ROLE OF SALICYLIC ACID FORMULATION FOR INDUCED RESISTANCE AGAINST CASSAVA ANTHRACNOSE DISEASE

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บทบาทของสูตรซาลิไซลิกแอซิดต่อการชักนำความต้านทานต่อโรคแอนแทรคโนสของมันสำปะหลัง

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ROLE OF SALICYLIC ACID FORMULATION FOR INDUCED RESISTANCE AGAINST CASSAVA ANTHRACNOSE DISEASE

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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งานวิจัยนี้ มีวัตถุประสงค์เพื่อประเมินประสิทธิภาพของสูตรซาลิไซลิกแอซิด (RSA protectors) ต่อการชักนำความต้านทานต่อโรคแอนแทรคโนสและศึกษากลไกการชักนำความต้านทานของมันสำปะหลังต่อเชื้อ *Colletotrichum gloeosporioides* หลังจากถูกกระตุ้นโดยใช้ RSA จำนวน 5 สูตรที่ได้รับจากบริษัท ซีเอส ทาปิโอก้า วิจัยและนวัตกรรม จำกัด มาใช้ในการทดสอบกับมันสำปะหลังพันธุ์ระยอง 72 เปรียบเทียบกรรมวิธีควบคุม คือ ซาลิไซลิกแอซิด 6% สารคาร์เบนดาซิม® และน้ำ โดยทำการทดสอบความเป็นพิษของสูตร RSA ที่ความเข้มข้น 50,100, 150 200, 500, 1000, 2,000 และ 60,000 mg/l พบว่า ที่ความเข้มข้น 60,000 mg/l มันสำปะหลังแสดงอาการใบไหม้ ซึ่งที่ความเข้มข้น 60,000 mg/l พบว่าขนาดแผลใหญ่ที่สุด 0.1x0.1 ถึง 0.8x1.03 cm จากนั้นทำการทดสอบความเข้มข้นที่เหมาะสมในการยับยั้งการเจริญเติบโตของเชื้อรา *C. gloeosporioides* พบว่าสารเคมีคาร์เบนดาซิม®สามารถยับยั้งการเจริญเติบโตของเชื้อได้สูงที่สุด 89.61% รองลงมาคือ RSA 3 ที่ความเข้มข้น 200 และ 500 mg/l สามารถยับยังการเจริญเติบโตได้ดีที่ 47.40 ±0.03% และ 59.41±0.01% ตามลำดับ จึงได้ทำการคัดเลือกความเข้มข้น 200 mg/l เพื่อใช้ศึกษากลไกการชักนำความต้านทานและการควบคุมโรคในสภาพเรือนทดลอง ทำการแช่ท่อนพันธุ์มันสำปะหลังเป็นเวลา 3 นาทีและฉีดพ่นสูตร RSA ที่อายุ 1 ถึง 3 เดือนก่อนทำการปลูกเชื้อสาเหตุโรค จากนั้นทำการศึกษากิจกรรมของเอนไซม์ คือ เบตา 1,3 กลูคาเนส, ไคติเนส, ฟีนิลอะลานีนแอมโมเนียไลเอส, ซาลิไซลิกแอซิดภายในพืช และปริมาณคลอโรฟิลล์ พบว่า มันสำปะหลังที่กระตุ้นด้วยสูตร RSA มีการกระตุ้นการทำงานของเอนไซม์เพิ่มขึ้นที่ 16.65 µg glucose released min^{-1} mg^{-1} protein, 0.275 µmol glcnac formed min^{-1} mg^{-1} protein, 7.957 µmol trans-cinnamic acid min^{-1} mg^{-1} protein, 22.51µg g^{-1} of fresh weight และ 0.215±0.017 µg/mm^2 ตามลำดับ และยังได้ทำการตรวจสอบการสะสมของสารไฮโดรเจนเปอร์ออกไซด์, ซูเปอร์ออกไซด์ แอนไอออนด้วยเทคนิคการย้อมสีพบว่า มันสำปะหลังที่กระตุ้นด้วยสูตร RSA มีการสะสมของของกิจกรรมไฮโดรเจนเปอร์ออกไซด์ และซูเปอร์ออกไซด์แอนไอออนเพิ่มขึ้นที่ 12 ชั่วโมงหลังทำการปลูกเชื้อ นอกจากนี้ ได้ทำการวิเคราะห์การเปลี่ยนแปลงของสารชีวเคมีภายในใบมันสำปะหลังที่ระดับมีอิพิเดอร์มิส และมีโซฟิลล์เซลล์ด้วยเทคนิค Synchrotron Radiation-based Fourier Transform Infrared Microspectroscopy (SR-FTIR) พบว่า มันสำปะหลังที่กระตุ้นด้วยสูตร RSA 3 แสดงให้เห็นการเปลี่ยนแปลงของโครงสร้าง

ใบมันสำปะหลังจากการเปลี่ยนแปลงทางชีวเคมีขององค์ประกอบของกลุ่มไขมัน เพคติน โปรตีน เอไมด์ I และโพลีแซ็กคาไรด์เพิ่มสูงขึ้น เปรียบเทียบกับมันสำปะหลังที่ถูกกระตุ้นด้วยสารเคมีคาร์เบนดาซิม®และน้ำ (กรรมวิธีควบคุม) จากนั้นทำการประเมินความรุนแรงของโรคแอนแทรคโนสที่ถูกกระตุ้นด้วยสูตร RSA ที่ 14 วันหลังปลูกเชื้อในสภาพเรือนทดลอง พบว่า มันสำปะหลังที่กระตุ้นด้วยสูตร RSA 3 มีระดับความรุนแรงของโรคต่ำที่สุดที่ 33.33% ซึ่งแตกต่างอย่างมีนัยสำคัญยิ่งทางสถิติ เมื่อเปรียบเทียบกับกรรมวิธีควบคุมที่มีระดับความรุนแรงสูงถึง 77.7% สุดท้ายทำการทดสอบประสิทธิภาพของสูตร RSA ในสภาพไร่ พบว่า สูตร RSA 3 สามารถลดการเกิดโรคแอนแทรคโนสของมันสำปะหลังได้อย่างมีประสิทธิภาพสูงที่สุดที่ 21.6 และ 45.83% ตามลำดับ จากผลการวิจัยนี้ชี้ให้เห็นว่า RSA 3 เป็นสารกระตุ้นมันสำปะหลังสำหรับควบคุมโรคแอนแทรคโนสในมันสำปะหลังโดยใช้กลไกการชักนำความต้านทานเพื่อลดความรุนแรงของโรค

สาขาวิชาเทคโนโลยีการผลิตพืช ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2563 ลายมือชื่ออาจารย์ที่ปรึกษา _____

RUNGTHIP SANGPUEAK : ROLE OF SALICYLIC ACID FORMULATION FOR INDUCED RESISTANCE AGAINST CASSAVA ANTHRACNOSE DISEASE. THESIS ADVISOR : ASST. PROF. NATTHIYA BUENSANTEAI, Ph.D., 127 PP.

FORMULATION OF SALICYLIC ACID/SR-FTIR/ANTHRACNOSE/CASSAVA

The objectives of this study were to evaluate the efficacy of SA formulations (RSA protectors) in inducing resistance against anthracnose disease and to study the mechanisms of induced resistance in cassava plants to *Colletotrichum gloeosporioides* after treated with effective SA formulation. Five formulas of RSA protectors obtained from CS Tapioca Research and Innovation Co., Ltd were tested in cassava varieties Rayong 72 and compared with control treatments which are 6% salicylic acid, carbendazim® and water. These were tested for toxicity of RSA formulations at concentrations of 50,100, 150, 200, 500, 1000, 2,000 and 60,000 mg/l. The result showed that at concentrations of 60,000 mg/l, cassava showed leaf toxic symptoms. In addition, at the concentration of 60,000 mg/l, the wound size was the largest at 0.1 x 0.1 to 0.8x1.03 cm. After that, the evaluation of appropriate concentration to inhibit the growth of *C. gloeosporioides* showed that carbendazim® was able to inhibit the growth most effectively at 89.61%, followed by RSA 3 formula at concentrations of 200 and 500 mg/l. They were able to inhibit fungal growth at 47.40±0.03% and 59.41±0.01%, respectively. Therefore, at the concentration of 200 mg/l, it was selected to study the mechanism of induced resistance and disease control under greenhouse conditions. Then, the enzyme activity was studied as follows: β-1,3-glucanases, chitinase, phenylalanine ammonia lyses, endogenous salicylic acid and

total chlorophyll content. It was found that cassava induced with RSA protectors was associated with increased stimulation of enzyme activity at 16.65 µg glucose released min^{-1} mg^{-1} protein, at 0.275 µmol glcnac formed min^{-1} mg^{-1} protein, 7.957 µmol trans-cinnamic acid min^{-1} mg^{-1} protein, 22.51µg g^{-1} of fresh weight and 0.215±0.017 µg/mm^2, respectively. The accumulation of hydrogen peroxide and superoxide anion was also investigated by histochemical detection. The results showed that cassava stimulated with RSA protectors had an increased accumulation and superoxide anion at 12 HAI. Furthermore, the analysis of biochemical changes in cassava leaf epidermis and mesophyll by using Synchrotron Based - Fourier Transform Infrared Microspectroscopy (SR-FTIR) technique showed that the RSA 3 formula can activate the biochemical compositional changes associated with lipid, pectin, protein amide I, and polysaccharide when compared with carbendazim® and water (control treatments). The disease severity of anthracnose was then assessed after being induced with RSA protectors at 14 days after inoculation under greenhouse condition. The results revealed that the RSA 3 had the lowest disease severity at 33.33%, which is a statistically significant difference when compared with control treatment which had a high level of 77.7%. Finally, the evaluation of the efficiency of RSA 3 protectors under field conditions demonstrated that RSA 3 showed the highest efficiency to enhance cassava growth-promotion and reduce anthracnose incidence of cassava at 21.6 and 45.83%, respectively. These findings suggest that RSA 3 is a cassava protector for controlling cassava anthracnose disease by mediating resistance mechanisms in order to reduce disease severity.

School of Crop Production Technology Student's Signature Rungthip Sangpueak

Academic Year 2020 Advisor's Signature

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
ASM	=	Acibenzolar-S- methyl
BLB	=	Bacterial leaf blight
BLS	=	Brown leaf spot
CAD	=	Cassava anthracnose disease
CBB	=	Cassava bacterial blight
CMV	=	Cucumber mosaic virus
CRD	=	Completely randomized design
CRRD	=	Cassava root rot disease
CV	=	Coefficient of Variation
DAI	=	Days after inoculation
DMRT	=	Duncan's Multiple Range Test
ET	=	Ethylene
H	=	Hour
HAI	=	Hours after inoculation
HR	=	Hypersensitive response
ISR	=	Induced systemic resistance
JA	=	Jasmonic acid
mg L^{-1}	=	Milligram per milliliter
µL	=	Microliter (s)
µm	=	Micrometer (s)

LIST OF ABBREVIATIONS (Continued)

min	=	Minute (s)
mL	=	Milliliter (s)
mM	=	Millimolar
NBT	=	Nitroblue tetrazolium
nm	=	Nanometer
NO	=	Nitrite oxide
O^{2-}	=	Superoxide anion
OD	=	Optical density
PA	=	Phosphorous acid
pal	=	Phenylalanine ammonia lyase
PBS	=	Phosphate-buffered saline
PCA	=	Principal Component Analysis
PR proteins	=	Pathogenesis-related proteins
PDA	=	Potato dextrose agar
ROS	=	Reactive oxygen species
rpm	=	Round per minute
SA	=	Salicylic acid
SAR	=	Systemic acquired resistance
SE	=	Standard error
SR-FTIR	=	Synchrotron based - Fourier transform infrared Microspectroscopy
Xoo	=	*Xanthomonas oryzae* pv. *oryzae*

CHAPTER I

INTRODUCTION

1.1 Background of the selected topic

Cassava (*Manihot esculenta* Crantz.) is one of the major crops in Thailand, especially in the northeastern region (Kunkeaw et al., 2010; Sangpueak, Phansak, and Buensanteai, 2018). It has been used mainly as primary starch in several agro-industries. Due to the increasing demand for food industry and renewable energy, Thailand has started to substitute the power production from agricultural products included palm oil or ethanol extraction from cassava in order to meet self-sufficiency of energy in the country (Hassan and Kalam, 2013; Sangpueak, Phansak, and Buensanteai, 2018). While the increasing of needs for cassava products, its planting area has also been increasing in northeastern of Thailand (Ratanawaraha, Senanarong, and Suriyapan, 2001; Sanginga and Mbabu, 2015). In the year 2016, the cassava farming area in Thailand was approximately 1.4 million hectares (Ratanawaraha et al., 2001). The planting of cassava has continuously changed according to the market demand. The increased planting areas and changing of cultivation practices lead to increase cassava diseases and reaching higher diseases severity (Ghini, Bettiol, and Hamada, 2011; Food and Organization, 2013; Sangpueak, Phansak, and Buensanteai, 2018). Since the year 2010, many outbreaks of cassava diseases have occurred in cassava planting areas in Thailand (Bellotti, Campo, and Hyman, 2012; Harris et al., 2015; Sangpueak, Phansak, and Buensanteai, 2018). The major diseases included leaf

spot caused by *Cercospora* sp., root rot disease caused by many fungal pathogens and cassava anthracnose disease (CAD) caused by *Colletotrichum gloeosporioides* (Bart et al., 2012; Duan et al., 2014; Sangpueak, Phansak, and Buensanteai, 2018). The CAD symptoms as circular, sunken lesions with orange spore masses were recognized on leaves and petioles of cassava (Fokunang et al., 2001; Sangpueak, Phansak, and Buensanteai, 2018). In Nigeria, the same symptoms have been reported with its high intensity (Owolade et al., 2005). In Thailand, an outbreak of CAD has been found during hot weather alternating with high rainfall; similar to that found in West Africa (Fokunang et al., 2001; Sangpueak, Phansak, and Buensanteai, 2018). The succession planting of cassava provides an opportunity to the *Colletotrichum* spore to reproduce throughout the year, hence increasing the chance of anthracnose outbreaks (Fokunang et al., 2001; Ojola, 2015; Sangpueak, Phansak, and Buensanteai, 2018). In the year 2018, Sangpueak et al. reported that CAD caused by *Colletotrichum* complex species including *C. capsici*, *C. lindemuthianum*, *C. aeschynomene*, *C. boninense* and *C. gloeosporioides*. Geographically, *C. gloeosporioides* species was found in all regions, but other species were found in some regions of Thailand. Over the past ten years, the anthracnose has caused large-scale economic damage in cassava planting areas around the world, especially in Thailand (Fokunang et al., 2001; Sangpueak, Phansak, and Buensanteai, 2018). At present, the practices for cassava diseases management in Thailand have been completed by cultural and chemical approaches (Thumanu et al., 2015). However, the use of chemical control is direct and popular control measures for anthracnose on cassava. But, the use of chemicals fungicide for long-term exposure will affect to improve the evolution of fungicide resistance and environmental contamination (Fokunang et al., 2001; Buensanteai, Yuen, and Prathuangwong, 2009).

Therefore, an alternative controlling approach is by using plant protectors induced plant immunity which have been proposed by using protectors induced plant immunity have been proposed (Utsumi et al., 2016). The induced systemic resistance of plant defense response to pathogens using plant protectors or elicitors is commonly used to prevent several plant pathogens (Kuć, 1982; Ramanathan, Vidhasekaran and Samiyappan, 2000; Walters, Ratsep, and Havis, 2013; Bektas and Eulgem, 2015). Plant protectors can be agro-chemical compounds, bio-molecules and various microorganisms, which trigger biochemical defense mechanism in plant healthier against pathogen infection (Mishra, Sharma, and Misra, 2012; Prakongkha et al., 2013; Thakur and Sohal, 2013; Wiesel et al., 2014; Bektas and Eulgem, 2015). The plant defense mechanisms and plant biosynthetic pathways are stimulated in protectors treated plants (Kuć, 1982; Cohen, Rubin, and Vaknin, 2011; Sillero et al., 2012; Spoel and Dong, 2012; Walters et al., 2013). Chitosan, *β*-glucan, yeast extracts, plant growth promoting rhizobacteria/fungi, as well as plant hormone or agro-chemicals, such as jasmonic acid (JA), phosphorous acid and salicylic acid (SA) are examples of plant protectors (Ménard et al., 2004; Ramanathan et al., 2000; Prakongkha et al., 2013; Gogbeu et al., 2015; Le Thanh et al., 2017). SA has been shown as a key signaling role in the stimulation of defense responses after pathogen infection (Durner, Shah, and Klessig, 1997; Mandal, Mallick, and Mitra, 2009; Prakongkha et al., 2013; He et al., 2017; Le Thanh et al., 2017). Mandal et al. (2009) reported the SA-treated tomato plants against *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). The results presented the induced resistance in tomato plants against *Fol* from systemic acquired resistance stimulated by SA. Le Thanh et al. (2017) reported that rice seed treatment and foliar sprays with exogenous SA reduced bacterial leaf blight (BLB) caused by bacterial

Xanthomonas oryzae pv. *oryzae* (*Xoo*) severity by more than 38%. He et al. (2017) studied the influence of SA on defense response to *C. gloeosporioides* in mango. In this study, *in vitro* assay showed that SA significantly inhibited the mycelial growth of *C. gloeosporioides*. The results in the severity of disease in treated fruits were significantly lower than the control fruits. Furthermore, it can increase the enzyme activities such as chitinase, β1,3-glucanase, phenylalanine ammonia lyase and polyphenol oxidase in mango fruit. Gogbeu et al. (2015) revealed that SA, phosphorous acid (PA) and the fungicide Sumi 8 could induce resistance against anthracnose disease. These results indicated that protectors could be a stimulating natural defense against CAD.

Although there have been studied on reporting the effectiveness of using resistance protectors for controlling CAD, but none have explained thoroughly the mechanism of cassava plant defense response. Thus, this research was conducted to study the defense mechanisms in cassava using different resistance SA protector formulations for the controlling of CAD in Thailand.

1.2 Research objectives of this study

1.2.1 To evaluate the efficacy of exogenous SA protector prototype formulations in inducing resistance to anthracnose disease in cassava plants.

1.2.2 To study the mechanisms of induced resistance in cassava plants to *C. gloeosporioides* after treated with effective SA protectors formulations.

1.3 Hypotheses of this study

1.3.1 The application of exogenous SA formulation could trigger induced systemic resistance (ISR) and protect cassava plants from anthracnose disease.

1.3.2 The mechanism of resistance in cassava could be associated with expression levels of genes involved in plant defense mechanisms.

1.4 Significance and designed paths of this study

14.1 Significance of this study

The significance of this study is due to largely increased in domestic demand for cassava. As a result, the cassava production in Thailand is not enough to meet the market demand. The causes of these declines are from climate change and extreme weather, drought, pests, nutrient deficiency and diseases (Bellotti et al., 2012). The loss from cassava diseases is a problem that should be realized today. The important diseases causing serious damage to cassava production in Thailand are cassava bacterial blight (CBB), brown leaf spot (BLS), root rot (CRRD) and anthracnose (CAD) (Sangpueak, Phansak, and Buensanteai, 2018). At current, there are severe epidemics in the major production areas such as Northeast and East parts of Thailand. Many farmers use an overdose of chemicals to control plant diseases, which may also result in negative farmers' health and environmental effects. Hence, to reduces the use of fungicide and chemical control plant diseases, induced resistance is the way of reducing the use of hazardous chemicals in agriculture. Plant inducers include plant immunity-inducing proteins, vitamins, volatile organic compounds, among others, and microbial inducers (Aranega-Bou et al., 2014; Dewen et al.,2017). However, the induced resistance method for plant disease is a new management method and lack of

commercial products in the Asian pesticide market and Thailand. Consequently, this study examined the efficacy of induced resistance to contribute the management of CAD in cassava.

1.4.2 The designed paths of this study

The application and efficacy of resistance salicylic acid elicitors (RSA protectors) that friendly with environment for induced resistance against CAD were evaluated in this study.

The study was divided into two main parts including eight successive experimental studies as follows:

(1) In chapter III, firstly, the effect of RSA formulations were assessed on disease severity of cassava after treated with formulations. Secondly, the effect of RSA formulations to structural responses and biochemical changes of lignin, pectin, amide I and lipid were analyzed using Synchrotron based - Fourier transform infrared (SR-FTIR) micro spectroscopy.

(2) Extending on this, in chapter IV, the effects of RSA formulations on cassava germination were studied. Subsequently, their capability was estimated on inducing resistance in cassava against CAD under greenhouse conditions. Thereafter, cassava defense genes after treated with the inducer were examined.

1.5 Scopes and limitation of the study

The purpose of this research is to study efficacy of resistance SA formulation prototypes (RSA protectors), application methods, rate and timing of application in inducing the resistance in susceptible cassava cv. Rayong 72 to CAD. Cassava defense mechanism against anthracnose disease after treated with RSA protectors and

inoculated with *C. gloeosporioides* was monitored. The biochemical changes associated with plant innate immunity were studied using traditional methods, such as salicylic acid, phenolic compound analysis combining with SR-FTIR micro spectroscopy. Moreover, this experiment also evaluated the effects of plant protectors on productivity of cassava at the field conditions.

1.6 Expected results of the study

1.6.1 Benefits expected for academia

1.6.1.1 The RSA formulation should be able to reduce yield loss from the anthracnose disease by approximately 10% of the total cassava production.

1.6.1.2 Understanding the resistance mechanisms in cassava plants after induced by the RSA protectors.

1.6.2 Benefits expected for the development of prototypes and application by the public

1.6.2.1 An effective formulation of the RSA protectors for controlling CAD in cassava farmer fields.

1.6.2.2 RSA protectors application technology could be transferred to farmers.

1.6.2.3 Providing an alternative method for controlling the CAD and reduce the use of chemical fungicides in cassava production.

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CHAPTER II

LITERATURE REVIEW

2.1 Roles and problems of current cassava production

2.1.1 Roles of cassava

Cassava is an annual plant in tropical and subtropical regions. The crop may have been cultivated in Colombia and Venezuela as early as 3,000-7,000 years ago. It is in the top 5 important plants in the world, after maize, rice, wheat, and potato (Rojanaridpiched et al., 2007; Lebot, 2008; Bedford et al., 2014; Oduah et al., 2015). The major cassava producers of the world are Brazil, Nigeria, Congo, and Indonesia, but they mainly produced for domestic consumption (Poramacom et al., 2013; Ohimain, 2015). In Thailand, it is recognized as the most important crop of the country in terms of economic agricultural product which 68% of cassava productions is exported to several countries (Poramacom et al., 2013). The most cassava production area is in the northeast region, for this reason, these areas become a collection center for tapioca and starch factories (Chuasuwan, 2017; Sangpueak, Phansak, and Buensanteai, 2018). Production of cassava in Thailand has been steadily increasing more than two decades ago and in 2016, the planting area of cassava was approximately 1,461,827 hectares yielding approximately 31 million tones/year. The demand for products is growing, leading to increase cultivated areas steadily every year (Chuasuwan, 2017; Sangpueak, Phansak, and Buensanteai, 2018).

