IMPROVEMENT OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) DEAMINASE ENZYME IN BRADYRHIZOBIA FOR PLANT GROWTH PROMOTION

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การปรับปรุงเอนไซม์ 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) DEAMINASE ในเชื้อแบรดี้ไรโซเบียมเพื่อการส่งเสริมการเจริญเติบโตของพืช

นางสาวสุกัลยา สารพัฒน์

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

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สุกัลยา สารพัฒน์ : การปรับปรุงเอนไซม์ 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) DEAMINASE ในเชื้อแบรดี้ไรโซเบียมเพื่อการส่งเสริมการเจริญเติบโตของพืช (IMPROVEMENT OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) DEAMINASE ENZYME IN BRADYRHIZOBIA FOR PLANT GROWTH PROMOTION) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 115 หน้า.

การตรึงไนโตรเจนภายใต้สภาวะพึ่งพาอาศัยระหว่างจุลินทรีย์และรากพืชเป็นความสามารถของเชื้อแบรดี้ไรโซเบียม/ไรโซเบียมโดยการสร้างปมที่รากและ/หรือลำต้นของพืชตระกูลถั่ว การตรึงไนโตรเจนดังกล่าวเป็นการเตรียมแหล่งไนโตรเจนให้กับถั่วและพืชอาศัย โดยเชื้อแบรดี้ไรโซเบียม/ไรโซเบียมทำการตรึงไนโตรเจนที่อยู่ในอากาศด้วยเอนไซม์ไนโตรจีเนส (nitrogenase enzyme) ส่งให้พืชอาศัยในรูปของแอมโมเนีย (NH_3) นอกจากนี้ เชื้อแบรดี้ไรโซเบียม/ไรโซเบียมบางสายพันธุ์ยังสามารถสร้างเอนไซม์ Aminocyclopropane-1-carboxylic acid (ACC) deaminase ที่สามารย่อยสาร ACC ซึ่งเป็นสารตั้งต้นในการผลิตเอทิลีนให้เป็นแอมโมเนียและแอลฟา-คีโตบูทีเรต (α-ketobutyrate) เอทิลีนจัดเป็นฮอร์โมนพืชชั้นสูงและมีบทบาทสำคัญอย่างมากต่อการมีชีวิตรอดของพืชเมื่อพืชต้องเผชิญกับสิ่งแวดล้อมที่ไม่เหมาะสมต่อการเจริญเติบโต อย่างไรก็ตาม ฮอร์โมนเอทิลีนทำให้เกิดผลด้านลบต่อพืชได้เมื่อถูกผลิตในปริมาณที่สูง แต่ปริมาณที่สูงของการผลิตฮอร์โมนดังกล่าวสามารถทำให้ลดลงได้โดยการควบคุมสาร ACC ด้วยกิจกรรมเอนไซม์ ACC deaminase ที่มีอยู่ในแบคทีเรีย ดังนั้น เพื่อปรับปรุงประสิทธิภาพของกิจกรรมเอนไซม์ ACC deaminase ที่มีอยู่ในเชื้อแบรดี้ไรโซเบียม SUTN9-2 ได้ดำเนินการสองวิธี คือ พันธุวิศวกรรม และ Adaptive Laboratory Evolution (ALE) การถ่ายทอด plasmid ที่มียีน *acdR* และ *acdS* จากเชื้อแบรดี้ไรโซเบียม SUTN9-2 เข้าไปในเชื้อ Wild type ทำให้ได้สายพันธุ์ปรับปรุงชื่อ SUTN9-2:pMG103::*acdRS* ในขณะที่การปรับปรุงกิจกรรมเอนไซม์ด้วยวิธี ALE ดำเนินการบนหลักการของการปรับตัว และเจริญเติบโตอย่างต่อเนื่องในสภาวะแวดล้อมที่ความเข้มข้นของACC สูง (3 mM ACC) ทำให้ได้สายพันธุ์ปรับปรุงชื่อ SUTN9-2 (ACCDadap) การปรับปรุงกิจกรรมเอนไซม์ ACC deaminase สามารถเพิ่มประสิทธิภาพกิจกรรมของเอนไซม์ ACC deaminase ได้ 8.9 เท่าในเชื้อสายพันธุ์ SUTN9-2:pMG103::*acdRS* และ 1.4 เท่า ในเชื้อสายพันธุ์ SUTN9-2 (ACCDadap) เมื่อเทียบกับเชื้อ Wild type อิทธิพลของการเพิ่มประสิทธิภาพให้กับกิจกรรมเอนไซม์ ACC deaminase ของเชื้อ SUTN9-2:pMG103::*acdRS* และ SUTN9-2 (ACCDadap)ได้ทำการทดสอบกับถั่วเขียว (*Vigna radiata* (L.) R.Wilczek SUT1) พบว่า การเพิ่มประสิทธิภาพเอนไซม์ ACC deaminase สามารถส่งเสริมการเข้า

สร้างปมในช่วง 12 วันแรกหลังจากการปลูกเชื้อ โดยเฉพาะการใช้เชื้อสายพันธุ์ SUTN9-2:pMG103::*acdRS* และสายพันธุ์ดังกล่าวยังช่วยให้การตรึงไนโตรเจนในถั่วเขียวสามารถดำเนินอยู่ได้มากกว่าสายพันธุ์อื่น ภายใต้สภาวะขาดน้ำ และช่วยส่งเสริมการเจริญเติบโตของถั่วเขียวหลังจากการรดน้ำกลับ (Rehydration) นอกจากนี้ สายพันธุ์ SUTN9-2 (ACCDadap) พบว่า มีการเปลี่ยนแปลงของนิวคลีโอไทด์บางตำแหน่งที่มีผลทำให้เกิดการเปลี่ยนแปลงของโครงสร้างในส่วนที่คาดว่าเป็นบริเวณที่ใช้จับสารตั้งต้น (ACC binding site) การกลายพันธุ์หรือการเปลี่ยนแปลงที่เกิดขึ้นนี้ อาจเป็นสาเหตุนำไปสู่การเพิ่มขึ้นของค่ากิจกรรมเอนไซม์ ACC deaminase ในเชื้อสายพันธุ์ SUTN9-2 (ACCDadap) นอกจากนี้ การปรับปรุงกิจกรรมเอนไซม์ ACC deaminase ของเชื้อ SUTN9-2:pMG103::*acdRS* สามารถส่งเสริมปฏิสัมพันธ์แบบพึ่งพา (Symbiosis) และเพิ่มความทนทานต่อการขาดน้ำ รวมทั้งยังส่งเสริมการฟื้นตัวจากการขาดน้ำให้กับถั่วเขียวได้ดีกว่าสายพันธุ์ที่มีกิจกรรมเอนไซม์ ACC deaminase ที่ต่ำกว่า และยังสามารถลดการสังเคราะห์เอทิลีนในข้าว (*Oryza sativa* L.) ซึ่งนำไปสู่การลดความเสียหายของเยื่อหุ้มเซลล์ และปริมาณคลอโรฟิลล์ เมื่อต้นข้าวได้รับความเครียดจากการขาดน้ำ ในขณะเดียวกัน การปลูกเชื้อที่มีกิจกรรมเอนไซม์ ACC deaminase ทั้งสายพันธุ์ Wild type และสายพันธุ์ที่ปรับปรุงกิจกรรมเอนไซม์ ACC deaminase ได้เพิ่มปริมาณน้ำภายในใบ (Relative water content (RWC)) และอัตราการรอดชีวิตของข้าว รวมทั้งอัตราการฟื้นตัวของต้นข้าวให้เพิ่มสูงขึ้น เมื่อเผชิญกับสภาวะขาดน้ำ ในขณะเดียวกัน ภายใต้การปลูกในสภาพแปลงและขาดน้ำ การปลูกเชื้อ SUTN9-2 (ACCDadap) สามารถปรับปรุงผลผลิตข้าวได้มากกว่าเชื้อ Wild type และไม่ได้ปลูกเชื้อ ดังนั้น ความทนทานต่อความเครียดที่เกิดจากการขาดน้ำในต้นข้าวและถั่วเขียว สามารถบรรเทาได้ด้วยการลดการสังเคราะห์เอทิลีนผ่านกิจกรรมเอนไซม์ ACC deaminase ของเชื้อ SUTN9-2 โดยเฉพาะสายพันธุ์ที่ได้รับการปรับปรุงประสิทธิภาพของเอนไซม์ ACC deaminase นอกจากนี้ แบคทีเรียสายพันธุ์ SUTN9-2 (ACCDadap) มีศักยภาพในการนำไปใช้เป็นหัวเชื้อชีวภาพที่ปลูกข้าวในสภาวะแปลงนา เพื่อส่งเสริมการเจริญเติบโต ผลผลิต และความทนทานต่อความการขาดน้ำให้กับพืชอีกด้วย

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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

SUKANLAYA SARAPAT : IMPROVEMENT OF 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) DEAMINASE ENZYME IN BRADYRHIZOBIA FOR PLANT GROWTH PROMOTION. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr. rer. nat, 115 PP.

ACC DEAMINASE/DROUGHT/*Bradyrhizobium*/*Oryza sativa*/*Vigna radiata*

Symbiotic nitrogen-fixation is an ability of *Bradyrhizobium/Rhizobium* by forming nodules in host legume roots and/or stem, which provide a nitrogen source as the ammonia (NH_3) form to the host plant from nitrogen-fixation process. Moreover, some bacterial strains in *Bradyrhizobium/Rhizobium* also produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme, which can degrade the ethylene precursor, ACC, converts to ammonia and α-ketobutyrate. Ethylene is a higher plant hormone and plays a vital role in plant survival when plants encounter unfavorable conditions. However, the hormone can present negative effects when it is produced in high concentration. Nevertheless, the significant amount of ethylene production can be decreased by controlling its substrate (ACC) by ACC deaminase activity contain in bacteria. Therefore, to improve the efficiency of ACC deaminase activity contained in *Bradyrhizobium* sp. SUTN9-2, two methods for improving, genetic engineering and adaptive laboratory evolution (ALE) based methods, were used. The transfer of a plasmid containing *acdR* and *acdS* into SUTN9-2 was genetic engineering improvement, while the ALE method was performed based on accumulation of an adaptive bacterial population that continuously grew in a specified growth condition for a long period of time. The improved strains succeeded in increasing ACC deaminase enzyme activity as 8.9–fold

in SUTN9-2:pMG103::*acdRS* and 1.4–fold in SUTN9-2 (ACCDadap) when compared with the wild type strain. The influences of the higher activity were observed in the host plant (*Vigna radiata* (L.) R.Wilczek SUT1). Both improved strains could enhance nodulation in the early stage of plant growth. Additionally, SUTN9-2:pMG103::*acdRS* could also maintain nitrogen fixation in water deficit conditions as well as promote plant biomass after rehydration. Moreover, the changes of nucleotide and amino acid in AcdS protein in SUTN9-2 (ACCDadap) were investigated. Some nucleotides were predicted to be located in the ACC binding site which was mutated. This mutation may lead to increasing ACC deaminase activity, which enhanced symbiotic interaction and drought tolerance, as well as have better recovery after rehydration than lower ACC deaminase activity. Moreover, these bacterial strains also reduce ethylene synthesis, lead to decreasing membrane destruction and chlorophyll contents in rice (*Oryza sativa* L.) when confronted with a water deficit situation. Meanwhile, the leaf relative water content (RWC), survival and recovery rates were improved as well as crop yield in field conditions. Therefore, drought stress tolerance in rice and mung bean plants could be improved by a controlling the ethylene synthesis via the ACC deaminase improvement in SUTN9-2. Moreover, the SUTN9-2 (ACCDadap) strain can be used as a bio-inoculant in field conditions to enhance growth, rice yield, and drought tolerance.

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LIST OF ABBREVIATIONS

ACC	=	1- aminocyclopropane-1-carboxy acid
ACC deaminase	=	1- aminocyclopropane-1-carboxy acid deaminase
SAM	=	S-adenosyl-Methionine
ACS	=	ACC synthase
ACO	=	ACC oxidase
GMO	=	genetically modified organisms
ALE	=	Adaptive laboratory evolution
ROS	=	Reactive oxygen species
H_2O_2	=	Hydrogen peroxide
HO•	=	Hydroxyl radical
O_2^-	=	Superoxide anion
$^1O_2$	=	Singlet oxygen
DNA	=	Deoxyribonucleic acid
RNA	=	Ribonucleic acid
SOD	=	Superoxide dismutase
TF	=	Transcription factors
ABA	=	Abscisic acid
ET	=	Ethylene
JA	=	Jasmonic acid
CKs	=	Cytokinins
SA	=	Salicylic acid

LIST OF ABBREVIATIONS (Continued)

IAA	=	Indole-3-acetic acid
GA	=	Gibberellin
PLP	=	Pyridoxal 5-phosphate
α-KB	=	α-ketobutyrate
NH_3	=	Ammonia
acdS gene	=	ACC deaminase structural gene
acdR gene	=	ACC deaminase regulator gene
acdRS genes	=	*acdS* and *acdR* genes
AcdS protein	=	ACC deaminase structural protein
Lrp	=	Leucine-responsive regulatory
PGPB	=	Plant growth promoting bacteria
PGPR	=	Plant growth promoting rhizobacteria
CRP	=	Cyclic AMP receptor
FNR	=	Fumarate nitrate reduction
ACC-DR	=	ACC deaminase domain region
AVG	=	2-aminoethoxyvinyl glycine
STS	=	Silver thiosulfate
WUE	=	Water use efficiency
PEG	=	Polyethylene glycol
OD	=	Optical density
YEM	=	Yeast extract mannital
LB	=	Luria-Bertani

LIST OF ABBREVIATIONS (Continued)

HM	=	HEPES-MES Salt medium
WT	=	Wild type
GC	=	Gas chromatograph
DAI	=	Days after inoculation
PWP	=	Permanent wilting point
RWC	=	Relative water content
MDA	=	Malondialdehyde
TCA	=	Trichloroacetic acid
TBA	=	Thiobarbituric acid
CFU	=	Colony forming unit
GASP	=	Growth advantage in stationary phase
NH_4NO_3	=	Ammonium nitrate
$MgSO_4$	=	Magnesium sulfate
nm	=	nanometer
rpm	=	Revolutions per minute
℃	=	degree celsius
(v/v)	=	volume/volume
kv/cm	=	kilovolt/centimeter
$\mu E\ m^{-2}\ S^{-1}$	=	Microeinsteins per square meter per second
µF	=	Microfarad
µg	=	Microgram
ml	=	Milliliter

LIST OF ABBREVIATIONS (Continued)

mM	=	Millimolar
pH	=	Potential of Hydrogen ion
DW	=	Dry weight

CHAPTER I

INTRODUCTION

1.1 Rationale and background

The possession ACC deaminase activity of bacteria can relieve the negative results of the high levels of ethylene production, which is stimulated by the environmental stresses, including both biotic and abiotic stresses. When plants confront with the stressors, they often deal with producing what is known as a stress ethylene, that is a cause of plant damages (Glick et al., 2007). The phytohormone, ethylene gas is synthesized from methionine via S-adenosyl-Methionine (SAM) and ACC acting as the intermediates, with ACC synthase (ACS) and ACC oxidase (ACO), respectively. The synthesis of ACC is the biosynthetic step that influence on the amount of ethylene synthesis in plant tissues (Duan, 2007). The ACC is absorbed by soil bacteria having ACC deaminase activity that bound to the roots of plants. Therefore, the function of the enzyme can be salvaged useful sources of carbon and nitrogen for metabolic needs (Thibodeaux and Liu, 2011). Previous study reported that ethylene can inhibit the nodulation process of *Bradyrhizobium/Rhizobium*. There is evidence suggesting that exogenous ethylene can block the invasion of the infection thread in the root cortex of the plant host (Spaink, 1997). Therefore, it is important to mention that the ethylene regulates the nodulation process (Tamimi and Timko, 2003). However, ACC deaminase enzyme in which produced by *Bradyrhizobium* or *Rhizobium* can increase the nodule number and can prevent the reduction of plant growths which is the common

response of plant to water deficit condition. For instance, the co-inoculation of rhizobia with rhizobacteria or plant growth promoting rhizobacteria (PGPR) containing the high levels of ACC deaminase activity can synergistically improve the rhizobium-legume interaction and also provide the alleviation of stress-induced water deficit. *Vigna radiata* inoculated with *Bradyrhizobium* sp. PRC008 could enhance plant biomass and decreased ethylene biosynthesis in combination with rhizobacteria (*Enterobacter* strains and/or *Chryseobacterium* strain) having high ACC deaminase activities, when compared with single inoculation of rhizobium under water deficit irrigation (Tittabutr et al., 2013). Likewise, the effect of co-inoculation was confirmed by the result which have found that under the drying soil, *R. leguminosarum* bv. viciae co-inoculated with *V. paradoxus* 5C-2, which contain enzyme ACC deaminase, could maintain and improve nodule number as well as yield in pea plants when compared with non-combination (Belimov et al., 2009).

