inoculated with *F. solani* strain SHRD1 has increased H_2O_2 levels at 24 h after inoculation. Our result suggests that Zacha elicitor formulation triggers an internal signal involved in oxidative bursts required for inducing defense-related genes. Hao et al. (2014) reported that defense induction of *Salvia miltiorrhiza* by SA contributed to enhancing the production of H_2O_2. Similar have been found in salicylic acid accumulation in SA-treated rice against *Xanthomonas oryzae* pv. *oryzae* (Le Thanh et al., 2017). *Fusarium* root rot infection in cassava plants led to increasing in PO, PPO, and catalase activity in Zacha treated plants was recorded after 24 h of inoculation.

These defense molecules are used for protection against pathogen invasion by an accumulation of lignins which leads to protection against different invading pathogens. (Acharya et al. 2011; Chandra et al. 2015). An increase in PO and PPO activity due to fungal infections is reported in many plants. CAT are important antioxidant enzymes mainly involved in the removal of reactive oxygen species. In conclusion, the defense enzymes induced upon plants treated with Zacha elicitor formulation of salicylic acid and its growth promotion activity might have reduced the disease incidence in cassava. Moreover, H_2O_2 content and four enzyme activities related to the defense system were performed in the inoculated cassava with the pathogen as well as the induction agent defense system SA elicitors in the early hours. In summary, the findings arising in this study indicate that the levels of H2O2 content and resistance enzymes such as polyphenol oxidase (PPO), polyphenol oxidase (PPO) and catalase (CAT) are the keys to the plant defenses against pathogens and the most extensively studied plant defense metabolites in plants in the treated are significantly higher than in the untreated. It is suggested that Zacha inducer is able to induce the natural plant resistance mechanisms. The use of Zacha as an environment-friendly stimulator is a promising approach to protect cassava against Fusarium root rot disease.

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CHAPTER IV

Salicylic acid formulation-induced resistance to Fusarium root rot in cassava (*Manihot esculenta* Crantz)

ABSTRACT

The experimental results showed that Zacha11 and Zacha15 prototype formulations inhibited the growth of *Fusarium solani* at approximately 34.83% and 39.67%, respectively. Furthermore, cassava plants treated with Zacha11 at a concentration of 500 ppm significant reduction in the disease severity of Fusarium root rot disease at 60 days after challenge inoculation. Salicylic acid (SA) activity, suggesting that SA-dependent signaling pathways are systemically triggered in cassava leaves by Zacha11 treatment. The monitoring of biochemical changes associated with activities of β-1,3-glucanase and chitinase in cassava plants inoculated with *F. solani* was significantly (DMRT, $P = 0.05$) higher than control. Maximum activities of PR-proteins were observed at 24 hours after inoculation in all induced plants; then, the activity decreased progressively. Moreover, the SR-FTIR spectral changes of Zacha11-treated epidermal tissues had higher integral areas of lipids, lignins, and pectins (1770-1700 cm^{-1}), amide I (1700-1600 cm^{-1}), amide II (1600-1500 cm^{-1}), hemicellulose and lignin (1300-1200 cm^{-1}) as well as cellulose (1155 cm^{-1}). Alteration in defensive carbohydrates, lipids, and proteins contributed to generate barriers against Fusarium invasion in cassava roots, leading to lower the root rot disease severity.

4.1 Introduction

Cassava (*Manihot esculenta* Crantz) is an important food crop and economic crop in Thailand (Verdier et al., 2004). Cassava production annually provides starch and energy source (Buensanteai et al., 2012). In the last few years, cassava has become an industrial crop because of the application of many industrial processes in which cassava could be crucial raw material. These increased demands for cassava products have brought about to an expansion of its cultivation in many areas. However, cassava yield is significantly reduced due to attacks by insects and diseases (Buensanteai et al., 2012). Cassava root rot (CRR) disease is attacked by several kinds of pathogens severely widespread in Thailand. *Fusarium* sp. is the critical pathogen of CRR disease (Bodah, 2017; Oliveira et al., 2013). The root rot pathogenic *Fusarium* is the primary threat for this crop and destroys the function of the cassava xylem to translocate upward water and nutrients. In most north of Thailand, *Fusarium* root rot disease occurred with a severe incidence of approximately 80% (Camila et al., 2018; Piyachomkwan and Tanticharoen, 2011).

Presently, resistance inducers have been widely assessed to prevent plant pathogens found on the resistance-induced concept (Buensanteai et al., 2009). Induced resistance in plants is involved in a plant innate immune system which conferred long-lasting resistance to a wide range of plant diseases. The SAR requires salicylic acid (SA) as a systemic signaling molecules associate with accumulation and produced defense-related enzymes and PR-proteins (Métrauxs, 2001). The induction of systemic resistance against root rot disease was carried in many kinds of plants. Therefore, the objectives of this research are to study the effect of SA formulation

(Zacha) on inducing defense mechanisms and observe the biochemical changes in induced cassava leaf tissues through SR-FTIR microscopy.

4.2 Materials and methods

4.2.1 Assessment of Zacha formulations on the growth of pathogen *Fusarium solani in vitro*

This design is used for experiments analysis of data with a split-plot in a completely randomized design (CRD) with four replications including Zacha11, Zacha15, Mancozeb, and a distilled water control., with four replications. The Zacha formulations are products of Bioactive Agro-Industry Co. Ltd., Thailand. A round hyphal slice of *Fusarium* at approximately 6 mm diameter was placed at the center of the petri plate containing PDA medium. Sterile Whatman filter paper discs at a diameter of 6 mm have dipped into a solution of Zacha at 500 ppm and put on the surface of the PDA medium near the edge of the petri plate. On the negative control, the hyphal round slices were identically handled, but distilled water was used instead of Zacha. Mancozeb (2,000 µg/ml) was used as a positive control. Repeat experiment at least 3 times until results are consistent. All petri dishes were kept for 7 days (room temperature). The hyphal growth was measured in diameter (mm) around the fungal discs of Fusarium.

4.2.2 Induced resistance against *Fusarium* root rot disease in cassava under greenhouse conditions

The experiment was conducted in a randomized complete block design (RCBD), four replications. Stalks of cassava cv. Rayong 72 were disinfected onto the surface with 1% NaOCl. Next, washed in with distilled water and dried at room

temperature. The stalks were then soaked for 10 minutes before planting with the solution of Zacha. After planting the cassava plants were inoculated with *Fusarium* suspension of 1 x 10^6 conidia mL^{-1}, such suspension was estimated for the root rot general symptoms after 60 days and mixing infested soil (100 mL/pot and plants) (Oliveira et al., 2013). Subsequently, at 15, 30, and 45 days after planting (DAP), cassava plants were sprayed with the solution of each elicitor. Each experiment was performed at least three times. Disease severity scores of *Fusarium* root rot were reported at seven days after inoculation (DAI), and disease score according to the following formula: 1 = no symptoms, 2 = stem, and root area affected by less than 25%, 3 = stem and root area affected by 25-50%, 4 = stem and root area affected by 51-75%, and 5 = stem and root area affected by more than 75% (modified from Sompong et al. 2013). After that calculated percentage of disease severity (DS) from Le Thanh et al. 2017. In reducing disease of disease severity for treated cassava may be adjusted from an equation: reduced severity of the disease = [(DS of the control group - DS of elicitor groups/DS of control group] × 100%. Then, the monitoring of biochemical changes associated with β-1,3-glucanase, chitinase, plant innate immunity accumulation of salicylic acid by using a spectrophotometer and characterized using SR-FTIR microspectroscopy.

4.2.2.1 Protein extraction for PR proteins analyses

Crude protein extract was extracted by the method as described by Prakongkha et al., 2013; Zur et al., 2013. Plant tissues (0.5 g) were ground in a cold mortar with a pestle using liquid nitrogen that homogenized mixed with 0.1 M sodium phosphate buffer (pH 6.5) (3 ml) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenate was centrifuged at 5000 rpm for 15 minutes at 4°C. The

supernatant was used as the enzyme extract for the assay of β-1,3-glucanase and chitinase activity. The protein content was examined using the standard Bradford protein assay (Bradford, 1976).

4.2.2.1.1 Determination of β-1,3 -glucanase and chitinase activity

The β-1,3-glucanase enzyme activity test was carried out in which a mixture containing of the protein homogenate (62.5 µl) mixed with an equal volume of 4% (w/v) laminarin in sodium acetate buffer pH 5.0 (0.05M) and incubated at 40°C for 10 min. After which stopped the reaction by adding dinitrosalicylic acid (375 µl) and dipping in boiling water for 5 min. The change in absorbance was recorded at 500 nm and the enzyme activity was expressed in nmol as the amount of released reducing sugar (D-glucose) per hour per milligram of soluble protein followed the method described by Pan et al., 1991; Prakongkha et al., 2013.

Assay of chitinase was done by the method as described by Reissig et al., 1955; Prakongkha et al., 2013. The reaction mixture consisting of the protein homogenate samples (0.4 ml) that an equal volume of colloidal chitin (0.1%, w/v) with sodium acetate buffer (pH 5.0) (0.05 M) after that incubated for 2 h at 37°C. The N-acetyl glucosamine (GlcNAc) produced from the reaction was determined by spectrophotometric reading at 585 nm.

4.2.2.2 Salicylic acid analysis in cassava plant

0.5 g of cassava plant tissues from each replication were randomly sampled, frozen with liquid nitrogen in a cold mortar, and ground into a fine powder with a cold pestle. Subsequently, 1 ml of extraction solution (90:9:1 volume of absolute methanol: glacial acetic acid: distillate water) was added to ground sample.

The crude extract was subsequently centrifuged at 12000 rpm for 20 min at 4°C and the supernatant was collected for the analysis. After that, the supernatant (500 µl) was mixed with an equal volume of ferric ammonium sulfate (0.02 M), incubated for 5 min at 30°C and the absorbance at 530 nm was read by a spectrophotometer to determine the SA content (Raskin et al., 1989; Prakongkha et al., 2013).