As a result, cassava production is not enough to market demand. The causes of this deficiency are from climate change, drought, pests, nutrient deficiency and diseases (Rosenzweig et al., 2001; Bellotti et al., 2012). The damage from cassava diseases is a problem that should be realized. The diseases are many and the pathogens can rest in the soil or stem debris for long periods time (Duchanee, 2015; Sangpueak, Phansak, and Buensanteai, 2018). Important serious diseases to cassava production in Thailand are cassava bacterial blight (CBB), brown leaf spot (BLS), root rot (CRRD) and anthracnose (CAD) (Sangpueak, Phansak, and Buensanteai, 2018). At present, there are severe epidemics in the major production areas such as Northeast and East of Thailand. Hence, cassava production has been decreasing during the past 3-4 years. Therefore, an effective management of cassava disease could reduce the infestation of pathogens and yield loss (Bellotti et al., 2012; Duchanee, 2015; Sangpueak, Phansak, and Buensanteai, 2018).

2.1.2 Important diseases of cassava

Cassava plants cultivated in almost districts in Northeast of Thailand are susceptible cultivars, resulting in high yield losses of more than 50%. The major diseases of the cassava in Thailand are cassava bacterial blight (CBB), cassava brown leaf spot (BLS), cassava root rot disease (CRRD), cassava anthracnose disease (CAD) and cassava mosaic disease (CMD).

2.1.2.1 Cassava bacterial blight (CBB)

CBB caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) is a serious problem in South America and Africa (Teri et al., 1978). Thailand can find this disease in every planting area during the rainy season. The disease symptoms include

die-back, leaf blight, and stem vascular necrosis. Rain splashing is an important way of dissemination within planting areas (Maraite, 1993; Bart et al., 2012).

2.1.2.2 Cassava brown leaf spot (BLS)

BLS disease is considered of an important cassava disease caused by *Cercosporidium henningsii* and found worldwide including Thailand (Pei et al., 2014). Symptoms are brown spots form between leaf veins, so their size and shape are limited by the veins. The center of the wound may be a hole in the leaf and become yellow halo and dry and may die early (Teri et al., 1978; Graziosi et al., 2016).

2.1.2.3 Cassava root rot disease (CRRD)

Cassava root rot is a disease problem in Africa and Thailand, It has impacted on cassava products is approximately 80% yield losses (Duchanee, 2015; Onyeka et al., 2005). CRRD is a complex of soilborne pathogens such as *Fusarium* sp., *Botryodiplodia theobromae, Sclerotium rolfsii*, *Neoscytalidium* sp., *Phytophthora* spp. and *Pythium* sp. (Onyeka et al., 2005; Bandyopadhyay et al., 2006; Aigbe and Remison, 2010; Kpémoua and Wydra, 2010; Duchanee 2015). The symptom is tissue breakdown of the mature tuberous roots and color changes with a foul odor (Bandyopadhyay et al., 2006; Kpémoua and Wydra, 2010; Duchanee, 2015).

2.1.2.4 Cassava anthracnose disease (CAD)

CAD caused by the Colletotrichum species complex. In 2018, Sangpueak et al. found that CAD in Thailand was caused by *C. capsici*, *C. lindemuthianum*, *C. aeschynomene*, *C. boninense* and *C. gloeosporioides*. The common species are *C. gloeosporioides* and *C. gloeosporioides* f. sp. *manihotis*. The main economic diseases of cassava Thailand is CAD in Thailand (Owolade et al., 2005; Sangpueak, Phansak, and Buensanteai, 2018). This depth details is presented in the next section.

2.1.2.5 Cassava Mosaic Disease (CMD)

Cassava Mosaic Disease is one of the most important diseases in Africa and many countries in the world. In Thailand, there was an epidemic of Sri Lankan cassava mosaic virus (SLCMV) in 2018, with the first reported outbreaks in Prachinburi, Surin, and Sri Saket provinces (Siriwan et al., 2020). SLCMV belongs to the genus Begomovirus in the family Geminiviridae (Minato et al., 2019; Wang et al., 2015). The symptoms of SLCMV were mosaic, curl, deformation, and insect vector transmission on young upper cassava leaves (Minato et al., 2019). That resulting in cassava yield losses in Thailand. Control of SLCMV in Thailand, because it is an emerging disease in Thailand, to avoid the spreading of the disease, the Department of Agriculture has the notification on the pest control area according to the Plant Quarantine Act B.E.2507 (and its amendment) as an official control to enforce the eradication program on cassava infected areas (Food and Agriculture Organization of the United Nations, 2019).

2.2 Cassava anthracnose disease

Colletotrichum species complex is a main pathogen of CAD because Thailand has a high rainfall as well as hot and humid conditions (Weir et al., 2012; Sangpueak, Phansak, and Buensanteai, 2018; Liu et al., 2019). Their characteristic symptoms are spots on leaves, petioles, shoots and stem. The severe infection can be found as the circular lesions with spore masses, wilt, die-back and defoliation (Fokunang et al., 2001; Hillocks and Wydra, 2002; Owolade et al., 2005b; Edwige et al., 2017; Sangpueak, Phansak, and Buensanteai, 2018). Moreover, the main distinctive symptom of CAD is the appearance of cankers on the stems of susceptible varieties. Moreover,

the main symptom is canker on the stems of susceptible varieties. The symptom may be formed at the petioles or on some part of the stem depending on the cassava varieties. Cankers can be exposing inner tissues of stems and result in distortions in the shape of infected stems (Fokunang et al., 2001; William et al., 2012). CAD is characterized by brown spots and the size of the lesions, at approximately 10- 30 mm in diameter (Makabila, 1987). Infected plants showed wilt symptoms and defoliation of leaves. Susceptible cultivars can show symptoms of broken petiole wilt severe defoliation and entire infected stems may dieback (William et al., 2012; Sangpueak, Phansak, and Buensanteai, 2018). The CAD is especially a violent infection during the rainy season in Thailand starting from early April to late October. In West Africa, anthracnose was reported to be more serious in humid rain forest areas, particularly in wet-season cassava crop, but was less important or absent in the dry savanna areas or during the dry season (Onyeka et al., 2005; Than et al., 2008). The CAD is widely epidemic in most cassava planting areas growing Kasetsart 50 and Rayong 72, both the most susceptible varieties (Rojanaridpiched et al., 2007). Moreover, this disease may decrease cassava production to more than 50% (Boonchanawiwat et al., 2016; Sangpueak, Phansak, and Buensanteai, 2018). In Nigeria, widespread incidence of the disease has been found to affect more than 80% of cassava grown in Ibadan in the Oyo state (Owolade et al., 2005b; Wokocha et al., 2011). Undoubtedly, the changes in the practices of cassava production have an effect on CAD outbreak from being a minor disease to become a major disease of economic importance (Hillocks and Wydra, 2002; Sangpueak, Phansak, and Buensanteai, 2018). Severe outbreaks can result in famine in communities and several countries.

2.3 Control of CAD

There are several ways to control CAD as follows:

2.3.1 Cultural methods

Cultural control is the use of practices having an effect on crop protection. Crop rotation is one of the most effective pest and disease control strategies. Generally, crop rotation is used to grow non-host crops until the population of pathogens reduce. Crop rotation methods together with soil plowing can help to disrupt the disease lifecycle. In addition, late planting could be reduced disease severity in the intensive cassava farming area (Ikotun and Hahn, 2015).

2.3.2 Use of CAD resistant cultivars

The reliable control and lowest cost were to use anthracnose resistant varieties, especially in localities severity of a CAD. For example in Nigeria, the farmers considered the use of cassava resistant varieties including TME 19, TME 53, TME113, TME 244, TME 475, and TME 523; I85/02015 and I8700028 to reduce CAD (Owolade et al., 2005b). However, in Thailand, severe outbreaks had not been officially reported, hence, it did not need to select or breed for CAD resistant varieties. Since 2005, there have been reports of outbreaks of this CAD in cassava in several varieties, such as Kasetsart 50, Rayong 90, Rayong 72 and CMR 35-22-196 (Prammanee et al., 2010; Houngue et al., 2018; The Office of Agricultural Economics, 2018).

2.3.3 Use of healthy cassava planting material

Healthy cassava sprouts help to escape early infections. In the case of infected cassava, the stems must be soaked with fungicides suggested by extension agents. Spores and other fungal materials can be destroyed by dipping or foliar spraying of suitable fungicides or resistance protector (Beeching et al., 2004; Gogbeu, 2015).

2.3.4 Chemical control

The attention and efforts on CAD control have mostly concentrated on the use of fungicides. For CAD control in cassava farms, Lozano et al. (1981); Théberge (1985), and Corbaz (1990) recommend that the foliar spray with copper compounds in cases of not having a severe disease. In Thailand, the Department of Agriculture has recommended the use of chemicals such as mancozeb for controlling this CAD. But the use of chemical fungicides is not practicable in long-term strategy due to high costs and residue contamination in the environment.

2.3.5 Biological control

Biological control is the use of beneficial microorganisms to prevent plant pathogens such as beneficial fungi or bacteria etc (Heydari and Pessarakli, 2010). The mode of action of microorganisms by entering the competition with the pathogen for space and nutrients, parasitism or predation, enhancement of plant defense system, and the production of antimicrobial compounds. Normally, several mechanisms functions can work together to maximize efficiency (Pal and Gardener, 2006; Peterson and Kaur, 2018). The CAD control necessitates to development of control methods that are ecology-conscious, impact on farmers' health, and cost-effective. Biocontrol agents have been studied on anthracnose disease for other plant species by Palaniyandi et al. (2011). The authors studied about *Streptomyces* sp. MJM5763 to against *C. gloeosporioides* in yam. The results found that treatment with MJM5763 and crude extract (CCFE) was effective in preventing anthracnose, leading to decrease disease incidence and severity *in vitro* and greenhouse conditions at 85-88% disease severity. Jahan et al., (2015) conducted an experiment to study the effect of BAU-Biofungicide (*Trichoderma harzianum*), *Trichoderma*-based IPM bio-pesticide and Bavistin

(Carbendazim) for control anthracnose in soybean. The report showed a treatment of BAU Bio-fungicide has a high percent reduction near to Bavistin-treated (76.25%).

2.3.6 Induced resistance

Plants are sensitive to a diversity of biotic stresses as fungi, bacteria, nematodes, phytoplasmas, viroid and viruses. That results to a key loss to economic crop yield (Beeching et al., 2004; Buensanteai et al., 2010). There are many options for farmers to prevent crops from diseases include resistant cultivars, crop rotation, biological and chemical control (Beeching et al., 2004; Buensanteai et al., 2010; Thakur and Sohal, 2013; Inchaya et al., 2016). Chemical fungicides are directly affected almost of antibiotics, but the use of fungicides in the long-term can affect the environment and farmers' health. So, induced resistance is a way of controlling plant diseases without side effects on humans and the environment. Induced resistance is also a part of the defense mechanism for long-lasting prevention against a broad spectrum of pathogens (Heil, 2002; Heil and Silva Bueno, 2007; Mandal et al., 2009; Buensanteai et al., 2010; Graham and Myers, 2011). There are two major defense systems for controlling plant diseases including induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Figure 2.1). ISR is a resistance process stimulated by biotic and abiotic factors and its action is characterized by indirect killing or inhibition of the pathogen but through the induction of plant defense to control disease (Choudhary et al., 2007; Buensanteai et al., 2009). That kind of resistance depends on jasmonic acid (JA) and ethylene (ET) signaling in the plants which are initiated by rhizobacteria (Buensanteai et al., 2009; Thakur and Sohal, 2013). SAR is an immune response in plants by nonpathogenic pathogens causing local cell death. This kind of resistance can be induced through the formation of salicylic acid signals and PR protein secretion. The

plants are primed to fastly and effectively to activate defense responses for pathogen attack (Conrath, 2006; Fu and Dong, 2013).

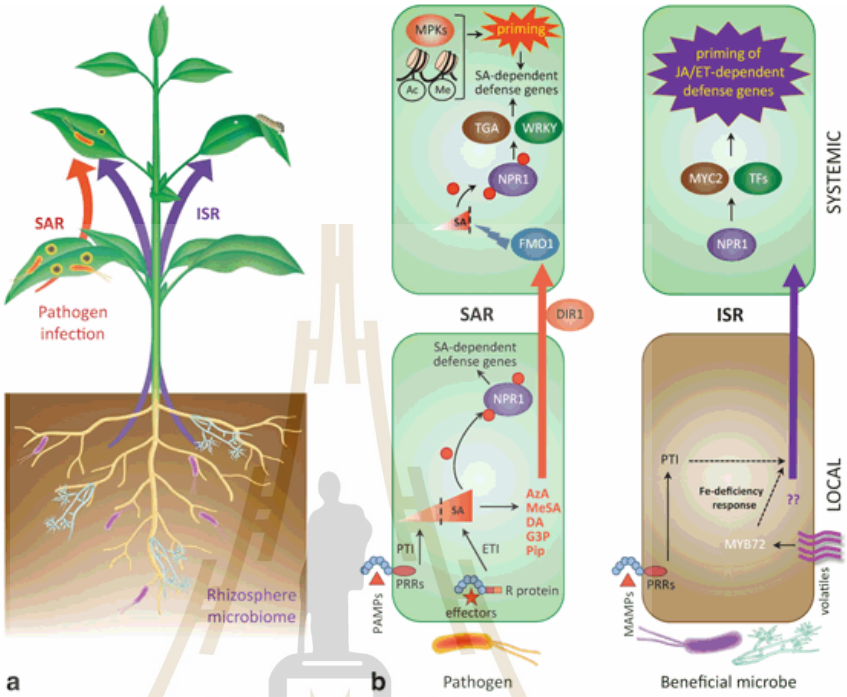

Figure 2.1 Major defense systems including induced systemic resistance (ISR) and systemic acquired resistance (SAR) (Pieterse and Van Wees, 2014)

Mechanisms responsible for plant defenses have differential recognition and can be crosstalk with the different signals of JA, ethylene, and SA. In this research, it will focus on systemic acquired resistance (SAR) by the accumulation of salicylic acid to stimulate defense mechanisms resulting in the hypersensitive response (HR). However, SA is the first chemical to induced PR genes, chitinases, and β-1,3-glucanases, etc. These key roles can prevent or delay the colonization of pathogens in the host cell (Sticher et al., 1997; Sudisha et al., 2011; Fu and Dong, 2013).

2.3.6.1 Characteristics of elicitors/protectors

Protectors or elicitors are agrochemical compounds, bio-molecules and various microorganisms, which trigger biochemical mechanisms in plants against pathogens (Buensanteai et al., 2008; Walters et al., 2013; Gogbeu, 2015; Le Thanh et al., 2017; Inchaya et al., 2016). The defense mechanism and biosynthetic pathways are stimulated in treated plants with protectors (Hammerschmidt and Kuć, 1995; Cohen et al., 2011; Spoel and Dong, 2012; Sillero et al., 2012; Walters et al., 2013). Plant growth-promoting rhizobacteria/fungi, β 1,3-glucanase, chitosan as well as plant hormones or agrochemicals as jasmonic acid (JA), phosphorous acid and salicylic acid (SA) are examples of protectors (Ramanathan et al., 2000; Ménard et al., 2004; Buensanteai et al., 2008; Gogbeu, 2015; Inchaya et al., 2016; Le Thanh et al., 2017). Natural compounds or protectors have a similar role to induce defense responses in plant signaling pathways. Protectors play an important role in biosynthetic pathways to enhance important compounds and immune responses in plants (Angelova et al., 2006; Buensanteai et al., 2008). They have low molecular weight and synthesized or released from polymeric precursors and from various resources that can trigger a physiological and biological responses (Buensanteai et al., 2008; Holopainen et al., 2009; Mejía-Teniente et al., 2010).

According to Radman et al. (2003) and Angelova et al. (2006), protectors are classified as biotic and abiotic ones depending on their origins and molecular structures. Biotic protectors are molecules of mild strains of pathogens and antimicrobial that can induce defense responses. The plant growth- promoting rhizobacteria/ fungi, yeast extract, polysaccharides, oligosaccharides, proteins,

glycoproteins, phytoalexin, and fatty acids are an example of biotic protectors (Hahn et al., 1994; Angelova et al., 2006; Buensanteai et al., 2008).

Abiotic protectors have more effects on the plants. All the factors which cannot be regarded as natural components of the environment of a plant cell, are considered as abiotic protectors. Most of them are metal ions, Salicylic acid, methyl jasmonate, calcium chloride, silver nitrate, copper sulfate, cinnamic acids, etc. (Pitta-Alvarez et al., 2000; Naik and Al-Khayri, 2016; Le Thanh et al., 2017). In this study, salicylic acid induced resistance against CAD pathogens was studied.

2.3.6.2 Salicylic acid (SA)

Salicylic acid (SA) is a phenolic derivative involved in the signalling pathway to induce local and systemic resistance in plants against pathogens (Hussain et al., 2015; Le Thanh et al., 2017). It can be synthesized from the cinnamate pathway by phenylalanine ammonia-lyase (PAL) (Figure 2.2) (Chen et al., 2009). That has been shown to play an important role in the expression of local resistance controlled by important genes to develop the induced resistance after the initial pathogen infection (Hammerschmidt and Kuć, 1995; Saikia et al., 2003; Kuć, 2006; Le Thanh et al., 2017). The resistance also has an effect on induced defense expression, H_2O_2 accumulation, and programmed cell death in plants (Levine et al., 1994; Kauss and Jeblick, 1996). Some studies showed that SA is a translocated signal for SAR, and play a role in the SAR gene expression (Vernooij et al., 1994; Pieterse et al., 1996; Seah et al., 1996). Ganesan and Thomas, (2001) studied the hydrogen peroxide accumulation response to salicylic acid (SA) in rice leaves. They found that rice plants stimulated with SA can activate the as element. That has been involved with the preventive mechanisms that operate during oxidative stress and the hypersensitive response (HR). These authors

also found that PR5 gene expression is one of the PR genes associated with SAR which is induced by SA. This induction seems to be independent of the H_2O_2 accumulation induced by SA. Mayers et al. (2005) reported that SA can induce the resistance to cucumber mosaic virus (CMV) in tobacco (*Nicotiana tabacum*), resulting from an inhibition of virus movement and induced signal pathway and also triggered by antimycin A. Ascencio-Ibáñez et al. (2008) reported that plants respond to pathogens by the salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) pathways. The gene profiling data showed that PR1, PR2, and PR5 transcripts, which are markers for the SA response, were elevated during Cabbage leaf curl virus (CaLCuV) infection. Gogbeu (2015) focused on phenolic compounds. In Gogbeu's research, SA, phosphorous acid (PA) and the fungicide Sumi 8 were used for elicitation of cassava plants. The results showed that cassava cultivars Yacé and Bonoua2 were more susceptible to *C. gloeosporioides*. The accumulation of phenolic compounds was more stimulated by SA and PA. Phenolic compounds accumulation has been correlated with the resistance of cassava to *C. gloeosoporoides.* In these cassava plants, especially those directly germinated in elicitation medium, symptoms of anthracnose and stem rot were reduced.

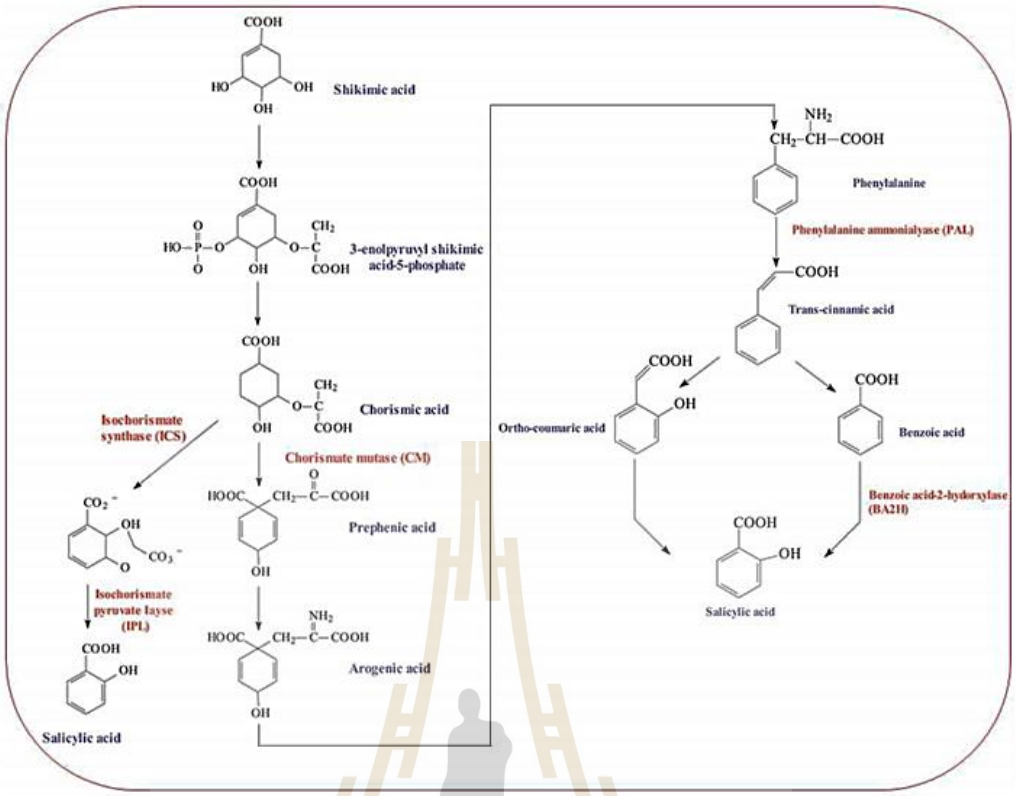

Figure 2.2 Salicylic acid (SA) biosynthesis pathway starting from Shikimic acid (Khan et al., 2015)

2.4 Technical approaches to study the biochemical changes of induced resistance mechanism

To study the biochemical changes associated with the cassava defense mechanism of induced resistance, several techniques were conducted as follows:

2.4.1 Real Time PCR

Real-time PCR is a method used for the detection progress of a PCR reaction in real-time. At the same time, this technique can be quantified of PCR products. Real-time PCR based on the detection of fluorescence produced by molecule increases from reaction proceeds. The reporter fluorescent molecular including dyes that bind to the

double-stranded DNA (i.e. SYBR® Green) or sequence-specific probes (i.e. Molecular Beacons or TaqMan® Probes). That can help for assays the accurate expression profiling of RNA transcripts and has become the most useful method for characterizing gene expression in plant research. Reports have been published of quantitative real-time (qRT-PCR) in plants to study the expressions of hormones (Benítez-Burraco et al., 2003) and to analyze the differential expression of genes involved in the SA- and JA/ET defense pathways at different time points on resistant and susceptible cultivars (Duan et al., 2014; Le Thanh et al., 2017). Djami-Tchatchou et al. (2013) studied about the expression of genes involving the defense responses and defense-related genes during process against the anthracnose disease by real-time PCR in avocado. Utsumi et al. (2016) showed that the expressions of plant defense-related genes, such as pathogenesis-related (PR) genes, cell wall-related genes, detoxification enzymes, genes related to the responses to the bacterium, mitogen-activated protein kinase (MAPK), genes related to salicylic acid, jasmonic acid, and ethylene pathways response to *C. gloeosporioides* using quantitative real-time reverse transcription PCR and expression profiling by the microarray in cassava cultivars Huay Bong 60.