In addition, *Bradyrhizobium* sp. strain SUTN9-2, which contain an ACC deaminase gene in its genomic DNA, could nodulate various leguminous plants, including *Aeschynomene americana*, *Glycine max*, *Arachis hypogaea*, *Vigna radiata*, *Macroptilium atropurpureum* (Noisangiam et al., 2012), and also was found that it is as an endophytic bacterium in the rice plants (*Oryza sativa*) (Piromyou et al., 2015). This leads us to emphasize that the high ACC deaminase activity may promote the growth of plants, and improve the stress-tolerant in leguminous and rice plants better than original strain, *Bradyrhizobium* sp. SUTN9-2. In order to improve the enzymatic activity of ACC deaminase, genetic engineering is the most promising approach. However, the limitation of releasing genetically modified organisms (GMO) in Thailand is still unclear and prohibited in several countries. Therefore, an alternative

method as metabolic evolution is more preferable. Adaptive laboratory evolution (ALE) approach is a strategy to use for improving the enzymatic activity of ACC deaminase without the transferring of plasmids or foreign genes into the bacterial cells. However, the engineering method was also need to investigation an overexpression ACC deaminase activity strain. Therefore, to achieve the goal, both improved strains, SUTN9-2 (ACCDadap) and SUTN9-2: pMG103::*acdRS* strains, were used to observe their effect on their host in comparison with original strain, SUTN9-2, under normal and water deficit conditions.

1.2 Objectives

1.2.1 Main objective

1.2.1.1 To improve the ACC deaminase activity in *Bradyrhizobium* sp. SUTN9-2

1.2.1.2 To investigate the roles of ACC deaminase on its host plants in aspects of plant growth promotion and plant symbiosis under the well-watered condition as well as the water stress tolerance under water deficit condition

1.2.2 Specific objectives

1.2.2.1 To obtain the improved bacterial ACC deaminase activity to be used as a bio-inoculant in field conditions

1.2.2.2 To assess the effects of the high ACC deaminase activity in SUTN9-2 on mung bean plants both the well-watered and water deficit conditions

1.2.2.3 To assess the influence of the high ACC deaminase activity in SUTN9-2 on rice plants under both the well-watered and water deficit conditions

CHAPTER II

LITERATURE REVIEWS

2.1 Plants responds and stress-induced ethylene biosynthesis

Plants cannot escape from the various biotic and abiotic stressful conditions when grow under field condition. Under the unstable rainfalls, plants confronted with the water deficiency cause of suffering and changing the morphology, physiology, biochemical and molecular levels (Pandey and Shukla, 2015). Several reports proposed that the water stress can affect the critical organelles leading to failing and inefficiency of metabolism pathways in plants such as photosynthesis disruption, pigment degradation, photorespiration increment, transpiration reduction and others (Cruz de Carvalho, 2008; Foyer, 2018; Karuppanapandian et al., 2011; Miller et al., 2010; Zhu, 2016). Moreover, the water deficit stress also induces alteration in the basic mechanism of plant cells, such as the generation of excess reactive oxygen species (ROS) which causes oxidative damage in cellular complements.

ROS are a by-product of aerobic organisms. The basic forms of cellular ROS are H_2O_2, HO•, O_2^- and $^1O_2$ and two of these forms (HO• and $^1O_2$) can harm and oxidize cellular components such as cell membrane, protein, lipids, DNA, RNA and ethers, leading to cell death (Karuppanapandian et al., 2011; Ullah et al., 2017). However, the excess ROS can activate ROS scavenging systems to eliminate the ROS accumulation result in ROS homeostasis in cellular compartments.

The ROS scavenging antioxidative systems, including enzymatic antioxidant

and non-enzymatic antioxidative systems have been developed to control the excess ROS. Normally, ROS scavenging systems request the cooperative system of various antioxidants such as O_2^- which is converted to H_2O_2 by SOD (superoxide dismutase) and then H_2O_2 is converted to H_2O by APX (Ascorbate peroxidase) and CAT (catalase), while the oxidative stress can activate osmoprotectant synthesis pathways such as proline which can prevent lipid, proteins, DNA and RNA damages from oxidative stress. It means that the activities can influence plant survival or it is the drought-tolerant traits (Cruz de Carvalho, 2008). Furthermore, the up generation of ROS caused by drought stress can activate plant hormones to deal with the unfavorable conditions.

The plant hormones are an important role in providing plant tolerance or resistance under water deficit condition (Hadiarto and Tran, 2011). Phytohormones are signaling molecules involved in the regulation of physiological and metabolic pathways by coordinating with other signaling pathways or molecules such as protein kinase, calcium signaling pathway, transcription factors (TF), ROS molecules as well as a crosstalk between the different plant hormones (Hadiarto and Tran, 2011; Kar, 2011; Miller et al., 2010; Ullah et al., 2017). Not only abscisic acid (ABA) is a pivotal role in adaptation to activate acclimation responses like stomata closure to reduce water loss, but also ethylene (ET) is a gaseous hormone which plays a role in plant defense to abiotic and biotic stress and requests the coordination with other plant hormones such as jasmonic acid (JA), cytokinins (CKs), salicylic acid (SA), auxin or indole-3-acetic acid (IAA), gibberellin (GA) and others (Peleg and Blumwald, 2011; Wani et al., 2016). Hormone ethylene is a vital role for plant survival during they encounter with unfavorable conditions to stimulate adaptive mechanisms (Druege, 2006; Wang et al., 2002). However, the plant hormone can present negative effects when it is produced in

high concentration. Glick et al., (2007) proposed that the evolution of ethylene can be elevated in a few hours after plants meet the unfavorable environmental conditions, which is a small peak and act as an initiation of plant respond (Figure 2.1A). The small peak exerts a plant signaling to stimulate gene transcriptions which provide stress tolerance strategy to duel against the unfavorable conditions, leading to positive effects on plants. Unfortunately, plants usually produce a large ethylene as a second peak during they face to environmental stresses for 1-3 days, leading to stress ethylene that is a cause of growth inhibition (Figure 2.1A). Furthermore, the stress ethylene also affect in plants by inducing and senescent acceleration plant cell death (Apelbaum and Yang, 1981; Chae and Lee, 2001; de Jong et al., 2002; El-Beltagy and Hall, 1974; Hall et al., 1977; Morgan and Drew, 1997). Ethylene was synthesized by inducing ACC synthase activity to produce 1-aminocyclopropane-1-carboxylic acid (ACC) which is an immediate precursor of ethylene synthesis before converting to ethylene gas by ACC oxidase and oxygen (Yang and Hoffman, 1984) (Figure 2.2 A). Normally, plants react to stress conditions by synthesizing a large ethylene which lead to stress ethylene phenomenon that is a negative role, because the large ethylene levels not only cause plant damages but also inhibit or reduce a plant growth (Apelbaum and Yang, 1981; Dubois et al., 2018; Kacperska and Kubacka-Zębalska, 1989; Stearns and Glick, 2003).

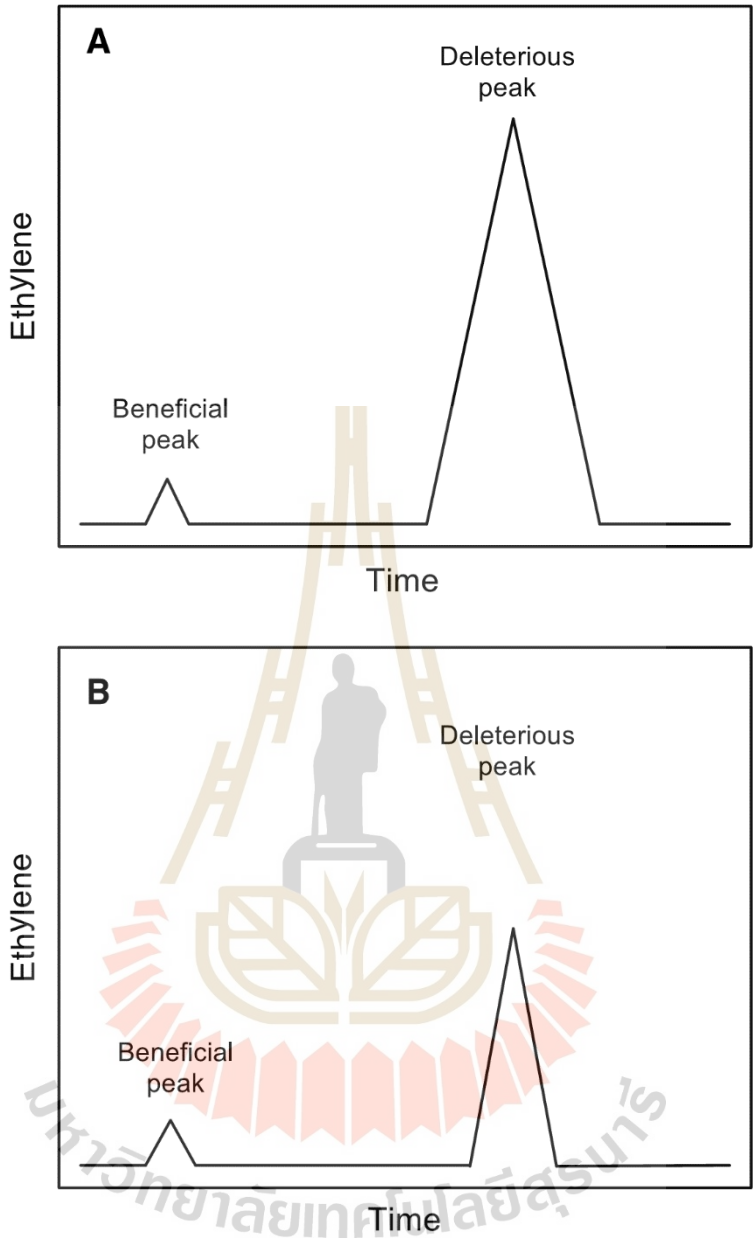

Figure 2.1 Ethylene production in higher plants against the unfavorable environmental condition. (**A**) The ethylene levels without bacterial ACC deaminase activity. (**B**) The influence of bacteria producing ACC deaminase activity on ethylene production (Glick et al., 2007).

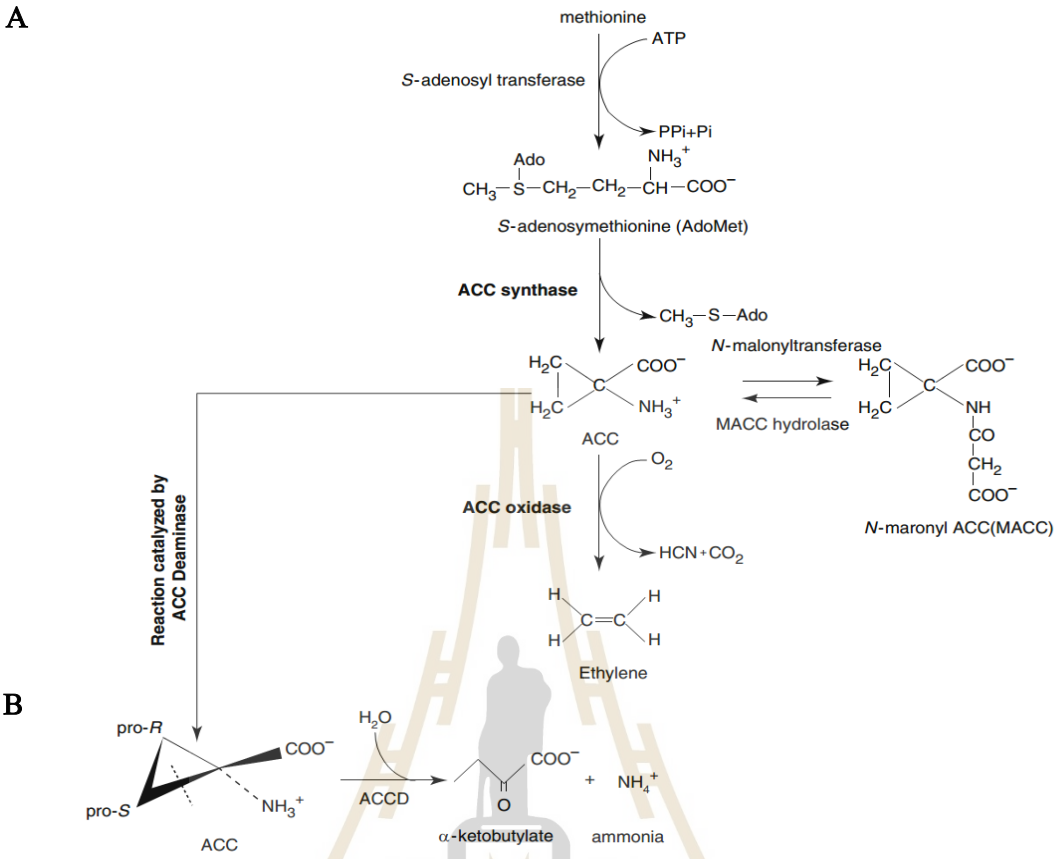

Figure 2.2 The enzymatic reaction of ACC deaminase react to its substrate, 1-Aminocyclopropane-1-carboxy acid (ACC) in ethylene biosynthesis pathway. (**A**) Ethylene biosynthesis pathway in higher plants. (**B**) The enzymatic reaction of ACC deaminase (Ose et al., 2003).

2.2 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase enzyme

The enzyme ACC deaminase requires the pyridoxal 5-phosphate (PLP) to utilize as a co-factor for enzymatic activity (Thibodeaux and Liu, 2011) (Figure 2.3A and B). The enzyme can cleave the C_{α}-C_{β} bond of an amino acid substrate, and it is a rare event in PLP–dependent enzyme catalysis because of the amino acid substrate (ACC) which does not contain the Cα proton. Then, the carboxylate group is converted to the α-ketobutyrate (α–KB) and ammonia (NH_3) product (Figure 2.3B). The catalytic pathways of most PLP-dependent enzymes start the reaction with an external aldimine between the amino group of the substrate and the PLP coenzyme (Thibodeaux and Liu, 2011) (Figure 2.3B). ACC deaminase is classified into a tryptophan synthase family because it enfolds into two domains in which the unit has an open twisted α/β structure similar to the β-subunit of tryptophan synthase.

However, ACC deaminase is dissimilar with the PLP dependent enzymes in the members of *β* family, which the PLP is buried deep in the molecule (Figure 2.4A). Among all of them have been published, the *β*-subunit of tryptophan synthase from *Salmonella typhimurium* (TRPSβ) and O acetylserinesulfhydrylase from *S. typhimurium* (OASS) as well as threonine deaminase from *Escherichia coli* (TD) have a similarity with yACCD (ACC deaminase of *Hansenula saturnus*). These molecules were classified in the β-family of the PLP-dependent enzymes, which suggested that the PLP-binding site in these enzymes shared the same fold which comprised two domains. Moreover, the structural attributes of yACCD shared 16% identities with the sequence comparison with TRPSβ. However, the sequence comparisons among yACCD, TD and OASS were very limited similarity, while ACC deaminase enzyme structure containing *Pseudomonas* is very similar to that of the yeast enzyme (Figure

2.4B). The bound PLP cofactor in the *Pseudomonas* enzyme forms an internal aldimine with Lys^{51} through its side chain *N* amino group. Residue binding at the PLP is also important for the catalytic reaction that various mutations have been introduced into many PLP enzymes. The enzyme can degrade several D-amino acid (e.g., D-serine and D-cysteine), which are a cyclopropanoid amino acid. Alpha- ketobutyrate and ammonia are yields of the reaction between ACC as a substrate and ACC deaminase enzyme (Glick, 2005; Glick et al., 2007; Karthikeyan et al., 2004; Saleem et al., 2007; Todorovic, 2008). ACC deaminase is first studied in *Pseudomonase* sp. ACP as a soil bacterium and yeast *H. satrunus* by Honma and Shimomura, (1978) (Saleem et al., 2007; Saraf et al., 2011). Numerous bacterial species and fungal species were found to produce ACC deaminase (Glick et al., 2007; Saleem et al., 2007). The results implied that may be nitrogen nutrition in the microbial habitat where containing ACC is important to their survival in terrestrial and/or non-terrestrial environments. However, not only ACC deaminase was found the microorganisms, but also the protein or enzyme was also fould in some plants (McDonnell et al., 2009; Todorovic, 2008).