4.2.2.3 Characterizations of biochemical changes of cassava leaf tissues using Synchrotron-based FTIR microspectroscopy

The experiment was carried out in CRD, three treatments including the most effective Zacha, water control, and fungicide control. Cassava leaf samples were collected at 7 DAI, embedded in OCT compound, then rapidly frozen in liquid nitrogen. Next, cassava leaves were moved to a -80^{o}C freezer for the cryo-sectioning process. Then, we used a cryostat with each frozen sample which transversely cut at a thickness of approximately seven µm and put on a barium fluoride window (13x2 millimeter) for analysis. Preparation for SR-FTIR microspectroscopy data analysis was recorded at the beamline of BL4.1 IR Spectroscopy and Imaging at Synchrotron Light Research Institute (Public Organization), Thailand (Le Thanh et al., 2017; Thumanu et al., 2017).

4.2.3 Data analysis

All experiments of the research were repeated for four replication. The similar results were obtained in all repeats of each experiment. Statistical analyses were performed using analysis of variance (ANOVA) with Duncan's multiple range test (DMRT) for multiple comparisons, the finding is considered statistically significant (p-value ≤ 0.05). SPSS software (version 16 for window) was used for statistical analysis.

4.3 Results

4.3.1 Efficacy of Zacha formulations on *in vitro* growth of *Fusarium solani*

The results of the anti-fungal activity showed that Zacha11 and Zacha15 prototype formulations could inhibit the mycelial growth of *F. solani* at 3 days after putting fungal slice (Table 4.1). At this time point, the percentage inhibition of Zacha11 and Zacha15 was approximately 34.83% and 39.67%, respectively, both statistically significant to that of the water control treatment. Similarly, the fungicide Mancozeb inhibited *Fusarium* growth at approximately 34.67%, significantly higher when compared with the negative control at 24.83%. At 5 DAPFS that the treated with Zacha11 and Zacha15 could also inhibit mycelial growth compared to the non-treated control were 18.90 and 27.90%, respectively. Likewise, the percentage of mycelial growth inhibition treated with those elicitors was 11.04 and 10.37% at 7 DAPFS, respectively. (Table 4.1).

Table 4.1 Anti-fungal activity of salicylic acid (Zacha formulations) against *Fusarium solani* using a dual culture test

Treatments	Growth Inhibition (%)[1/]			
	1 DAPFS[2/]	3 DAPFS	5 DAPFS	7 DAPFS
Zacha 11 (500 ppm)	3.82±1.10 a	34.83±0.29 b	18.90±0.17 c	11.04±0.13 b
Zacha 15 (500 ppm)	3.82±1.10 a	39.67±0.29 a	27.90±0.17 b	10.37±1.28 b
Mancozeb	3.82±1.10 a	34.67±0.29 b	30.90±0.17 a	15.48±0.13 a
Distilled water control	0.00±0.10 b	0.00±0.00 c	0.00±0.00 d	0.00±0.00 c
F-test	**	**	**	**
cv%	2.41	0.07	0.06	0.35

[1/] Mean ± SE (standard error) followed by the same letter do not differ significant according to DMRT at P = 0.05. [2/] DAPFS: days after putting fungal slices.

4.3.2 Efficacy of Zacha on inhibiting *Fusarium* root rot disease severity under greenhouse conditions

Treatment of seed soak and foliar spray with the Zacha at the concentration of 500 ppm significantly reduced the severity of *Fusarium* RRD at 7 DAI compared with negative control, that again confirmed in a study of induction resistance has occurred. Under greenhouse conditions, Zacha 11 treatment showed the lowest disease severity (9.00%), followed by Zacha 15 and mancozeb treatments caused a significant decrease in disease severity percentage when compared with distilled water control treatment (13.00, 13.00, and 37.00%, respectively) (Table 4.2).

Table 4.2 Efficacy of salicylic acid (Zacha formulations) on the severity of Fusarium root rot disease caused by *F. solani* under greenhouse conditions at 7 days after inoculation

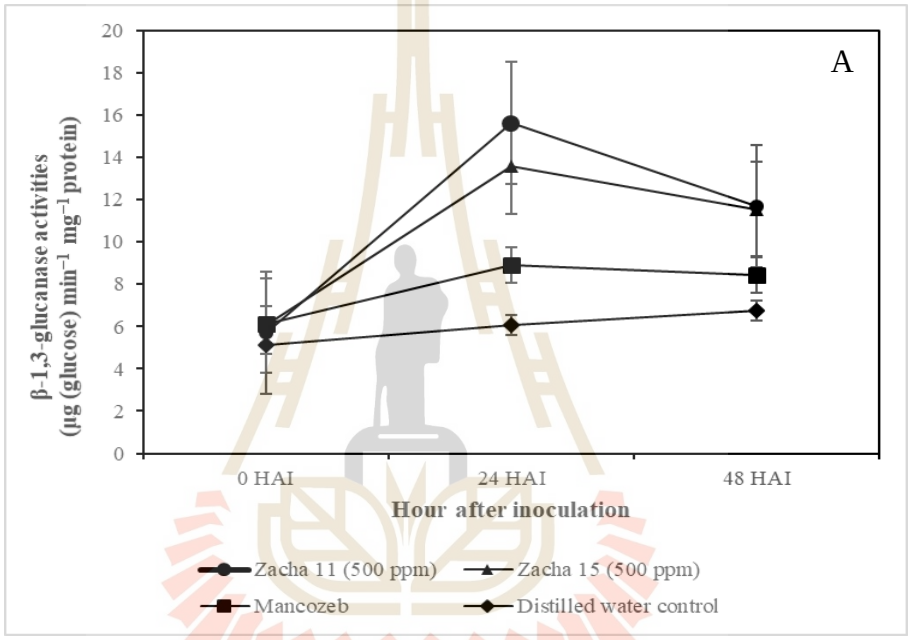
Treatment	Disease severity (DS, %)[1/]	Disease severity reduction (%)
Zacha 11 (500 ppm)	9.00±14.75 b	75.68
Zacha 15 (500 ppm)	13.00±5.70 b	64.86
Mancozeb	13.00±12.55 b	64.86
Distilled water control	37.00±14.83 a	-
F-test	*	
cv%	37.80	

[1/] Mean ± SE (standard error) followed by the same letter do not differ significant according to DMRT at $P \leq 0.05$

4.3.2.1 Effects of elicitors in the activities of PR proteins

Present study highlighted that PR-proteins of cassava plants treated with elicitors. The results indicate that the treatment with Zacha11 have an effect on

the β-1,3-glucanase activity increased significantly at 24 hours after inoculation was 15.62 µg (glucose) min^{-1} mg^{-1} protein when compare with control (Figure 4.1 A). Likewise, the chitinase activity increased significantly in Zacha11 treatments was 25.71 µmol (glcnac) min^{-1} mg^{-1} protein at 24 hours after inoculation than that of control plants (Figure 4.1 B).



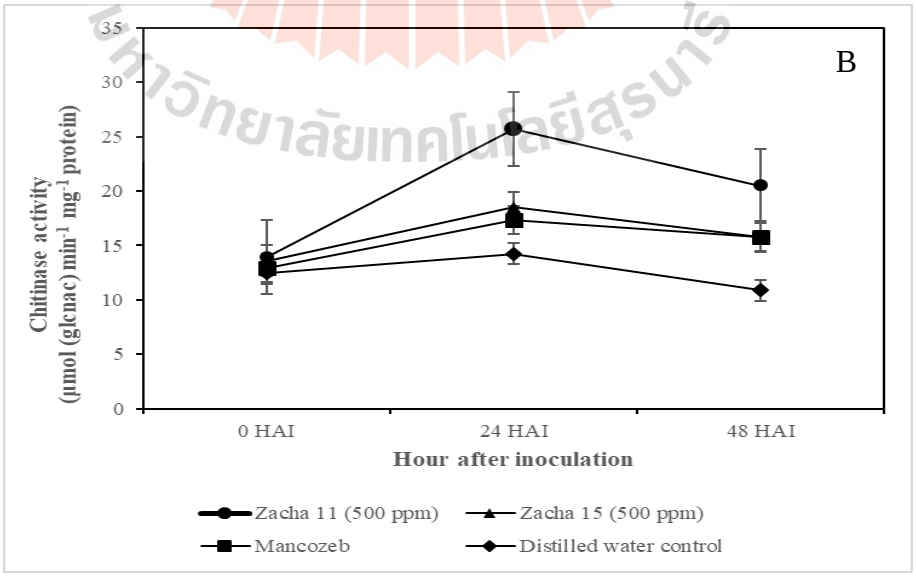


Figure 4.1 Change of pathogenesis related (PR) proteins activities in cassava plants against challenged inoculation with Fusarium root rot disease; A) determination of β-1,3-glucanase activity; B) chitinase activity

4.3.2.2 The accumulations of salicylic acid

Results indicated that in cassava plants treated with Zacha formulation, salicylic acid content significantly increased at 24 hours after inoculation of 69.95 µg g^{-1} fresh weight. In contrast, nontreated but pathogen-inoculated cassava showed an accumulation of SA was significantly smaller (68.08 µg g^{-1} fresh weight) (Table 4.3).

Table 4.3 Accumulation of salicylic acid activities in leaves of cassava with Zacha formulations foliar treatments and after challenge inoculation with cassava root rot disease

Treatments	Salicylic acid (µg g^{-1} /fresh weight)[1/]	
	During time (hr.)	
	0	24
Zacha 11 (500 ppm)	37.37±0.14 a	69.95±0.20 ab
Zacha 15 (500 ppm)	37.07±0.14 b	69.45±0.20 b
Mancozeb	37.30±0.45 a	70.16±0.21 a
Distilled water control	37.18±0.14 b	68.08±0.17 c
F-test	*	*
%CV	0.70	1.29

[1/] Mean ± SE (standard error) followed by the same letter do not differ significant according to DMRT at $P \leq 0.05$

4.3.2.3 Biochemical changes of induced cassava leaf tissues using SR-FTIR microspectroscopy

The conformational changes of protein amide at the range of wavelength 1700-1600 cm^{-1} indicated detail on protein secondary structure like a beta-sheet (peak at 1635 cm^{-1}), alpha-helix (peak at 1653 cm^{-1}), and beta-turn (peak at 1685 cm^{-1}) (Figure 4.2). Results also indicated that the average infrared spectra of epidermis tissues of cassava treated with Zacha11 were shown the biochemical components at the peak of 1737 cm^{-1} assigned to C=O ester vibration from pectin. Other vibrational peaks of 1658, 1607, and 1571 cm^{-1} were assigned to Amide I and Amide II. The cassava plants treated with Mancozeb had differences in the vibrational peaks of 1464, 1147, and 1112 cm^{-1}, which were assigned to C–H bending and C–O stretching of hemicelluloses and lignin (Table 4.4).