2.4.2 Fourier Transform Infrared (FTIR) microspectroscopy

FTIR microspectroscopy is a technique capable of producing images from biological tissue samples. The absorption of infrared light is measured by a microscopic through the tissue section and the infrared absorption spectrum is determined pixel-by-pixel Synchrotron-based Fourier Transform Infrared (SR-FTIR) microspectroscopy. This is a new tool developed for bioanalytical technique that is rapid, direct, non-destructive tissue and can analyze samples at the micron level or micro-sample areas (Yu, 2004; Thumanu et al., 2015). Advantages of synchrotron light are small effective

source size, capable of exploring the molecular chemistry within the microstructures of samples and high ratio (Yu, 2004; Kastyak-Ibrahim et al., 2012; Wang et al., 2015). SR-FTIR is a new technique to study plant tissues and used to detect the interactions between plant microbes and plant pathogen responses. Moreover, that can use to characterize functional groups of plant cellular components and identify microorganisms, etc. (Dokken et al., 2005; Yu, 2008; Thumanu et al., 2015, 2017). A report on the use of FTIR in plants was published. McCann et al. (1992) demonstrated that FTIR spectroscopy can detect the changes of pectic polymers on drying onion cell walls compare with the chemical extraction sequence. The results found that FTIR spectroscopy is a powerful and rapid assay for cell wall components and cross-links by identifying polymers and functional groups. Yu (2008) used SR-FTIR microspectroscopy to analyze protein molecular structure in canola seeds. The cluster analysis and principal component analysis showed non-differences between the yellow and brown canola seed tissues but in different ratios. Thumanu et al. (2015) studied about *Bacillus subtilis* CaSUT007 which can be induced to stimulate the growth of cassava using FTIR microspectroscopy. FTIR analysis showed that cassava leaves after treated with CaSUT007 has changes in the epidermis and mesophyll tissue related to changes in growth and development on cassava plants. Thumanu et al. (2017) have been investigated the change of chili structure after treated with *Bacillus subtilis* strain D604 by SR-FTIR. The result revealed that the changes in the biochemical component in the cell were observed in lipids, lignin, pectin, and polysaccharides in the treated plants and inoculated with the pathogen.

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CHAPTER III

EFFECT OF SALICYLIC ACID FORMULATIONS ON INDUCED PLANT DEFENCE AGAINST CASSAVA ANTHRACNOSE DISEASE

ABSTRACT

This study was to investigate defense mechanisms on cassava induced by 5 salicylic acid formulations (RSA protectors) against anthracnose disease. Our results indicated that the RSA formulations no. 3, 6, 8, 10 and 16 could be reduce anthracnose severity in cassava plants up to 33.3-50.0% under the greenhouse condition. Then, the *β*-1,3-glucanase and chitinase enzyme activities of defense mechanism were significantly increased at 24 HAI and decrease at 48 HAI after *Colletotrichum gloeosporioides* challenge inoculation, respectively, after cassava treated with RSA formulations. In addition, SR-FTIR spectra was used for biochemical change and defense mechanism analysis. The results revealed that the cassava biochemical changes in the C=H stretching vibration (3000-2800 cm^{-1}), pectin (1740-1700 cm^{-1}), Amide I protein (1700-1600 cm^{-1}), Amide II protein (1600-y1500 cm^{-1}), lignin (1515cm^{-1}) as well as Mainly C–O–C of polysaccharides (1300-1100cm^{-1}) in the leaf epidermal and mesophyll tissues treated with RSA formulations, compared to those treated with fungicide Carbendazim® and distilled water after the challenged inoculation with *C. gloeosporioides*. Hence, the results indicate that biochemical changes in cassava leaf treated with salicylic acid RSA formulations played an

important role in the enhancement of structural and chemical defense mechanisms leading to reduced anthracnose severity.

Keywords: anthracnose disease, cassava, formulation of salicylic acid, SR-FTIR

3.1 Introduction

Anthracnose disease is widespread in cassava plants grown in various countries including Nigeria, Tanzania, Africa, India, and Thailand (Magdalena, Ernest, and Robert, 2012; Sungpeak et al., 2017). In Nigeria, the harsh ascendant of the disease was found that influence above 80% of cassava yield. Economic yield losses out of cassava anthracnose disease (CAD) in Nigeria an obstruction to approximation because of disease severity depend very much on environmental factors (Onyeka et al., 2008). In Africa, CAD outbreak is the main issues on account of its can damage in cassava. A violent infection could affect a decrease in total crop products (Wokocha, Nneke, and Umechuruba, 2010).

The susceptibility varieties of cassava to CAD can affect difference of disease severity. The damage caused by the disease has been reported in Congo, with severe destruction of up to 90% of cassava production. The disease is caused by *C. graminicola*, *C. gloeosporioides* and *C. gloeosporioides* f.sp. *manihotis* (Liu et al., 2019). In Thailand, anthracnose outbreaks have occurred in the northeastern region, it causes the loss of up to 80% of cassava products (Sangpueak, Phansak, and Buensanteai, 2018). The physical symptoms of CAD are wilt, defoliation, necrotic lesions on leaves, bases of leaf petioles and stems and dieback (Sangpueak et al., 2018; Liu et al., 2019).

Although CAD has been listed as the importance disease of cassava outbreak in Africa, little effort has been made in developing its effective control measures because the disease is considered less serious compared to cassava mosaic and bacterial blight diseases. As a result general controlling practices, such as crop sanitation, crop rotation, using clean propagation stalks, and quarantine measure have been recommended (Fokunang, Ikotun, Dixon, and Akem, 1997). Applying fungicides is also encouraged when it is necessary but using resistant varieties is considered the best choice to control the CAD. It is only recently that such resistant varieties have been identified in Africa (Legg and Bouwmeester, 2010). In Thailand, because of a more humid condition, CAD has become a serious threat. This combining with the outbreak of pink mealy bug (*Phenacoccus manihotis*) which can predispose cassava to the anthracnose infection make it devastating if the disease is not properly controlled (Fokunang et al., 1997). Apart from the available recommended methods, using induced resistance has been shown to be promising in controlling the CAD (Sompong, Wongkaew, Tantasawat, and Buensanteai, 2012). In more general, induced resistance be able to categorize in two major types, including systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR could stimulate by treatment with inducer, including non-pathogenic pathogens and some chemicals, in which salicylic acid (SA) is an intermediate in this pathway (Żur et al., 2013; Nair and Umamaheswaran, 2016; Muthulakshmi and Lingakumar, 2017). ISR develops from the settlement of roots by soil bacteria or plant growth promoting rhizobacteria and intercede by a jasmonate and ethylene pathway. Induction resistance could bring about to the direct motivation of tissue defenses, cause stronger elicitation of resistance to disease, following pathogen attack (Jendoubi, Harbaoui, and Hamada, 2015).

Salicylic acid (SA) has an essential role in the induction of defense response to stress responses (War, Paulraj, War, and Ignacimuthu, 2011; Prakongkha, Sompong, Wongkaew, Athinuwat, and Buensanteai, 2013). Furthermore, it has a role in regulating the components of the signaling pathway. Moreover, that can be contributed to cross-talk with many different paths act as intermediary plant resistance (Lu, 2009; War et al., 2011; Lu, Greenberg, and Holuigue, 2016). Strawberry treated with SA at different concentrations have higher shoot and root weight covering to chlorophyll content is higher in stress condition (Bradford, 1976; Karlidag, Yildirim, and Turan, 2009). The induced treatment in various plants by phosphate salts, phosphorous acid or salicylic acid produced prevention against multiple pathogens. Treatment of cassava leaves and roots by salicylic acid induced an accumulation of phenolic compounds in leaves. With supporting data from induced resistance research, many commercial activator enhancers have been enhancers have been listed and used in crop production, e.g. Bion and Actigard (Syngenta), Elexa (chitosan; Safe Science). The work of Gogbeu et al. (2015) reported that SA, phosphoric acid, jasmonic acid, vitamin C and ascorbic acid have high effective protectors mechanism to control cassava diseases seem to be the latest development in farmer field. For induced resistance mechanism, there are several techniques either traditional and molecular techniques that can be used to investigate the structural and functional changes of plant defense response against plant diseases (Alonso-Simón et al., 2011; Gogbeu, Sekou, Kouakou, Dogbo, and Bekro, 2015). FTIR spectroscopy is one of the processes that is rapid, low cost and simple to use for the analysis of plant tissues. Synchrotron radiation based on Fourier-transform infrared Spectro microscopy (SR-FTIR) has applied to detect direct samples and undestroyed at the micron level

(Alonso-Simón et al., 2011; Wang et al., 2015). This technique has benefits synchrotron light brightness can use to study the chemical structure inside the molecular of biological tissue without destroying physical structures inside cells (Yu, 2004). It has been successfully employed in the differentiation of pathogens and investigations of plant interactions and plant chemical variations (Thumanu, Sompong, Phansak, Nontapot, and Buensanteai, 2015). Then, this study was to investigate the biochemical and behavior changes of cassava after being induced by RSA protector formulations using the SR-FTIR technique together with plant defense enzyme activities.

3.2 Materials and methods

3.2.1 Salicylic acid formulation preparation

Five salicylic acid formulation (RSA protectors) preparations, RSA no. 3, 6, 8,10 and 16 were prepared by dissolving them in sterile water and adjusted to the final concentration 200 mg/l salicylic acid (active ingredient). The formulations are the products of the CS Tapioca Research and Innovation Co., Ltd, Thailand. The 200 mg/l SA concentration was selected to be used to use from the preliminary results showing that it was the most effective among those tested (data not shown).

3.2.2 Efficacy of RSA protectors in inducing resistance to anthracnose disease in cassava under a greenhouse condition

By design the randomized complete block design with four replications, cassava stalks cv. Rayong 72 washed with 1% sodium hypochlorite solution for 2 min, followed by washing with sterile water three times, after that drying for 5 min at room temperature. Subsequently, the cassava stalks were soaked for 5 min in the RSA

protectors preparation before planting. Soaking the stalks in distilled water and Carbendazim® 50% (10 ml/ 20l) WP (Bentus®: Sotus International Co., Ltd, Thailand) 6% salicylic acid (200 mg/l) are negative and positive controls. After planting, the protector preparations, water and Carbendazim® were sprayed on to the plants, until run off, for three more times at 1,2 and 3 months. At 24 hours after the last foliar spray, spore suspensions of *C. gloeosporioides* at 1×10^6 conidia/ml concentration was sprayed on to the healthy cassava leaves until run off. After the inoculation the leaves were put in plastic bags within 48 hours under greenhouse. Disease severity scoring of CAD was collected at 14 days after the inoculation as follows by: 1 = not show the symptoms of anthracnose disease, 2 = show the symptom less than 25% on the leaf, 3 = show the symptom 25 - 50% on the leaf, 4 = show the symptom 50 - 75% on the leaf, and 5 = show the symptom over 75% on the leaf (Sangpueak et al., 2018). After the scoring, the leaves were collected to detect for biochemical changes associated with plant defense using FTIR microspectroscopy.

3.2.3 Differential level of β-1,3-glucanases and chitinases activities

3.2.3.1 Protein concentration

Protein extract method described by Nair and Umamaheswaran (2016). Cassava leaf was kept at 0, 24 and 48 hours after infection (HAI). Grind 1 g of tissue in liquid nitrogen and 5 ml extraction buffer that contained of 0.1 M sodium phosphate buffer (pH 6.5) and polyvinyl pyrrolidone (PVP) was added. The sample protein content was examined using the method from Bradford, (1976), using bovine serum albumin (BSA) to be a standard.

3.2.3.2 *β*-1,3-glucanases activity

The crude protein extract (62.5 µl) was mixed within 1% (w/v) laminarin (Sigma-Aldrich, USA) in 0.05M sodium acetate buffer (pH 5.0) after that incubated in 10 min at 40°C (Zur et al., 2013). Stop reaction by the increase of 375 µl (1% dinitrosalicylic acid) and boiling 5 min in water. After that, cooling at 28°C (room temperature) and diluted 1:20. The reaction of enzyme was detection by measured at 500 nm followed by Inchaya et al. (2016); Zur et al. (2013). Enzyme activity is expressed in µg glucose released min^{-1} g fresh weight mg^{-1} protein.

3.2.3.3 Chitinase activity

Chitinase activity was assayed by using crude protein extract (400 µl) mixed with 0.1% (w/v) colloidal chitin in 0.05 M sodium acetate buffer pH 5.0 at ratio 1:1 (v/v). Then, incubation mixture at 37°C for 2h. Detection of N-acetyl glucosamine (GlcNAc) by absorbance at 585 nm followed by Prakongkha et al. (2013). For standard curve can be calculated using a standard curve prepared from GlcNAc.

3.2.3.4 Statistical analyses

The results of *β*-1,3-glucanases activity and chitinase activities were dissolved one-way analysis of variance (ANOVA) using SPSS version 14. The New Duncan's Multiple Range Test (DMRT) was used to differentiate treatment at $P \leq 0.05$.

3.2.4. Biochemical changes analysis using SR-FTIR microspectroscopy

3.2.4.1 Sample preparation for SR-FTIR microspectroscopy

Only the 5th top leaves from that of the most effective protector treatments were collected for SR-FTIR microspectroscopic analysis. Cassava leaves at the same position from that of the negative and positive controls were also included

for the comparison. The analysis proceeded in a completely randomized design (CRD) within three replications. The samples were subsequently cut to 1x1 cm size and embedded in the OCT compound (optimal cutting temperature), followed by Quick-freezing in liquid nitrogen. Then, the samples were stored into a -80^{o}C as long as to do cryo-sectioning process. Subsequently, each of cassava leaf samples was cut crosswise to 10 μm using a microtome of cryostat (Leica 3050 S, Germany) and stick on BaF2 window size 13 x 2 mm for SR-FTIR microspectroscopy.

3.2.4.2 Data measurement of SR-FTIR microspectroscopy

The determination was operated with a mapping by using a size of aperture at 10 × 10 μm with a resolution of 4 cm^{-1}, with 64 scans. By used spectra was received with FTIR spectrometer (Vertex 70 from Bruker Optics, Ettlingen, Germany) together with IR microscope (Hyperion series 2000, Bruker) with MCT detector refrigerate in liquid nitrogen through the determination range from 3000 to 800 cm^{-1}. Spectral equipment control was carried on by OPUS 7.2 software (Bruker Optics Ltd, Ettlingen, Germany) at the Synchrotron Light Research Institute (SLRI).

3.2.4.3 Cassava image analysis

IR imaging of cassava tissues was created and analyzed using Cytospec 1.3.4 (Cytospec Inc, NY, USA). The peak was transferred to the second derivative by using 13 smoothing points and vector normalized for the differing sample. The image structuring can be using a univariate model of generates up to peak intensity and peak area typically have meanings as chemical group maps. Hierarchical Cluster Analysis (HCA) was operated to separate the biochemical different components sample overranges of (3000-2800 and 1800-900 cm^{-1}). The 2D cluster maps saved as figure files with a particular color to define a cluster. The spectra of cassava tissue from

treated and non-treated plants were assays by principal cluster analysis (PCA) to differentiate the different biochemical composition of the tissue.

3.2.4.4 PCA analysis

Spectra from each cluster were assayed using PCA for differentiating of biochemical elemental of tissue. The process was using 2nd derivative and vector normalized (Savitzky-Golay method, 3rd polynomial, 9 smoothing points) from the Unscrambler software version 9.7 (CAMO, Norway)

3.3 Results

3.3.1. Efficacy of RSA protectors in inducing resistance to CAD

At 14 days after the last foliar spray with four RSA protectors of 200 mg/l SA concentration, anthracnose severity on cassava treated with formulations RSA3 were among the lowest at 33.3%, comparable to that treated with Carbendazim®. The severity observed on the negative control, where the cassava was treated with water, was among the highest at 77.7% (Table 3.1).

Table 3.1 Efficacy of RSA protectors on severity of cassava anthracnose disease caused by *Colletotrichum gloeosporioides* under a greenhouse condition at 14 day after inoculation.

Treatments[1/]	**Disease severity (%)***
RSA3 (200 mg/l)	33.3[c]
RSA6 (200 mg/l)	55.5[abc]
RSA8 (200 mg/l)	44.4[bc]
RSA10 (200 mg/l)	50.0[abc]
RSA16 (200 mg/l)	33.3[c]
E-TER ® (1 kg/l)	66.6[ab]
6% Salicylic acid (200 mg/l)	33.0[c]
Carbendazim® (10 ml/20 l)	66.6[ab]
Water (control)	77.7[a]
F-test	*
CV%	0.82

[1/] Final concentration of active ingredients. [2/]Mean ± standard deviation. Each value represents a mean of three replicates. [3/] 6% SA= 6% of salicylic acid solution in 70% ethanol and adjusting the final volume with water to 50,100,200 and 500 mg/l for compare with formulations. Mean values in each column followed by a different lower-case-letter are significantly different by Duncan's multiple range test at P = 0.05.

3.3.2 *β*-1,3-glucanases activity

The induction of *β*-1,3-glucanase by treated with RSA protectors and inoculation with pathogen showed the increased response of *β*-1,3-glucanase level of all of RSA protectors. It's has increased at 24 HAI and decrease at 48 HAI

significantly different when compared with control. Especially, RSA 3 have increased at 24 HAI at 16.65 µg glucose released min^{-1} mg^{-1} protein was significantly higher compared with non-treated plant (Figure 3.1).

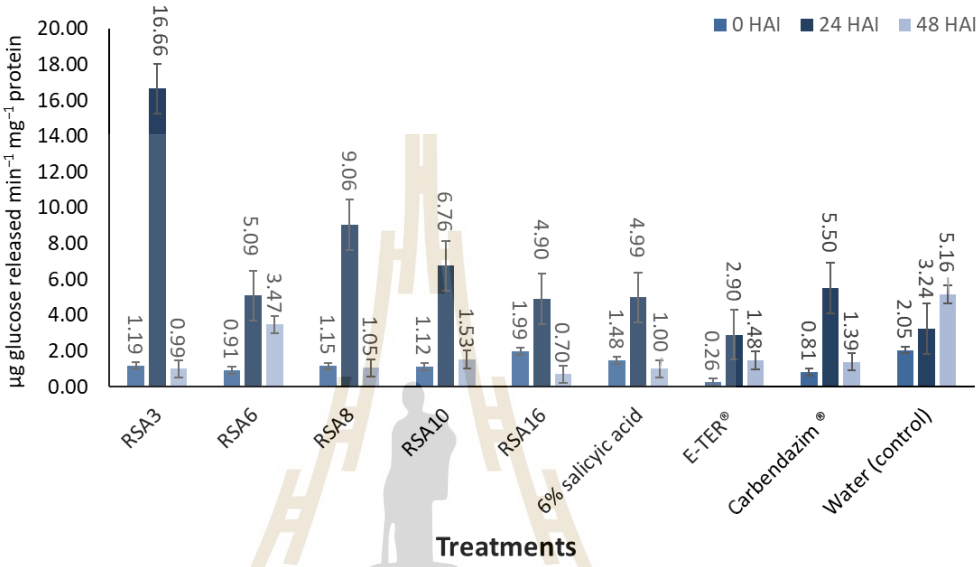


Figure 3.1 Enhancement of *β*-1,3-glucanase activities in cassava leave soaking and foliar spray treatment at 0,24 and 48 HAI with *C. gloeosporioides*.

3.3.3 Chitinase activity

Chitinase activity of cassava leaf after treated with RSA formulation and inoculation with fungal pathogen. The results show that chitinase activity was observed all of the treatments of RSA protectors increased rapidly at 24 HAI and decrease at 48 HAI significantly different when compared with non-treated plant (control). 6% salicylic acid can be induced the accumulation of chitinase has the highest level at 0.287 µmol glcnac formed min^{-1} mg^{-1} protein followed by RSA 6 and RSA 3 have chitinase level at 0.275 and 0.275287 µmol glcnac formed min^{-1} mg^{-1} protein respectively (Figure 3.2).