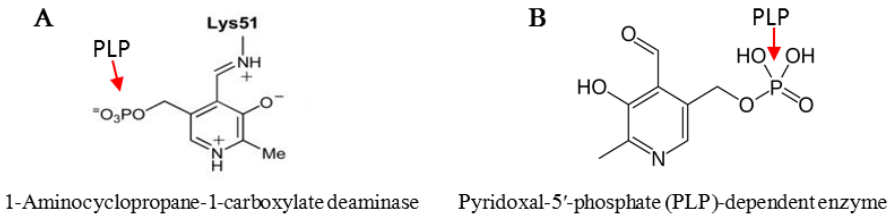

Figure 2.3 Structural biochemistry or chemical bonding of pyridoxal-5′-phosphate (PLP)-dependent enzyme containing phosphate group of PLP. (**A**) ACC deaminase structure (Thibodeaux and Liu, 2011). (**B**) Common structure of PLP-dependent enzyme (http://en.wikipedia.org/wiki/Pyridoxal_phosphate).

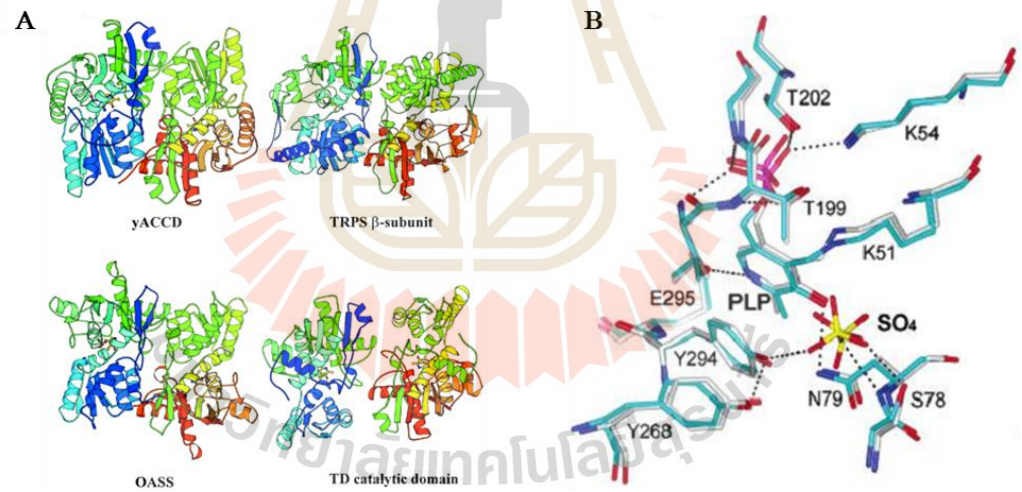

Figure 2.4 The stereoscopic drawing of the β family of PLP-dependent enzymes. (**A**) Ribbon representation protein structure of yACCD (from yeast *Hansenula saturnus*), TRPS*β* (from *Salmonella typhimurium*), TD (from *E. coli*), and OASS (from *S. typhimurium*) (Yao et al., 2000). (**B**) Stereoscopic view of the shared active sites of the *Pseudomonas* (cyan) and the yeast (gray) Hydrogen bonds are indicated by dotted lines (Karthikeyan et al., 2004).

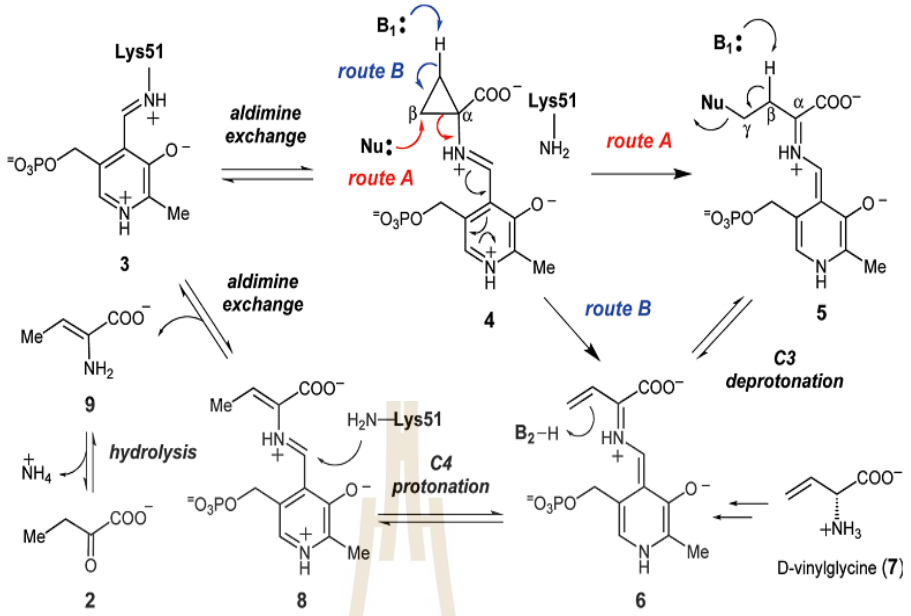

Figure 2.5 Putative reaction mechanism of bacteria ACC deaminase enzyme involving acid-catalyze ring scission (Thibodeaux and Liu, 2011).

2.3 Mechanism of ACC deaminase

Microorganism producing ACC deaminase activity can convert ACC to be ammonia and α- ketobutyrate (Glick et al., 1998; Honma and Shimomura, 1978), and utilize ACC as nitrogen and carbon sources of the microorganism (Van de Poel and Van Der Straeten, 2014; Vanderstraeten and Van Der Straeten, 2017). Thus, the microorganism like soil bacteria containing ACC deaminase activity have been screened and wildly studied because they can decrease the second peak of ethylene as stress-induced ethylene production to be lower, resulting to the optimum concentration as a result of plant growth promotion (Figure 2.1B). ACC deaminase enzyme encode from *acdS* gene and it is controlled by leucine-responsive regulatory (Lrp) protein which transcribed from *acdR* gene. Moreover, the enzyme is classified as a trait of plant

growth promoting bacteria (PGPB). Consistent with the mentioned above. Saleem et al., (2007) and Glick, (2014) reviewed and explained the benefits and mechanisms as well as gene regulation of the bacteria containing ACC deaminase enzyme using to promote crop plants or relieve the suffering from various types of stress agricultures including salinity, drought, waterlogging, temperature, pathogenicity, wilting of flowers, nodulation and contaminants of heavy metals and organic pollutants as well as air pollutants.

Nowadays, the numerous knowledges were elicited as well as applied in agriculture. Metabolism of ACC deaminase was firstly observed and identified from *Pseudomonas* sp. ACP and *H. saturnus* by Honma and Shimomura, (1978), which both the organisms were isolated from soil. Their major characteristics are able to use ACC as a nitrogen source. Moreover, they also proposed a result indicating that the enzyme is a pyridoxal-5′-phosphate (PLP) protein, which possess a unique reaction when compared with other PLP-dependent enzymes. The products of the enzyme reaction are α-ketobutyrate and ammonia, which the chemical mechanisms are a rare event in the PLP-dependent enzyme catalysis (Figure 2.5) (Hontzeas et al., 2006; Thibodeaux and Liu, 2011; Zhao et al., 2003). In addition to the details of ACC domains as mentioned above, the key amino acid residues have been investigated as well as the crystal structure and its chemical mechanisms. Previous studies demonstrated that Lys^{51} residue as a binding site of PLP (internal aldimine form) possessed important roles in the transformation of internal aldimine to external aldimine forms and required for the abstraction of a hydrogen atom from the Cβ of ACC to open the ACC cyclopropane ring. Moreover, other studies have shown that Ser^{78} residue contained a functioning in the nucleophilic addition to slave the ACC cyclopropane ring in the second route, and

Tyr^{294} showed a role in the forming of external aldimine by binding ACC with hydrogen bond while both residues, Glu^{295} and Leu^{322}, involved in the recognition of substrates. The amino acid residues acted as high conservation, which emphasized that the protein residues are necessary residues around the PLP-binding site of the enzyme ACC deaminase.

2.4 Regulation of bacterial ACC deaminase (*acdS*) gene expression

Soil bacteria, such as *Pseudomonas* sp. as early genus was used to study in the ACC deaminase regulation. The ethylene precursor, 1-aminocyclopentane carboxylic acid (ACC), is produced from SAM via catalysis of ACS activity, which is absorbed by soil bacteria which bound around the roots of plants and was degraded by ACC deaminase to α-ketobutyrate and ammonia (Figure 2.6) (Glick et al., 1998). In addition, ACC deaminase was also considered in gene regulation. The enzyme ACC deaminase transcribes from the *acdS* gene and it is regulated by *acdR* gene (LRP- like protein-coding region) which locate in the up-stream of *acdS* gene in the opposite direction (Glick et al., 2007; Grichko and Glick, 2000). Moreover, the bacterium was found that its DNA segment possesses a CRP (cyclic AMP receptor) binding site, an FNR (Fumarate nitrate reduction regulatory protein) binding site, and an *acdR* which encode Lrp (leucine-responsive regulatory protein) protein. The *acdR* locates at the up-stream of the ACC deaminase gene (*acdS*). The CRP- and FNR- binding sites are regulated by *acdR* in different conditions. The *acdR* encoding the Lrp protein which binds either CRP- or FNR- box depend on aerobic- or anaerobic- condition. However, bacteria containing ACC deaminase must be induced by the ACC, which is absorbed into bacterial cells. Briefly, the absorbed ACC into bacterial cell, the ACC then bind the

AcdB protein (encoding glycerophosphory diester phosphodiesterase) which becomes a complex, the complex binds either CRP box or FNR box depend on the envelopment. Both boxes are the transcriptional regulation of *acdS* (Figure 2.7) (Glick et al., 2007). However, certain reports revealed that the physical organization of *acdR* and *acdS* genes have varieties of the orientation in *Proteobacteria* (Blaha et al., 2006; Prigent-Combaret et al., 2008). Therefore, it can be hypothesized that different bacteria containing ACC deaminase may have varieties in their gene regulation, gene physical organization, and DNA sequences. Bacterial ACC deaminase activity and its role can promote plant growth under environmental stress conditions. Surprisingly, Nukui et al., (2006) showed that ACC deaminase of *Mesorhizobuim loti* strain MAFF303099 did not show ACC deaminase activity, but it contains ACC deaminase (*acdS*) gene in its genomic DNA. It led to an examination to study the regulation of the gene encoding the ACC deaminase. They found that the *acdS* gene expression was regulated by symbiotic genes, NifA2 protein. In addition, *R. leguminosarum* displayed a silent ACC deaminase gene which its ACC deaminase gene was revealed that the levels of *acdS* gene expression was very low without ACC. However, it could be induced by ACC concentrations as 1 μM (Ma et al., 2003a; Ma et al., 2003b).

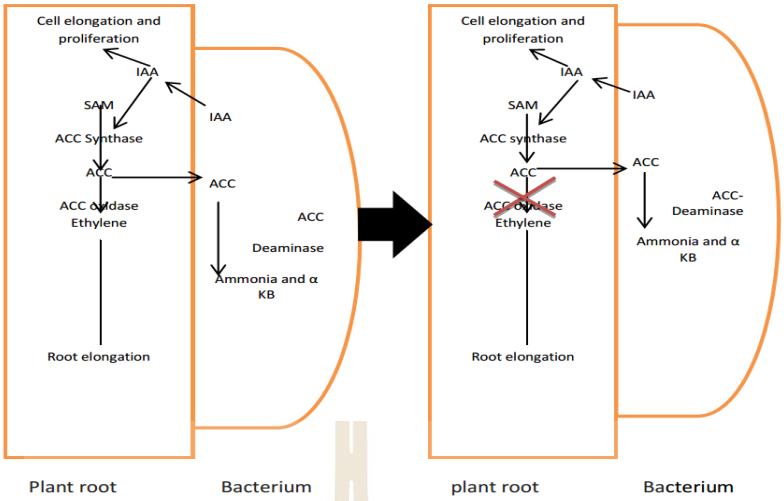

Figure 2.6 Mechanism of the ethylene reduction by ACC deaminase containing bacteria (Abduelafez, 2012).

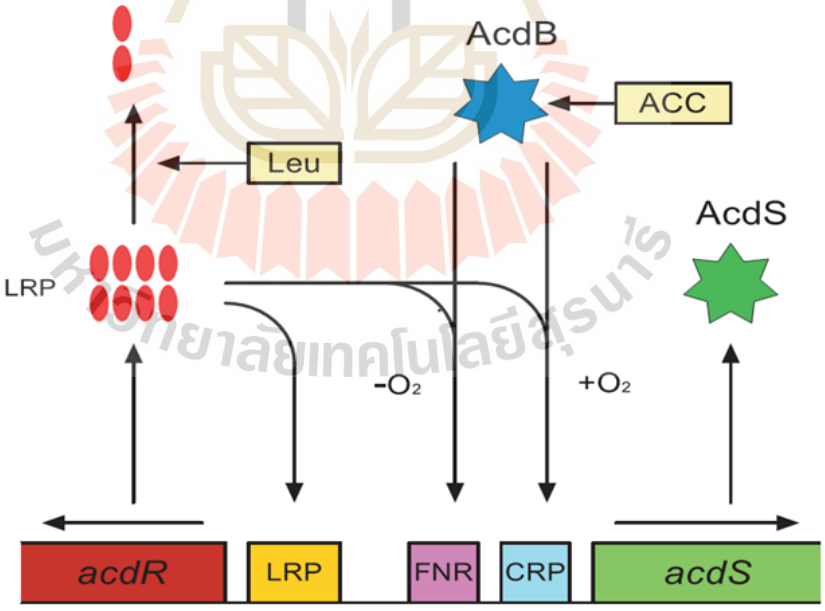

Figure 2.7 Scheme of the transcriptional control of ACC deaminase (*acdS*) gene expression in *Pseudomonas putida* UW4 (Glick et al., 2007).

2.5 Expression levels

Bacteria containing ACC deaminase are divided into two general types: the first is a symbiotic microorganism, which normally relates to the process of specific structure such as nodule and internal plant tissue of host plants, and the second group is free-living microorganism which presents near root plants. From previous studies confirm that the symbiosis is often possessed lower ACC deaminase activity than free-living microorganisms. Therefore, the close relationship between plants and the symbiont might be influence on the ACC deaminase activity in the symbiotic microorganism evolutions. Whereas, the free-living bacteria are a binding non-specificity in plant tissues which require higher ACC deaminase activity to facilitate the ability of competition against other microorganisms to utilize the plant secretions, which is a food source (Glick, 2005; Glick, 2014). Therefore, the levels of ACC deaminase in the symbiont just contain at a level to overcome at a point of the ethylene level which interferes to the infection of the symbiotic bacteria process. However, inoculation with *R. leguminosarum* biovar viciae (the presence of ACC deaminase overproduction mutation) did not enhance the nitrogenase activity, so it was suggested that ACC deaminase is necessary in the early stages of nodule development (Ma et al., 2003a).