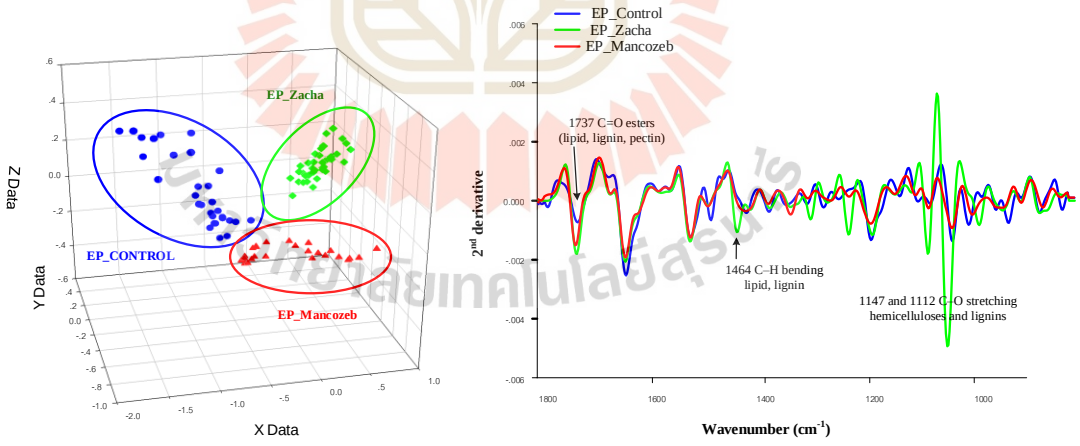


Figure 4.2 Principle component analysis (PCA) and average 2nd derivative spectrum of epidermis of leaf tissues

Table 4.4 General band assignments of the FTIR spectra of biological tissue

Spectral ranges	Peak name	Vibration peak assignments
3000-2800	C-H stretching vibration	Symmetric stretching vibration and C-H asymmetric of mainly lipid groups combining to protein
1740-1700	C=O esters	Stretching vibration of C=O ester of bond of pectin or their esters, lipid, lignin
1700-1600	Amide I	Amide I due to C=O stretching of α-helix protein, contribution from C-N stretching (C-N stretch (10%), N-H bending (10%), C=O stretch (80%))
1658, 1607 and 1571	Amide II	Amide II due to N-H bending and C-N stretching of protein (N-H bend (60%), C-N stretch (40%))
1470-1350	C-H bending	CH3 from mainly lignin and lipids and C-H bending from CH2
1320-1200	C-O Stretching hemicellulose and lignin	C-C, C-O skeletal
1147 and 1112	C-O-C glycoside	C-O-C glycoside ether mainly hemicelluloses

4.4 Discussion

F. solani was extremely a common root rot pathogen in cassava plants (Bodah, 2017). The *Fusarium* is genetically diverse and universal in soil, with a frequency of isolation at approximately 45% of root rot-infected cassava fields (Aigbe and Remison 2010). Application of exogenous SA (Zacha) prototype formulations through seed and foliage could induce the resistance in cassava plants against *Fusarium* RRD. This disease resistance as dependent upon formulations concentrations was already similar observation for comparisons between different pathosystems (Faoro et al., 2008). Obtaining the best of efficiency induction was achieved that the concentration at 500 ppm of Zacha formulations was used to compare with Mancozeb and water control. Zacha11 formulation could reduce disease severity, similar efficacy compared with the untreated control. In this research, the inhibition percentage of induced treatments was at approximately 35-40%. The results are in corresponding to some studies on induced resistance against *Fusarium* disease in banana plants at 53.1% (Fernández-Falcón et al., 2003), in tomato plants at 5.3-85.8% (Amer et al., 2014). The possible mode of action to inhibit the pathogen might be that the Zacha11 could be directly attacking the pathogen in conjunction with the induction of systemic resistance against *F. solani*. We found that the highest salicylic acid accumulation resulted from the treated and a foliar spray with Zacha11, the response was more pronounced in plants inoculated with root rot disease, suggesting pathogen recognition and a subsequent plant defense response. Increased expression of the SA marker after pathogen challenge was similar to the induction of SA and jasmonic acid activities that play a role in protecting plant cell, indicative of plant priming for resistance and elevated production of some defense

enzymes peroxidase (POX) and phenylalanine ammonia lyase (PAL) proving SAR against root rot disease caused by *F. solani* (Buensanteai et al., 2009 Mandal et al., 2009; War et al., 2011).

These studies also showed that the treated and a foliar spray with Zacha11 systemic acquired resistance (SAR) against Fusarium root rot of cassava. SAR by Zacha11 (salicylic acid formulation) involves increasing physical of the host cell wall, inducing defense mechanisms and causing physiological and biochemical changes leading to the synthesis of β-1,3-glucanase and chitinase in cassava tissues (van Loon and van Strien, 1999; Chen et al., 2000; Kim et al., 2001; Ramamoorthy and Samiyappan, 2001). Our results demonstrate that Zacha11-treated plants showed higher levels of resistance connect with enhanced activity in β-1,3-glucanase and chitinase that lead to the direct expression of resistance mechanisms (Smit and Dubery, 1997; Egea et al., 2001; Körösi et al., 2011). According to Prakongkha et al. (2013) that show activities level of PR-proteins increased significantly seven days after treatment and much more seven days after challenge inoculation with pathogen.

The SR-FTIR microspectroscopy has been applied as a tool to characterize changes in the biochemical of tissues at a high resolution and sensitivity, and analysis was used to the spectral differences of epidermal tissues characterized. Moreover, SR-FTIR microspectroscopy identified compositionally and concentration changes of proteins, polysaccharides, pectins, and lipids of cassava leaves which characterized the roles of plant induction of defense by Zacha11 formulation. The obtained results of FTIR spectra interestingly revealed that cassava leaves treated with Zacha11 and challenge inoculated with *F. solani* exposed an alteration of pectins and lignins, essential components of polysaccharides, and of amide I structure of a protein. There

are many results on the importance of their enhanced levels in plant resistance to various diseases caused by phytopathogens. Still, their role in defense responses has been described in recent publications. The lignin and pectin alteration plays a crucial role in disease resistance to the cassava cell-wall reinforcement in cassava leaf tissues (Heil and Bostock, 2002). Also, the conversion of ∝-helix structure of amide I protein into the type of β-sheet structure leads to expose the high-affinity binding site for receptors on cell membranes to signal transduction systemically (van't Slot et al. 2003). Besides, increased levels of carbohydrates and proteins were recorded and shown the relationship between elicitors and host plants, including tomato and safflower against the infection and invasion of root rot *Fusarium* (Amer et al., 2014). The results of the study were consistent with Thepbandit et al. (2021), salicylic acid elicitor (100 ppm) control bacterial leaf blight caused by *Xoo* and the changes on the biochemical components on rice plants by using Synchrotron radiation-based Fourier Transform Infrared (SR-FTIR) microspectroscopy revealed that the cell membrane identified as fatty acid (2825 cm^{-1}), nucleic acid, and phospholipid groups (1162 and 1040 cm^{-1}) had spectra higher than the untreated samples. This finding is supported by previous studies such as Thumanu et al., 2017. The findings of this research are consistent with previous studies following Le Thanh et al. (2017), the present study, demonstrates that the higher ratios of pectin and lignin were observed in plants sprayed with salicylic acid. SA treated on rice plants shown higher β-sheet structure and lipids.

In conclusion, salicylic acid content was correlated with the resistance level to disease severity of root rot, related to the expression of SAR genes, and can determine the selective activation of defense responses during pathogen infection and invasion that can

change physiological, biochemical through molecular levels of the plant. Elicitor formulations (Zacha) could cause the structural changes of the cassava epidermal cells. In a further study, leaf samples were collected at different harvesting times to understand the interactions of plant disease compare with spectral differences that may be appeared at the same time as growth, development, or incidence disease in cassava.

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CHAPTER V

Efficacy of salicylic acid and a bacillus bioproduct in enhancing growth of cassava and controlling root rot disease

ABSTRACT

The research aims to evaluate the efficiency of the formulated salicylic acid and a bacillus bioproduct in controlling cassava root rot disease and enhancing its growth under greenhouse and field conditions. The results revealed that cassava stalk soaking and foliage spraying with 500 mg/L of SA in Zacha11 formulation or a bioproduct of JN2-007 *Bacillus subtilis* could increase cassava plant growth at 60 days after planting under a greenhouse condition. In addition, Zacha11 and JN2-007 treated cassava plant cv. CMR-89 gave significantly higher growth parameters than those treated with bacillus commercial products and mancozeb. Among them, Zacha11 gave the maximum stem height (11.67 cm), root length (18.91 cm) and number of roots (49.50) compared to water as a negative control which gave 4.66 cm, 9.92 cm and 21.83, respectively. Fusarium root rot severity indices of all treatments were reduced but not significantly different. Moreover, at 7 days after inoculation, plants treated with Zacha11 and JN2-007 had 53.33% and 48.33% disease severity reduction respectively compared to the 66.67 % reduction for plants treated with mancozeb which experiment done under a field condition at 2 locations using two different cassava varieties and naturally occurring cassava diseases yielded similar

results in that all the elicitors could suppress root rot disease in both cultivars as well as bacterial leaf blight. They also reduced the severity of anthracnose, and brown leaf spot found in the field. Between Rayong 72 and CRM-89 plants treated with Zacha11, only Rayong 72 gave significantly higher yield parameters than those treated with other elicitors. In location 2, Rayong 72 treated with Zacha11 gave 8.4 tubers per plant, 4.95 kg tuber weight per plant, 49.17 kg/10 m^2 tuber yield, and 20.23% starch content compared to that of the negative control which were 3.49, 1.73 kg/plant, 17.33 kg/10 m^2, and 14.80% respectively. Thus, it is possible that our formulated salicylic acid and Bacillus bioproduct can be used for controlling of cassava diseases and increasing cassava productivity under farmer field.