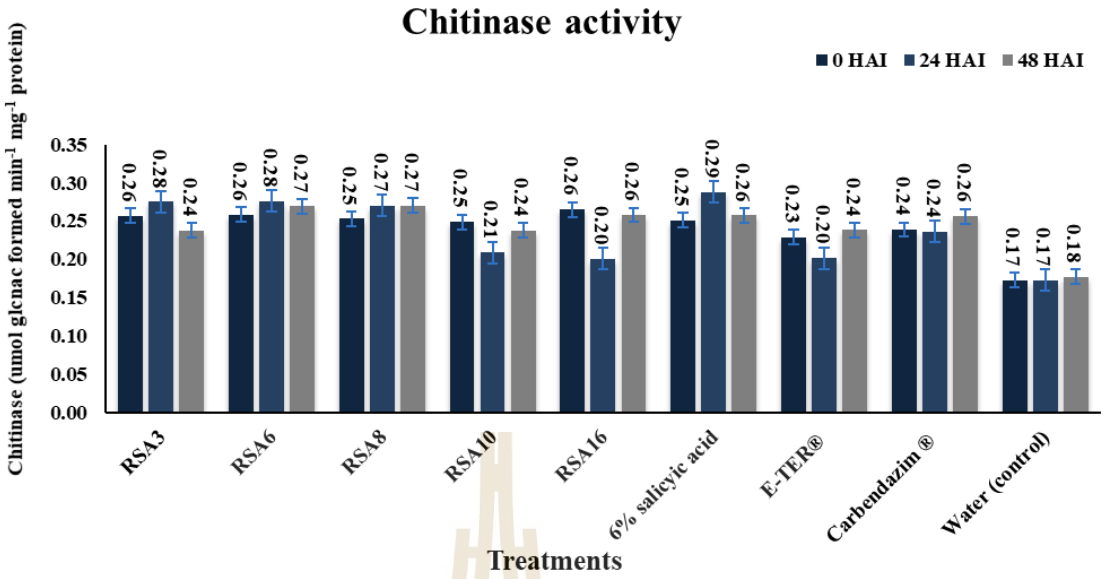
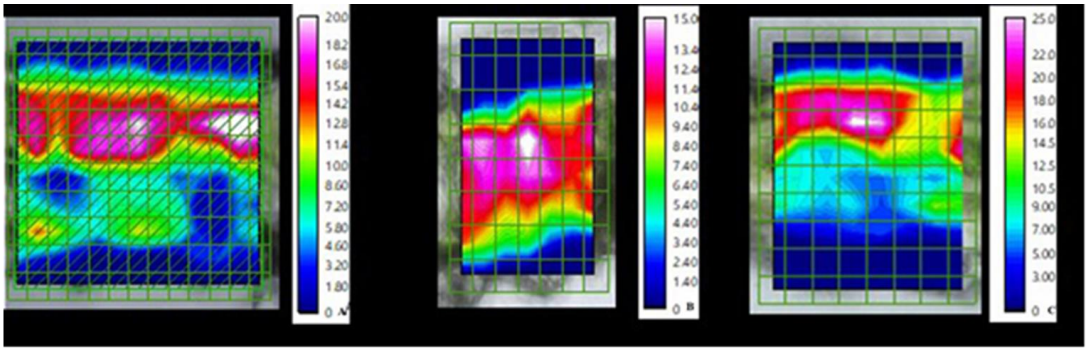


Figure 3.2 Enhancement of chitinase activities in cassava leave soaking and foliar spray treatment at 0, 24 and 48 HAI with *C. gloeosporioides*

3.3.4 Biochemical changes analysis using SR-FTIR microspectroscopy

The purpose of this experiment to study the effect of RSA 3 formulations compared to that of the fungicide Carbendazim® of cassava defense. The biochemical and cell configuration of cassava leaf in epidermal and mesophyll tissues by using SR-FTIR. Biochemical changes were compared in the treated and non-treated cassava leaf. The IR images indicated absorbance intenseness of imaging were relative to color change: blue (show the lowest of chemical imaging) < green < red which show the highest of chemical imaging (Figure 3.3 A-C).



A) Cassava treated with water (the control) B) Cassava treated with elicitor RSA® 3 C) Cassava treated with fungicide

Figure 3.3 The IR images displaying absorbance intensities from chemical mapping were proportional to color change: blue (the lowest) < green < red (the highest). A) Cassava treated with water (the control). B) Cassava treated with protector RSA 3. C) Cassava treated with fungicide

HCA was applicable to separate spectra depending on 5 clusters in the among of range 3000 – 900 cm^{-1}, and changed the color of different groups. The chemical imaging of microstructures of cassava leaves is shown in Figure 3.4 (A-F). Leaf tissues of the negative and positive controls were also observed to correlate the changes of spectral initiate in the proteins, lipids, and polysaccharides to that of the protector treated tissues.

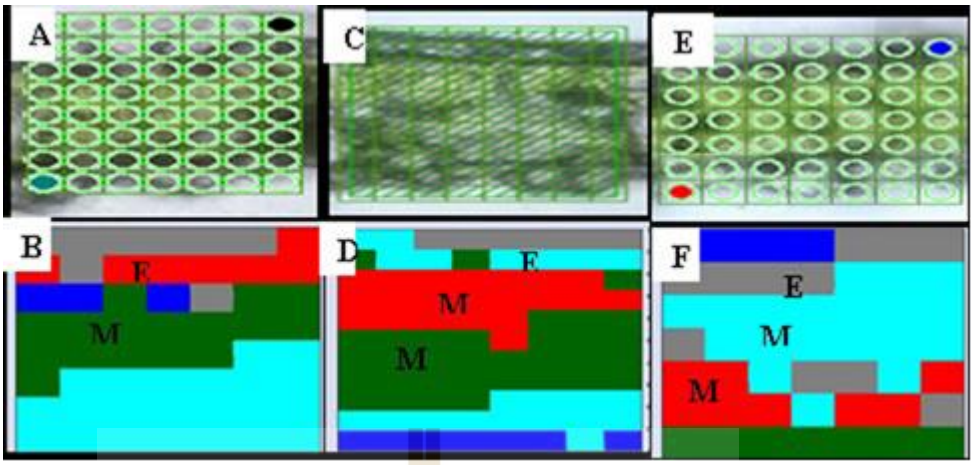


Figure 3.4 Maps of representative of functional group area obtained under the spectral region of cassava leaves cv. R72. (A, B) Map of SR-FTIR microspectroscopy and HCA map of cassava treated with protectors RSA 3. (C, D) Map and HCA map of cassava treated with water (the control). (G, H) Map and HCA map of cassava treated with fungicide

Spectra and secondary derivative average spectrum in the phase of 3000–2800 and 1800–900 cm^{-1} were differences of the epidermal and mesophyll tissues (Figure 3.5). Amide II protein (1600-1500 cm^{-1}), no significant difference was found in the cassava epidermis of those treated with the RSA 3 formulations compared to those of the positive and negative controls. The differences C=H stretching vibration (3000-2800 cm^{-1}), pectin (1740-1700 cm^{-1}), Amide I protein (1700-1600 cm^{-1}), lignin (1515cm^{-1}), Mainly C–O–C of polysaccharides (1300-1100cm^{-1}) were found at some vibrational peaks of (Table 3.2 and Figure 3.5B).

Mesophyll tissue of cassava leaves treated with RSA 3 also had a significant difference in the cassava epidermis of those treated with the RSA 3 formulations

compared to those of the positive and negative controls. At the vibrational peaks of pectin, Amide I protein, Amide II protein, lignin, Mainly C–O–C of polysaccharides while spectrum of C-H stretching was no significant difference in mesophyll tissue were calculated in Table 3.3 (Figure 3.5D).

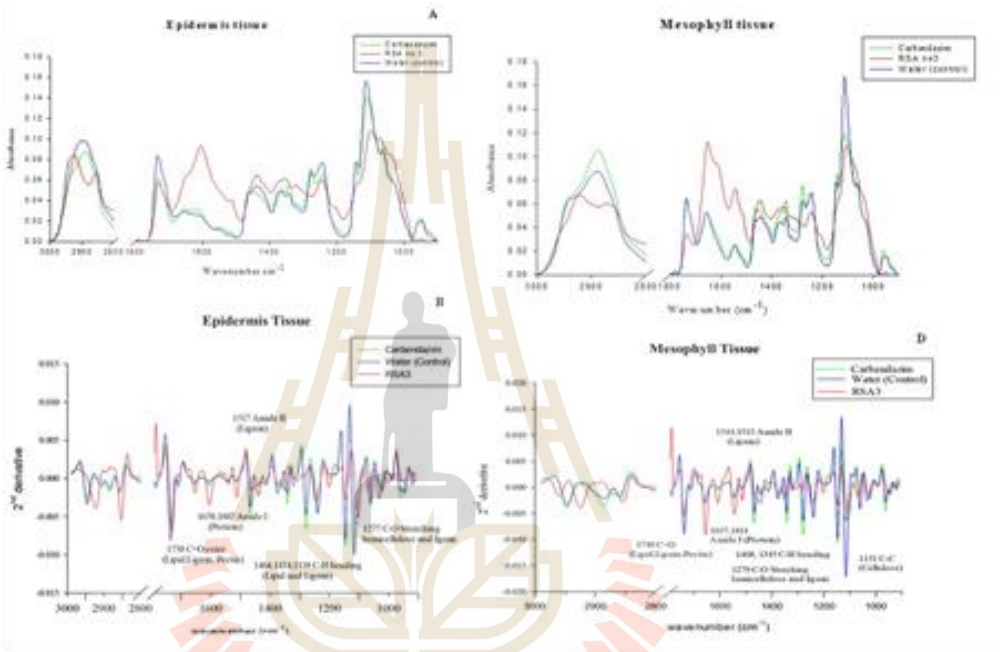


Figure 3.5 Original and second derivative average spectra in cassava cv. R72 treated with 200 mg/l salicylic acid formulation (RSA 3) compared with carbendazim® or water and challenge inoculated with *C. gloeosporioides* at 14 DAI under a greenhouse condition. Representative original average spectra in the range of 3000–2810 cm^{-1} and 1800–900 cm^{-1}. (A) original average spectra in epidermis tissues of cassava leaf, (B) second derivative in epidermis tissues of cassava leaf, (C) original average spectra in mesophyll tissues of cassava leaf, (D) second derivative in epidermis tissues of cassava leaf mesophyll tissues

Table 3.2 The integral area of average spectra from epidermis of cassava leaf treated with salicylic protector (RSA 3) and challenge inoculation with *Colletotrichum gloeosporioides*.

Treatments	Functional groups					
	C=H stretching vibration (3000-2800 cm^{-1})	**Pectin (1740-1700 cm^{-1})**	**Amide I protein (1700-1600 cm^{-1})**	**Amide II protein (1600-1500 cm^{-1})**	**lignin (1515 cm^{-1})**	**Mainly C–O–C of polysaccharides; very complex and depends upon contributions from polysaccharides, cellulose, hemicellulose, and pectin (1300-1100 cm^{-1})**
RSA 3	0.0400 ±0.008[a]	0.0260±0.002[ab]	0.0223±0.003[a]	0.0016±0.000	0.0076±0.001[a]	0.0443±0.006[b]
Carbendazim®	0.0236±0.003[b]	0.0253±0.001[b]	0.0073±0.000[b]	0.0013±0.001	0.0010±0.000[b]	0.0893±0.004[a]
Water	0.0256±0.001[b]	0.0280±0.001[a]	0.0040±0.000[b]	0.0010±0.000	0.0006±0.000[c]	0.0823±0.002[a]
F-Test	*	*	**	ns	**	**

Note: Different letters indicate significant differences ($p \leq 0.05$)

Table 3.3 The integral area of average spectra from mesophyll of cassava leaf treated with salicylic protector (RSA 3) and challenge inoculation with *Colletotrichum gloeosporioides*.

Treatments	Functional groups					
	C=H stretching vibration (3000-2800 cm^{-1})	**Pectin (1740-1700 cm^{-1})**	**Amide I protein (1700-1600 cm^{-1})**	**Amide II protein (1600-1500 cm^{-1})**	**lignin (1515 cm^{-1})**	**Mainly C–O–C of polysaccharides; very complex and depends upon contributions from polysaccharides, cellulose, hemicellulose, and pectin (1300-1100 cm^{-1})**
RSA 3	0.0380±0.002	0.0226±0.001^{b}	0.0536±0.004^{a}	0.0213±0.002^{b}	0.0070±0.000^{a}	0.0496±0.001^{c}
Carbendazim®	0.0373±0.002	0.0346±0.002^{a}	0.0140±1.734^{b}	0.0073±0.000^{a}	0.0000±0.000^{b}	0.1026±0.009^{b}
Water	0.0323±0.004	0.0363±0.003^{a}	0.0136±0.002^{c}	0.0076±0.001a	0.0000±0.000^{b}	0.1320±0.009^{a}
F-Test	ns	**	**	**	**	**

Note: Different letters indicate significant differences ($p \leq 0.05$)

This information represents course changes of biochemical in cassava after being treated of RSA 3 formulations, elucidating the response to defense mechanisms against anthracnose disease. To used PCA technique was applied to analyze biochemical changes. This technique is useful in analyses of bio spectroscopic data by making it visible of similar spectra in scores and loading plots (data not showed) between treated and nontreated leaves. In addition, the data analysis represents that the PC1 and PC2 regularly is the most difference clustering two or three groups. The red points representing RSA 3 treatment could be easily distinguished from the blue and green points of the water control and Carbendazim. The PC1 and PC2 loading of RSA 3 treatment were shown that separation between PC1 and PC2 corresponded to total variance of 49% from PC1 and 14% in epidermis tissue (Figure 3.6 A and C). For mesophyll tissue Found that the separation between PC1 and PC2 corresponded to a total variance of 64% from PC1 and 13% (Figure 3.6 B and D). The results show structural and physiological changes associated with the defense mechanism in cassava.

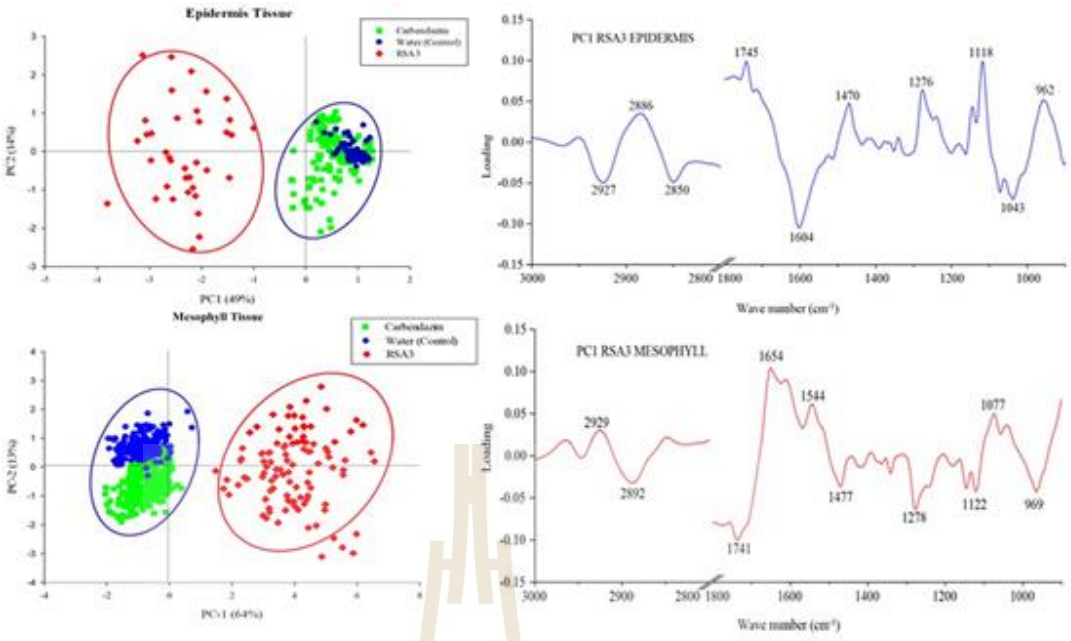


Figure 3.6 PCA analysis in cassava cv. R72 cassava leaves treated with 200 mg/l Salicylic acid formulation (RSA 3) and compared with Carbendazim® or water and challenge inoculated with *C. gloeosporioides* at 14 DAI under a greenhouse condition. (A and B) 2D scatter plot of score from PCA analysis of RSA 3 in epidermis and mesophyll tissue; (C and D) loading plots from PCA analysis of epidermis and mesophyll tissue

3.4 Discussion

The application of 5 RSA formulations by sprayed could be induce cassava leaf resistance to anthracnose caused by *C. gloeosporioides*. These resistances were depending upon the concentration of each protector formulation, similar to operates in different plant pathosystems (Anderson et al., 2013). In this present study, the most efficacy for induction was obtained at concentrations at 200 mg/l of RSA 3 were used to compare with fungicide and water control. RSA 3 can reduce disease severity by

33.3% compared with the control. Similar results were observed to previous studies, In hydroponic tomato plants, with the treatment of SA at 200 µM showed to control 9% of leaf yellowing of Fusarium wilt, compared with the control which exhibited disease incidence reaching 70%, at two weeks after inoculation (Jendoubi et al., 2015). Exogenous application of 50 µM SA at 15 days before inoculation reduced the severity of leaf curl virus in tomato (Ong and Filomena, 2016). SA-treated plants tolerance disease severity at approximately 60%, significantly lower than that of the control at about 78%. In sweet green pepper plants, the spray of SA at a concentration of the diameter of leaf lesions, prevented the development of wilt disease caused by *Fusarium oxysporum* (Yousif, 2018).

Also, salicylic acid can be induced, accumulate methodically are linked to systemic acquired response (SAR) (Gao et al., 2015). These results show that the change of β -1,3-glucanase and chitinase at 24 HAI after last sprayed with RSA formulation and inoculation with fungal pathogen that had been related to plant defense mechanisms against pathogen attack and can destroy the constituents of cell walls of *C. gloeosporioides* in the attacked cassava tissue (Gupta et al., 2012; Santos et al., 2004). These enzymes an essential role for induced cassava resistance to pathogens (Thakur et al., 2013). It can be substantiated that protector can enhance the resistance of PR proteins and also act as increase plants resistance

(Jayaraj et al., 2004; El-kereamy et al., 2011).

In addition to plants defence depend on the contribution of the cassava cell wall to against an infection to pathogens. This research focuses on the composition changes of cell wall in exogenous RSA formulation treated cassava by using SR-FTIR microspectroscopy. Because of the synchrotron light that gives the intensity and

source brighter than conventional sources. In addition, it allows the analysis results to have a ratio between signal and noise ratio (S/N) better without losing spatial resolution (Baker et al., 2014). And also helps to reduce the analysis time compared to using conventional IR Source (Wang et al., 2016). The SR-FTIR mapping displays the integrated area of proteins, lipids, and polysaccharides at absorption between 3000–2800 and 1800–900 cm^{-1} (Wang et al., 2016). The peak variation between treatments indicated that the changes in the intensity of lignin, lipids, Amine I of protein and Amine II of lignin in epidermis and mesophyll tissues. The epidermis and mesophyll tissues treated by RSA 3 formulations showed spectra higher than those of the control significantly at the vibrational peaks of C=H stretching vibration, pectin, Amide I protein, lignin, Mainly C–O–C of polysaccharides. That can be help strength cassava cell wall structure. Consistent research of 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 amide I, *β*-sheet structure and lipids. This physical defence may include the amplification of cell wall thickenings such as the blockage of plant vessels. (Jendoubi et al., 2015). The results show physiological and chemical changes with the defense mechanism in cassava against anthracnose disease (Figure 3.7).

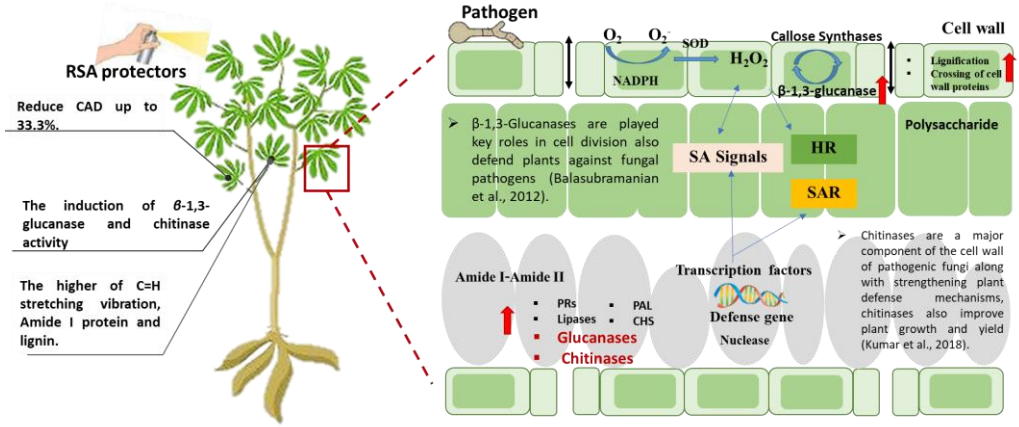


Figure 3.7 Physiological and chemical changes with the defense mechanism in cassava after treated with RSA protector and challenge inoculate with anthracnose pathogen

3.5 Conclusion

Cassava treated with RSA formulation could be reduce anthracnose disease caused by *C. gloeosporioides* on cassava under greenhouse condition up to 33.3-50.0%. This protector can be the induction of *β*-1,3-glucanase and chitinase activity by treatment with protector is correlated by the increased resistance of the cassava tissues against infection by the *C. gloeosporioides* pathogen. Moreover, SR-FTIR microspectroscopy could be used for detecting biochemical changes at a most resolution. Its showed that the structural and biochemical of different cassava tissues after treatment of different protector. This research revealed that the changes of structural in cassava tissue have interaction with plant defense responses after stimulated with the salicylic acid formulation. This research indicated that the changes of the cassava structural tissue have interaction with plant defense responses after stimulated with the salicylic acid formulation in cassava. Besides, one solution of

products containing salicylic acid, especially RSA 3 would be to introduce an alternative product to prevent yield loss of cassava-related infections from anthracnose disease pathogen.

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CHAPTER IV

INFLUENCE OF SALICYLIC ACID PROTECTORS ON HYDROGEN PEROXIDE PROMOTES SALICYLIC ACID ACCUMULATION IN CASSAVA

ABSTRACT

The aim of this study is to investigate the influence of five salicylic acid protectors (RSA protectors) on chlorophyll content, ROS accumulation, phenylalanine ammonia-lyase (PAL) enzyme activity, and endogenous salicylic acid (SA) accumulation in cassava leaves. The results found that after cassava enhanced by RSA protector and challenged with elicitor three times, the highest total chlorophyll content was determined in cassava leaves treated with RSA 16 and RSA 3 at 200 mg/l significantly increased at 0.215±0.017 and 0.194±0.005 µg/mm^2. In addition, the production of reactive oxygen species (ROS) have a higher accumulation of SA biosynthesis in the cassava leaves treated with RSA formulations. It was associated with SAR induction activated by *Colletotrichum* infection. The blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining indicated that the increase of accumulation of O_2^- and H_2O_2 in each of treated cassava leaves tissue reached a maximum at 12 and 24 48 hours after *Colletotrichum* inoculation (HAI) and then reduce to a low level in 48 HAI while at this every time-point is no significant difference was found in the water control treatment. Moreover, PAL activity of cassava leaves after treated with RSA formulation and inoculation with pathogen

showed the increased response of PAL activity level at 24 HAI at 7.957, 6.340, 6.357, 6.933, 8.577 and 6.261 μmol trans-cinnamic acid min^{-1} mg^{-1} protein respectively, and decreased to low-level in 48 HAI. Furthermore, endogenous salicylic acid concentration showed that the RSA 3 formulation can enhance the higher level of endogenous SA content at 24 HAI to approximately 22.74% and decreased in 48 HAI. These data could be confirm the enhanced ROS response before SA biosynthesis is able to trigger for systemic acquired resistance (SAR) system in cassava treated by effective RSA protector and challenge inoculation with anthracnose pathogen infection.