2.6 Bradyrhizobia/Rhizobia containing ACC deaminase

Bradyrizobium/*Rhizobium* producing ACC deaminase activity plays a significant role for increasing the yield as well as plant growth under stress conditions, which can form the legume–rhizobia symbiosis and nitrogen fixation process (Murset et al., 2012). There were several rhizobia species contain ACC deaminase activity such

as, *Mesorhizobium loti* MAFF30309, *M. loti* ICMP3153, *Bradyrhizobium* sp. BTAi1, *B. diazoefficiens* USDA 110, *R. leguminosarum* 128C53K, *R. leguminosarum* bv.viciae3841, and *S. meliloti* SM11 played a crucial role to protect the rhizobia from the inhibitory effects of ethylene synthesis in their host, leading to improvement the nodulation process (Prigent-Combaret et al., 2008). Previous studies showed that high ethylene synthesis could decrease the number of nodules, for instance, ethylene could inhibit the nodulation development of alfalfa, sweet pea and *Trifolium repens* (Goodlass and Smith, 1979; Peters and Crist-Estes, 1989). On the other hand, rhizobacteria ACC deaminase could decrease the negative effects and increase the number of nodules. For example, Alfalfa was nodulated by *S. meliloti strain* Rm1021 (which did not present *acdS*), which transferred the inserted *acdS* gene of *R. leguminosarum* bv. viciae in a carrier plasmid into its cell (Ma et al., 2004). A similar experiment was conducted by Tittabutr et al., (2008) who introduced *acdS* (the plasmid is multiply copy) of strain *Sinorhizobium* sp. BL3 into *Rhizobium* sp. TAL1145. The results displayed both the increased ACC deaminase activity strains, resulting to improvement the number of nodule, weight of nodule and root in its host plant.

2.7 Adaptive laboratory evolution (ALE)

The ALE experiment is a strategy that could change the metabolism of the microorganism without any additions of plasmids or foreign genes or heterologous gene into bacterial cells, but using bacteria cells with growth-based selection under specific condition (Dragosits and Mattanovich, 2013). The process of neutral evolution is an important mode of genetic changes and forms the basis for the molecular clock (Bloom et al., 2007). The ALE experiment was applied by Jin et al., (2016) to investigate the

acdS providing from the soil, they proposed that amino acid residue at 55 to 91 of *acdS* are ACC deaminase domain region (ACC-DR) located on the small domain (helix 6) in the extra loop as the binding site of an ACC deaminase structure (Yao et al., 2000). Therefore, it is possible that the amino acid residues (58 to 169) located on the part of the small domain of ACC deaminase enzyme are an important role or influence on improving or optimizing the function of the enzyme activity. However, the adaptation was applied combining metabolic engineering to improve the fermentative production of bacteria and yeasts after they were engineered by mutating some pathways (Jeffries, 2006). For example, *E. coli* strain C was engineered to produce primarily succinate or malate in mineral salt media using simple fermentation (Jantama et al., 2008). Because of the strain was deleted central anaerobic fermentation gene (*ldhA*, *adhE*, *achA*) from the pathway, the strain grew poor in the mineral media.

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CHAPTER III

EFFECTS OF INCREASING 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) DEAMINASE ACTIVITY IN *Bradyrhizobium* sp. SUTN9-2 ON MUNG BEAN SYMBIOSIS UNDER WATER DEFICIT CONDITION

3.1 Introduction

The lowering of ethylene biosynthesis by degrading 1-aminocyclopropane-1-carboxilic acid (ACC), which is the precursor of ethylene in higher plants, is a major influence of microbial ACC deaminase, leading to plant growth promotion, especially under environmental stresses (Gamalero and Glick, 2015; Glick, 2014a; Saleem et al., 2007a; Saraf et al., 2010). Under the stress conditions, plants often respond by producing the higher amount ethylene, which is caused by the stress-induced ACC accumulation, leading to stress ethylene phenomenon. Large amount of ethylene promote tissue damage, reduce plant growth, which ultimately cause yield losses (Apelbaum and Yang, 1981a; Dubois et al., 2018a; Kacperska and Kubacka-Zębalska, 1989a; Stearns and Glick, 2003a). Therefore, bacteria containing ACC deaminase activity can modulate the higher ethylene production in plants, which provides an appropriate level for plant growth. To illustrate how soil bacteria producing ACC deaminase can decrease ethylene production in plants, Glick et al., (1998a) created a model to display it, in which they proposed that ACC, a product of *S*-adenosyl- methionine

(SAM) via catalysis of ACC synthase activity in plant tissues, is absorbed by soil bacteria which live around the plant roots or inside the plant tissue, and then the ACC is degraded by ACC deaminase contained bacteria to be α-ketobutyrate and ammonia. Thereby, the limiting of ACC level can be controlled in upstream of ethylene production in ethylene biosynthesis pathway, leading to a decrease in the negative effects of the high ethylene production. In addition to the reduction of plant growth as mentioned above, the ethylene hormone as a factor has been observed to be able to interfere with the nodule formation in rhizobia-legume interactions. The endogenous ethylene occurring from application of exogenous ACC or exogenous ethylene affects nodule number by reducing the nodule number in several legume plants such as *Medicago sativa*, *Pisum sativum*, *Lotus japonicus*, *Macroptilium atropurpureum* and *Trifolium repens* (Goodlass and Smith, 1979b; Lee and LaRue, 1992a; Lee and LaRue, 1992b; Nukui et al., 2000; Oldroyd et al., 2001a; Penmetsa and Cook, 1997; Peters and Crist-Estes, 1989b). Contrastingly, the leguminous plants that were treated with ethylene synthesis inhibitors such as L-α-(2-aminoethoxyvinyl) glycine (AVG) and silver thiosulfate (STS) as well as in an ethylene-insensitive mutant plant of *M. truncatula* (named *sickle*). Furthermore, the ethylene synthesis inhibitors and the *sickle* mutation could increase their nodule number more than untreated and its wild type. Tittabutr et al., (2008b) demonstrated that the improvement of ACC deaminase activity by transferring plasmid containing multiple copies of native *acdS* (*Rhizobium* sp. TAL1145) and exogenous *acdS* (*Sinorhizobium* sp. BL3) into TAL1145 strain can improve ACC deaminase activity which leads to ACC tolerance and promotes nodule number as well as biomass of *Leucaena leucocephala* more than parental- and *acdS*-mutant strains. Moreover, the mutant of *Sinorhizobium* sp. BL3 strain defective in *acdS*

showed the loss of nodulation competitiveness ability in mung bean when compared with wild type strain, while a strain with increasing copy showed a higher nodule formation than wild type strain at 1:1 ratio of bacterial cells delaying. Another experiment also indicated the benefit of ACC deaminase on nodule senescence (Piromyou et al., 2015b). Additionally, previous studies have demonstrated that the bacteria producing ACC deaminase activity can prevent the reduction of plant growths which is the common response of plant to water deficit condition. For instance, *Pseudomonas* strains could enhance drought stress tolerance in mung bean (*Vigna radiata* L.) (Kumari et al., 2016) as well as eliminate or alleviate ethylene stresses as a result of water deficit in several pea cultivars (Arshad et al., 2008; Zahir et al., 2008) which leads to improvement and promotion of plant growth when compared with non-inoculation under drought condition. In addition to *Pseudomonas* strains, other rhizobacteria such as *Enterobacter*, *Bacillus*, *Pantoea* and *Ochrobactrum* could also improve plant growth and induce drought tolerance in several plants including velvet bean (*Mucuna pruriens* (L.) DC. (Saleem et al., 2018), mung bean (Silambarasan et al., 2019), black gram (*V. mungo* L.) and garden pea (*Pisum sativum* L.) (Saikia et al., 2018), respectively. Apparently, co-inoculation of rhizobia with rhizobacteria or plant growth promoting rhizobacteria (PGPR) containing the high levels of ACC deaminase activity can synergistically improve the rhizobium-legume interaction and also provide the alleviation of stress-induced water deficit. *V. radiata* inoculated with *Bradyrhizobium* sp. PRC008 could enhance plant biomass and decreased ethylene biosynthesis in combination of rhizobacteria (*Enterobacter* strains and/or *Chryseobacterium* strain) having high ACC deaminase activities when compared with single inoculation of rhizobium under water deficit irrigation (Tittabutr et al., 2013b).

Likewise, the effect of co-inoculation was confirmed by the result which have found that under drying soil, *R. leguminosarum* bv. viciae co-inoculated with *Variovorax paradoxus* 5C-2 (producing ACC deaminase activity) could maintain and improve nodule number as well as yield in pea plants when compared with non-combination (Belimov et al., 2009b).

Lately, Belimov et al., (2019) demonstrated that *R. leguminosarum* bv. viciae 1066S containing ACC deaminase activity improved the shoot biomass, nodulation, nitrogen fixation, water use efficiency (WUE) and nutrient uptake in pea exposed to water deficit condition. Meanwhile, *Rhizobium* strains encoding ACC deaminase enzyme could enhance soybean (*Glycine max* L.) germination under drought condition, which was induced by 4% polyethylene glycol (PEG) (Igiehon et al., 2019). Thus, it is important to mention that the activity of ACC deaminase enzyme has essential roles in plant growth and root infection, especially under unfavorable conditions. Unfortunately, rhizobia producing ACC deaminase activity have been less investigated in the aspect of ACC deaminase in correlating with the ethylene production, nitrogen fixation and plant biomass after rehydration on mung bean plants under water deficit condition.

Although, the improvement of ACC deaminase enzyme activity by genetic engineering is a promising strategy but the improvement faces a limitation of releasing genetically modified organisms (GMO) in Thailand, and it is still prohibited. In this study, two strategies were designed to improve ACC deaminase containing *Bradyrhizobium* sp. SUTN9-2 using the first strategy of genetic engineering method by transferring plasmid containing *acdR* and *acdS* genes into SUTN9-2. The *acdR* and *acdS* genes encoded for ACC deaminase regulator (AcdR) protein and ACC deaminase

structural (AcdS) protein, respectively, which are responsible for synthesizing the enzyme ACC deaminase (Glick et al., 2007; Grichko and Glick, 2000b; Li and Glick, 2001). These two genes are presented in chromosome of SUTN9-2 in the opposite direction (Piromyou et al., 2017; Piromyou et al., 2015b). The AcdR protein is a leucine-responsive regulatory (Lrp) protein, which is a specific regulator to control the *acdS* gene expression. The second strategy was the adaptive laboratory evolution (ALE) approach, which is an approach that could change metabolism of the microorganism without any additions of plasmid or foreign gene or heterologous gene into bacterial cells, but the bacterial cells were collected with growth-based selection under specific condition (Dragosits and Mattanovich, 2013a). Therefore, the objective of this study was to investigate the effect of the ACC deaminase improvements by both genetic engineering and ALE-based methods on symbiosis of mung bean, and also to determine its efficiency for alleviation against water deficit condition. In addition, the amino acid changes of ACC deaminase derived from ALE was also investigated.

3.2 Objective

To investigate the effects of the high ACC deaminase activity in *Bradyrhizobium* sp. SUTN9-2 on mung bean plants

3.2.1 Specific objectives

3.2.1.1 To improve ACC deaminase activity in SUTN9-2

3.2.1.2 To examine the effects of the improved ACC deaminase activity strains on mung bean symbiosis

3.2.1.3 To improve plant growth using improved ACC deaminase activity strains on mung bean plants under water deficit condition

3.3 Materials and methods

3.3.1 ACC deaminase activity improvements in SUTN9-2 strain

3.3.1.1 Adaptive laboratory evolution (ALE) approach

To perform ACC deaminase improvement by ALE approach, the starter culture of *Bradyrhizobium* sp. strain SUTN9-2 (Piromyou et al., 2015b) (1% of starter (v/v)) was cultured in minimal medium broth supplemented with 10% (v/v) of HM broth and 0.5 mM ACC (Sigma-Aldrich, USA) at final concentration to propagate the first starter culture of SUTN9-2 adaptive strain. The bacteria culture was cultivated at 30˚C, 200 rpm until the optical density (OD) reached 0.2 at 600 nm before using as a next starter. Similarly, the microbial cells (1% (v/v)) was cultured in minimal medium broth supplemented with 10% (v/v) HM broth containing 1.0 mM ACC (final concentration) for creating a new propagation, and then was cultured at 30˚C, 200 rpm until the OD reached 0.2 at 600 nm. The higher ACC concentrations were including 1.5, 2.0, 2.5 and 3.0 mM similarly conducted after the microbial cells reached 0.2 at 600 nm of each ACC concentrations in parallel serial cultures (Figure 3.1). This improvement was stopped at 3.0 mM ACC (considered by value of ACC deaminase activity) and the bacterial strain was named SUTN9-2 (ACCDadap).

3.3.1.2 Plasmid construction and transformation

The full fragment of *acdRS* (1804 base pair) was amplified fromSUTN9-2 genome using acdRXbaI-f (5´TGCATCTCTAGAATCTCTCATGATG CGGATAGC-3´) and acdSBamHI-r (5´CTGGCAAGGATCCGTCCTCGCAAAACG ATTGCT-3´) primers. PCR condition was conducted by following 1 cycle of 95˚C, 3min for initial denaturation step; 35 cycles of 95˚C, 1 min; 55˚C, 1 min; 72˚C, 1 min and 1 cycle at 72˚C for 5 min (T100™ Thermal Cycler, Bio-Rad Laboratories, Inc.

USA). The fragment of *acdRS* was digested by *Xba*I and *Bam*HI and ligated into pMG103-nptII-cefo^r (Wongdee et al., 2016) at the complementary sites, to obtain recombinant pMG103::*acdRS*-S9-2::nptII-cefo^r plasmid (Figure 3.2). This recombinant plasmid was transferred into *Escherichia coli* (DH5α) and spread on LB containing 20 μg/ml cefotaxime for selection. Then, pMG103::*acdRS*-S9-2::nptII-cefo^r was extracted using alkaline lysis method (Bimboim and Doly, 1979) and directly transformed into competent cell of SUTN9-2. The competent cell was prepared by culturing SUTN9-2 in YEM broth at 30˚C with shaking at 200 rpm until the bacterial cells reached 0.4 at OD600, and the cell was collected by centrifugation at 5,000 rpm, 4˚C for 15 minutes. After discarding the medium, sterile deionized water was used for washing (3 times) and then washed twice with 10% glycerol. The competent cells were immediately stored at -80˚C or used freshly after the cells were resuspended in 10% glycerol. The transformation of the plasmid was conducted by electroporation (15 kv/cm, 100Ω, and 25 μF; BTX™ Gemini X2 Electroporation System). The colonies of SUTN9-2 containing pMG103::*acdRS*-S9-2::nptII-cefo^r were cultured on YM supplemented with 20 μg/ml cefotaxime. SUTN9-2 harboring pMG103::*acdRS*-S9-2::nptII-cefo^r plasmid is named as SUTN9-2:pMG103::*acdRS*.

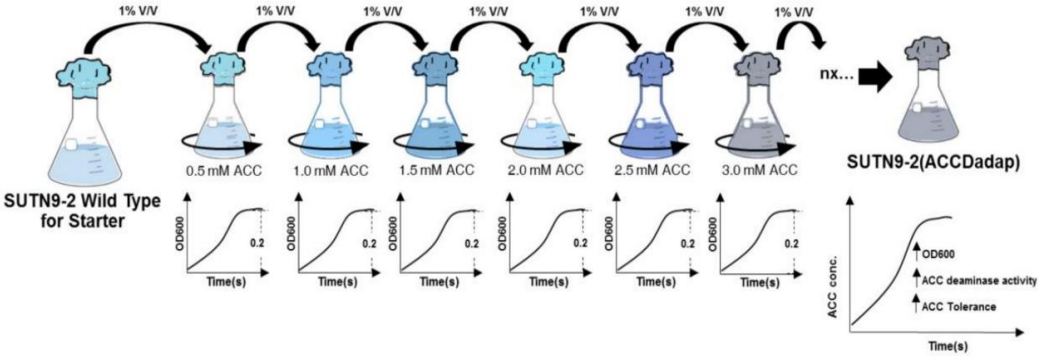

Figure 3.1 Improvement of enzyme ACC deaminase activity containing *Bradyrhizobium* sp. SUTN9-2. ACC deaminase in SUTN9-2 (WT) was improved by adaptive laboratory evolution (ALE). The approach was conducted based on the sequential serial passage under a compulsive condition of ACC at 0.5, 1.0, 1.5, 2.0, 2.5 until 3.0 mM. The investigations of optical density, ACC deaminase activity and ACC tolerance were used to indicate the improved ACC deaminase activity strain. The improved strain is named SUTN9-2 (ACCDadap).