5.1 Introduction

In Thailand, cassava has been recognized as one of the most important major economic crops (Chaisinboon and Chontanawat, 2011; Jakrawatana et al., 2015; Treesilvattanakul, 2016). The average cassava yield of the country has been approximately 20 ton/ha, which is lower than the expected official yield. The constrains contributing to yield reduction are many, but those from several fungal, bacterial and viral diseases have been estimated to be as high as 20-80% (Camila et al., 2018; Piyachomkwan and Tanticharoen, 2011). One of the most severe diseases in cassava is root rots especially *Fusarium* spp., *Lasiodiplodia theobromae*, *Neoscytalidium hyalinum*, *Sclerotium rolfsii* and *Phytophthora* spp (Charaensatapon et al., 2014; Duchanee, 2015). The conventional practices for cassava disease control in Thailand have been using chemicals, which are effective to a certain extent but directly affect the environment and the health of consumers (Panuweta et al., 2013;

Sriket et al., 2015). Therefore, resistance elicitors could be a better alternative and have been widely assessed to manage plant diseases. *Bacillus subtilis*, can enhance growth and have shown their ability to induce disease resistance in several economic crops (Prathuangwong and Kasem, 2004; Prathuangwong and Buensanteai, 2007). Exogenous salicylic acid (SA) has also been used as a promoter for plant growth and found to generate a wide range of metabolic and physiological responses in plants thereby affecting development and their growth (Hayat and Ahmad, 2007; Le Thanh et al., 2017). The result of development and plant growth can increase the amount of harvested plant yield and indirectly production. It also can trigger the SA-dependent signaling pathway in plant defence against pathogens in plant cells and stimulates the induction of plant defence mechanisms in numerous plants (Hayat et al., 2010; Sangpueak et al., 2018). Although there have been numerous reports in using several resistance elicitors for managing plant diseases, none of them have been tested on the cassava root rot disease. So, this research was studied on the role of salicylic acid and a bacillus bioproduct in controlling cassava root rot disease under both greenhouse and field experiments.

5.2 Materials and methods

5.2.1 Preparation of the salicylic acid elicitor (Zacha)

An exogenous salicylic acid (Zacha), a product of Bioactive Agro Industry Co., Ltd was used in this experiment. Zacha is a prototype formulation developed by the Plant Molecular Biology Laboratory, Suranaree University of Technology, Thailand.

5.2.2 Preparation of the bioproduct elicitor and culture condition of *Fusarium solani*

Bioproduct (*B. subtilis:* JN2-007) is a product of Bioactive Agro Industry Co., Ltd and developed by Nikaji et al. (2015) from the Plant Molecular Biology Laboratory, Suranaree University of Technology, Thailand. The product was adjusted to $1x10^7$cfu/ml concentration with sterile distilled water. *F. solani* isolate SHRD was obtained from stock culture in potato dextrose broth (PDB) with 30% glycerol stored at -80°C. The culture was transferred onto a potato dextrose agar (PDA) plate then incubated at 28 ± 2°C for 7 days. Subsequently, agar blocks of the growing mycelia were transferred into the PDB flask (125 ml) and incubated in a shaker at room temperature for three days, and used for the experiment. (Malandrakis et al., 2018; Patil et al., 2011).

5.2.3 Evaluation of the elicitors' efficacy in promoting plant growth and reducing root rot severity under greenhouse condition

The experiment was conducted in a randomized complete block design (RCBD), four replications. Cassava stalks (accession number CMR-89) were surface-sterilized with 1% NaOCl. Then, washing with sterile water and let dry. The experiment was set up as 2 identical sets, each set consisted of six treatments including the solution of Zacha11 elicitor (salicylic acid 500 mg/L), JN2-007 elicitor ($1x10^7$ cfu/ml concentration), 3 positive controls (Larminar; $1x10^9$ cfu/mg WP, Sarcon; SA 1,000 mg/L, and Mancozeb 80% WP) and negative control (water) treatment. The cassava stalks were soaked in the solutions for 10 minutes before planting in black plastic pots (12 inches diameter), in four replications with three stalks per each replication. At 15, 30 and 45 days after planting (DAP), the cassava

plants were sprayed again with the same solution of each treatment. Subsequently at 60 DAP, one set of the plants were inoculated with *Fusarium* suspension of 1 x 10^6 conidia/ml by mixing it into the soil (100 mL/pot), while plants in the other set were uprooted and growth parameters were recorded. Disease severity scores based on the affected area are as follows: 1 = no symptoms, 2 = area affected by less than 25%, 3 = area affected by 25-50%, 4 = area affected by 51-75%, and 5 = area affected by more than 75% (Duchanee, 2015; Onyeka et al., 2005; Sompong et al., 2012; Wokocha et al., 2010). Then the percentage of disease severity index was calculated using the formula slightly modified from that of Le Thanh et al. (2017) as follows: Disease severity (%) = [Σ (class frequency × score of rating class)]/[(total number of scored plants) × (maximum disease score)] × 100. The root rot disease reduction was assessed at seven days after inoculation (DAI) and was calculated using the formula: Reduced disease severity of the disease = [(DS of the control group - DS of elicitor treated groups/DS of the control group] × 100%. The severity assessment was done by measuring the affected cassava root area (Duchanee, 2015; Le Thanh et al., 2017; Sompong et al., 2012). The experiment was conducted three times.

5.2.4 Evaluation of the elicitors' efficacy in promoting plant growth and reducing root rot severity under field condition

Field experiments are experiments carried out at Suranaree University of Technology, Nakhon Ratchasima, Thailand, in two locations; DMS: 14°52'44.7"N 102°00'14.6"E and 14°51'43.2"N 102°01'57.5"E. The experimental areas were thoroughly ploughed two times and approximately 45 cm high beds were made. Cassava stalks of cv. Rayong 72 and accession number CMR-89 (aged 8-12 months old with 15 cm length) were then planted vertically with 1×1 m spacing. Weed

management was undertaken and granular fertilizer (15-15-15) was applied at 125 kg/ha (1, 2 and 3 months after planting (MAP) to all treatments). The same concentrations of elicitors tested under the greenhouse condition were also used under the field condition. The experiment was conducted in a randomized complete block design (RCBD), four replications. Application of the elicitors was done by stalk soaking for 10 minutes before planting and spraying to cassava leaves (125 L/ha) three times at 1, 2 and 3 MAP. Water was used as a negative control (untreated) whereas Larminar (commercial bacillus), Sarcon (commercial salicylic acid) and Mancozeb, served as positive controls. The cassava plants were recorded for severity from natural infection using the disease scores similar to that described in the previous experiment. After that, number of tubers (tubers/plant), tuber weight (kg/plant), fresh tuber yields and starch contents (%) were collected from the size of sampled area 1×10 m^2/replications. All treatment data were collected at 9 months after planting (MAP) (Polthanee et al., 2014; Promkhambut, 2016; Terry and Hahn, 2009). Each experiment was performed at least three times.

5.3 Results

5.3.1 Elicitors' efficacy in promoting plant growth and reducing root rot severity under greenhouse condition

Enhancing effect of the elicitors on growth and reduction of disease severity on cassava accession number CMR-89 cultivars were observed. The cassava plants treated with Zacha11 (500 mg/L) and JN2-007 ($1x10^7$cfu/ml) had all growth parameter significantly higher than that of the water negative control and the commercial elicitors and mancozeb (Table 1). At 60 DAP, the cassava plants treated

with Zacha11 and JN2-007 gave the maximum stem height (11.67 cm and 9.71 cm), root length (18.91 cm and 17.91 cm) and number of roots (49.50 and 47.16); significantly different from that of the negative control treatment (4.66 cm, 9.92 cm and 21.83, respectively). The severity and its reduction of Fusarium root rot were assessed on cassava roots. The results indicated that root rot severity on cassava treated with Zacha11 (35.00±5.77%) or JN2-007 (38.75±4.87%) were not different from those treated with commercial elicitors but differently lower than that of the negative control. (75.00±15.81%) (Table 5.1). Similar results were observed in root rot reduction percentage.

Table 5.1 Efficacy of elicitors application in promoting cassava plant growth and reducing Fusarium root rot severity under greenhouse condition

Treatment[1/]	Growth parameter [2/]			Disease severity index[2] (%)	Disease reduction[2] (%)
	Stem height (cm.)	Root length (cm.)	Number of roots		
T1	11.67 ± 1.59 a	18.91 ± 2.31 a	49.50 ± 1.44 a	35.00 ± 5.77 b	53.33 ± 7.70 b
T2	9.71 ± 0.91 a	17.91 ± 1.16 a	47.16 ± 1.52 a	38.75 ± 4.78 b	48.33 ± 6.38 b
T3	7.25 ± 1.54 ab	13.75 ± 0.86 ab	40.22 ± 0.453 ab	40.00 ± 4.08 b	46.67 ± 5.44 b
T4	8.83 ± 1.19 ab	13.50 ± 0.90 ab	39.50 ± 1.16 ab	37.50 ± 6.45 b	50.00 ± 8.61 b
T5	7.33 ± 1.15 ab	13.41 ± 0.90 ab	37.83 ± 1.69 ab	25.00 ± 4.08 c	66.67 ± 5.44 a
T6	4.66 ± 1.23 b	9.92 ± 1.08 b	21.83 ± 1.80 b	75.00 ± 15.81 a	0
F-test	*	*	*	**	*
CV (%)	15.42	8.27	3.76	16.32	12.67

[1/] (T1) Zacha11, (T2) JN2-007, (T3) commercial bacillus, (T4) commercial salicylic acid (T5) mancozeb and (T6) water used as a negative control. The elicitors and mancozeb were applied to cassava Acc. No CMR-89 by stalk soaking and foliage spray at 15, 30 and 45 days after planting *Fusarium solani* inoculation was done by mixing into the soil at 60 days after planting. [2/] Means in the column followed by the same letter are not significantly different according to Duncan's multiple range test at P = 0.05. Each value represents a mean of four replicates.

5.3.2 Effects of the elicitors on growth, diseases and yield under field condition

In location 1, disease severity indices of brown leaf spot and anthracnose on cassava cv. Rayong 72 were not different in all treatments indicating the no effect of elicitors and mancozeb on these two diseases (Table 5.2).

Table 5.2 Efficacy of elicitors treatment on severity of naturally occurring diseases on two cassava cultivars under field condition of location 1 in Nakhon Ratchasima.