4.1 Introduction

In this recent years, cassava disease is the problem of cassava cultivation in Thailand has become increasingly every year. One of the serious diseases is anthracnose disease causal by *C. gloeosporioides* is a fungal disease affecting cassava production (Fokunang et al., 2001). Cassava is the most cultivated food processing industrial crop in the country and as such the use of chemicals in the control of cassava diseases is not recommended. For control disease and reduced to the use of chemical fungicides. Practical application of induced resistance is a good way to plant diseases (Walters and Fountaine, 2009). That will be the focus on systemic acquired resistance (SAR) for the protection of crops. These controlled by a signaling pathway that dependent on the SA accumulation in plants and enhanced by biotic and abiotic inducers or plants elicitors (Conrath, 2006; Fu and Dong, 2013). Plant elicitors/protector/activator/inducer are a stress activator that induces the accumulation of antibiotically active and the production of phytoalexins in plants (Nuutila and

Oksman-Caldentey, 2003; Angelova, Georgiev, and Roos, 2006). These compounds are protective responses express in plant cellular defense mechanisms when induced by a plant protector or elicitor (Nuutila and Oksman-Caldentey, 2003; Mejía-Teniente et al., 2010).

Elicitors are another interesting group is plant hormones that affect the growth and development of plants (Dempsey and Klessig, 2017). Especially salicylic acid, which is an essential hormone that controls many characteristics of plant growth and development as well as plant innate immunity, induced resistance in the local and systemic systems upon pathogens attacks (Raskin, 1992; Ding and Ding, 2020). That SA influence reactive oxygen species (ROS) by oxidative bursts originating in plant tissue by H_2O_2 bursts conducted by NADPH oxidases and extracellular peroxidases in subcellular locations (Herrera-Vásquez, Salinas, and Holuigue, 2015). The main ROS are singlet oxygen ($^1O_2$), superoxide radicle ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicle (•OH) (Sharma et al., 2020). It has been suggested the SA was caused an increase in the activity was a systemic signal resistance to pathogens in several plants (Duan et al., 2014). Also, that caused an increase of phenylalanine ammonia-lyase (PAL) activity, a key phenolic biosynthesis enzyme that increases plant resistance to fungal pathogens (Sharma et al., 2019). This study to elucidate of cassava mechanisms used a salicylic acid inducer for SA enhances, ROS levels, PAL activity *in vivo* after pathogens infection.

4.2 Materials and methods

4.2.1 Fungal pathogen preparation

The virulent isolates of *C. gloeosporioides* isolate NKSTKLS02 were obtained from plant pathology and biopesticide laboratory, Suranaree University of

Technology, Thailand. After that, re-culture on water ager medium (WA) incubated at room temperature 48h. The pure colonies that were transferred onto potato dextrose agar (PDA) incubated at room temperature 2 weeks before use. *Colletotrichum* spore suspension was obtained by filtered through layers with sterile distilled water and adjusted to 10^6 spores/ml using a hemocytometer and add 1 or 2 drop per 100 ml of drop spore suspension of Tween-20 before used.

4.2.2 Plant inoculation

Healthy cassava leaves of susceptible cultivar, Rayong 72 were soaking 5 min before planting and foliar spray 3 times at 1 to 3 months with five salicylic formulations including RSA 3, RSA 6, RSA 8, RSA 10 and RSA 16 200 mg/l provided promotion cassava growth and protection to anthracnose disease. Compare with 6% salicylic acid at 200 mg/l, plant elicitor (E-TER® 1 kg/20l), chemical fungicide (Carbendazim ® 10 ml /20l) and water are negative and positive controls. At 24 h after the last foliar spray, spore suspensions of *C. gloeosporioides* at 1×10^6 conidia/ml concentration was sprayed on to the healthy cassava leaves until runoff. After the inoculation, the leaves were cover with plastic bags within 48 h under the greenhouse. The leaves were collected to detect biochemical changes associated with plant defense.

4.2.3 Determination of leaf chlorophyll content

Cassava leaves tissues (e.g., 4 mm radius) into a 1.5 ml microtube containing 1 ml of dimethylformamide (DMF). Five replicates for leaves incubated overnight at 4°C. After that mix 300 µl of sample solution with 600 µl of DMF into the new tube. The absorbance read in a spectrophotometer by a microplate reader (Bio-Tek, USA) at 647 nm and 664.5 nm. The chlorophyll content was calculated according to the

following formulas proposed by Jake Harris and Baulocmbe, 2015 (Harris and Baulcombe, 2015).

Chlorophyll a content (µg/ml) = (12 x $A_{664.5}$)-(2.79 x A_{647})

Chlorophyll b content (µg/ml) = (20.78 x A_{647})-(4.88 x $A_{664.5}$)

Total chlorophyll content (µg/ml) = Chl a + Chl b

Sample area (for 3 leaf discs at 4 mm radius) (mm2) = 3 x πr^2

Total chlorophyll content (µg/mm^2) = (Chl a + Chl b)/Sample area

4.2.4. Histochemical detection

The detection of O_2^- and H_2O_2 accumulation was operated by using nitro blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining followed protocol by Kumar et al., 2014 and Daudi and Brien, 2012 (Daudi and O'Brien, 2012; Kumar et al., 2014).

4.2.4.1 NBT staining

Cassava leaves after treatment and inoculation at 0, 12, 24 and 48 h were collected to submerged in NBT solution (0.1 g NBT in 50 mM sodium phosphate buffer pH 7.5). After that store in a brown bottle overnight at room temperature. Then remove the chlorophyll by boiled in 95% ethanol for 15 min or the chlorophyll completely and soaked in 60% glycerol for photograph.

4.2.4.2 DAB staining

The infection leaves were incubated in DAB solution (50 mg in 100 ml of 20mM Tris buffer saline, pH 7.5) keep overnight at room temperature. Then leaves were boiled in 95% ethanol to remove the chlorophyll completely and soaked in 60% glycerol for photos taken.

4.2.5 Enzyme Activities

4.2.5.1 Phenylalanine ammonia lyase (PAL) Activity

Cassava leave extracts were prepared according to Goâmez-vaâsquez et al., (2004) by homogenizing 2 g of cassava leaves with liquid nitrogen and add 4 ml of 0.1 M sodium borate buffer (pH 8.8). Centrifuged at 4°C for 15 min at 20,000 g. The reaction mixture was 200 ml of the enzyme extract in 0.5 ml of 0.1 M sodium borate buffer (pH 8.8). Added to an equal volume of the enzyme extract into 0.02 M D-phenylalanine. The extinction coefficient of cinnamic acid is 16,596 l mol^{-1} cm^{-1} after incubation for 30 min at 30°C and the absorbance measurements at 290 nm (Bio-Tek, USA). One unit (U) of enzyme activity was defined as the amount of enzyme forming 1 pmol of trans-cinnamic acid from L-phenylalanine per min per mg of protein.

4.2.5.2 Indigenous salicylic acid analysis (SA)

The method as follows by (Raskin, Turner, and Melander, 1989) by grind cassava leaves samples (0.5 g) with liquid nitrogen. Add 1 ml of extraction buffer (90:9:1 volume of absolute methanol, glacial acetic acid, and distillate water). After that centrifuged at 12000 rpm and 4°C for 15 min. Then transfer 500 µl of supernatant into a new tube mixed with an equal volume of 0.02 M ferric ammonium sulfate, incubated at 30°C for 5 min. The absorbance was read at 530 nm by a spectrophotometer (Bio-Tek, USA). The read absorbance will be compared with the reference standard to obtain the actual amount of SA in the cassava leaves sample.

4.3 Results

4.3.1. Total chlorophyll content

After cassava challenged with elicitor three times, the highest total chlorophyll content was determined in cassava leaves treated with RSA 16 and RSA 3 at 200 mg/l

significantly increased at 0.215±0.017 and 0.194±0.005 µg/mm^2. when compared with 6% salicylic acid 200 mg/l, Carbendazim ® (10 ml/20l) and untreated plant (Table 4.1).

Table 4.1 Total chlorophyll content of cassava leaves as treated by salicylic formulation at 3 months.

Treatment	Chlorophyll a[2/] (µg/ml)	Chlorophyll b[2/] (µg/ml)	Total Chlorophyll[2/] (µg/mm^2)
RSA3 (200 mg/l)	22.701± 0.533^{ab}	6.674±0.600^{ab}	0.194±0.005^{ab}
RSA6 (200 mg/l)	11.693±4.431^{d}	2.644±1.262^{f}	0.095±0.021^{d}
RSA8 (200 mg/l)	15.910±0.618^{c}	5.906±0.257^{bc}	0.144±0.006^{c}
RSA10 (200 mg/l)	20.547±1.687^{b}	6.967±0.918^{ab}	0.182±0.017^{b}
RSA16 (200 mg/l)	25.175±1.592^{a}	7.259±0.868^{a}	0.215±0.017^{a}
6% salicylic acid (200 mg/l)	17.44±1.272^{c}	5.989±0.632^{bc}	0.155±0.011^{d}
E-TER ® (1 kg/20 l)	16.405±1.145^{c}	5.259±0.086^{cd}	0.143±0.008^{c}
Carbendazim ® (10 ml/20l)	10.093±0.635^{d}	3.910±0.198^{e}	0.092±0.198^{d}
Water (Negative control)	12.500±0.350^{d}	4.601±0.042^{ef}	0.113±0.003^{c}
F-Test	**	**	**
CV%	19.273	8.266	0.002

[1/](Percentage of untreated-total chlorophyll content of treated plants)/(total chlorophyll content of untreated plants. [2/]Mean ± standard deviation. Each value represents a mean of three replicates. Mean values in each column followed by a different lower-case-letter are significantly different by Duncan's multiple range test at P = 0.05.

4.3.2 Histochemical localization of O_2^- and H_2O_2 on in cassava leave

Production of reactive oxygen species (ROS) before SA biosynthesis in cassava leaves after treated with salicylic formulations can be induction of SAR activated by challenged with *Colletotrichum*. NBT staining indicated that the increase of accumulation of O_2^- in each of treated leaves tissue reached a maximum at 12 and 24 HAI with the pathogen and decreased to a low level by 48 HAI. While at this every time-point is no significant difference was found in the water control treatment (Figure 4.1).

DAB staining of leaf tissues shows the accumulation of H_2O_2 at different times. The result indicated that treated plant with RSA 3, RSA 8, RSA 10, RSA 16, 6% including salicylic acid at 200 mg/l and plant elicitor (E-TER® 1 kg/20 l), spray for three times, the results found that this effective RSA formulation can induced a maximum of H_2O_2 at 12 HAI and decreased in 24 and 48 HAI. Moreover, the treatment of RSA 6, chemical fungicide as Carbendazim® 10 ml/20l and water found the accumulation of H_2O_2 very low-level and delay in 24 and 48 HAI (Figure 4.2). These data can confirm the enhanced ROS synthesis before SA synthesis be able to stimulate for SAR induction in cassava after Colletotrichum pathogen infection.

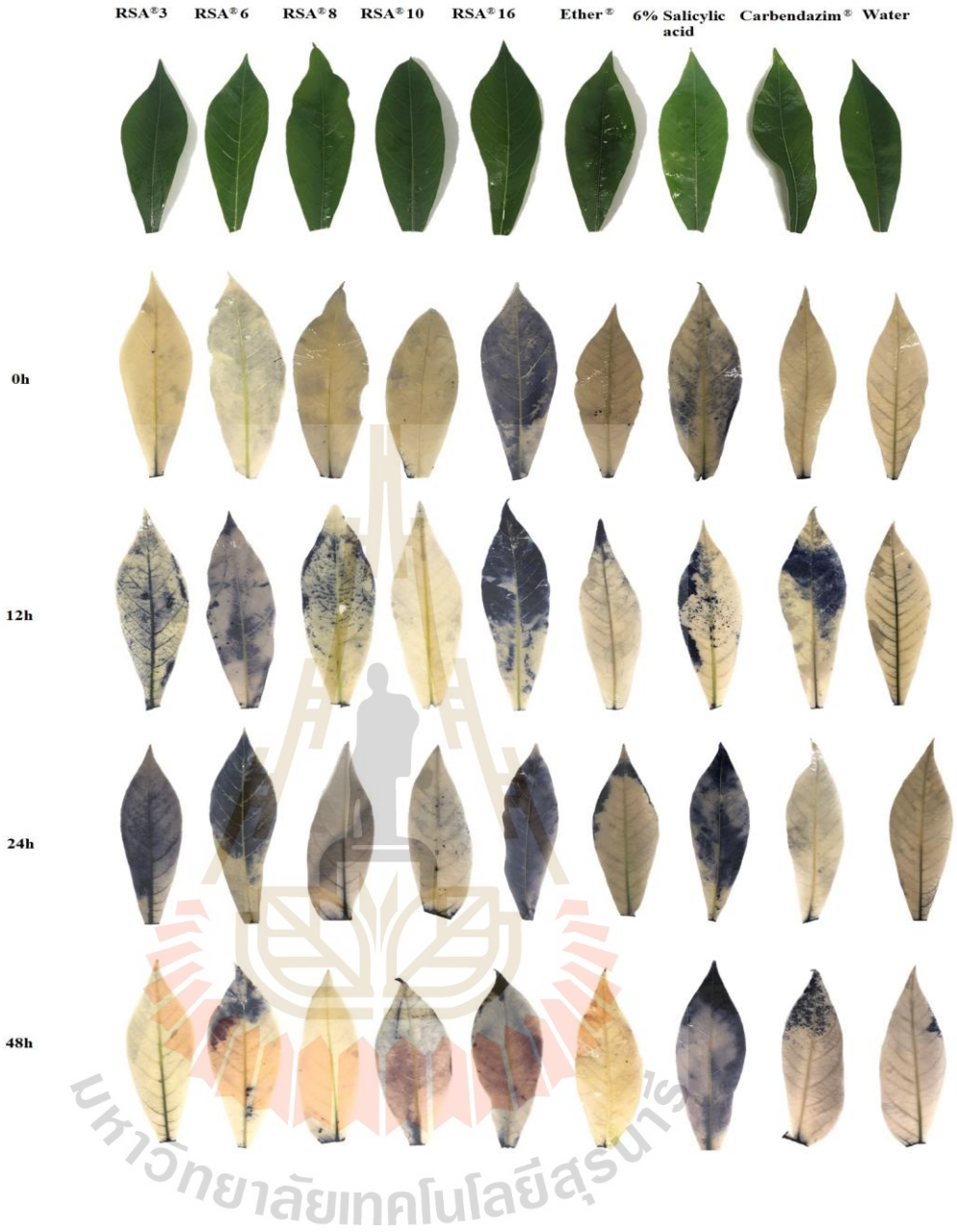

Figure 4.1 NBT staining in treated cassava at 0, 12, 24 and 48 HAI with *C. gloeosporioides*

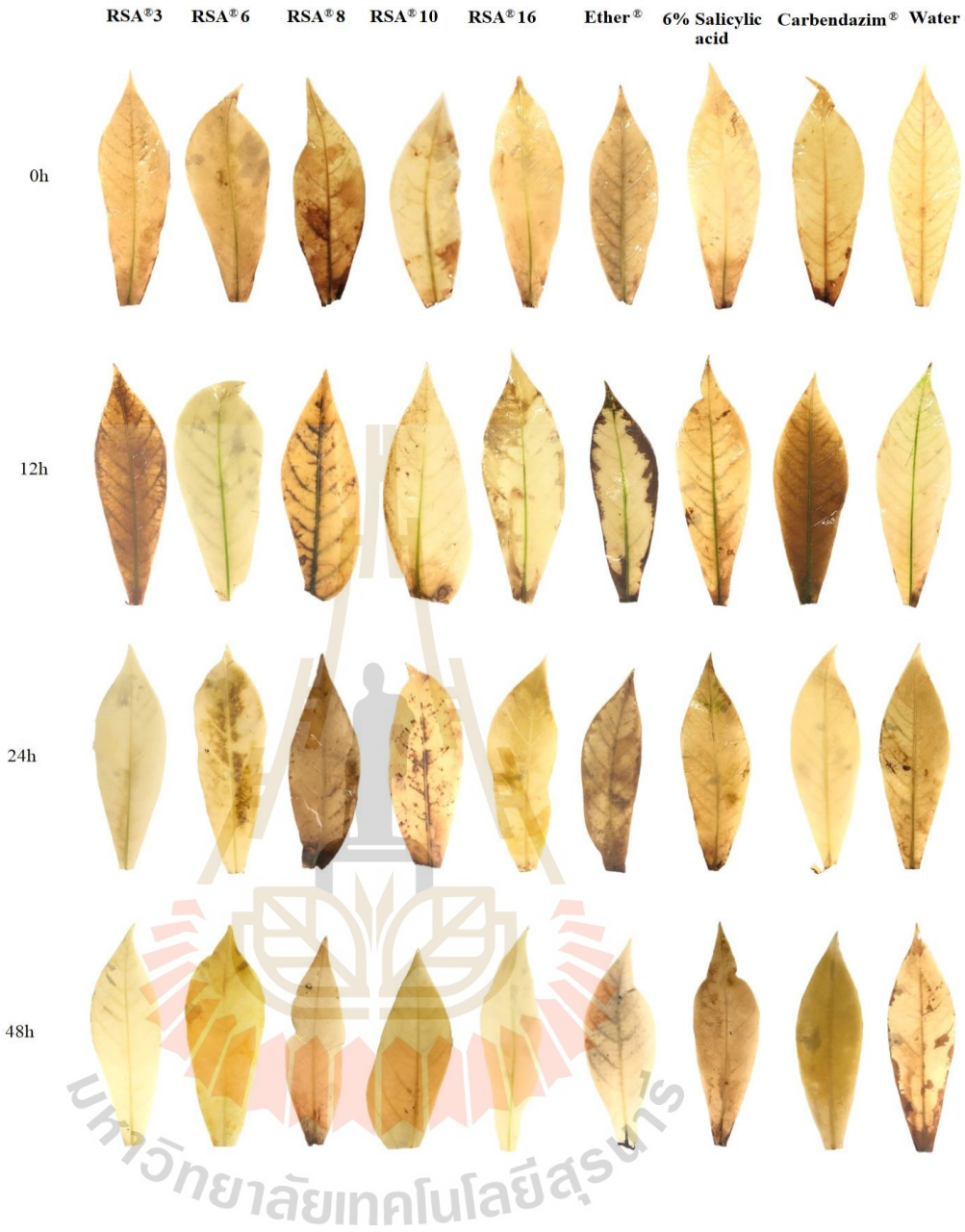

Figure 4.2 DAB staining in treated cassava at 0, 12, 24 and 48 HAI with *C. gloeosporioides*

4.3.3 Phenylalanine ammonia lyase (PAL) Activity

PAL activity of cassava leaves after treated with RSA formulation and inoculation with pathogen. The leaves of cassava treated with RSA 3, RSA 6, RSA 10, RSA 16 with 6% salicylic acid at 200 mg/l, plant elicitor (E-TER® 1 kg/20l) and chemical fungicide (Carbendazim® 10 ml/20 l) showed the increased response of PAL activity level at 24 HAI at 7.957, 6.340, 6.357, 6.933, 8.577 and 6.261 µmol trans-cinnamic acid min^{-1} mg^{-1} protein respectively, and decreased to low-level in 48 HAI. But there was no change of PAL activity level in the control treatment (water) (Figure 4.3).

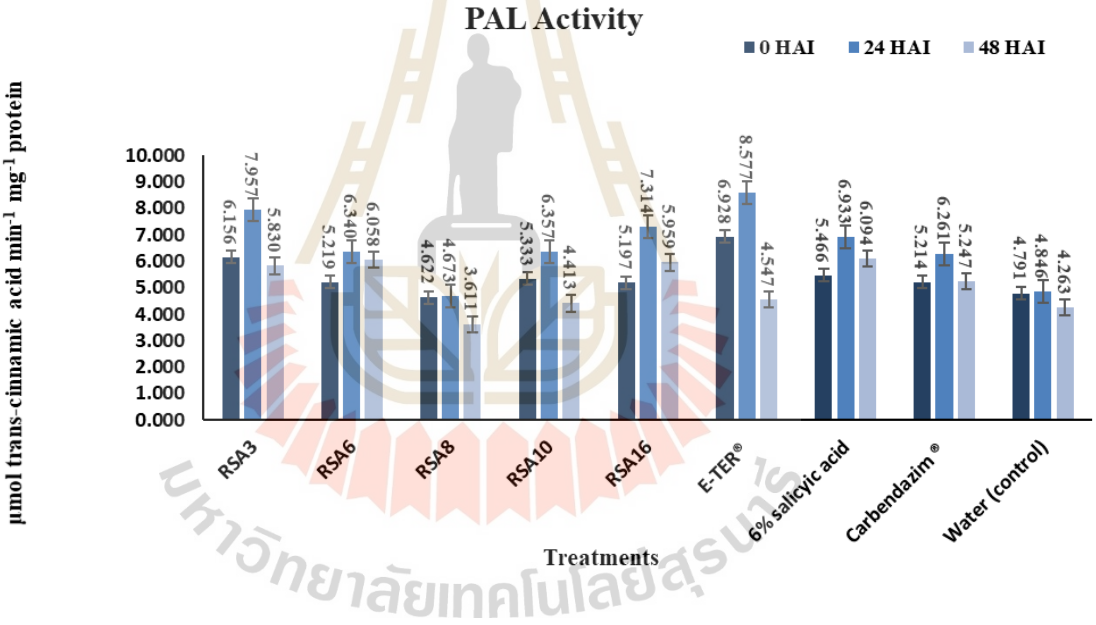

Figure 4.3 PAL activity of cassava leaves after treated with salicylic formulation at 0, 24 and 48 HAI

4.3.4 Accumulation of endogenous salicylic content

Salicylic acid concentration in cassava leaves after treated with five RSA protectors at 200 mg/l compared with 6% salicylic acid at 200 mg/l, plant elicitor (E-TER® 1 kg/20l), chemical fungicide (Carbendazim® 10 ml/20 l) and water are

negative and positive controls. The result showed RSA 3 can enhance the higher level of salicylic content at 24 HAI to approximately 22.74% and decreased to a low level in 48 HAI (Table 4.2). This was accompanied by a similar change in PAL activity level.