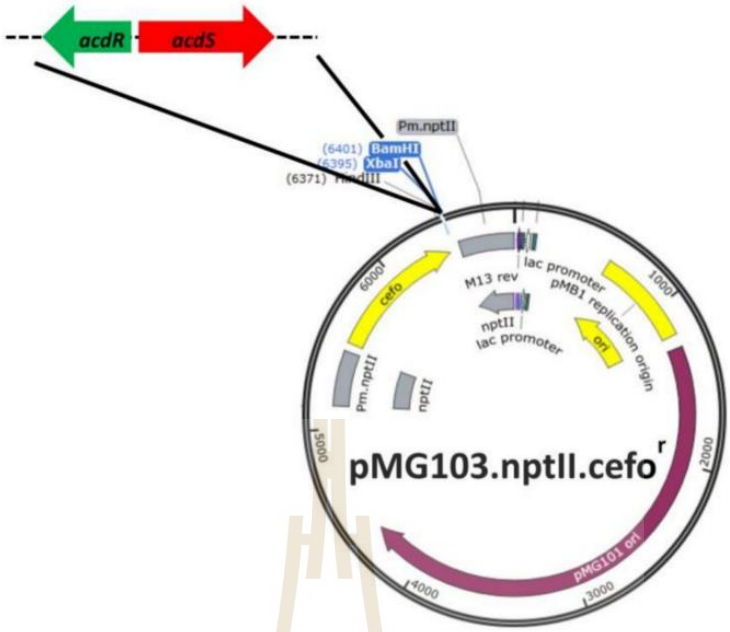

Figure 3.2 Physical map of vector containing *acdR* and *acdS* fragment was amplified from *Bradyrhizobium* sp. SUTN9-2 genome. The fragment gene was inserted between the cefotaxime resistant gene (cefo^r) and neomycin phosphotransferase II gene (nptII) of pMG103 vector.

3.3.2 Bacterial culture conditions and bacterial cell preparations

Bradyrhizobium sp. SUTN9-2 (WT), SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* were cultured in 100 ml HEPES-MES Salt medium (HM) (Cole and Elkan, 1973; Kanbe et al., 2007) broth at 30˚C, 200 rpm, with shaking for 7 d prior to use. The medium for growth of SUTN9-2:pMG103::*acdRS* strain was HM supplemented with 20 µg/ml cefotaxime. To induce ACC deaminase expression, the bacterial cells were transferred into minimal medium (Soedarjo et al., 1994; Tittabutr et al., 2008b) supplemented with 10% (v/v) HM and 3.0 mM ACC and then were incubated at 30˚C, 200 rpm for 24 hours (Siddikee et al., 2011).

3.3.3 ACC deaminase activity measurement

Bacterial cells were cultured in 15 ml HM broth at 30˚C, 200 rpm shaking for 7 days until they reached early stationary phase. The bacterial cells were collected by centrifugation at 5000 rpm, 25˚C for 15 minutes and washed twice with minimal medium. The bacterial cells were resuspended in 15 ml of minimal medium supplemented with 10% (v/v) HM containing 1 mM ACC, and then incubated at 30˚C, 200 rpm, for 40 hours. The enzyme activity was performed as described by Tittabutr et al., (2008b). The protein content was determined by Lowry's method (Lowry et al., 1951a).

3.3.4 Investigation of ACC tolerance ability

The bacterial cells (0.1% v/v) including SUTN9-2 (WT), SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* were cultured in 3 ml yeast extract-mannitol (YEM) (Somasegaran et al., 1985) broth containing ACC at a final concentration of 3.0 mM after normalized by optical density (OD600 nm) at 1.0. The bacterial cultures were cultivated at 30˚C without any shaking for 15 days. The viable cells were determined by viable plate count on YEM medium.

3.3.5 Plant material and inoculation of bacterial strains

Seeds of *Vigna radiata* (L.) R. Wilczek SUT1 were surface sterilized with 95% (v/v) ethanol for 10 second following 3% (v/v) sodium hypochlorite for 5 min and washed 5-10 times with sterilized distilled water. The seeds were soaked for overnight in the sterilized distilled water. The seeds were germinated on sterilized wet tissue paper for 2 days at room temperature. The seedlings (2 plants/jar) were grown in modified Leonard's jars which contained sterilized sand, and were watered with a nitrogen-free nutrient solution (Somasegaran et al., 1985). The seedlings were grown at 28±2˚C, 60%

relative humidity with 12 hours light/dark cycle under a white fluorescent light (approximately 300 μE m^{-2} S^{-1}). Before inoculation, the bacterial cells were washed twice with sterilized 0.03 M $MgSO_4$, and then 1 ml of each bacterial culture after adjustment to the same optical density (OD_{600} = 1.0) was inoculated into the two-day-old seedling of mung bean plants. Twelve days after inoculation (DAI), the nodules and plant biomasses of mung bean were dried at 70˚C for 48 hours and then recorded.

3.3.6 Water deficit and Rehydration conditions

Mung bean seedlings were grown under normal condition for two weeks after sowing, and water deficit situation was conducted by discarding nitrogen-free nutrient solution from Leonard's jars and was performed in the same normal condition at 28±2˚C, 60% relative humidity with 12 hours light/dark cycle under a white fluorescent light (approximately 300 μE m^{-2} S^{-1}). The mung bean plants which showed wilt symptom at 7 days after discarding water were harvested. Then, the ethylene emission from each plant was measured by a Gas chromatograph (GC) (Model 310, SRI Instruments Ltd., UK) equipped with a flame ionization detector (6' x 1/8" SS column; Valco Instruments Co. Inc) according to the method described by Tittabutr et al., (2013b). To determine the effect of bacteria containing ACC deaminase activity on plant growth after rehydration condition, mung bean seedlings were imposed into the water-deficit situation at initial planting in modified Leonard's jars. The progressive drying of sand was controlled at 10% of permanent wilting point (PWP) by adding 1 ml of nitrogen-free nutrient solution into sand to control the humidity of sand. After 12 DAI, the mung bean plants were rehydrated with nitrogen-free nutrient solution for twenty days in the same conditions as mentioned above and then harvested the recovered plants at 32 DAI.

3.3.7 Nitrogenase activity measurement

Detached root nodules of mung bean were incubated in 15 ml of rubber-capped plastic tubes and 5% (v/v) of air was withdrawn from the tubes and then replaced with pure acetylene. The samples were incubated at 25˚C for 1 hour for conversion of acetylene to ethylene. One ml of airspace was injected into the GC machine, which mentioned above, to determine ethylene gas. The nitrogenase activity was presented as nmol ethylene $hr.^{-1}$ mg^{-1} nodule dry weight (Somasegaran et al., 1985).

3.3.8 Investigation of nucleotide, annotated amino acid sequences and three-determination structure of ACC deaminase enzyme

The nucleic acid sequences of *acdR* and *acdS* in SUTN9-2 (ACCDadap) strain were amplified using PCR condition as mentioned above. The PCR product was ligated into the pTG19-T PCR cloning vector (Vivantis Technologies Sdn. Bhd.) to use for sequencing after transformation in the *E. coli* strain DH5α and plasmid extraction. The DNA sequences of *acdR* and *acdS* containing the vectors were performed by Macrogen Inc. (Korea). The sequences were analyzed by alignment using Multiple sequence alignment with hierarchical clustering (Corpet, 1988), a published piece of software (http://multalin.toulouse.inra.fr/multalin/), and were compared with nucleic acid- and amino acid sequences of SUTN9-2 (WT) strain. The protein structure of ACC deaminase in SUTN9-2 (ACCDadap) strain was predicted and created using Phyre^2 software (Ittisoponpisan et al., 2019; Kelley et al., 2015) and PyMOL Molecular Graphics System. Moreover, 3DLigandSite-Ligand binding site prediction server (Wass et al., 2010) was used to predict binding sites of ACC deaminase in SUTN9-2 (ACCDadap) strain compared with ACC deaminase in SUTN9-2 (WT), *Pseudomonas* sp. ACP and *P. putida* UW4 strains.

3.4 Results

3.4.1 ACC deaminase activity of SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* strains

SUTN9-2 wild type possessed ACC deaminase activity at 3.88 μmol of α-ketobutyrate hr^{-1} mg^{-1} protein while ACC deaminase activity of the improved strains increased 1.4-fold (5.58 μmol of α-ketobutyrate hr^{-1} mg^{-1} protein) in SUTN9-2 (ACCDadap) and significantly increased 8.9-fold (34.72 μmol of α-ketobutyrate hr^{-1} mg^{-1} protein) in SUTN9-2:pMG103::*acdRS* when compared with SUTN9-2 (WT) (Table 3.1). It was confirmed that the enzyme activity correlated with the ability of ACC tolerance, both the improved strains were observed based on the survival in high ACC concentration at 3.0 mM. The results showed a significant increase in the colony forming unit (CFU) of both improved strains up to 1.21-fold in SUTN9-2:pMG103::*acdRS* and 1.08-fold in SUTN9-2 (ACCDadap) when compared with SUTN9-2 (WT) after they were cultured for 15 days in YEM supplemented with 3.0 mM (Table 3.2).

Table 3.1 ACC deaminase activity of *Bradyrhizobium* sp. SUTN9-2 strain and its development strains, *Bradyrhizobium* sp. SUTN9-2 (Wild Type strain), SUTN9-2 (ACCDadap) (Adaptive strain) and SUTN9-2:pMG103::*acdRS* (Increase copy number of *acdRS*).

Bacterial strains	**ACC deaminase activity µmol of α-ketobutyrate h^{-1} mg^{-1} protein**
SUTN9-2	3.88±0.16b
SUTN9-2 (ACCDadap)	5.58±0.08b
SUTN9-2:pMG103::*acdRS*	34.72±1.09a

Significant at $P \leq 0.05$ is indicated by mean standard error bar (n = 3).

Table 3.2 The number of bacterial cells in YEM containing 3.0 mM ACC for 0 day and 15 days.

Bacterial strains	**Number of bacterial cells (log10 CFU ml^{-1})**		
	0 day	**15 days**	**-fold**
SUTN9-2:pMG103::*acdRS*	6.26±0.0364	9.11±0.0052a	1.21
SUTN9-2 (ACCDadap)	6.22±0.0310	8.14±0.0021b	1.08
SUTN9-2	6.17±0.0094	7.51±0.0195c	-

Data are average of three replicates ± standard error (S.E.); differing letters indicate significant differences at $P \leq 0.05$.

3.4.2 Effect of *Bradyrhizobium* sp. SUTN9-2 containing improved ACC deaminase activity on mung bean symbiosis

Since ethylene has been proved to be able to inhibit the nodule formation, especially in early process of infection, to prove this hypothesis. The mung bean plants were inoculated with both improved and WT strains. The results demonstrated that the nodule number of mung bean increased up to 1.4-fold and 1.7-fold with SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* strains, respectively, when compared with SUTN9-2 (WT) (Figure 3.3A). The nodule dry weight also increased in both of the improved strains up to 1.3-fold with SUTN9-2 (ACCDadap) and significantly increased up to 1.9-fold with SUTN9-2:pMG103::*acdRS* when compared with SUTN9-2 WT (Figure 3.3B). The results showed a significant increase in nitrogenase activity up to 1.5-fold with SUTN9-2 (ACCDadap) and 1.6-fold with SUTN9-2:pMG103::*acdRS* when compared with SUTN9-2 WT (Figure 3.3C). However, there were no statistically significant differences in the plant dry weight of mung bean plants inoculated with bacteria when compared with SUTN9-2 WT (Figure 3.3D).

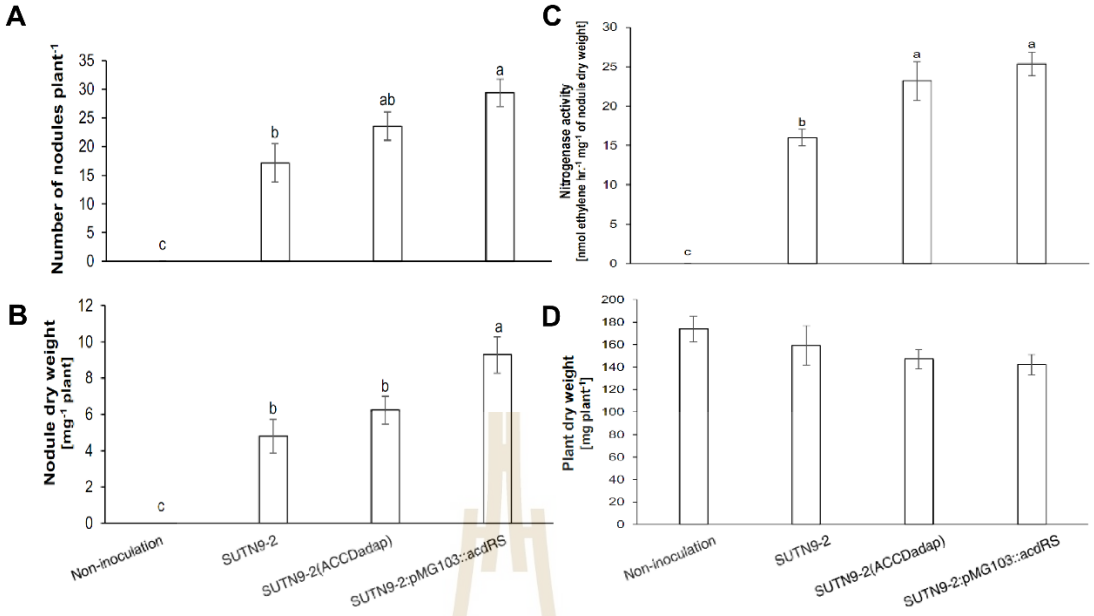

Figure 3.3 Effects of *Bradyrhizobium* sp. SUTN9-2 and its improved ACC deaminase activity strains on mung bean. The average of (**A**) number of nodule, (**B**) nodule dry weight, (**C**) nitrogenase activity and (**D**) plant dry weight of mung bean plant at 12 DAI inoculated with *Bradyrhizobium* sp. SUTN9-2 and its modified strains, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* compared with un-inoculation. Data are presented as the mean of seven replicates and the vertical bars indicate standard error. Significant differences were shown with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests.

3.4.3 Efficiency of the SUTN9-2 containing modified ACC deaminase strains against water deficit and rehydration conditions

In order to assess the efficiency of the improved ACC deaminase activity against water deficit stress on mung bean growth and its nitrogenase activity, watering was stopped at 12 DAI, and then plant morphology was observed. The results indicated that the nodule number inoculated with each bacterial strain differed in neither normal nor water deficit conditions (Figure 3.4A). However, the nodule dry weight, plant dry weight and nitrogen fixation were reduced by water deficit when compared with normal irrigation (Figure 3.4B, C and D). Under water deficit condition, there were no statistically significant differences in the plant dry weight but the improved strains trended to increase plant growth (nodule and plant dry weights) (Figure 3.4B and C) when compared with wild-type strain and non-inoculation. SUTN9-2:pMG103::*acdRS* could retain the higher nitrogenase activity than SUTN9-2 (ACCDadap) under water deficit condition, while SUTN9-2 (ACCDadap) could promote nitrogen fixation better than SUTN9-2:pMG103::*acdRS* in normal condition (Figure 3.4D). Under the water deficit condition, the high level of ethylene emission was detected, especially in that of non-inoculation, while all plants inoculated with bacteria significantly produced lower level of ethylene than non-inoculated plant. The lowest level of ethylene emission was detected in plant inoculated with SUTN9-2:pMG103::*acdRS*. There was no significant difference in ethylene emission among plants grown under normal condition and the level of ethylene emission was lower than that of plants grown under water deficit condition (Figure 3.4E). The influence of highly increase in ACC deaminase improvement in rhizobium SUTN9-2:pMG103::*acdRS* could significantly improve the plant dry weight when compared with other treatments after rehydration (Figure 3.5D)

which was consistent with the plant phenotypes (Figure 3.5E). However, there were no significant differences in nodule number, nodule dry weight, and nitrogen fixation among the three inoculated plants (Figure 3.5A-C).