Treatment1/	Disease severity index (%)2/							
	Cassava cv. Rayong 72				Cassava accession number CMR-89			
	Brown leaf spot	Anthracnose	Bacterial blight	Root rot	Brown leaf spot	Anthracnose	Bacterial blight	Root rot
T1	33.33	42.67	0.00 b	0.00 b	14.67 ab	23.33	0.00 b	0.00 b
T2	37.33	50.67	0.00 b	0.00 b	17.33 ab	27.33	0.00 b	0.00 b
T3	21.33	42.00	0.00 b	0.00 b	15.33 ab	22.67	0.00 b	0.00 b
T4	20.00	39.67	0.00 b	0.00 b	15.00 ab	20.00	0.00 b	0.00 b
T5	18.67	37.33	0.00 b	0.00 b	7.33 b	17.33	0.00 b	0.00 b
T6	38.00	51.33	11.33 a	14.67 a	25.33 a	37.33	7.33 a	24.67 a
F-test	ns	ns	*	*	*	ns	*	*
CV (%)	30.64	13.45	38.28	40.07	30.30	29.15	28.59	30.87

1/ Cassava plants were treated with (T1) Zacha11, (T2) JN2-007, (T3) commercial bacillus, (T4) commercial salicylic acid (T5) mancozeb and (T6) water used as a negative control under field conditions; DMS: 14°52'44.7"N 102°00'14.6"E. 2/ Means in the column followed by the same letter are not significantly different according to Duncan's multiple range test at P = 0.05. Each value represents a mean of four replicates.

For bacterial blight and root rot diseases, the indices were 11.33% and 14.67%, respectively, on plants in the negative control treatment, while symptoms of the two diseases were not observed on the elicitors and mancozeb treated cassava. On cassava cv. CMR-89, severity indices of brown leaf spot on the elicitor treatments including Zacha11 (14.67%), commercial SA (15.33%) and JN2-007 elicitors (17.33%), were at the middle position, but no statistical difference between the negative control (25.33%) and the mancozeb treated plants (7.33 %). No differences were observed for the anthracnose disease severity on cassava of all treatments. Similar to what observed on Rayong 72, bacterial blight and root rot diseases were not found on the elicitor-treated CMR-89, while plants in the negative control treatment showed 7.33% and 24.67% disease severity, respectively (Table 5.2). In location 2, severity indices of brown leaf spot on Rayong 72 were not different in all treatments; but for anthracnose, plants treated with Zacha11 gave the lower severity index (50.00%), significantly different to the negative control (72.67%) (Table 5.3). For bacterial blight and root rot, similar results to that found in location 1. No diseases were found on the treatments of elicitors and mancozeb, but severity indices of 7.33 and 23.33% were recorded on plants in the negative control treatment, respectively. The reactions of treated cassava cv. CMR-89 to brown leaf spot and anthracnose disease severities of all elicitor treatments were not significant or significantly different to that of the negative control treatment. However, indices of elicitor treatments of bacterial blight and root rot were zero percent, statistically lower than those of the negative control (Table 5.3).

For growth and yield parameters in location 1; in Rayong 72, Laminar and Sarcon gave the high plants (165.93 and 165.47cm) but there was no statistical difference to other treated ones, except that of the negative control treatment which

gave the lowest height (150.27 cm). In terms of tuber quantity in Rayong 72, JN2-007 gave the highest number of tubers per plant (8.80), seconded by Zacha11 (8.60) and commercial SA (8.53), followed by macozeb (6.80), which were significantly higher than that of the negative control (2.33). There were no differences among the tuber weight (5.11-7.84 kg/plant) and yields (51.07-78.40 kg/10 m^2) of plants treated with elicitors and mancozeb, but they were all significantly higher than negative control (2.11 kg/plant and 21.07 kg/10 m^2). For the starch content, Zacha11 treated plants gave the highest starch percentage (25.20%), significantly different to the negative control treatment at only 14.17%. For CMR-89, Zacha11, JN2-007, Laminar, Sarcon and mancozeb treatments did not differently affect plant height of CMR-89 (131.53, 124.00, 129.80, 124.50 and 125.00 cm, respectively) but the treated plants were all significantly taller than those in the negative control treatment. There were no differences of tuber weight per plant and tuber yield in plants of all treatments including those of the negative control. For starch content Zacha11 treated plants also gave the highest compared to those of the other treatments (Table 5.4). Similar results were also found in location 2, in which elicitors treatment seem to have less effect on CMR-89's height but commercial SA appeared to give the highest Rayong 72 plants (147.13 cm) compared to that of the negative control (122.60 cm) (Table 5.5). In terms of yields, Rayong 72 plants in all treatments gave significantly higher tuber number per plant than that in the negative control treatment but no differences were found in CMR-89 in all treatments including that of the negative control. Likewise, there were no differences in all other yield parameters of CMR-89 treated plants compared to that of the negative control; but for Rayong 72, plants treated with Zacha11 gave the highest tuber weight per plant (4.95 kg), tuber yield (49.47 kg/ha), and starch content (20.23%)) (Table 5.5).

Table 5.3 Efficacy of elicitors treatment on severity of naturally occurring diseases on two cassava cultivars under field condition of location 2 in Nakhon Ratchasima.

Treatment1/	Disease severity index (%)2/							
	Cassava cv. Rayong 72				Cassava accession number CMR-89			
	Brown leaf spot	Anthracnose	Bacterial blight	Root rot	Brown leaf spot	Anthracnose	Bacterial blight	Root rot
T1	34.00	50.00 bc	0.00 b	0.00 b	32.00	68.67 ab	0.00 b	0.00 b
T2	34.00	72.67 a	0.00 b	0.00 b	36.67	67.33 ab	0.00 b	0.00 b
T3	31.33	60.67 ab	0.00 b	0.00 b	37.33	75.33 a	0.00 b	0.00 b
T4	32.67	61.33 ab	0.00 b	0.00 b	29.33	67.07 ab	0.00 b	0.00 b
T5	26.67	36.00 c	0.00 b	0.00 b	26.67	62.00 b	8.00 ab	0.00 b
T6	45.33	72.67 a	7.33 a	23.33 a	41.33	79.33 a	8.67 a	23.33 a
F-Test	ns	**	*	*	ns	*	*	*
CV (%)	20.06	26.86	28.58	30.11	16.16	9.69	34.28	20.11

1/ Cassava plants were treated with (T1) Zacha11, (T2) JN2-007, (T3) commercial bacillus, (T4) commercial salicylic acid (T5) mancozeb and (T6) water used as a negative control under field conditions; DMS: 14°51'43.2"N 102°01'57.5"E. 2/ Means in the column followed by the same letter are not significantly different according to Duncan's multiple range test at P = 0.05. Each value represents a mean of four replicates.

Table 5.4 Effect of elicitors on plant height, number of tubers, tuber weight, fresh tuber yields and starch contents on two cassava cultivars under field condition of location 1 in Nakhon Ratchasima.

Treatment[1/]	Cassava cv. Rayong 72[2/]					Cassava accession number CMR-89[2/]				
	Plant height (cm.)	Number of tubers (tubers/plant)	Tuber weight (kg/plant)	Yields (kg/10 m^2)	Starch contents (%)	Plant height (cm.)	Number of tubers (tubers/plant)	Tuber weight (kg/plant)	Yields (kg/10 m^2)	Starch contents (%)
T1	159.53 ab	8.60 ab	6.57 a	65.73 a	25.20 a	131.53 a	9.87 a	4.76	47.60	20.40 a
T2	157.67 ab	8.80 a	7.84 a	78.40 a	17.47 ab	124.00 a	8.73 a	4.33	43.33	13.97 b
T3	165.93 a	8.53 ab	5.95 a	59.53 a	18.10 ab	129.80 a	9.93 a	3.72	37.20	15.80 ab
T4	165.47 a	8.55 ab	5.84 a	58.36 a	17.39 ab	124.50 a	8.87 a	3.32	33.24	15.63 ab
T5	160.87 ab	6.80 b	5.11 a	51.07 a	19.43 b	125.00 a	9.47 a	2.92	29.20	14.47 b
T6	150.27 b	2.33 c	2.11 b	21.07 b	14.17 c	109.87 b	4.60 b	2.36	23.60	12.30 c
F-test	*	**	*	*	**	*	*	ns	ns	**
CV (%)	3.59	39.03	37.99	37.99	21.37	6.88	26.32	29.76	29.76	19.95

[1/] Cassava plants were treated with (T1) Zacha11, (T2) JN2-007, (T3) commercial bacillus, (T4) commercial salicylic acid (T5) mancozeb and (T6) water used as a negative control under field conditions; DMS: 14°52'44.7"N 102°00'14.6"E. [2/] Means in the column followed by the same letter are not significantly different according to Duncan's multiple range test at P = 0.05. Each value represents a mean of four replicates.

Table 5.5 Effect of elicitors on plant height, number of tubers, tuber weight, fresh tuber yields and starch contents on two cassava cultivars under field condition of location 2 in Nakhon Ratchasima.

Treatment[1/]	Cassava cv. Rayong 72[2/]					Cassava accession number CMR-89[2/]				
	Plant height (cm.)	Number of tubers (tubers/plant)	Tuber weight (kg/plant)	Yields (kg/10 m^2)	Starch contents (%)	Plant height (cm.)	Number of tubers (tubers/plant)	Tuber weight (kg/plant)	Yields (kg/10 m^2)	Starch contents (%)
T1	132.40 bc	8.40 a	4.95 a	49.47 a	20.23 a	134.27 ab	9.40	2.93	29.33	17.80
T2	129.80 bc	8.20 a	3.88 ab	38.80 ab	15.73 b	140.47 a	9.27	2.92	29.20	15.03
T3	147.13 a	7.20 a	3.00 bc	30.00 bc	15.00 b	128.53 b	8.73	2.85	28.53	17.20
T4	132.00 bc	7.09 a	3.15 bc	31.49 bc	15.05 b	128.43 b	7.57	2.47	24.36	15.48
T5	138.27 ab	6.67 a	2.60 bc	26.00 bc	14.87 b	130.13 b	7.67	2.59	25.87	15.80
T6	122.60 c	3.49 b	1.73 c	17.33 c	14.80 b	125.00 b	7.47	2.35	23.47	13.77
F-test	**	*	**	**	*	*	ns	ns	ns	ns
CV (%)	6.79	35.67	38.08	38.08	14.62	4.51	28.76	30.43	30.43	25.41

[1/] Cassava plants were treated with (T1) Zacha11, (T2) JN2-007, (T3) commercial bacillus, (T4) commercial salicylic acid (T5) mancozeb and (T6) water used as a negative control under field conditions; DMS: 14°51'43.2"N 102°01'57.5"E. [2/] Means in the column followed by the same letter are not significantly different according to Duncan's multiple range test at P = 0.05. Each value represents a mean of four replicates.