Table 4.2 Effectiveness of SA formulation on the accumulation of endogenous salicylic acid in cassava leaves.

Treatment[1/]	**Endogenous salicylic acid (µg g^{-1} of fresh weight)**[2/]			**Increase of SA activity (%) at 24 HAI**
	SA 0hai	**SA 24 HAI**	**SA 48 HAI**	
RSA3 (200 mg/l)	18.34	22.51	21.89^{a}	22.74
RSA6 (200 mg/l)	18.80	19.44	18.4539^{ab}	2.99
RSA8 (200 mg/l)	19.57	20.39	17.4145^{c}	5.19
RSA10 (200 mg/l)	19.34	19.18	22.2649^{a}	2.20
RSA16 (200 mg/l)	20.22	20.80	20.7999^{ab}	2.91
6% salicylic acid (200 mg/l)	18.35	21.28	22.2275^{a}	16.77
E-TER ® (1 kg/20l)	18.19	21.13	20.3175^{ab}	17.34
Carbendazim ® (10 ml/20l)	19.91	21.01	19.1408^{ab}	5.59
Water (Negative control)	18.29	18.78	21.1882^{ab}	2.72
F-test	ns	ns	**	-
CV%	7.58	28.60	26.04	-

[1/]Cassava plants were treated by foliar sprays three times at 1 to 3 months with RSA elicitor at 200 mg/l compared with 6% salicylic acid at 200 mg/l, plant elicitor (E-TER® 1 kg/20 l), chemical fungicide (Carbendazim® 10 ml/20 l) and water are negative and positive controls. Cassava leaves were challenged with *C. gloeosporioides* isolate NKSTKLS02. [2/] Endogenous salicylic acid was evaluated pre-inoculation and 24 h post-inoculation. Each value represents a mean of three replicates. Mean values in each column followed by a different lower-case-letter are significantly different by Duncan's multiple range test at P = 0.05.

4.4 Discussion

Based on the results of this work, the efficacy of RSA protectors to induce resistance in susceptible cassava varieties by soaking cassava stalk for 5 minutes before planting together with spraying 1 time per month for 3 months. It was found that RSA 3 protectors can increase the accumulation of chlorophyll content, lead to an increase of photosynthetic product and growth promotion in cassava. Similar results were observed in cucumber seedlings were treated with foliar 1 mM SA applications was the highest chlorophyll content in salt stress condition (Yildirim, Turan, and Guvenc, 2008). It also found that the use of fungicide mix with SA was significantly increased in the total chlorophyll, phenolic content, and activities of defense enzymes response to *Bipolaris sorokiniana* infection in wheat (Naz et al., 2018). It also plays a role in inducing disease resistance to *C. gloeosporioides* by the increase O_2^- and H_2O_2 at the site of attempted invasion during the early stages of cassava leaves tissue with pathogen interactions (Figure 4.4). Salicylic elicitor were regularly induced the highest generation of O_2^- and H_2O_2 level at 12 HAI in leaves tissue (Gómez-Vásquez et al., 2004). These molecules play an important role in stimulating hypersensitive reaction (HR) and systemic acquired resistance (SAR) and constitute induces of SA accumulation in the next level of the defense mechanism (Eloy et al., 2015). After the rapid changes in O_2^- and H_2O_2 could also promote PAL activity in cassava tissue at 24 HAI. Moreover, the accumulated of PAL activity is a major response to pathogen invasion with *C. lagenarium* at 24 HAI in Muskmelon plants (Ge, Guest, and Bi, 2014). PAL is the primary key enzyme in the phenylpropanoid pathway for biosynthesis and is the most popular enzymes for defense responses to biotic and abiotic stress (Kim and Hwang, 2014; Yadav et al., 2020). By is involved in the

synthesis of salicylic acid (SA), an essential signal in plant resistance. Alteration of salicylic acid in cassava cells occurred after 24 HAI (Dogbo et al., 2012). Similar have been found in salicylic acid accumulation in SA-treated rice against *Xanthomonas oryzae* pv. *oryzae* (Le Thanh et al., 2017). All of these studies indicate that the use of salicylic acid stimulants was effective to the enhanced the activity of cassava defense-related enzymes reducing cassava anthracnose disease severity.

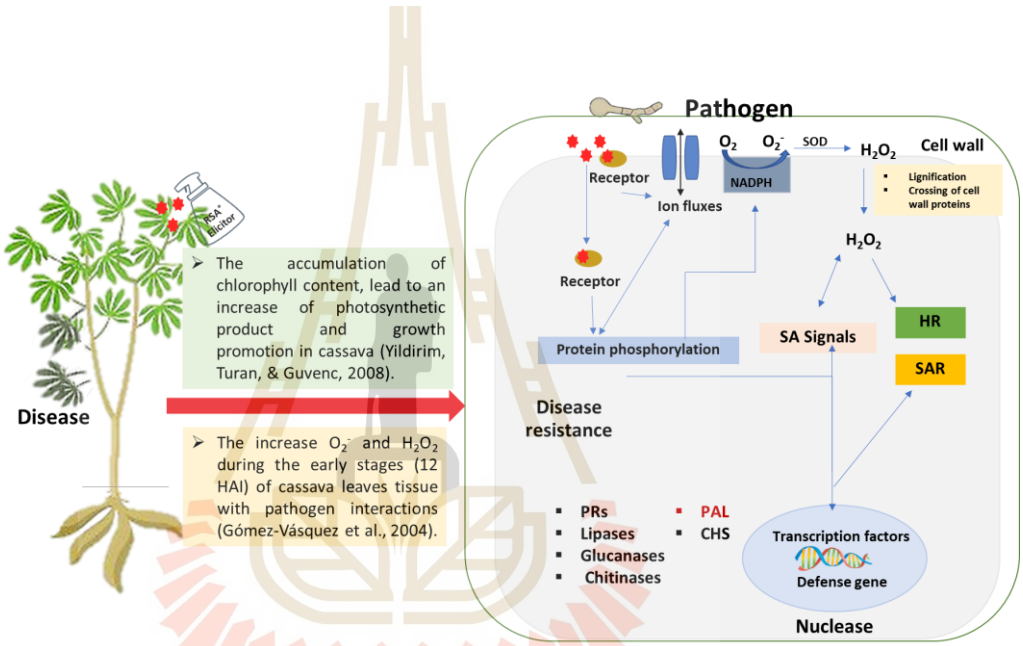

Figure 4.4 Schematic diagram of cassava after treated with RSA protector show the change of O_2^- and H_2O_2 for induced of cassava defense-related enzymes resistance to pathogens

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CHAPTER V

EFFECT OF SALICYLIC ACID AS A PLANT GROWTH REGULATOR AND PLANT PROTECTOR ON CASSAVA

ABSTRACT

In this research, the effects of salicylic acid (SA) as a plant growth regulator and disease protector were investigated. The four SA formulations including RSA3, RSA6, RSA8, and RSA10 at 10 concentrations were preliminarily tested for their phytotoxicity and inhibitory efficacy on mycelia growth of *Colletotrichum gloeosporioides*, the causal agent of cassava anthracnose. Subsequently, the RSA3, RSA6, and RSA10 at 200 mg/l SA were chosen for a further experiment under field conditions to see their effects on anthracnose disease severity, plant growth, and the yield on cassava Rayong 72 cultivar. The cassava plants were treated with the protector by stalk dipping for 5 mins before planting, followed by three foliar sprays at 1, 2, and 3 months after planting. Cassava treated with carbendazim®, 6% SA, and water were used as positive and negative controls, respectively. For plant growth determination, at 3 months of RSA shelf-life evaluation, the results found that the RSA 3 gave significantly growth better than those cassava treated with other formulations of pure SA or water significantly different from cassava treated with carbendazim® at 6 months old, cassava treated with RSA3 still ranked the growth better but were not significantly different from those cassava treated with pure SA, while cassava treated with carbendazim® ranked the shortest, at 9 months old, while

those treated with pure SA gave the better growth, seconded by RSA 3. For the anthracnose severity assessment, at 3 months old, pure SA (SA 6%) treated cassava gave the lowest severity of 15%, while indices of cassava treated with carbendazim® (16.66%), and RSA 3 (17.50%) ranked the second-lowest. At 6 months, the plants treated with RSA 3 had the lowest severity (21.66%) compared to that of the plants treated with water (54.16%) At 9 months old, plants treated with RSA 3 still gave the lowest disease severity of 45.83% compared to those treated with water (56.66%). For the effects of protector on yield, RSA 3 treated cassava gave the highest starch content (20.26%), averaged number of storage roots per plant (9.55), and storage roots fresh weight per plant of 9.06 kg equivalent to 90,666.62 kg/ha total yield compared to 55,888.87 kg/ha of the water treated plants.

Keywords: salicylic acid, plant growth regulators, plant protector, cassava yield

5.1 Introduction

Severe outbreaks of cassava anthracnose from *C. gloeosporioides* usually occur in major planting areas of Thailand. The outbreaks can be found in every stage of cassava growth. However, severe symptoms of anthracnose are usually recorded when cassava plants are 3-6 months old. Typical symptoms of anthracnose are leaf necrotic lesions, wilt, defoliation, and dieback of shoot tip (Magdalena, Ernest, and Robert, 2012; Sangpueak, Phansak, and Buensanteai, 2018). In susceptible varieties, dieback symptom is most common and can cause damage to approximately 50 to 80% of the cassava storage roots (Fokunang et al., 2001; Sangpueak et al., 2018). The application of chemical fungicides is a common practice for controlling anthracnose

disease because resistant cultivars are still lacking in Thailand. To reduce the use of chemical fungicides, plant protectors are a good option for anthracnose control.

Plant protector or resistance activators or protectors are beneficial compounds that could stimulate a range of chemical and biochemical defenses in susceptible host plants (Buensanteai, Yuen, and Prathuangwong, 2009; Walters and Fountaine, 2009). The plant protectors can be biotic or abiotic, depending on the origin and their molecular structure (Thakur and Sohal, 2013; Jamiołkowska, 2020). To date, protectors that have been extensively tested are salicylic acid (SA), methyl salicylate, benzothiadiazole, benzoic acid, and chitosan which induce the production of phenolic compounds and defense enzymes in host plants (Thakur and Sohal, 2013; Abdel-Monaim, 2017). One of the main signaling molecules for plant defense is SA. The SA plays a key role in resistance to microbial pathogens as well as an establishment of local and systemic acquired resistance (LAR and SAR), an accumulation of pathogenesis-related (PR) proteins for controlling pathogens including *Fusarium oxysporum*, *Alternaria alternata*, *C. gloeosporides*, *Xanthomonas* spp., and viruses (Loake and Grant, 2007; Palmer, Shang, and Fu, 2017; Ding and Ding, 2020). The role of SA is also on promoting plant growth and development, including seed germination, root initiation, stomatal closure, and floral induction (Larqué-Saavedra and Martin-Mex, 2007; Koo, Heo, and Choi, 2020). Based on this assumption, exogenous SA at very low concentrations can be applied to produce a positive response in susceptible host plants (Rivas-San Vicente and Plasencia, 2011; Koo et al., 2020). Foliar spray of SA can help plants to overcome the effects of drought, increase the restoration process in plants. An application of SA also plays a constructive role in decreasing the adverse effects of biotic and abiotic stresses on

plants and increases their growth and yield (Souri and Tohidloo, 2019; Khan, Bano, and Babar, 2020). Based on the context mentioned above, this research was carried out to study the effect of SA as a plant growth regulator and plant protector on cassava.

5.2 Materials and methods

5.2.1 Preparation of SA treatments

Four SA formulations (RSA protectors) including RSA 3, RSA 6, RSA 8, and RSA10 were prepared by dissolving them in sterile water and adjusted to the final concentration of 50, 100, 150, 200, 500, 1,000, 2,000, and 60,000 mg/l (The active ingredient of RSA protectors was 6% salicylic acid). The formulations are the products of the CS Tapioca Research and Innovation Co., Ltd, Thailand. The RSA protectors were compared with 6% salicylic acid dissolved in 70% ethanol and adjusting the final volume with water to 100 ml for the stock solution, then dilute the final concentration of the stock solution (6% salicylic acid) with water similar concentration of RSA protectors before used.

5.2.2 Preparation of Colletotrichum inoculum

Colletotrichum gloeosporioides isolate NKSTKLS02 was selected from the collection of virulent isolates of *Colletotrichum* species collected from cassava fields in 10 districts of eight provinces of Thailand from September 2013 to July 2015 as a part of CAD surveillance activities (Sangpueak et al., 2018). *C. gloeosporioides*, has been identified as the major causal agent of cassava anthracnose during that survey. The fungus was cultured on potato dextrose agar (PDA) plates (potato 200 g/l, dextrose 15 g/l, agar 15 g/l) at 28±2°C for 14 days. This fungal stock culture was used throughout the research.

5.2.3 Potential toxic levels of RSA protectors on cassava leaves

The experiment was conducted in a completely randomized design (CRD), with four replications for 4 formulations and 8 concentrations of RSA protectors. The fifth leaves of 3 months old Rayong 72 cassava plants were used for the test. The leaves were washed with tap water and air-dried for 5-10 min at room temperature, and placed in the moist box, 3 leaves each. Subsequently, 50 µl of RSA at the concentrations of 50, 100, 150, 200, 500, 1,000, 2,000 and 60,000 (mg/l) were pipetted onto each leaf. For the untreated control, the cassava leaves were handled identically, but distilled water was used instead of RSA protectors. Toxicity levels were recorded at 3 days after the treatment. Incidence of length burn injuries on cassava leaves was assessed at the treated sites (Wiman et al., 2019). The experiment was repeated twice.

5.2.4. *In vitro* evaluation of RSA protectors on inhibition of *C. gloeosporioides* mycelial growth

The experiment was carried out in CRD, with four replications for 4 formulations, and 4 concentrations of RSA protectors. The effect of RSA protectors on the mycelial growth of *Colletotrichum* was estimated in a radial growth inhibition assay. Five mm PDA discs from actively growing colonies of *C. gloeosporioides* were placed on the surface of PDA plates containing different concentrations of RSA protectors of 50, 100, 200, and 500 mg/ml. PDA plates without SA were included as a negative control, and those with carbendazim® (20ml/20l; methyl benzimidazole-2-yl-carbamate 50% w/v) were served as positive controls. Mycelial growth was determined by measuring colony diameters at 5 days after putting fungal disease and the inhibition efficacy (I) was calculated using the following formula

(Do Chi and Kunasakdakul, 2013).

$$I\ \% = [(C2-C1) / C2] \times 100$$

Where C2 = Mean diameter of the control colony and C1= Mean diameter of the *Colletotrichum* colony in the presence of the tested compound. The experiment was repeated twice, with three replicates per treatment. The concentration of RSA protectors that best inhibiting the fungal mycelial growth was selected for further experiments.

5.2.5 Effect of RSA protector application on growth, anthracnose disease severity, and yield of cassava under field conditions

The field experiment was carried out in randomized complete block design (RCBD) with four replications at Suranaree University Farm, Suranaree University of Technology, Nakhon Ratchasima, Thailand during June 2018-April 201, using Rayong 72 as the test cultivar. The RSA protector formulations and concentrations that less toxic to cassava and best inhibiting the fungal mycelia growth were selected for the experiment. Before planting the cassava stalks were soaked with RSA protectors for 5 minutes before planting followed by three foliar sprays at 1,2 and 3 months after planting. For the negative control, distilled water was used instead of RSA protector, and chemical fungicide, carbendazim® (20 ml/20l), was used as a positive control. The experiment was done in a cassava field that had been heavily infested with anthracnose disease in the previous season, therefore no extra *Colletotrichum gloeosporioides* inoculum was added into the field.

Data were collected on plant height and disease severity scoring at 3, 6, and 9

months after planting, as follows: 1 = no symptoms, 2 = less than 25% of leaf area was affected, 3 = 25 to less than 50% of leaf area was affected, 4 = 50 to less than 75% of leaf area was affected, and 5 = more than 75% of leaf area was affected (Sangpueak et al., 2018). The percentage of disease severity index was calculated using the formula slightly modified from that of Wokocha et al. (2010). Collected the data of plant height at 3,6, and 9 months. At harvest, the number of cassava storage roots/ plant, fresh yields, and starch contents was collected when the plants were 9 months old (Terry and Hahn, 1980; Polthanee, Janthajam, and Promkhambut, 2014).

5.2.6 Statistical analysis

The data were analyzed and subjected to Analysis of Variance (ANOVA) using SPSS software, version 16. The significance of treatments was determined by the magnitude of the F value (P = 0.05). Treatment means were separated by Duncan's Multiple Range Test (DMRT)

5.3 Results

5.3.1 Potential toxic levels of RSA protectors on cassava leaves

The toxicity test of SA (RSA protectors) formulations showed that at the concentration range of 0-500 mg/l, no necrotic lesions were observed on the tested sites of the leaves, but at 1,000 to 60,000 mg/l the SA made leaf tissue burn and turn into necrotic brown lesions. The burn was most evident at 60,000 mg/l giving the lesion size as big as 0.1 x 0.1 to 0.8 x 1.03 cm (Figure 5.1). Burn symptom of cassava leaf tissues caused by SA at high concentrations is due to the high acidity of the solution. Therefore, low concentrations of SA from 0-500 mg/l were selected for further experiment.

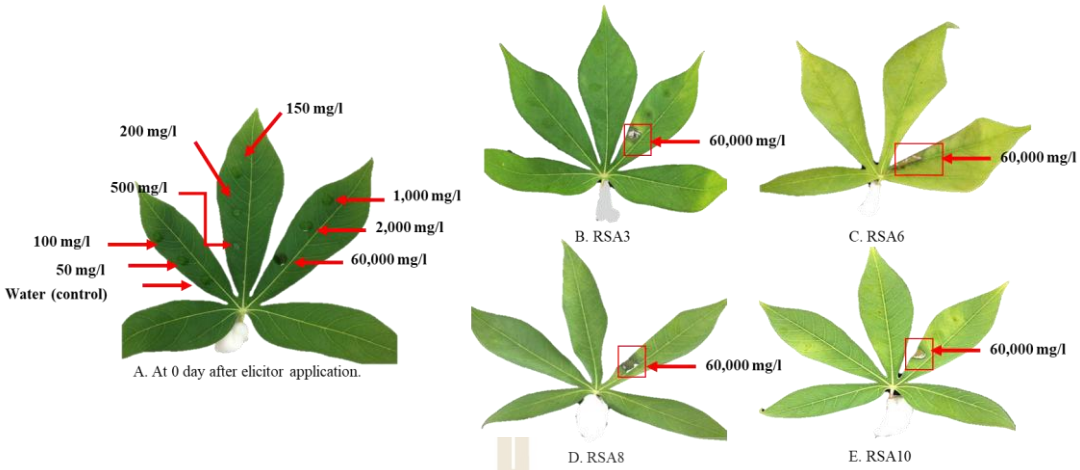

Figure 5.1 RSA protectors toxicity test on Rayong 72 cassava leaf. A. 0 day after protector application, B.-E. RSA 3, RSA 6, RSA 8, and RSA 10 at 3 days after protectors application.

5.3.2 *In vitro* evaluation of RSA protectors on inhibition of *C. gloeosporioides* mycelial growth

From Table 5.1 it can be seen that SA at all tested concentrations could inhibit the mycelial growth of *C. gloeosporioides*. Among the chemicals, Carbendazim® even at the concentration of 20 ml/20l could almost completely inhibit the mycelia growth at 89.61±0.03%. For the SA formulations, RSA 3 was the most inhibitory giving 32.46±0.02% inhibition at 100 mg/l concentration and 47.40±0.03% and 59.41±0.01%, at 200 and 500 mg/l, respectively. From the results of this experiment and the results of biological and physiological responses in chapters, III and IV were selected RSA 3 at 200 mg/l to be used in the next experiment.

Table 5.1 Percentage of mycelial growth inhibition of *C. gloeosporioides* by the SA protectors amended in the PDA medium after 5 days of incubation.

Treatments	Mycelial growth inhibition (%)			
	50 mg/l[1/2/]	**100 mg/l**	**200 mg/l**	**500 mg/l**
RSA3	25.32±0.03[b]	32.46±0.02[b]	47.40±0.03[b]	59.41±0.01[b]
RSA6	21.75±0.05[b]	26.62±0.05[b]	37.66±0.06[c]	49.02±0.06[cd]
RSA8	12.33±0.06[c]	13.63±0.04[c]	27.59±0.02[d]	43.18±0.03[d]
RSA10	16.88±0.09[bc]	24.02±0.13[b]	44.15±0.06[b]	51.29±0.01[c]
6% Salicylic acid[3/]	0.97±0.06[d]	2.59±0.01[d]	10.71±0.01[e]	34.41±0.08[e]
Carbendazim ® (10 ml/20l)	89.61±0.00[a]	89.61±0.03[a]	89.61±0.03[a]	89.61±0.03[a]
Water (control)	0.00±0.03[d]	0.00±0.00[d]	0.00±0.00[f]	0.00±0.00[f]

[1/] Final concentration of active ingredients. [2/]Mean ± standard deviation. Each value represents a mean of three replicates. [3/] 6% SA= 6% of salicylic acid solution in 70% ethanol and adjusting the final volume with water to 50,100,200 and 500 mg/l for comparison with formulations. Mean values in each column followed by a different lower-case-letter are significantly different by Duncan's multiple range test at P = 0.05.