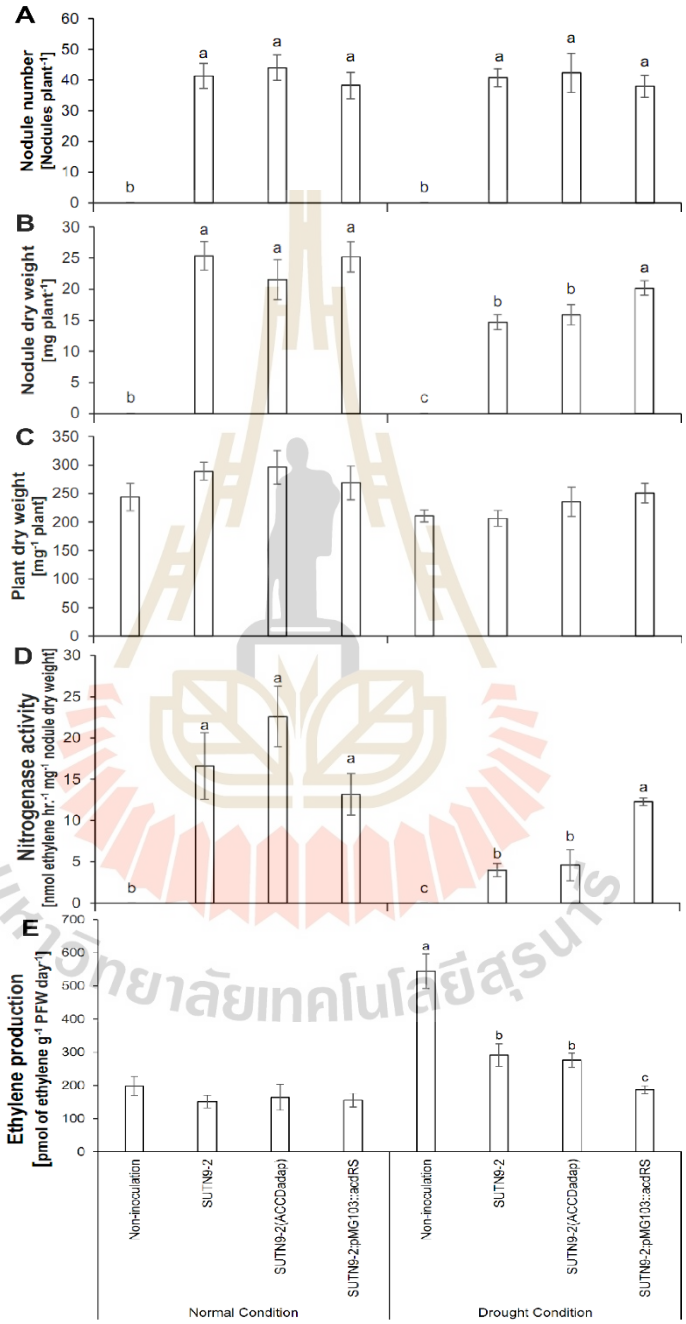

Figure 3.4 Effects of *Bradyrhizobium* sp. SUTN9-2 and its improved ACC deaminase activity strains on mung bean compared with non-inoculation under normal

and drought conditions. The average of (**A**) number of nodule, (**B**) nodule dry weight, (**C**) plant dry weight, (**D**) nitrogenase activity and (**E**) ethylene production of mung bean at 19 DAI inoculated with *Bradyrhizobium* sp. SUTN9-2 and its modified strains, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS*. Data are presented as the mean of seven replicates, and the vertical bars indicate standard error. Significant differences were shown with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests. The statistic comparison was performed separately in normal condition and drought condition.

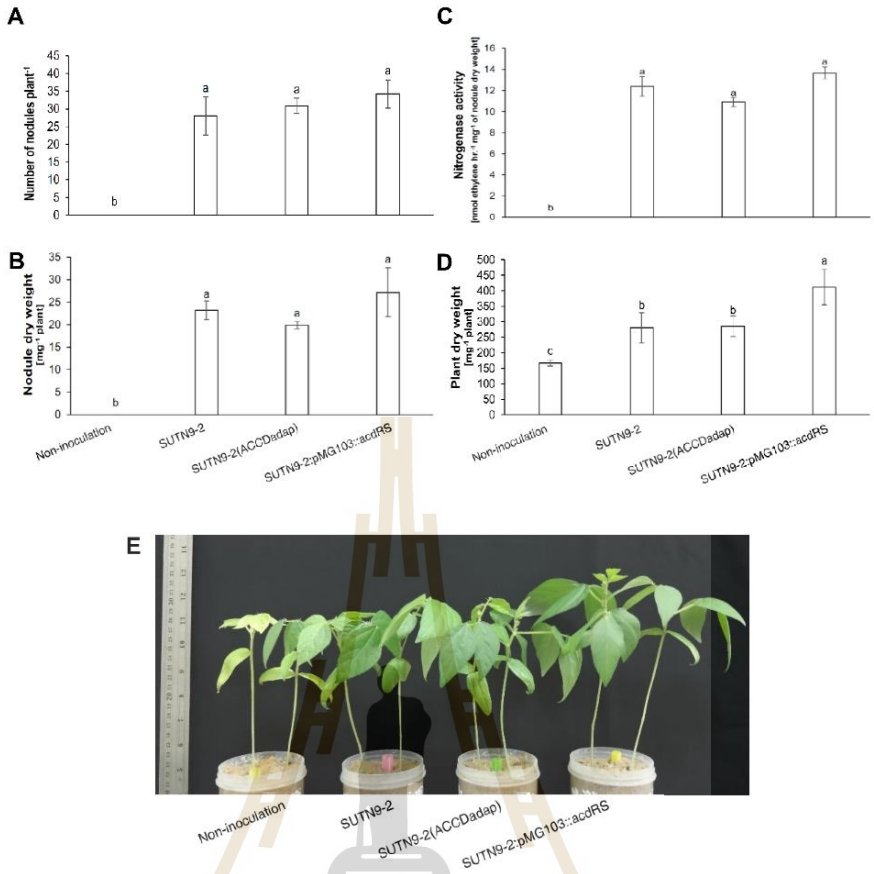

Figure 3.5 Effect of bacteria producing ACC deaminase activity and influence of improvement of ACC deaminase activity on mung bean after rehydration for twenty days. The average of (**A**) number of nodule, (**B**) nodule dry weight, (**C**) nitrogenase activity, (**D**) plant dry weight and (**E**) phenotypes of mung bean at 32 DAI inoculated with *Bradyrhizobium* sp. SUTN9-2 and its modified strains, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* compared with un-inoculation under normal- and deficit conditions. Data are presented as the mean of seven replicates and the vertical bars indicate standard error. Significant differences were shown with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests.

3.4.4 The influence of adaptive laboratory evolution (ALE) on ACC deaminase gene (*acdS*) and its protein structure

The sequence alignment of *acdS* gene in SUTN9-2 (ACCDadap) showed some changes of the nucleic acid sequence encoding ACC deaminase enzyme (Figure 3.6A). The guanine base (G), located at 419 region of *acdS* gene in SUTN9-2 WT was changed to be adenine base (A) in SUTN9-2 (ACCDadap). Another point is that cytosine base (C) located at 843 region in SUTN9-2 WT was changed to be thymine base (T) in SUTN9-2 (ACCDadap). The alteration of the nucleic acid sequence at 419 in the SUTN9-2 (ACCDadap) affected the encoding of amino acid residue. While the *acdR* gene in SUTN9-2 (ACCDadap) did not appear in the DNA base sequence change when compared with SUTN9-2 WT (data not shown). The results of protein residue alignment demonstrated that the amino acid residue encoding ACC deaminase in SUTN9-2 (ACCDadap) showed 99% identity when compared with SUTN9-2 WT. This result could be explained by changing of nucleic acid sequence from G to A at the 419 region of *acdS* gene. The modification affected the change of serine (Ser/S) protein residue in SUTN9-2 WT to an asparagine (Asn/N) residue in SUTN9-2 (ACCDadap) at the 140 region of the amino acid residue encoding ACC deaminase enzyme (Figure 3.6A and B). However, the change at the position 843 of nucleic acid sequence did not affect the encoding of amino acid residue of ACC deaminase enzyme in SUTN9-2 (ACCDadap). In addition, the changes of amino acid residue encoding ACC deaminase enzyme in SUTN9-2 (ACCDadap) caused the difference in the configuration of the enzyme. The results showed that the protein structure of ACC deaminase (AcdS protein) in SUTN9-2 (ACCDadap) contained alpha helix and beta strand structures at 43% and 15%, respectively while SUTN9-2 WT showed 42% and 16%, respectively in

alpha helix and beta strand structures. Moreover, the AcdS protein sequence in SUTN9-2 WT shared the sequence identity at 71% and 70% with *Pseudomonas* sp. ACP and *P. putida* UW4, respectively (Figure 3.7).

The changes of the amino acid residue as a result of nucleotide sequences change led to the enrichment modifications of the binding site of the protein structure of ACC deaminase enzyme in SUTN9-2 (ACCDadap). Altogether, the results of the predicted binding site showed that the SUTN9-2 (ACCDadap) possessed new predicted binding sites as a glycine positioned on the residue 161 (Gly^{161}), which was not observed in SUTN9-2 WT (Table 3.3). Therefore, it indicated that the change of the amino acid residue, asparagine, located at 140 (Asn^{140}) region, might have impact on the forming or folding of protein structure (Figure 3.6C).

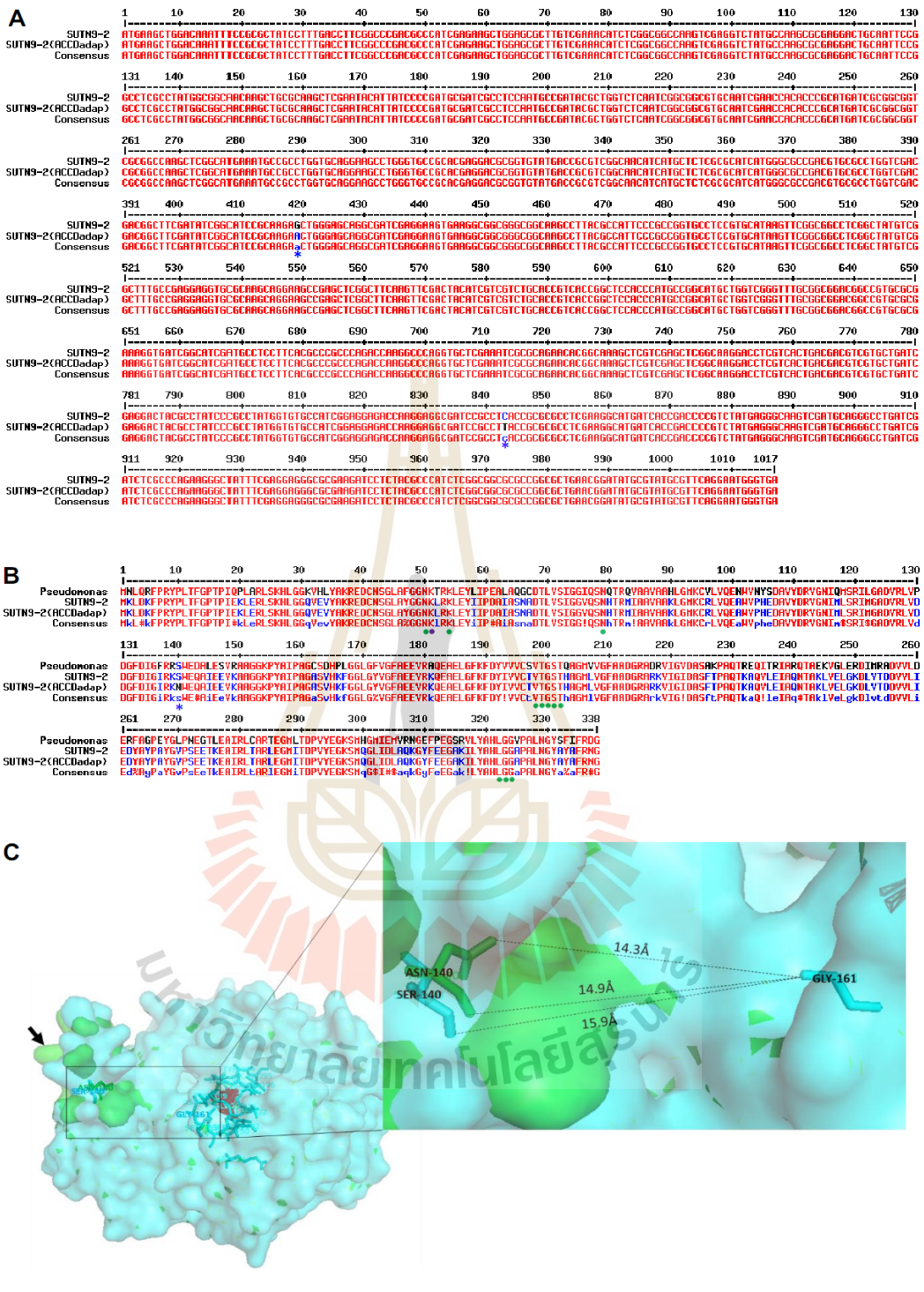

Figure 3.6 Sequence alignments of nucleotide and amino acid of *acdS* gene which encode ACC deaminase enzyme. (**A**) The alignment comparison of nucleotide sequences of *acdS* gene containing SUTN9-2 (ACCDadap) with

SUTN9-2 (WT). The blue asterisk (*) is a nucleotide sequence that changed from SUTN9-2 (WT) located at 419 and 843 positions. (**B**) The comparison alignment of amino acid sequences of ACC deaminase containing *Bradyrhizobium* sp. SUTN9-2 (ACCDadap) with SUTN9-2 (WT). The Lys^{51} residue is marked with a purple circle as a binding site of PLP. The green circles were the predicted binding site of ACC deaminase containing SUTN9-2 (ACCDadap) compared with its wild type strain *P. putida* UW4 and *Pseudomonas* sp. ACP. The blue asterisk (*) is a position of amino acid that changed from serine (Ser/S) in SUTN9-2 WT and *Pseudomonas* sp. strains to be an asparagine (Asn/N) in SUTN9-2 (ACCDadap) at the 140 residue region. (**C**) The superposition of three-dimensional structure of ACC deaminase producing SUTN9-2 WT (aqua color) and SUTN9-2 (ACCDadap) (green color). The arrow points the folding of ACC deaminase structure which changed from a native structure (aqua color) to a modified structure (green color) as a result of asparagine (Asn/N) which located at 140 region impacted on the folding of GLY^{161} amino acid residue, leading to a prediction as a binding site (green color).

Table 3.3 The predicted binding site of ACC deaminase containing SUTN9-2 (ACCDadap) strain compared with its wild type strain (SUTN9-2) and two other strains, *Pseudomonas* including *P. putida* UW4 and *Pseudomonas* sp. ACP. The data were obtained from Phyre[2] software and analyzed by 3DLigandSite-Ligand binding site prediction server.

***Bradyrhizobium* sp. SUTN9-2**		***Bradyrhizobium* sp. SUTN9-2 (ACCDadap)**		***P. putida* UW4 and *Pseudomonas* sp. ACP**	
Residue	Amino acid	Residue	Amino acid	Residue	Amino acid
50	ASN	50	ASN	50	ASN
51	LYS	51	LYS	51	LYS
54	LYS	54	LYS	54	LYS
79	ASN	79	ASN	79	ASN
-	-	*161	GLY	-	-
163	SER	163	SER	163	SER
196	CYS	196	CYS	196	CYS
197	THR	197	THR	197	THR
198	VAL	198	VAL	198	VAL
199	THR	199	THR	199	THR
200	GLY	200	GLY	200	GLY
201	SER	201	SER	201	SER
202	THR	202	THR	202	THR
294	TYR	294	TYR	294	TYR
295	GLU	295	GLU	295	GLU
322	LEU	322	LEU	322	LEU
323	GLY	323	GLY	323	GLY
324	GLY	324	GLY	324	GLY

* is a predicted binding site of AcdS protein which was only plus in the adaptive bacterial strain, SUTN9-2 (ACCDadap).