5.4 Discussion

From greenhouse experiment, the results clearly show that cassava stalk soaking and foliage spraying with 500 mg/l of SA in Zacha11 formulation or a bioproduct of JN2-007 *Bacillus subtilis* could increase cassava plant growth at 60 DAP. Zacha11 and JN2-007 treated cassava plants cv. CMR-89 gave significantly higher growth parameters than those treated with commercial products and mancozeb. Such effects were also observed under field conditions but more pronounced in Rayong 72 than in CMR-89. Under the field condition of location 2, Rayong 72 treated with Zacha11 gave the highest values of all yield parameters assessed except that of stem height. Such finding indicates differential response of cultivars to the elicitor treatment both in terms of types, formulation, and concentration of the active ingredients. It is clearly showed in stem height parameter in that commercial SA which has twice as much SA (1000 mg/ml) compared to that of Zacha11 (500 mg/ml) gave the tallest stem height to Rayong 72 but not to CMR-89. The higher concentration of SA recommended in the commercial SA formulation seem to have no additive effect on tuber yield and starch content of Rayong 72 because the 500 mg/l SA rate in our formulation performed better in terms of increasing the yield. Lower concentration of SA not only save the cost of treatment but also lower down the risk of SA phytotoxicity. SA has been known to be phytotoxic to many crops when used at high concentration. Enhancing effects of exogenous SA application on plant growth have been reported in several crops (Gawade and Sirohi, 2011; Gharib, 2006; Khandaker et al., 2011); therefore, our finding can add up cassava into the list of such crops. Also, salicylic acid (SA) is one plant hormone that plays an important role in plant defence and has a key role in the signal transduction pathways (Vallad and Goodman, 2004). Moreover, these defense genes associated with defence enzymes in plant cells were activated by early and

secondary signaling molecules and then trigger resistance against pathogen infection in the plant (Buensanteai et al., 2009; Hinarejos et al., 2016).

From our greenhouse experiment, all the tested elicitors were equally effective in reducing *Fusarium* root rot severity but slightly less compared to that of Mancozeb. However, if we consider the benefit of SA and *Bacillus* as growth or yield promoter which showed under the field experiment, as well as the drawback of using chemical fungicides, such marginal effects of fungicides can be ruled out. Under the field condition; because the *Fusarium* was not inoculated therefore the root rot symptoms observed on plants in the negative control treatment could be from many other soil-borne pathogens. The absence of root rot and bacterial blight symptoms observed on the elicitors treated plants indicates that the nature of SA and *Bacillus* in inducing disease resistance is broad-spectrum to cassava diseases. It has been known that exogenous SA application could decrease leaf symptoms caused by *Glomerella cingulata* in apple plants (Zhang et al., 2016). Chávez-Arias et al. (2020) reported that foliar spray application of 100 mg/L of SA elicitor in cape gooseberry seedlings reduced disease severity and vascular wilt caused by *F. oxysporum*f.sp. *physali*. Exogenously applied salicylic acid (0.2 mM) reduced *Rizoctonia solani* infection by 73% on potato tubers in the greenhouse (Hadi and Balali, 2010).

Similarly, *Bacillus* spp. are well documented for their capability in enhancing plant growth and inducing systemic resistance (Kloepper et al., 2004). In addition, it could physiological systems to promote growth, trigger plant biochemical and colonize in roots (Prathuangwong and Kasem, 2004; Ryu et al., 2004; Prathuangwong and Buensanteai, 2007). These results are similar to that studied by Song (2014) who suggested that the *Bacillus* species had good potential as a microbial agent for the

biocontrol of the ginseng root rot caused by *Fusarium cf. incarnatum*. Hinarejos et al. (2016) showed that *B. subtilis* IAB/BS03 could reduce disease severity of two foliage diseases including *Botrytis cinerea* and *Pseudomonas syringae* on tomato. Root dip treatment with *B. subtilis* formulations showed an increase in root length when compared to untreated control while disease control was achieved better with seed application (Narasimhan and Shivakumar, 2016). Meanwhile, soaking and foliar spray application of salicylic acid and beneficial bacteria have an increase of yields of many crops based on Hadi and Balali (2010) on potato, Javaheri et al. (2012) on tomato, Jonathan et al. (2015) on cassava and Yildirim et al. (2006) on cucumber. These increases in yields may be closely linked to the increase in plant growth characteristics, i.e. plant height, number of roots, root weight and yields. According to Tucuch-Haas et al. (2017), salicylic acid regulates plant growth and may increase crop yield when applying 1 µM increases the production of total dry biomass, grain yield, as well as nutrients contents in tissue and grain when the canopy of maize seedlings is sprinkled with SA as compared to the control under field conditions.

Normally, the elicitor formulations of Zacha11 and JN2-007 could increase cassava growth, tuber weight, yield, and starch contents suppress root rot disease as well as bacterial leaf blight. Moreover, planting locations (different soil types and environment) and cultivars of cassava are one of the important factors that affect the increased productivity. However, to obtain high and sustainable yields, the crop should be well managed by the use of high-yielding varieties and good production practices. More research is needed to elucidate further the physiological and biochemical of cassava productivity in relation to inducers, particularly under field conditions.

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CHAPTER VI

OVERALL DISCUSSION AND CONCLUSION

6.1 Overall discussion

There are two main objectives in this research: (1) the efficacy of salicylic acid formulation for induced resistance against Fusarium root rot in cassava plants was evaluated (Chapter III) by investigate the effect of our formulated salicylic acid and a bacillus bioproduct for inducing resistance on H_2O_2 content, peroxidase, polyphenol oxidase, and catalase activities against Fusarium root rot disease in cassava; (2) to study the induced resistance mechanisms in cassava plants against the CRRD after being treated with the elicitors (Chapter IV and V) including (2.1) *in vitro*, inhibition of growth of Fusarium solani was surveyed by using salicylic acid prototype formulations. Then, monitoring of biochemical changes associated with plant innate immunity (PR-proteins activity) and salicylic acid analysis by using a spectrophotometer and their characterization using SR-FTIR microspectroscopy and (2.2) under both greenhouse and field conditions, the efficacy of exogenous salicylic acid and a bacillus bioproduct in controlling cassava root rot disease was evaluated.

Evaluation of physical changes and phytotoxicity of Zacha elicitor formulations. Evaluation of physical changes and phytotoxicity of Zacha elicitor formulations. During storage at 9 months could be defining color groups of elicitors were two groups (Yellow and non-color group). In the study, Zacha's elicitor formulations have a low pH level also not found the sedimentation, liquid separation, and contamination. The toxicity test

effects on cassava leave the use of the Zacha elicitor at 200 ppm without causing toxic effects on leaves. The effect of elicitors on mycelial growth inhibition to different concentrations of abiotic and biotic elicitors compared with chemical fungicides show the potential to directly inhibit the mycelial growth of *F. solani* isolate SHRD1. In this study, all of a concentration of JN2-007 elicitor, Zacha11, and Zacha15 at a concentration of 500 ppm can reduce mycelial growth. Chemical antioxidants on the mycelial growth of *F. solani* and *S. rolfsii*. Especially, salicylic acid with a concentration of 4 mM was the most effective that reduced the mycelial growth of *F. solani* and *S. rolfsii* at the average of 20.00 and 29.63%, respectively (Mohamed et al., 2012). Salicylic acid (80 mM), when added to Czapek Dox agar medium after 8 days had totally suppressed the root rot of fenugreek by inhibited mycelial growth of *F. solani* was significantly (Ramteke, 2019). Similar results could be obtained by Saad et al. (2014) using 200 ppm SA, which completely inhibited *F. solani*. Other authors (Abdel-Moniam et al., 2012; Sedghi and Gholi-Toluie, 2013) observed that the in vitro growth of fungal pathogens (*Fusarium* spp). SA (10-25 ppm) revealed that inhibit the mycelium growth of *R. solani*, *Alternaria solani*, *B. cinerea*, *P. aphanidermatum* and *Fusarium* spp. completely in PDA- plate assay (Saikia et al., 2003; Jabnoun-Khiareddine et al., 2015). All formulations of *Bacillus* spp (*B. subtilis*, *B. cereus*, and *Pseudomonas fluorescent*) exhibited varied percentages of mycelial inhibition of *F. solani* caused by root rot disease of cantaloupe (Sallam et al., 2013). Antagonistic bacteria for their ability to control *F. solani* were tested for the ability to inhibit by dual culture on PDA plates showed high inhibition of mycelium obtained 53.33% (Zulfikar et al., 2018). Also, 15 strains of *Bacillus* spp could inhibit mycelial growth at 45.19% of *Sclerotinia sclerotiorum* when compared with control (Vinodkumar et al., 2017). *Bacillus* spp produces antibiotics that can suppress more pathogens. Kim et al. (2008) revealed that *B.*

subtilis against mycelium growth of fungi may be due to the production of hydrolytic enzymes that can degrade cell walls.

Costet et al. (2002) found that *F. solani* isolate SHRD1 invasion induced oxidative stress in cassava by the generation of ROS such as H_2O_2, which can directly attack membrane and antioxidant enzyme as PO, PPO and CAT activities, these enzymes are involved in defense reactions. Zacha11 at a concentration of 500 ppm and challenged inoculation showed that significantly enhanced H_2O_2 content, PO, PPO, and CAT activities and increased till 24 HAI when compared with uninfected control. The accumulation of reactive oxygen species (ROS), which include the hydroxyl radical (OH), superoxide radical (O_2^-), and hydrogen peroxide (H_2O_2) (Costet et al., 2002; Apel and Hirt, 2004) were defence reactions that activated plants in response to pathogen attack which contributes to rapid localized cell death (Dempsey and Klessig, 2017; Singh et al., 2019). Besides, that to involve in cross-linking in cell walls, an increase of total SA accumulation, defence-related proteins, gene expression, defence genes and induced systemic resistance (Mellersh et al., 2002; Shetty et al., 2003; Apel and Hirt, 2004; Choudhury et al., 2017). Peroxidase (PO) and polyphenol oxidase (PPO) are involved in defense reactions against plant pathogens. Moreover, catalase (CAT) involved in the formation of lignin, and other oxidative phenols that contributed to generate barriers against pathogens invasion in plants and also the regulation of H_2O_2 levels in plant tissues (Figure 6.1) (Sudhakar et al., 2001; Thipyapong et al., 2004; Guan and Scandalios, 2006; Hassan et al., 2007; Anahid et al., 2013).