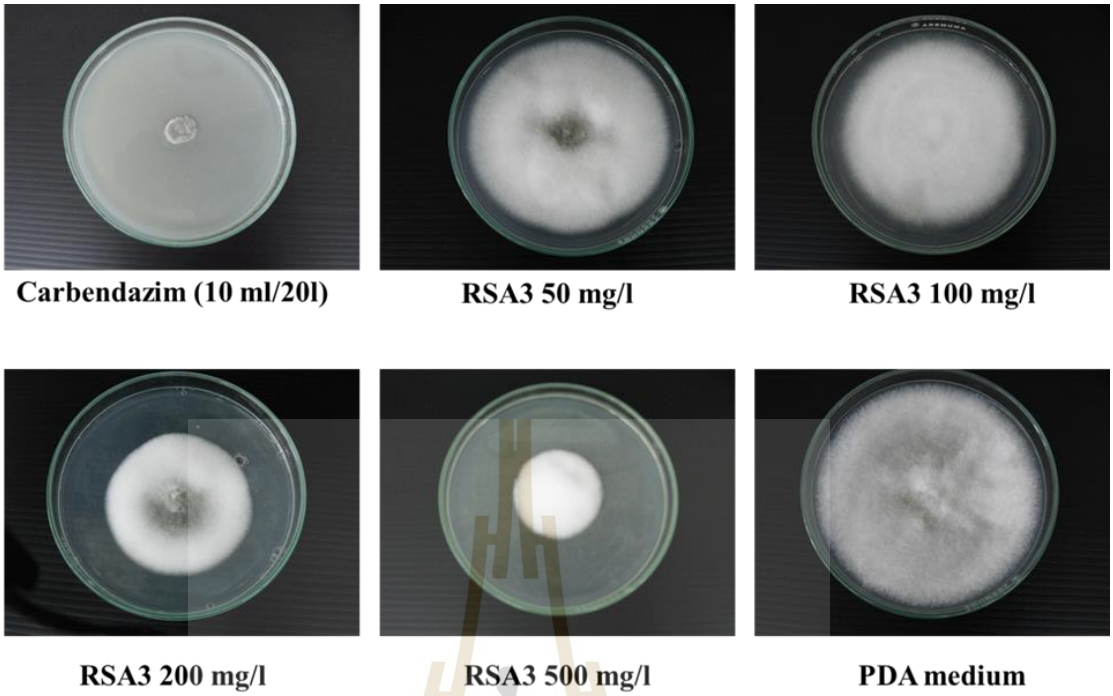

Figure 5.2. Mycelium inhibition of *Colletotrichum gloeosporioides* by different SA concentrations of RSA 3 formulation amended in PDA at 5 days after incubation.

5.3.3 Effect of RSA protector application on anthracnose disease severity, and yield of cassava under field conditions

The effects of SA formulations on growth appear to depend on the cassava age, at 3 months old RSA 3 gave significantly taller plants (99.96±3.49 cm) than those treated with other formulations of pure SA (87.66±3.20 cm) or water (86.16±0.72 cm) but not significantly different from plants treated with Carbendazim® (104.33±4.09 cm) (Table 5.2). At 6 months old, Carbendazim®, seems to lose its growth-stimulating effect giving plants not taller (126.36±6.34 cm) than those treated with water (123.40 ±2.23 cm), but those treated with RSA 3 still ranked the tallest (142.43±2.13 cm)

which were not significantly different from those treated with pure SA (142.20±1.73 cm). At 9 months old, Carbendazim® showed the negative effect on growth, giving the shortest plant height of 149.46±5.06 cm while those treated with pure SA gave the tallest of 188.80±2.36 cm, seconded by RSA 3 (178.30±9.65 cm).

Table 5.2 Effect of salicylic acid formulations application on the height of Rayong72 cassava plants at 3, 6, and 9 months after planting under field conditions.

Treatments [2/]	**Plant height** [1/] **(cm)**		
	3 months [2/]	**6 months**	**9 months**
RSA3 (200 mg/l)	99.96±3.49 [ab]	142.43±2.13 [a]	178.30±9.65 [ab]
6% Salicylic acid [3/] (200 mg/l)	87.66± 3.20 [b]	142.16±1.73 [a]	188.80±2.36 [a]
Carbendazim® (10 ml/20l)	104.33±4.09 [a]	126.36±6.34 [b]	149.46±5.06 [b]
Water (control)	86.16±0.72 [b]	123.40±2.23 [b]	158.56±6.80 [bc]
F-Test	*	**	**
CV%	13.10	24.35	7.17

[1]= mean of three replicates; **= P<0.01. [2/] Final concentration of active ingredients. [2/]Mean ± standard deviation. Each value represents a mean of three replicates. [3/] 6% SA= 6% of salicylic acid solution in 70% ethanol and adjusting the final volume with water 200 mg/l for comparison with formulations. Mean values in each column followed by a different lower-case-letter are significantly different by Duncan's multiple range test at P = 0.05.

When the plants were assessed for anthracnose severity, their reactions to protector treatment seem to be different depending on the plant age (Table 5.3). At 3 months old, pure SA (SA 6%) treated cassava gave the lowest severity of 15%, while indices of those treated with Carbendazim® (16.66%), and RSA3 (17.50%) ranked

second and were significantly different, and those treated with water (control) gave the highest severity (38.26%). But at 6 months old, both 6% SA and carbendazim® appear to be less effective than when the plants were 3 months old, giving severity indices of 25.00% and 27.50% respectively, which were significantly higher than that of the plants treated with RSA 3 (21.66%) which was the lowest. At this age, plants treated with water had the highest index of 54.16%. At 9 months old, plants treated with RSA3 and Carbendazim® gave the lowest disease severity of 45.83% and 46.66% respectively, significantly lower than that of the plants in control (water) treatment (56.66%).

Table 5.3 Effect of salicylic acid formulations application on disease severity index of Rayong 72 cassava plants at 3, 6, and 9months after planting under field conditions.

Treatments[1/]	**Disease severity index (%)**		
	3 months	**6 months**	**9 months**
RSA3 (200 mg/l)	17.50[b]	21.66[b]	45.83
6% Salicylic acid [2/] (200 mg/l)	15.00[b]	25.00[b]	54.16
Carbendazim®(10ml/20l)	16.66[b]	27.50[b]	46.66
Water (control)	38.26[a]	54.16[a]	56.66
F-Test	*	**	ns
CV%	28.82	28.21	36.81

[1/] Final concentration of active ingredients. Each value represents a mean of three replicates. [2/] 6% SA= 6% of salicylic acid solution in 70% ethanol and adjusting the final volume with water 200 mg/l for comparison with formulations. Mean values in each column followed by a different lower-case-letter are significantly different by Duncan's multiple range test at P = 0.05.

At harvest when yields were assessed, it appeared that the plants responded differently to the treatment, depending on the formulations. Among the four, RSA 3 treated cassava gave the highest starch content (20.26%), averaged number of storage roots per plant (9.55), and fresh weight per plant (9.06 kg), (Table 5.4). When the total yield of all treatments was compared, RSA 3 treated plants gave the highest of 90,666.62 kg/ha, seconded by those treated with SA6% (65,222.18 kg/ha) or Carbendazim® (62,888.87kg/ha), and those treated with water gave the lowest (55,888.87 kg/ha) (Table 5.4).

Table 5.4 Effect of salicylic acid formulations application on yield of Rayong72 cassava under field conditions.

Treatments[1/]	**Starch content (%)**	**Number of storage roots per plant (storage root/plant)**	**Fresh weight per plant (kg/plant)**	**Yield (kg/ha)**
RSA3 (200 mg/l)	20.26	9.55[a]	9.06[a]	90,666.62[a]
6%Salicylic acid[2/] (200 mg/l)	15.00	8.00[ab]	6.52[b]	65,222.18[b]
Carbendazim® (10ml/20l)	15.13	8.55[ab]	6.28[b]	62,888.87[b]
Water (control)	14.73	6.88[b]	5.58[b]	55,888.87[b]
F-Test	ns	**	**	**
CV%	20.38	22.24	16.47	0.014

[1/] Final concentration of active ingredients. Each value represents a mean of three replicates (ten cassava trees/replication). [2/] 6% SA= 6% of salicylic acid solution in 70% ethanol and adjusting the final volume with water 200 mg/l for comparison with formulations. Mean values in each column followed by a different lower-case-letter are significantly different by Duncan's multiple range test at P = 0.05.

5.4 Discussion

Based results of this work, the high concentrations of SA at 1,000 to 60,000 mg/l were toxic to cassava causing burns and necrosis to the leaf tissues. The results are in line with the reports of (Bai, Dong, Kong, Xu, and Liu, 2015; Guo, Liu, Liang, Li, and Fu, 2019). These authors reported that exogenous SA treatment at high concentrations could affect plant growth and seed germination. Therefore, if SA is to be applied to any crops, its safe concentration should be determined first. From our experiment, it has been proved that the concentrations between 200-500 mg/l are safe for cassava. As a plant protector, SA has proved in our experiment that it can be as effective as carbendazim for controlling anthracnose, depending on its formulation. Among the four formulations, RSA3 appeared to be the most effective whenever the severity assessment was done, while other formulations and 6% SA, the effectiveness was much lower and not that consistent depending on the assessment times. The effectiveness of SA in reducing anthracnose severity could come partly from its inhibitory effect on *C. gloeosporioide*s, mycelial growth also found in our experiment and others (Panahirad, Zaare-Nahandi, Mohammadi, Alizadeh-Salteh, and Safaie, 2014; Zhang et al., 2016; Palmer et al., 2019) but that could not be the only mode of action because, at 200 mg/l, RSA 3 was far less effective than carbendazim in inhibiting mycelial growth but was equally effective in reducing disease severity. Previous researches have shown that the application of SA by root feeding and foliar spray could induce plant resistance against *F. oxysporum* in tomato and banana, *Ralstonia solanacearum* in pepper, *Xanthomonas oryzae* in rice, *Botrytis cinerea* in the bean, cassava brown streak disease, and *Colletotrichum gloeosporioides* in cassava and several diseases on many crops (Rajkumar, Lee, and Freitas, 2008; Mandal,

Mallick, and Mitra, 2009; Koo et al., 2020). The SA, a phenolic derivative in the plant, plays a crucial role in plant signalling and defence against biotic and abiotic stresses (Zhao, Lu, and Yang, 2017). The SA can act as a signal molecule leading to the expression of systemic resistance to plant pathogens. It can induce pathogenesis-related proteins and other compounds, which impart increased resistance to pathogens attacks (Durner, Shah, and Klessig, 1997; Andersen, Ali, Byamukama, Yen, and Nepal, 2018). Because the salicylic formulations used in this study were among formulations containing different inert ingredients. So, the efficacy of each salicylic formulations causing different results.

Apart from being a plant protector, SA in our experiment has also been proved to be a good plant growth promoter in cassava. Similar to its effect on disease resistance, formulations seem to play an important role in determining the SA effectiveness in promoting growth (Zhao, Lu, and Yang, 2017). While RSA3 treated plants gave almost twice as much yield compared to that of the plants treated with water (negative control consistent with the research results of Khan et al. (2013) reported SA plays a role in increased leaf area and dry weight production in corn and soybean. According to the results of this study, RSA 3 was recommended to soaked cassava stem at the concentration of 200 mg/l of water for 5-10 minutes and spray every month for the first 3 months. That can help to reduce the incidence of cassava anthracnose disease.

5.5 References

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CHEPTER VI

OVERRALL DISCUSSTION AND CONCLUSTION

6.1 Overall discussion

The objectives of this study were: (1) to evaluate the efficacy of exogenous SA elicitor prototype formulations in inducing resistance to anthracnose disease in cassava plants, (2) to study the mechanisms of induced resistance in cassava plants to *C. gloeosporioides* after treated with effective SA elicitor formulations. Abiotic resistance elicitors including resistance SA elicitor formulations products (RSA protectors) and 6% SA compared with chemical fungicides. This study explored the changes in enzyme activities in cassava plant defense responses with a biochemical assay using synchrotron fourier transform infrared micro-spectroscopy. The changes of lignin, pectin, amide I and lipid were characterized in Chapter III and IV. The standard pathological techniques were used to evaluate the efficacy of exogenous SA elicitor against anthracnose pathogens under *in vitro* and *in vivo* conditions in Chapter V.

According to in vitro and *in vivo* studies, the most effective for induction was obtained at the concentration of 200 mg/l of RSA 3 and was used to compare with fungicide and water as a negative control. Regarding the low concentration of RSA formulation at 200 mg/l could directly inhibit mycelial growth of *C. gloeosporioides*. Similar results were observed in mango fruits with low concentration SA significantly reduced mycelial growth of *C. gloeosporioides* (Joyce et al., 2001; He et al., 2017).

In tomato, found that SA (1-25 mM) was used for antifungal activity against tomato pathogens *i.e. Fusarium oxysporum* f. sp. *lycopersici*, *F. oxysporum* f. sp. *radicislycopersici*, *F. solani*, *Alternaria solani*, *C. coccodes*, *Verticillium dahliae*, *Pythium aphanidermatum*, *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Botrytis cinerea*, and *Sclerotinia sclerotiorum* (Jabnoun-Khiareddine et al., 2015). Nonetheless, the RSA formulation at concentration of 200 mg/l showed non-toxic effects on cassava leaves. It was also found that soaking the cassava stem with RSA 3 and then foliar spray for three times at 1, 2, and 3 months after planting, and inoculated with a spore suspension of *C. gloeosporioides* can reduce anthracnose severity in cassava plants up to 33.3-50.0% under greenhouse conditions.

Not only directly reduced anthracnose severity, the RSA also plays an important role in plant immunity by indicating the alteration in activities of different enzymes after infection as follows. The production of reactive oxygen species (ROS) in the cassava leaves treated with RSA formulations was associated with SAR induction activated by *Colletotrichum* infection. The blue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB) staining indicated that the increasing of O_2^- and H_2O_2 were maximum at 12 and 24 hours after inoculation (HAI) and then reduced to a low level in 48 HAI. The change of ROS response is reported to enhance and induce *β*-1,3-glucanase and chitinase activities and also enhance in cell walls (Shetty et al., 2009).

Cassava treated with RSA 3 at 200 mg/l showed the increased response of *β*-1,3-glucanase level at 24 HAI at 16.65 μg glucose released min^{-1} mg^{-1} protein and was significantly higher compared with non-treated plant. For chitinase activity, the 6% SA induced the accumulation of chitinase to the highest level at 0.287 followed by

RSA 6 and RSA 3 have chitinase level at 0.275 and 0.275287 μmol glcnac formed min^{-1} mg^{-1} protein, respectively. Similarly, in cotton, exogenous salicylic acid has induced chitinase and *β*-1,3-glucanase accumulation which resulted in the prevention of callus cells from *Verticillum dahlia* (Li et al., 2003). In tomato, susceptible cultivar foliar sprayed with SA treatment significantly increased the accumulation activities of PAL, Chinese, and *β*-1,3-glucanase resistance to *Botrytis cinerea* (Li et al., 2015).

Phenylalanine ammonia lyase (PAL) activity in treated cassava at 0, 24 and 48 HAI found that the RSA 3 has PAL level increased in 24 HAI at 7.3739 μmol trans-cinnamic acid min^{-1} mg^{-1} protein and decrease at 48 HAI, significantly different when compared with negative control at 4.846 μmol trans-cinnamic acid min^{-1} mg^{-1} protein, respectively. Dogbo et al., 2012 also found that cassava stimulated with SA were demonstrated PAL activity increased significantly in 24 HAI at 40.13 mmol min^{-1} mg prot higher than that of the control. Also, the activity of PAL was increased in the SA treatment and pathogen inoculation and the peak appeared 24 h after pathogen challenge in wheat (Gholamnezhad et al., 2016). Because PAL is important in the plant defense involved in the biosynthesis of SA as a key signal involved in plant systemic resistance (Gómez-Vásquez et al., 2004; Dogbo et al., 2012; Kim and Hwang, 2014). This is the first step in the phenylpropanoid pathway and produces a variety of secondary metabolites (Chen et al., 2017; Han and Kahmann, 2019; Heldt and Piechulla, 2011). PAL is an inducible enzyme that responds to biotic and abiotic stresses such as pathogens.

These processes are known as systemic acquired resistance (SAR). SAR is a signaling mechanism that provides broad-spectrum and confers long-lasting resistance to infections plant (Figure 6.1) (Chaturvedi and Shah, 2007; Gao et al.,2015). One of

the earliest responses activated is reactive oxygen species (ROS) including H_2O_2, superoxide, and singlet oxygen species induced cell death in plants (Kawano, 2003; Dempsey and Klessig, 2017). In addition, the cross-linked of cell wall proteins also increased the activation of protein kinases and the expression of defense genes (Zhang et al., 2010). Some of these genes encode peroxidases, proteinase inhibitors, and biosynthetic enzymes, such as phenylalanine ammonia-lyase (PAL) (Xu and Brosché, 2014; Herrera-Vásquez, Salinas, and Holuigue, 2015). PAL is the first enzyme in the phenylpropanoid pathway and is a key enzyme of this pathway that is induced by pathogen infection (Gao et al., 2015). This process involves cell activation mechanisms and promotes responses with salicylic acid protectors and the ability to control *C. gloeosporioide* causal agent anthracnose disease in cassava.

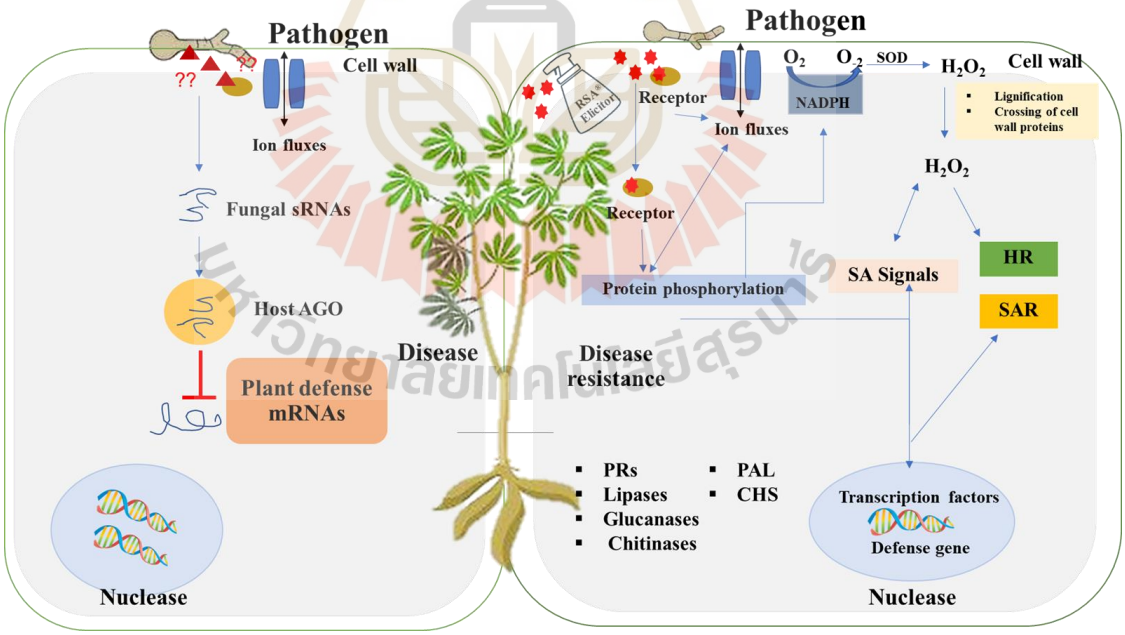

Figure 6.1 Role of RSA formulation induced systemic resistance in cassava against anthracnose disease.

Moreover, the biochemical change results from SR-FTIR spectra were compared in the treated and non-treated cassava leaf. The peak variation between treatments indicated that the changes in the intensity of lignin, lipids, Amine I of protein, and Amine II of lignin in epidermis and mesophyll tissues. The epidermis and mesophyll tissues treated by RSA 3 formulations showed spectra higher than those of the control significantly at the vibrational peaks of C=H stretching vibration, pectin, Amide I protein, lignin, Mainly C–O–C of polysaccharides. This observation suggests that RSA 3 formulations can activate the salicylic acid-defense pathway (SA pathway) (Chavarriaga-Aguirre et al., 2016). This is associated with systemic acquired resistance (SAR) activated upon infection of the pathogens attack. The SAR stimulates the expression of resistance genes (Miedes et al., 2014). These genes can create phytoalexins, reactive oxygen intermediates, hypersensitive responses (HRs), (PR) proteins and cell wall fortification were enhanced lignin modification or cell wall composition impacts the strength of the cell wall (Chavarriaga-Aguirre et al., 2016; Maherawati et al., 2017) . This work indicated that reinforcing cell strength by extra deposition of lignin on the wall avoided pathogen attacks.

The RSA formulation has effect on cassava anthracnose disease severity under field condition. The use of salicylic acid formulation soaked 5 minutes before planting and spraying at 1, 2 and 3 months after planting has an effect on growth rate, yield, and disease incidence of cassava under field conditions. The result found that at 3 months after planting, the treatments with Carbendazim® was an effect on plant height significant difference when compared with the treatments at all ranged 104.31±4.09 cm which followed by the RSA 3, the plant height was 99.96±3.49 cm, respectively. At 6 months after planting, the results found that the formulation 10 was the height

ranged non-significantly different when compare with RSA 3 and 6% SA were 146.96±3.57 cm, 142.43±2.13 and 142.2±1.73 cm, respectively. At 9 months after planting, the result showed that 6% SA was the highest range followed by RSA 3 was 188.83±2.36 cm and 178.36±9.65 cm, respectively, which had a significant difference when compared with negative control. Assessing the severity of cassava anthracnose disease every 3 months found that at 3, 6, and 9 months after planting all of the salicylic acid treatments were significantly reduced disease severity when compared with non-treated plants. For the anthracnose severity at 9 months treatment of RSA 3 and Carbendazim® were shown the lowest disease at 45.83% and 46.66%, while the negative control treatment was significantly higher disease severity at 56.66%. It also found that RSA 3 was the difference increase levels of cassava starch content at 20.26%, 9.55 of tubers per plant, and 9.06 kg/plant of tuber fresh weight and highest significantly different when compared with the negative control treatment. All of the results indicated that the use of RSA 3 at 200 mg/l soaked for 5 minutes before planting and spraying at 1,2 and 3 months after planting. That plays direct or indirect roles as a preventative and reduces anthracnose disease in cassava.

6.2 Conclusion

The results represented that RSA 3 plays important role as direct or indirect roles as a growth regulator and induced systemic acquired resistance to control anthracnose disease in cassava. The RSA 3 can inhibit the growth of *Colletotrichum* and decrease disease occurrence. By induced systemic resistance including endogenous salicylic acid, H_2O_2, β-1,3-glucanase, chitinase, and PLA activity (Figure 6.2). RSA 3 also enhance the mechanical strength of plant or cell wall composition

and reduces the hazard of pathogen infection. It is also saved and environmentally sustainable and safe in the cassava farming system.