3.5 Discussion

Previous studies reported that bacteria containing ACC deaminase were divided into two general types: a symbiotic relationship and a free-living microorganism. The symbiotic bacteria showed ACC deaminase activity lower than free-living microorganisms (Glick, 2005a; Glick, 2014a). SUTN9-2 possessed approximately ACC deaminase activity at 3.48 μmol of α-ketobutyrate h^{-1} mg^{-1} protein, which is lower than *P. putida* UW4 (20.48 μmol of α-ketobutyrate h^{-1} mg^{-1} protein) acting as a free-living bacterial strain (Ma et al., 2003a). This study presented the approaches for improving ACC deaminase activity by ALE strategy (SUTN9-2 (ACCDadap) strain) compared with a genetically engineered SUTN9-2 strain overproducing ACC deaminase (SUTN9-2:pMG103::*acdRS* strain). The presence of ACC deaminase overexpression mutation strain showed ACC deaminase activity as 34.72 μmol of α-ketobutyrate h^{-1} mg^{-1} protein. So, it was suggested that the overexpression mutation strain may contain 8 to 10 copies of its own pMG103::*acdRS* plasmid in bacterial cells, while SUTN9-2 (ACCDadap) showed 5.58 μmol of α-ketobutyrate h^{-1} mg^{-1} protein. Under continuous evolution, SUTN9-2 (ACCDadap) strain was accumulated and selected at high ACC concentration (3 mM) as a specified growth condition, leading to improvements of bacterial growth and ACC tolerance. This phenomenon based on a life cycle of bacteria can be explained that the prolonged periods in stationary phase or starvation period are a duration of mutant selection with increasing the fitness express growth advantage in stationary phase (GASP), and then they can grow and scavenge the parent cells, ultimately as a main population (Bacun-Druzina et al., 2011). The ACC deaminase activity of the improved strains, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS*, was not only confirmed by measurement of α- ketobutyrate (Table

3.1) but the abilities of ACC tolerance and/or utilization of ACC were also used to guarantee the enzymatic improvement. Since bacteria producing ACC deaminase enzyme can use ACC as a nitrogen (N) source (Glick et al., 1998a; Vanderstraeten and Van Der Straeten, 2017b), this can confirm the improved enzyme activities. The ACC deaminase improved bacterial strains could grow better than SUTN9-2 WT, which were implied by the increment of their cells in a high concentration of ACC during their growth in the YEM medium which were less affected by ACC. Therefore, it could be confirmed that the improved methods not only improved the enzymatic activity of ACC deaminase but also enhanced the ACC tolerance more than wild type strain which can be indicated by number of bacterial cells (Table 3.2 and Table 3.1). High concentration of ACC is toxic to bacterial cell. However, it could be hypothesized that the increased ACC deaminase activity would increase the efficiency of cell to detoxify high ACC concentration. The preliminary results showed that the growth of SUTN9-2 WT was limited by high ACC concentration (3 mM ACC), while ACC deaminase activity improved strains could tolerate to high ACC concentration more than SUTN9-2 WT as indicated by viable cell number (Table 3.2).

The ACC deaminase activity and its levels of activity or level of expression played an important role in nodulation process or symbiosis relationship, resulting in the symbiont being able to overcome a point of the ethylene level which interferes in the infection, leading to increasing the number of nodules (Figure 3.2A) and nodule dry weight (Figure 3.2B) as well as nitrogenase fixation (Figure 3.2C). The direct evidences for confirmation in the influence of ACC deaminase activity on nodulation process were exposed by the results of the deletion mutant of gene encoding enzyme ACC deaminase (*acdS*) as well as introduction of exogenous *acdS* gene. They found that the

defective mutant strain in ACC deaminase reduced its nodulation while the nodulation ability increased in rhizobia containing an exogenous ACC deaminase gene when compared with its wild type strain that did not show ACC deaminase activity or presented a lower activity (Ma et al., 2004; Ma et al., 2003a). However, in the aspect of plant growth promotion, the mung bean plants inoculated with bacteria were not improved in plant biomass when compared with non-inoculation (Figure 3.2D). Since the plants in this experiment were 12 DAI, it was possible that the nitrogen for plant growth might be covered by nutrients in seed and resulting in showing no response of bacterial inoculation on the plant biomass.

Under normal condition, the increase of ACC deaminase activity contained in bacteria did not alter plant growth and symbiosis (Figure 3.3). These results were similar to the experiment of Ma et al., (2003a), the plant inoculation with *R. leguminosarum* bv. viciae (high-expression mutation of ACC deaminase activity) did not show a significant increase in nodule number, shoot dry weight, and nitrogenase activity when compared to wild type strain when grew under normal condition. Interestingly, the increase of ACC deaminase activity acted against plant stress tolerance in its host plant, which was demonstrated by that SUTN9-2:pMG103::*acdRS* strain could increase nodule dry weight and nitrogenase activity efficiently under stressed condition (Figure 3.3B and D). However, nodule number (Figure 3.3A) and plant dry weight (Figure 3.3C) at 19 DAI were not promoted by the increase of ACC deaminase activity when compared with wild type strain under water deficit conditions.

The relationships between nodule senescence deriving from some other factors include the large amounts of reactive oxygen species (ROS) and the decrease of antioxidants such as ascorbate and glutathione in the nodule which affected the

accumulation of ROS (Puppo et al., 2005). Plant hormones can also regulate the nodule senescence including abscisic acid and ethylene through the carbon metabolism. The ratio of sugar/nitrogen changing in nodule is a signal to activate the abscisic acid which often occurs in plants. The rate of change was supported by a lower carbon together with high nitrogen metabolite availability in nodule (Tittabutr et al., 2015). Moreover, the change can induce the nodule senescence resulting in lowering nitrogen fixation in mung bean inoculated with SUTN9-2:pMG103::*acdRS* at 19 DAI under normal condition. Usually, the nodule senescent by environmental stress occurs faster than developmental senescence since the ethylene, which is a senescence hormone was highly induced under environmental stress condition (Puppo et al., 2005; Tittabutr et al., 2015). In previous studies, they found that water deficit situation caused the reduction of carbon supply (malate). Thus, the carbon supply could not migrate into bacteroid because of photosynthesis reduction as a result of stomatal closure, leading to CO_2 availability and transpiration reductions (González et al., 1998; Larrainzar et al., 2009). These studies suggested that insufficient supply of carbon metabolism directly affected nitrogen fixation and could also induce ethylene biosynthesis (Gazzarrini and McCourt, 2001). Berrabah et al., (2018) demonstrated that exogenous ethylene (25 to 250,000 ppm) could reduce nitrogen fixation of *M. truncatula* and *L. japonicas* after incubation for 3 days but could not affect the *sickle* plant. They explained that ethylene stimulated plant defenses, resulting in necrotic areas in the central part of root nodules. In our study, the nitrogenase activity of plant inoculated with bacteria showed the lowering of nitrogenase activity in water deficit condition except mung bean inoculated with SUTN9-2:pMG103::*acdRS*. Since SUTN9-2:pMG103::*acdRS* produced a high ACC deaminase activity, leading to the moderation of nodule senescence by decreasing

the substrate of ethylene synthesis, plant inoculated with SUTN9-2:pMG103::*acdRS* showed high nitrogenase activity in water deficit situation (Figure 3.3D and E).

Moreover, SUTN9-2:pMG103::*acdRS* led to the improvement of the plant dry weight after rehydration (Figure 3.4D). Staudinger et al., (2016) postulated that *M. truncatula* inoculated with *S. medicae* WSM419 containing ACC deaminase activity showed the reduction of proteins involved in ethylene synthesis such as ACC oxidase, which converts ACC to ethylene in ethylene biosynthesis pathway, when compared with non-inoculation under water deficit condition. The reduction of ethylene production let to a delayed leaf senescence, resulting in the lowering of leaf abscission. Furthermore, they also suggested that the bacterial inoculated plant was provided the carbon source in forms of sugar rather than starch. So, the rehydration of plant inoculated with bacteria was recovered faster than non-inoculation, because the utilization of carbon source as sugar form was easier than starch form. Moreover, the accumulation of sugar form still persisted after rehydration which did not happen in the non-inoculation. Therefore, the nodulation of rhizobacteria could improve the recovery by controlling the plant metabolism such as plant hormones, the proportion of anion/cation, C and N stabilization as well as the ability of change protein. Additionally, Irigoyen et al., (1992a) demonstrated that the violence of water deficit levels affected the levels of ethylene emission, and the violence was often accompanied with lipid peroxidation and hydrogen peroxide (H_2O_2) that correlated with the trend of ethylene production in alfalfa. Therefore, it is possible that bacteria having ACC deaminase activity can decrease H_2O_2 content as a cause of nodule senescence. These reasons supported that the overproduction of ACC deaminase activity in SUTN9-2:pMG103::*acdRS* could retain the nitrogenase activity in drought stress condition.

Hence, we suggested that the improvement of ACC deaminase activity using the genetic engineering was more efficient than the adaptive evolution for enhancing nodulation at seedling stage and alleviation in drought stress condition. However, adaptive evolution strain has tendency to promote the nitrogen fixation under normal condition.

The ALE approach led to the increase of ACC deaminase activity by changing the DNA sequence of *acdS* gene but it did not affect the nucleotide sequence of *acdR* gene. Based on previous studies, AcdR is a transcriptional regulatory protein of *acdS* to synthesize the ACC deaminase enzyme when it is induced by ACC (Glick et al., 2007; Grichko and Glick, 2000b; Leonard et al., 2001; Li and Glick, 2001). Therefore, under ALE approach which the mutant was selected based on increasing the fitness express growth with ACC, the AcdR protein needed to be stable. Since the stability of *acdR* gene led to the existence of ACC deaminase expression, the nucleotide sequence of *acdR* gene present in SUTN9-2 (ACCDadap) should not be modified. Thus, the change of nucleotide sequence of *acdS* may has a greater effect on improvement of ACC deaminase activity than *acdR* in this study. Moreover, the protein three-dimensional structure was used as an evidence to confirm the hypothesis which can be explained by new distance (14.3 Å and 14.9 Å) of amino acid residues between Asn^{140} (green color) and Gly^{161} (aqua color) containing SUTN9-2 (ACCDadap) while SUTN9-2 WT showed the further distance (15.9 Å) between Ser^{140} (aqua color) and Gly^{161} (aqua color) (Figure 3.5C). In addition, an asparagine (Asn) possesses higher molecular weight (residue mass) than a serine (Ser), which locate at 140 region. Therefore, we suggested that the amino acid residue could impact the forming or folding of protein structure (Gly^{161}). Moreover, the three-dimensional structure also showed that the predicted binding site as Gly^{161} located at the small domain fold of ACC deaminase

which lies on the front side of the pyridine in the loop of binding site, leading to benefit for binding ACC resulted in improvement of ACC deaminase activity (Karthikeyan et al., 2004; Yao et al., 2000a). The results of three-dimensional alignment showed that the protein structures are different, when they are compared with protein residues of bacteria (Figure 3.7A). The difference of the protein structures appeared by colorful lines, which did not superimpose in comparison with ACC deaminase structure of yeast (Figure 3.7B). Therefore, the higher ACC deaminase activity of SUTN9-2 (ACCDadap) was an effect of the changes in the enrichment of protein structure. The changes of the enrichment also affected the electron density of the binding site of the ACC deaminase enzyme. Hence, it was suggested that the modification of the protein residues at Ser^{140} in original strain to be Asn^{140} in the improved strain affected the protein residue at Gly^{161} around active site, resulting in changing the inner diameter size of the cavity of the enzyme. Therefore, the changing of the cavity of the enzyme led to additional binding site in the SUTN9-2 (ACCDadap) strain (Table 3.3). The predicted binding site data showed conservation in the amino acid residues containing *Bradyrhizobium* sp. and *Pseudomonas* sp., and it was also indicated that the amino acid residues were important for enzymatic activity. Moreover, based on the alignments of three-dimensional structure, it could be accepted that the small domain of ACC deaminase containing bacteria (amino acid residues at 58 to 169) might be a region of the diversity or an establishment of the enzymatic activity of ACC deaminase producing microorganism (Jin et al., 2016). Yao et al., (2000a) proposed that amino acid residues at 55 to 91, located on the small domain (helix 6) in the extra loop as a binding site of ACC deaminase enzyme, were fixed after growth in medium broth supplemented with ACC for several selection rounds. However, this hypothesis should be clarified by the

site-directed mutation experiments in the further study. Since SUTN9-2 was isolated from *Aeschynomene americana* (Noisangiam et al., 2012; Piromyou et al., 2015b), and it was confirmed as an endophyte in rice tissues by Piromyou et al., (2017), to develop SUTN9-2 strains as an inoculum using in the crop rotation between mung bean and rice, the bacterial strains should be evaluated in the aspect of endophyte in rice plant.

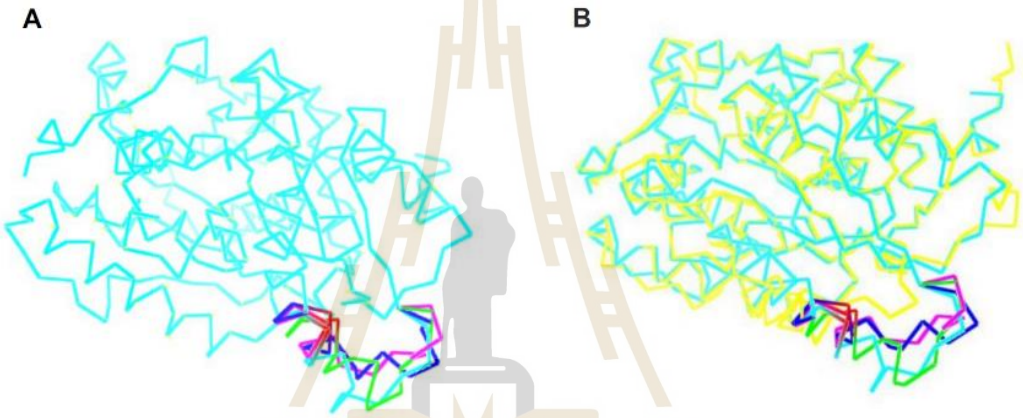

Figure 3.7 The three-dimensional structures of ACC deaminase enzyme display in different colors. (**A**) The superposition of ACC deaminase structure of SUTN9-2 WT (aqua color) and SUTN9-2 (ACCDadap) (green color) compared with the other bacteria, *P. putida* UW4 (blue color) and *Pseudomonas* sp. ACP (pink color) in ribbon model. (**B**) The superposition of ACC deaminase structure of SUTN9-2 WT (aqua color) and SUTN9-2 (ACCDadap) (green color), *P. putida* UW4 (blue color) and *Pseudomonas* sp. ACP (pink color) compared with yeast, *Hansenula saturnus* (yellow color).

Figure 3.8 The secondary protein structures of ACC deaminase containing *Bradyrhizobium* sp. SUTN9-2 (ACCDadap) compared with SUTN9-2 WT and two other strains of *Pseudomonas*, including *Pseudomonas putida* UW4 and *Pseudomonas* sp. ACP. The red circles are the predicted binding site of amino acid residues of ACC deaminase that were predicted by Phyre[2] software and analyzed by 3DLigandSite-Ligand binding site prediction server. The arrow is a position of amino acid residue that changed from serine (Ser/S) in SUTN9-2 wild type to be an asparagine (Asn/N) in SUTN9-2 (ACCDadap) at the 140 residue region.