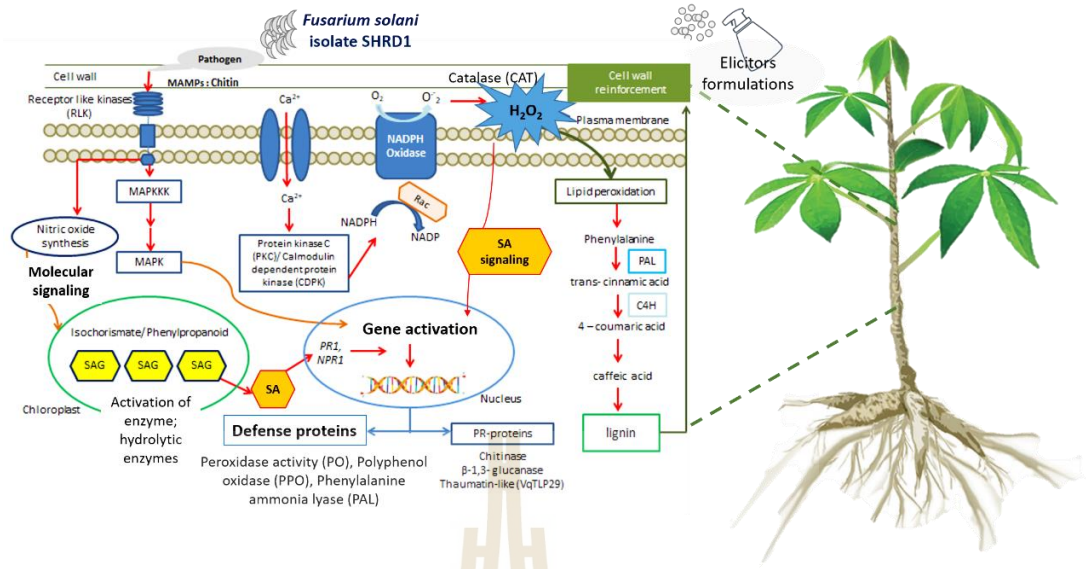

Figure 6.1 Induction of systemic resistance against Fusarium root rot disease in cassava (*Manihot esculenta*)

In the study, the efficacy of inhibiting Fusarium root rot disease severity using Zacha elicitor formulations can reduction in the disease severity of Fusarium root rot disease at 60 days after challenge inoculation to compare with chemical fungicide and water control (Figure 6.2). The efficacy of inducers on Fusarium wilt disease in chickpea when SA is applied through stem injection at 2000 µg/mL to the disease severity reduced by 40% (Saikia et al., 2003). SA application (1.0 mM) to rice seed soaking and foliar spraying application can reduced disease severity caused by *X. oryzae* pv. *oryzae* (*Xoo*) significantly, approximately 55.35% compared to negative control (Le Thanh, 2015). Also, 1 mM SA treatment showed up a smaller percentage of diseased leaves than non-priming plants which reduced the Cassava bacterial blight diseased severity to 18% and 25% in HB60 and HN, respectively (Yoodee et al., 2018). PR proteins are associated with disease resistance that described in various species of plant (Bolwell et al., 2001; Kombrink et al., 2001; Anand et al., 2004; Velazhahan and

Muthukrishnan, 2004). β-1,3-glucanase is a major component of the cell walls of many fungi and chitinase can break down chitin (Wessels and Sietsma, 1981; Monteiro and Ulhoa, 2006; Mishra 2010; Prakongkha et al., 2013). Induction of chitinase activity increased in inoculated plants with *Rhizoctonia solani* and non-inoculated plants treated with the SA at 2, 4, 6 and 8 days then decreased at 10 days from application. Prakongkha et al. (2013) revealed that foliar sprayed with or without chitosan and BTH, activities level of PR-proteins increased significantly seven days after treatment and much more seven days after challenge inoculation with *Sphaceloma ampelinum*. Furthermore, inoculation of bacterial blight in rice plants with acibenzolar-S-methyl (ASM) and *Xoo*-inoculated increased PR-proteins activities that induced resistance can be triggered persisted for at least 3 days (Babu et al., 2003).

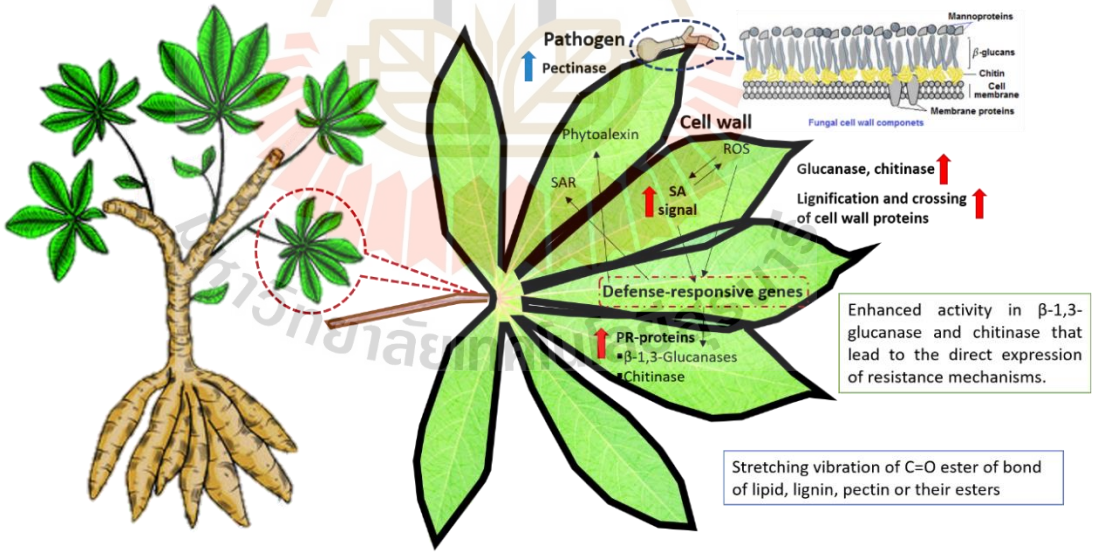

Figure 6.2 A proposed schematic model of induced resistance in cassava plants against *F. solani* after being treated with Zacha

Salicylic acid (SA) is one plant hormone that has a key role in the signal transduction pathways (Vernooij et al., 1994; Singh et al., 2004; Walter et al., 2007; Esmailzadeh et al., 2008; Buensanteai et al., 2009) and plays an important role in plant defense which are common plant biochemical responses associated with systemic acquired resistance (SAR) activation (Vallad and Goodman, 2004; Grant and Lamb, 2006; Archana et al., 2011; Cohen et al., 2011; Anwar et al., 2013; Sina et al., 2015).

Zacha11 were shown the biochemical components at the peak of 1737 cm^{-1} assigned to C=O ester vibration from pectin. Also found peaks of 1464, 1147, and 1112 cm^{-1}, which were assigned to C–H bending and C–O stretching of hemicelluloses and lignin which is a major component of plant cell membranes and important for both operation and plants structure. Furthermore, the chemical structure of plant secondary metabolites has played an important role in plant defense (Yu, 2005; Dokken and Davis, 2007; Prathuangwong and Buensanteai, 2007; Buensanteai et al., 2012; Skotti et al., 2014; Thumanu et al., 2015; Le Thanh et al., 2017). Lignin is a cell wall component that is covalently linked to hemicellulose and cross-links to different plant polysaccharides which play an important role against many lytic enzymes produced by plant pathogens during host tissue colonization (Menden et al., 2007; Banerjee et al., 2010; Bethke et al., 2014). The accumulation of polysaccharides and pectin associated with cell wall thickening that are complex composites of cellulose, cross-linking glycans, protein, and pectin substances (Figure 6.2) (Kacurakova et al., 2000; Kenneth and Lawrence, 2005; Zhao and Dixon, 2014; Lahlali et al., 2015; Thumanu et al., 2017).

Le Thanh et al. (2017) found that biochemical component ratios revealed that twice as much pectin and lignin were present in rice plants treated with SA as shown by the peaks at 1629, 2851, and 1735 cm^{-1}. Similarly, SR-FTIR revealed changes biochemical

composition when inducing resistance by the *B. subtilis* strain D604 in the chili leave cells which higher integral areas for the C=O ester from pectin, lignin or lipids (1770-1700 cm^{-1}) (Thumanu et al., 2017). FTIR spectroscopy to report on the defense mechanism of wheat against Fusarium head blight caused by *F. graminearum* in comparing resistance and susceptible cultivars (Lahlali et al., 2015). Other authors reported that the spectra of FT-IR for all the samples in the region 4000-400 cm^{-1}. The pronounced peaks belonging to the vibration of C=O, C-OH, C-N, and N-H were present in the spectra of diseased roots of sesame (Gokulakumar and Narayanaswamy, 2008).

Formulated salicylic acid (Zacha) at concentration 500 mg/L and a bioproduct of *B. subtilis* (JN2-007) by soaking cassava stalk and foliage spraying are highly efficient in controlling cassava root rot disease and enhancing its growth under greenhouse and field conditions. Moreover, SA has an effective role in the potential plant growth of the regulator which can regulate various physiological and metabolic processes and it plays an important role in the defense mechanism against plant diseases and impact of yield and quality of cassava starch (Promkhambut et al., 2016; Youssef et al., 2017; Hasanuzzaman et al., 2019) (Figure 6.3).