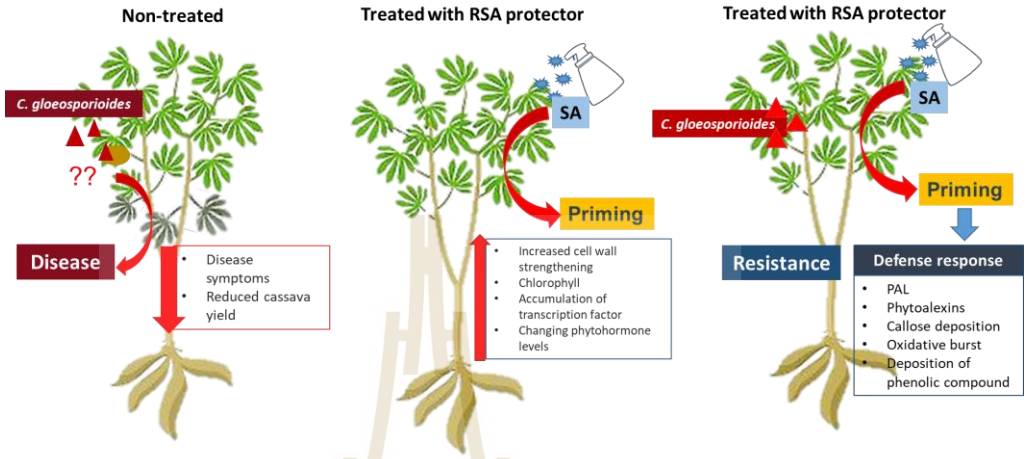

Figure 6.2 Events associated with non-induced and induced resistance in cassava.

6.3 Suggestion

The results of this research indicated that RSA may be used as a factor to induce resistance on cassava against anthracnose disease with direct and non-direct inhibit functions. Further studies are required to investigate on PR proteins quantitative also gene expression for clarification of plant-pathogen interaction and cassava growth-promoting using RSA formulation. Furthermore, RSA formulation showed high potential to be produced in the industry scales and required for several region field trials in Thailand to confirm the formulation performance. In addition, RSA can use as a safety fungicide for an environment which would be a good strategy for sustainable agriculture on cassava production.

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APPENDIX

I. MEDIUMS

1.1 Water Agar (WA)

Agar	15	g
Water	1	L

1.2 Potato Dextrose Agar (PDA)

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

II. CHEMICALS

2.1 6% Salicylic acid (100 ml)

Salicylic acid	6.00	g
95% Ethonal	60	ml
Water	40	ml

2.2 0.5 M Sodium acetate buffer (1000 ml)

Stock A 0.2M Acetic acid

Acetic acid	11.60	g
Water	1000	ml

Stock B 0.2M Sodium acetate (1000 ml)

Sodium acetate	16.4	g
Water	1000	ml

Stock A		Stock B		pH
41.0	ml	9.0	ml	4
20.0	ml	30.0	ml	4.8
10.5	ml	39.5	ml	5.2

2.3 Dinitro salicylic acid (100 ml)

3,5 Dinitro salicylic acid	1	g
ddH_2O	50	ml
Mix it together		
Sodium potassium tartrate tetrahydrate	30	g
2N Sodium hydroxide	20	ml
Add ddH_2O dilute to a final volume of	100	ml

2.4 2N Sodium hydroxide (1000 ml)

Sodium hydroxide	80	g
ddH_2O	1000	ml

2.5 1M Sodium borate buffer (1000 ml)

Boric acid	61.83	ml
Sodium hydroxide	10	ml
Adjust pH 8.8 and add ddH_2O until volume	1000	ml

2.6 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 7, 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

2.7 0.02 M Ferric ammonium sulfate

Ferric ammonium sulfate	4.82	g
Water	500	ml

2.8 10% Folin-ciocalteu reagent

Folin-ciocalteu reagent	10	ml
Water	100	ml

2.9 Salkowski's reagent

Prepare stock solution of 0.5 M FeCl3 (1.35 g in 10 ml water)

Use 1 ml of this stock to mix with 49 ml of 35% $HClO_4$

2.10 12mM L-phenylalanine (1000 ml)

L-phenylalanine	1.99	g
Water	1000	ml

2.11 1M Potassium iodide (1000 ml)

Potassium iodide	16.6	g
Water	1000	ml

2.12 1% Trichloroacetic (100 ml)

Trichloroacetic	1	g
Water	100	ml

2.13 0.09% Laminarin (100 ml)

Laminarin	0.09	g
0.1 Sodium acetate buffer	100	ml

2.14 DNSA Reagent (50 ml)

Sodium hydroxide	5	g
Add ddH_2O	45	ml
3,5 Dinitro salicylic acid	0.5	g
Sodium carbonate	0.025	g
Phenol	0.1	g

Add ddH_2O to final volume 50 ml and keep in amber colored bottle

2.15 40% Sodium potassium tartrate (SPT)

SPT	20	g
ddH_2O	50	ml

2.16 N-Acetyl-D-Glucosamine (100 ml)

N-Acetyl-D-Glucosamine	0.1	g
ddH_2O	100	ml

2.17 Cinnamic acid (1000 ml)

Cinnamic acid	2	mg
ddH_2O	1000	ml

2.18 1M Hydrogen peroxide (100 ml)

30% Hydrogen peroxide	10.20	ml
ddH_2O	89.8	ml

2.19 DAB staining solution

DAB	50	mg
Sterile H_2O	45	ml

Add 0.2M HCL to dissolve DAB and cover tube with aluminium foil since DAB is light sensitive.

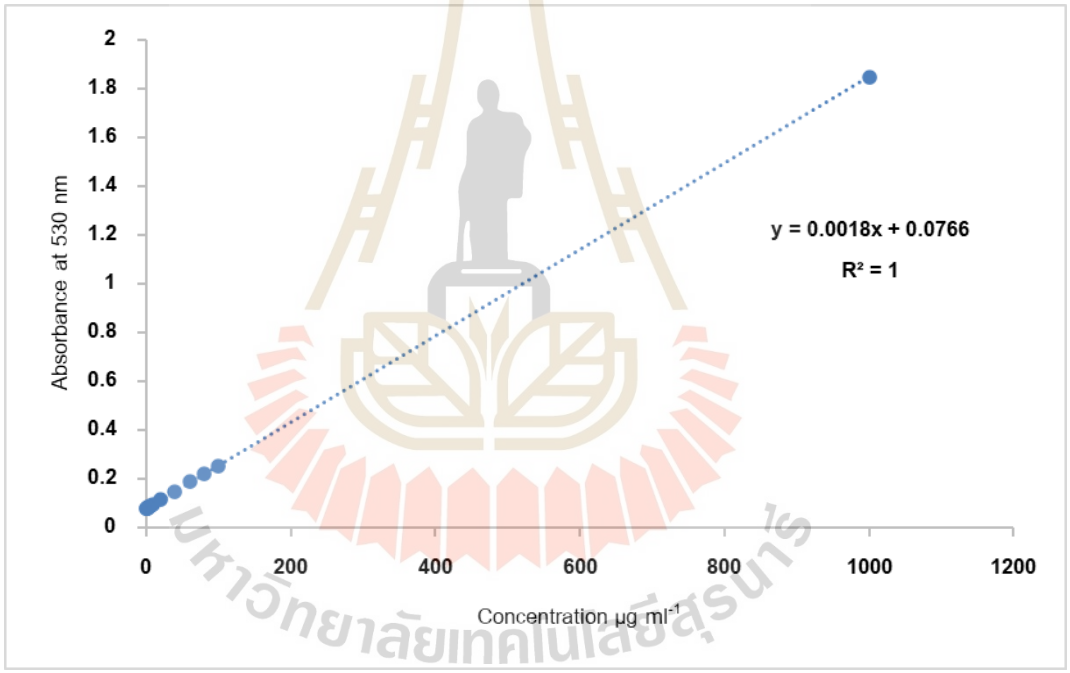
Add 25 ul tween 20 (0.05% v/v) and 2.5 ml 200mM Na_2HPO_4 to the stirring DAB solution.

2.20 0.2% NBT staining solution

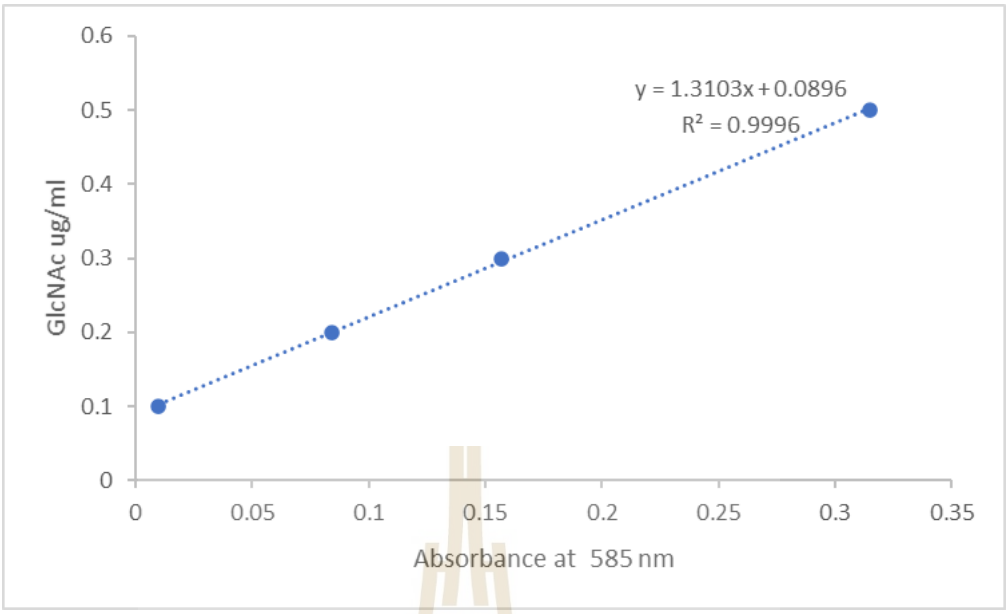
Nitroblue tetrazolium 0.1 g

50mM Sodium phosphate buffer (pH 7.5) and make volume to 50 ml

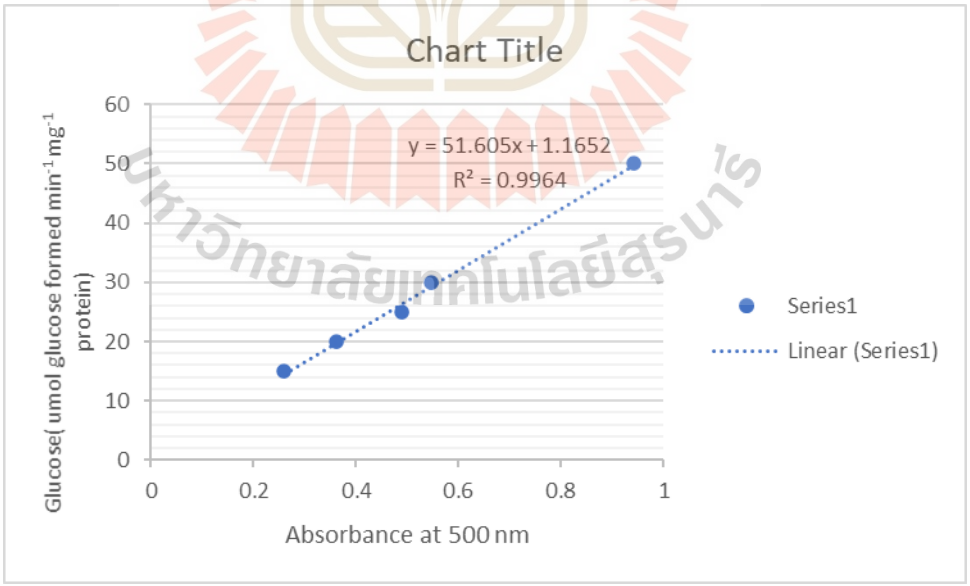
III Standard curve



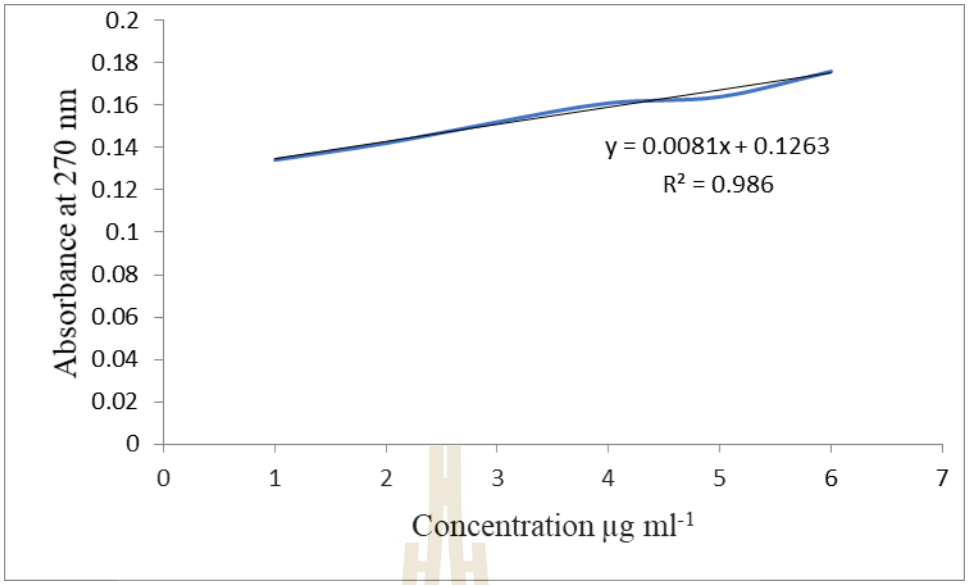
Attached figure 1 Standard curve of salicylic acid (SA) at the absorbance 530 nm.



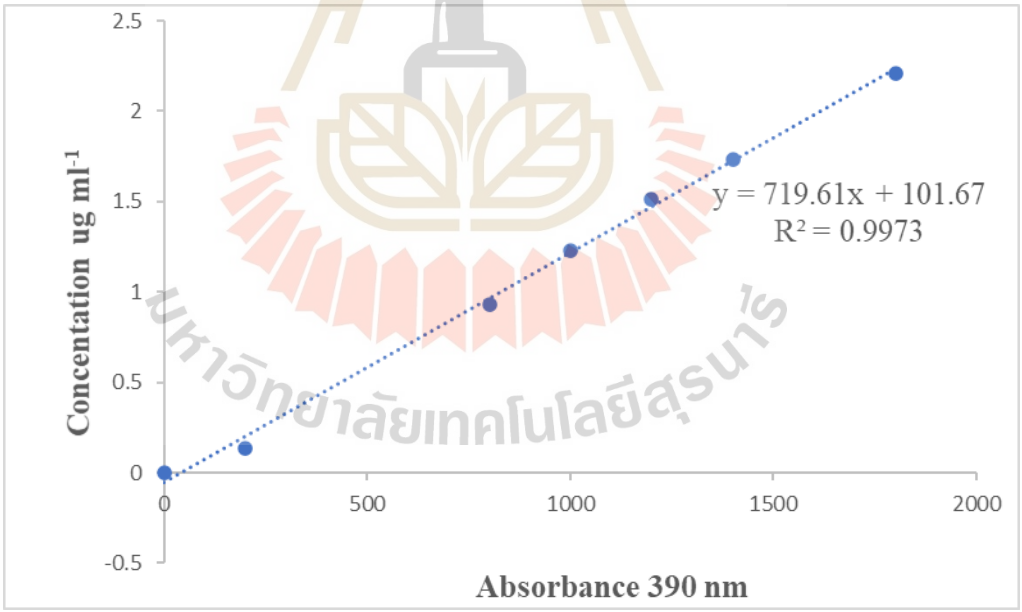
Attached figure 2 Standard curve of N-Acetyl-D-Glucosamine at the absorbance 585 nm.



Attached figure 3 Standard curve of D-Glucosamine at the absorbance 500 nm.

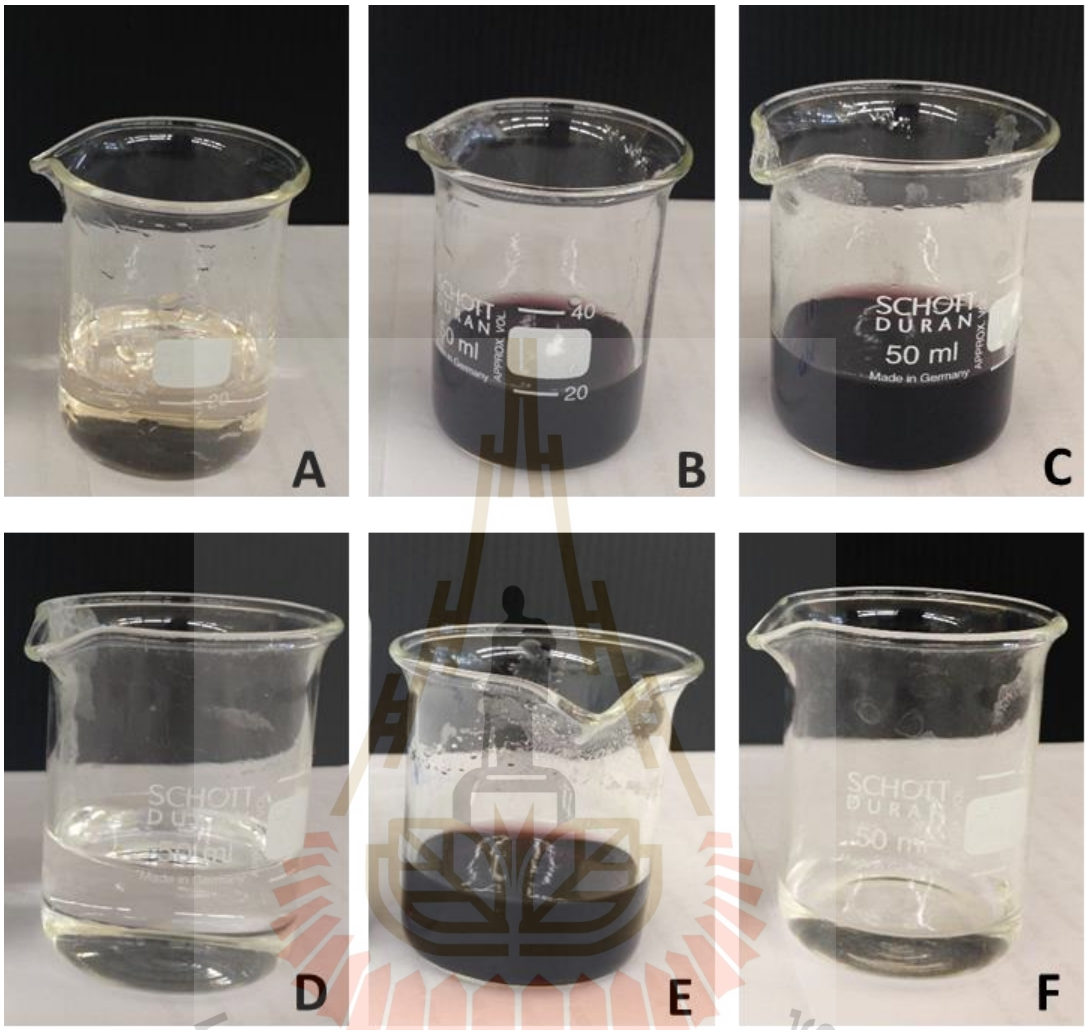


Attached figure 4 Standard curve of protein concentration at the absorbance 270 nm.



Attached figure 5 Standard curve of hydrogen peroxide at the absorbance 390 nm.

IV RSA product evaluation

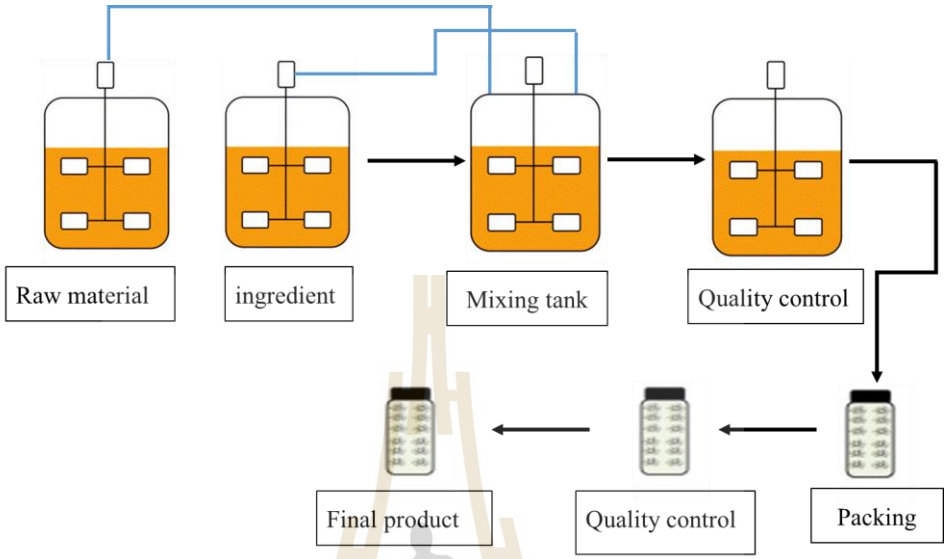


Attached figure 6 The color properties of the 5 formulations of RSA protector at 6 months A) RAS 3 B) 6% Salicylic acid.

Attached Table 1 The physical properties of the 5 formulations of RSA protector at 3 and 6 months**.**

No. RSA protector	Color	Turbidity/ transparency	Sedimentation	pH		separate mixtures of substances	Contamination of fungus
				3 months	6 months		
RSA 3	Yellow Orange group (19-D)	transparency	×	2.71	2.68	×	×
6%Salicylic acid	Non color	transparency	×	2.12	2.78	×	×

V RSA Manufacturing Planning and Control Systems



Attach figure 7. Schematic process and steps of assessment the product**.**

Attached Table 2 Costs of the controlling disease production

Items	**Cost**[1/]		
	Carbendazim	**Commercial elicitor**	**RSA**
Protection product	38	33.24	1.44
Labor	150	150	150
Total	188	183.24	154.4

[1/]Units are in Thai baht (THB) per rai, in conventional cassava farming.

BIOGRAPHY

Miss Rungthip Sangpueak was born on May 03, 1989, in Prachinburi Province, Thailand. she graduated with a Bachelor and Master's degree in school of crop production technology from Suranaree University of Technology. In 2016, 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 [PHD59I0084].

Moreover, in 2017 she publicly first paper in the Journal of Phytopathology in the title of Morphological and molecular identification of Colletotrichum species associated with cassava anthracnose in Thailand. In the last year of the Ph.D. program, she was an exchange student at Gembloux Agro-Bio Tech, University of Liège, Belgium, from May 2019 to November 2019.