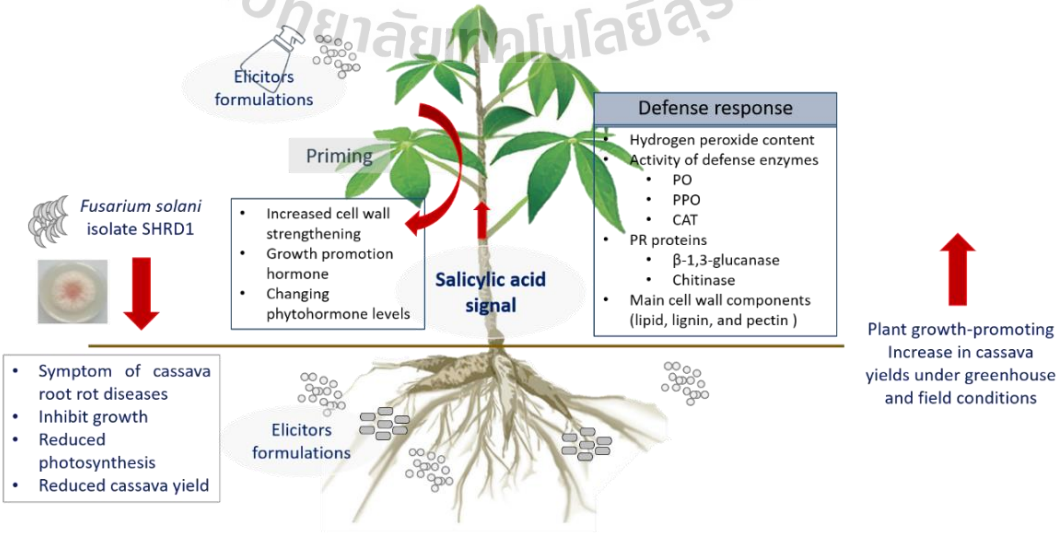

Figure 6.3 A model describing the application of elicitors formulations to cassava plants control root rot disease caused by *F. solani* isolate SHRD1; Zacha11 and JN2-007 elicitor leading to weaker pathogenesis on cassava plant by changing its biochemical components, induced pathogen resistance, and also enhancing plant growth and increasing cassava productivity.

6.2 Conclusion

Overall, the results of this study of the Zacha11 on induced resistance for cassava plant defense focuses on understanding the mechanisms as a growth regulator and induced systemic acquired resistance to control Fusarium root rot disease. The experiments confirmed that Zacha elicitor can inhibit the mycelium growth of *F. solani* isolate SHRD1, reducing the disease severity, inducing resistance including H_2O_2 content, peroxidase, polyphenol oxidase, catalase, activities of PR-proteins, endogenous salicylic acid. Moreover, the biochemical changes on cassava tissue (proteins, polysaccharides, pectins, and lipids of cassava characterized the roles of plant induction of defense). Finally, Zacha11 (167 mL/20 L of water for foliar spray) and JN2-007 (100 mL/20 L of water for foliar spray) can be used for controlling diseases and increasing cassava productivity by soaking the cassava stalks for 10 minutes before planting and spraying cassava plants at 1, 2 and 3 months after planting under greenhouse and farmer field.

6.3 Suggestion

Although the study has significantly advanced the mechanistic understanding of induced pathogen resistance against Fusarium root rot disease in cassava, several

aspects still deserve further experimental investigation. Firstly, more studies are needed to clearly the mechanism of Zacha elicitor on induced resistance interaction between plant and fungal pathogens. Second, the effectiveness of Zacha elicitor on induced resistance which a component of integrated disease management to Fusarium root rot disease in the field condition and the way of its commercial inducer formulation can be studied. Finally, the effectiveness of inducer on induced resistance on another cassava cultivar that guides strategies for eco-friendly and sustainable long-term cassava productivity (Table 6.1).

Table 6.1 Comparison of commercial plant elicitor products

Elicitor products	Active ingredient	Target	Price	
SARCON®; Thailand	Salicylic acid & Orthocilicic acid	Cassava, vegetable and rice disease	50 THB/Rai	
LARMINAR®; Thailand	*Bacillus subtilis*	Vegetable diseases, Rice disease and Plant growth	33.60 THB/Rai	
Dithane ™ NT M-45; Thailand	Mancozeb	Broad Spectrum Protectant Fungicide	22.40 THB/Rai	
Zacha11; Thailand	Salicylic acid	Cassava diseases	22.85 THB/Rai	

Although several reports of SA and BS demonstrate the potential to control root rot disease but in Thailand, we never developed as a commercial elicitor for large-scale agricultural use for reducing disease severity which modes of action for induction of systemic disease resistance to pathogens. Therefore, the developments of plant elicitor product have the potential and possibility to improve cassava production in Thailand.

6.5 Reference

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APPENDIX

I. Mediums

1.1 Potato Dextrose Agar (PDA)

Potato	200	g
Dextrose	20	g
Agar	15	g
Water	1,000	ml

II. Chemicals

2.1 1% Sodium hypochlorite (NaOCl)

NaOCl	1	ml
Water	100	ml

2.2 0.1% (w/v) trichloroacetic acid (TCA); ice-cold

Trichloroacetic acid	0.1	g
Water	100	ml

2.3 10 mM potassium phosphate buffer (pH 7.0)

Dipotassium hydrogenphosphate	93	mg
Potassium dihydrogen phosphate	63	mg

Prepare 80 mL of ddH_2O in a suitable container.

Add 0.093 g of K_2HPO_4 to the solution. Add 0.063 g of KH_2PO_4 to the solution. Add ddH_2O until volume is 0.1 L and store at room temperature.

2.4 1M Potassium iodide (1,000 ml)

Potassium iodide	16.6	g
Water	1,000	ml

2.5 1M Sodium phosphate buffer (1000 ml)

Stock A

Sodium Phosphate, Monobasic 69 g

Add ddH_2O and adjust pH 7, add ddH_2O until volume 500 ml

Stock B

Disodium phosphate 71 g

Add ddH_2O and adjust pH 6.5, add ddH_2O until volume 500 ml

Mix 423 ml of stock A with 577 ml of stock B autoclave 121^{o}C 15 min and store at room temperature

2.6 0.05 M pyrogallol

Stock 1M

Pyrogallol 1.2611 g

0.5 ml of stock 1M, add ddH_2O until volume 10 ml.

2.7 1M Hydrogen peroxide (100 ml)

30% Hydrogen peroxide 10.20 ml

ddH_2O 89.8 ml

0.33 ml of 30% Hydrogen peroxide, add ddH_2O until volume 10 ml.

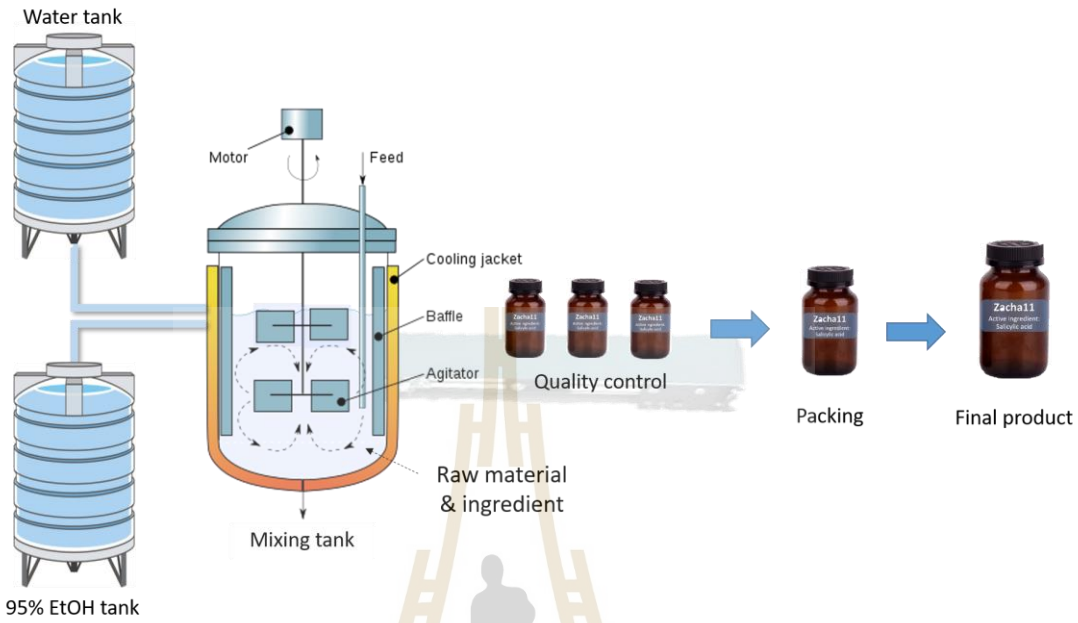
2.8 Colloidal chitin (0.1%, w/v)

Colloidal chitin 0.1 g

3N HCL 100 ml

Dissolve in 100 ml of 3N HCL while stirring in a fume hood at room temperature. Add chitin-HCL mixture slowly into pre-cooled water to obtain the colloidal chitin.

III. Zacha Manufacturing Planning and Control Systems



Attach figure 6 Schematic process and steps of assessment the product.

BIOGRAPHY

Mr. Chanon Saengchan was born on February 10, 1989, in Nakhon Phanom Province, Thailand. He graduated with a Bachelor degree in School of Crop Production Technology from Suranaree University of Technology in 2011. Then, in the same year, he decided to further study for Master's degree and graduated in 2015 from School of Crop Production Technology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. In 2015, she was accepted for a Ph.D. program under the supervision of Asst. Prof. Dr. Natthiya Buensanteai at the School of Crop Production Technology, Institute of Agricultural Technology, Suranareee University of Technology, Thailand. This program was supported by the Research and Researchers for Industries (RRI) and The Thailand Research Fund (TRF) under grant [PHD58I0070].

In 2018, he was an attend workshop of International Workshop on Cross Border Transmission of Crop Diseases and Insect Pests and Biological Safety Technology for South and Southeast Asia Countries in Yunnan Agricultural University in China, from June 10-29, 2018. In the same year, he was an exchange student at Department of Plant Pathology, University of Nebraska at Lincoln, Nebraska, USA during July 24, 2018 - January 24, 2019. Moreover, he was a participant International Training Workshop on Symbiosis Farming of Rice and Aquatics for Belt & Road Countries, September 11 to 25, 2019, Nanchang, China.