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CHAPTER IV

APPLICATION OF RICE ENDOPHYTIC *Bradyrhizobium* STRAIN SUTN9-2 CONTAINING MODIFIED ACC DEAMINASE WITH RICE PLANT UNDER WATER DEFICIT CONDITION

4.1 Introduction

Drought is a major limitation in rice production (Farooq et al. 2009). Generally, under water deficit, plants suffer together with the battle against the limited water availability lead to the changes in morphological, physiological, biochemical, molecular responses and ultimately yield reduction (Blum 1996; Pandey and Shukla 2015; Ullah et al. 2017). Briefly, plants respond to water deficiency in soil by root perception before sending corresponding signals to other parts of the plants, particularly leaves. To decrease water loss, plants required stomata closure. However, this phenomenon is a cause of the photosynthesis reduction of photosynthesis, increasing of or stopping that leads to photorespiration and the accumulation of reactive oxygen species (ROS), leading to the cell membrane damage (lipid peroxidation) (Ahmad 2016). Furthermore, plant hormones also play an important role as signaling molecules in plant physiological responses against the unfavorable conditions (Abeles et al. 2012; Ahmad and Prasad 2011; Bleecker and Kende 2000; Dubois et al. 2018). Ethylene, a plant hormone, plays a vital role in plant survival when plants encounter with unfavorable

hormone, plays a vital role in plant survival when plants encounter with unfavorable conditions to stimulate adaptive mechanisms (Druege 2006; Wang et al.2002). However, the hormone can present negative effects when it is a high concentration. The ethylene production in plants elevates in a few hours after exposure to unfavorable environmental conditions, as a small amount that initiates plant defense mechanisms (Druege 2006; Wang et al. 2002). The evolution of ethylene production in plants can elevates in a few hours after plants exposure to the unfavorable environmental conditions, which is a small peak and as a small amount that initiates plant defense mechanisms (Glick et al. 2007). Nevertheless, the significant amount of ethylene production can be decreased by controlling its substrate, 1-Aminocyclopropane-1-carboxylic acid (ACC) which is considered as a rate-limiting in ethylene production (Apelbaum and Yang 1981; Glick et al. 2007; Honma and Shimomura 1978; Yu et al. 1979). Microorganism containing ACC deaminase catalyzes a cleavage and deamination of ACC to form ammonia and α-ketobutyrate (Glick et al. 1998; Honma and Shimomura 1978), which are utilized as a nitrogen and a carbon source of the microorganism, respectively (Van de Poel and Van Der Straeten 2014; Vanderstraeten and Van Der Straeten 2017), these microorganisms have potential to reduce ACC content in plant cell and reduce stress ethylene. ACC deaminase enzyme encoded from *acdS* gene and it is controlled by leucine-responsive regulatory (Lrp) protein, encoded from *acdR* gene and this property is classified as a trait of plant growth promoting bacteria (Glick et al. 2007; Grichko and Glick 2000; Li and Glick 2001).

Rice (*Oryza sativa* L.) is the staple food and a major economic crop of many Asian countries, including Thailand. Around 5.6 million hectares in the northeastern region of Thailand is the main rice production area, and approximately 74.6 percent of

this region are outside of the irrigation area (Rice Department 2016). Based on these data, it was implied that half of rice productivity was cultivated in the rain-fed farming system. However, the productivity is generally related to the amount and distribution of rainfall, in which this is a risky situation according to the climate change, especially, heat and drought conditions. Therefore, it is interesting to apply the strategy of the bacteria-containing ACC deaminase activity associated with rice plants which often encounter in the rain delay for a long time or the distribution of rice growing area outside the irrigation areas in the Northeast of Thailand. Previous studies demonstrated that endophytic bacteria-containing ACC deaminase activity was a remarkable key to enhance plant tolerances against stresses or can alleviate deteriorate effects when their host expose to environmental stress (Hardoim et al. 2008; Santoyo et al. 2016). In maize seedling under drought stress conditions, ACC deaminase producing *Burkholderia phytofirmans* PsJN and *Enterobacter* sp. FD17 promoted biomass, photosynthesis, and photochemical efficiency of PSII when compared with non-inoculation (Naveed et al. 2014a; Naveed et al. 2014b; Sun et al. 2009). Furthermore, both endophytes also improved the leaf relative water content and decreased the membrane permeability of the maize seedlings when compared with non-inoculation.

However, those studies were more focused on salinity stress, while the water deficit or drought experiments were less investigated in rice production. Recently, rice endophytic *Bradyrhizobium* sp. SUTN9-2 was used to investigate the plant growth promotion in rice seedling, they documented that SUTN9-2 (wild type strain) was a bio-inoculant to promote rice growth at seedling stage (7 and 14 days after inoculation) in both N-free and with NH_4NO_3 under *in vivo* condition (Greetatorn et al. 2019). In addition, our previous results on SUTN9-2, which possessed the improved ACC

deaminase activity enhanced symbiotic interaction, plant biomass and drought tolerance in mung bean (Sarapat et al. 2020). We hypothesized that the improved ACC deaminase activity strains, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* containing high ACC deaminase activity with 1.4 and 8.9 folds than wild type, respectively, may provide some beneficial effects to the rice plants under water deficit conditions. Both strains were improved their ACC deaminase activities by different strategies, including adaptive laboratory evolution (ALE) and genetically modified organism (GMO) approaches (Sarapat et al. 2020). In this study, we examined the potential of using rice endophytes to promote rice growth under water deficit as well as well-watered conditions under laboratory, greenhouse, and field conditions.

4.2 Objective

To investigate the effects of the improved ACC deaminase activity in *Bradyrhizobium* sp. SUTN9-2 on rice plants under normal and water deficit conditions

4.2.1 Specific objectives

4.2.1.1 To examine the effects of the improved ACC deaminase activity strains in the aspect of PGPB trait on rice plants

4.2.1.2 To improve the efficiency and effectiveness of the improved ACC deaminase activity strains on rice plants in order to cope with the water deficit stress

4.3 Materials and methods

4.3.1 Bacterial strains and inoculant preparations

The *Bradyrhizobium* sp. SUTN9-2 (Piromyou et al., 2015b) and its ACC deaminase improvement strains from the previous study, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* (Sarapat et al. 2020), were grown in 100 ml HEPES-MES Salt medium (HM) (Cole and Elkan, 1973; Kanbe et al., 2007) broth at 30˚C, 200 rpm, with shaking for 7 days prior to use. The medium for growth of SUTN9-2:pMG103::*acdRS* strain was HM and supplemented with 20 μg/ml cefotaxime. After 7 days, the bacterial culture was washed twice with minimal medium by centrifugation at 5,000 rpm, 25˚C for 15 minutes, and then induced with 3.0 mM ACC in minimal media, which contained 10% (v/v) of HM medium, for 24 hours at 30˚C, 200 rpm (Siddikee et al., 2011). Finally, the induced bacterial culture was collected and washed twice with 0.03 M $MgSO_4$ at 5,000 rpm, 25˚C for 15 minutes. Before inoculation, the bacterial suspension was adjusted to the same optical density (OD_{600}nm = 1.0).

4.3.2 Plant materials and growth conditions

Rice plant (*Oryza sativa* L. var.*indica* cv. Pathumthani 1) was obtained from Lopburi Rice Seed Center, Thailand. The seeds were surface sterilized with 95% (v/v) ethanol for 5 min followed by 3% (v/v) sodium hypochlorite for 10 min after washing 2-3 times of a sterile distilled water and were washed again for 5-10 times followed by soaking in the sterile distilled water for 48 hours. The seed were germinated on sterilized wet germination paper for 2 days at room temperature. The seedlings were soaked in each bacterial suspension (SUTN9-2 wild type, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS*), while a control treatment (un-inoculated plant) was soaked in 0.03 M $MgSO_4$ before planting. In laboratory experiments, the inoculation

and un-inoculation seedling (3 plants per tube) were transferred and grown in pot (8 cm of diameter and 17 cm of depth) which contained a sterilization of soil and sand mixture (1:1, v/v) as planting material under controlling condition of a white fluorescent light (approximately 300 μE m^{-2} S^{-1}) at 28±2˚C at 60% relative humidity, 12 hours light/dark cycle. The physical and chemical parameters of soil for mixing were summarized in Table 4.1. The Hoagland nutrient solution (Mae and Ohira 1981) was used for watering. While, greenhouse condition was performed at Suranaree University of Technology Farm (SUT farm; latitude 14˚88´83.49´´N, longitude 102˚00´42.47´´), Nakhon Ratchasima, Thailand. The rice seedlings (10 plants per pot) were planted in plastic pots (30 cm of diameter and 23 cm of depth) containing 8 kg of the sterilized soil. The pots were placed in the completely randomized design in factorial arrangement with five replicate pots. The soil in this experiment was the same collection with the mixed soil from laboratory experiment. The rice plants were grow at 23± 2˚C day/19± 2˚C night, respectively; 82% average relative humidity under natural light. Rice seedlings were irrigated with the same volume of tap water. Finally, the rice seedlings of each bacterial strain inoculation were tested in the field condition of the SUT farm (latitude 14˚87´79.71´´, longitude 102˚00´69.34´´) during dry season (November to February). The randomized complete block design (3 replications) was used in this experiment, and the same soil was used in this condition. The size of the experimental plot was 100×300 cm, which contained 3 rows and each plant spaced 20 cm in the same row while spaced 25 cm in beside rows.

Table 4.1 Soil physical and chemical properties.

Soil property	**Soil**
Texture	Sandy loam
Electrical conductivity (ds/m, 1:5 (soil:water))	0.21
pH (1:1)	7.5
OM (%)	0.78±0.09
Available P (ppm)	26.37±2.90
K (ppm)	74.00±8.64
Ca (ppm)	2,773.37±640
Na (ppm)	31.00±2.08
Field capacity (%)	14.21±0.75
Permanent wilting point (%)	9.16±0.17
Field density test	0.36±0.01

4.3.3 Water deficit and recovery treatments

Fourteen days after inoculation (DAI) of rice seedlings were exposed to water deficit stress. In laboratory condition, supplementation of Hoagland nutrient solution was stopped until the seedlings showed leaf wilting as well as in greenhouse condition, the water in pot was removed. While, water stress treatment under the field condition was performed at tillering stage by withholding tap water. The leaf wilting or leaf rolling of non-inoculation was used as a sign to obverse as the water deficit. The watering was conducted after the all of treatments displayed the leaf wilting. The Hoagland nutrient solution was supplied to rice seedling in laboratory experiments,

while greenhouse and field experiments were recovered with water. Moreover, the rice plants in field conditions after recovery were further grown in the well- watered condition until the maturity stage.

4.3.4 Determination of ethylene emission

The bacterial inoculation and un-inoculation seedling (3 plants per tube) were transferred and grew into a glass test tube (15×150 mm) as described by Greetatorn et al., (2019). After 14 DAI, the glass test tubes containing 3 rice seedlings were induced water stress by discarding the Hoagland nutrient solution and replaced with the nutrient solution containing polyethylene glycol (PEG8000) (Santa Cruz Biotechnology, Inc., USA). Tubes were tightly closed with a rubber septum prior to seal with parafilm. After incubation for 5 days at 25˚C under laboratory condition as described above, the ethylene productions were determined by method described by Fukao et al., (2006). Briefly, the 5 ml of headspace air of each tube was injected into a Gas chromatograph (GC) (Model 310, SRI Instruments Ltd., UK) equipped with a flame ionization detector (6' x 1/8" SS column; Valco Instruments Co. Inc). The PEG solution was provided at -5.11bars which the osmotic pressure (OP) of PEG 8000 solution was calculated using the following equation of (Michel, 1983) : $OP = 1.29\times C^2\times T - 140\times C^2 - 4.0\times C$ (C, PEG concentration; T, temperature). In greenhouse condition, after the rice seedlings were exposed to water deficit stress for 20 days and showed the visible wilting. The seedlings were incubated in an 8×12 $inch^2$ vacuum plastic bag (1 plant per bag), and then were vacuum-packed by a vacuum packing machine. The vacuum sample bags were incubated for 3 days under light as explained in PEG stress induction. The ethylene emission was detached by injecting 5 ml of airspace of each sample bag into a Gas chromatograph (GC) (Tittabutr et al., 2013a).

The ethylene emission of each sample was calculated per one milliliter and presented as pmol mg^{-1} fresh weight day^{-1} compared with a standard curve generated with pure ethylene. Five replicates were served for each treatment.

4.3.5 Determination of malondialdehyde (MDA) content

The content of malondialdehyde was determined using the thiobarbituric acid reaction. Briefly, the rice samples (14 DAI) were induced water stress by PEG for 5 days (19 DAI) according to ethylene induction condition as mentioned above. The whole leaves of rice seedling (25 mg) were grounded by mortar and liquid nitrogen with a pestle until the plant sample as a fine powder. Continuously, the powder was transferred into a 15 ml tube for mixing with 5 ml of 50 mM sodium phosphate buffer (pH 7.5) and then was centrifuged at 15,000 rpm for 15 min. The supernatant (1ml) was collected to react with 4 ml of 20% Trichloroacetic acid (TCA) containing 0.5% Thiobarbituric acid (TBA). The reaction mixture was incubated at 95˚C for 30 min and the reaction was stopped by cooling in an ice bath, and then was centrifuged at 10,000 rpm for 10 min (Du and Bramlage 1992; Hodges et al. 1999). MDA content was recorded in 532 and 600 nm (nonspecific turbidity) and was calculated comparing with a standard curve generated with pure MDA (Sigma- Aldrich, USA). The MDA concentration was expressed by following a formula:

$$\text{MDA (nmol mg}^{-1}\text{ protein)} = \frac{OD_{532} - OD_{600}}{\text{mg plant dry weigh}}$$

Five replicates were used for each treatment. The protein content was determined by following Lowry's method (Lowry et al., 1951b).

4.3.6 Leaf relative water content (RWC)

Leaf RWC measurement of rice seedling was performed after rice seedlings (14 DAI) exposed to water deficit for 5 days (19 DAI). Their leaves were cut

by a third of the leaf (10 cm of length) and measured the fresh weight before the leaf seedlings were soaked in a petri dish containing double-distilled water at 4˚C for 24 hours in darkness. The soaked leaves were measured as a fully turgid weight while dry weight was determined after drying the leaf plants at 70˚C for 72 hours (Naveed et al. 2014a). These used for preparing rice samples of the well-watered condition. Five replicates were served for each treatment, and RWC was calculated according to Teulat et al. (2003) as described:

$$\text{RWC (\%)} = \left[\frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fully turgid weight} - \text{Dry weight}}\right] \times 100$$

4.3.7 Chlorophyll a, b and carotenoid content in leaf

Measurement of photosynthetic pigment was performed after rice seedlings (14 DAI) exposed to water deficit for 5 days (19 DAI) in stress samples. The whole leaf rice of both well-watered and water stress conditions (three replicates) were used for measuring pigment content (Lichthenthale 1987). Briefly, leaf samples were lyophilized with a freeze drier, and then grounded in a mortar containing liquid nitrogen with a pestle. The fine powder was transferred into test tube supplemented with 96% (v/v) ethanol followed with vortex. The extracted solutions were incubated at room temperature for 16 hours and centrifuged at 1,300 rpm for 1 min, 4˚C. The supernatants were used for estimation of chlorophyll a, b, and carotenoid content using a spectrophotometer recorded with 664. 2 nm, 648. 6 nm, and 470 nm, respectively. The pigment content was expressed in terms of mg^{-1} plant dry weight. The amounts of each pigment content were calculated according to the following equations:

$$\text{Chlorophyll a (mg mg}^{-1}\text{ DW)} = \frac{[13.36A_{664.2} - 5.19A_{648.6}] \times 8.1}{\text{DW}}$$

$$\text{Chlorophyll b (mg mg}^{-1}\text{ DW)} = \frac{[27.43A_{648.6} - 8.12A_{664.2}] \times 8.1}{\text{DW}}$$

$$\text{Carotenoid (mg mg}^{-1}\text{ DW)} = \frac{[4.785A_{470} + 3.657A_{664.2} - 12.76A_{648.6}] \times 8.1}{\text{DW}}$$

$$\text{Total Chlorophyll (mg mg}^{-1}\text{ DW)} = \frac{[5.24A_{664.2} - 22.24A_{648.6}] \times 8.1}{\text{DW}}$$

4.3.8 Rice survival rate and drought scoring

After the rice seedlings (14 DAI) were exposed to water stress for 5 days (19 DAI), the seedlings were assessed the survival rate after watering for 3 days. Moreover, the rewatering of rice plants was also conducted in the greenhouse condition. However, the condition did not assess with the survival rate, but it was evaluated by the scoring system which defined by the Standard Evaluation System for rice (IRRI 1996). The scoring system was divided into drought resistance, drought recovery score, and leaf rolling score, in which the details of each category were described in Table 4.2 (IRRI 1996). The five replicates of each treatment were served.

Table 4.2 Scoring for assessing rice morphology, drought resistance and leaf rolling during water deficit and after rewatering conditions under greenhouse experiment.

Characteristics	Score	Description
Leaf rolling score	0	Leaves are healthy
	1	Leaves start be to folded
	3	Leaves are folded (Deep-V-shaped)
	5	Leaves are fully cupped (U-shaped)
	7	Leaves margins touching (O-shaped)
	9	Leaves are tightly rolled
Drought recovery score	1	90%-100% of plants recovered
	3	70%-89 of plants recovered
	5	40%-69% of plants recovered
	7	20%-39% of plants recovered
	9	0%-19% of plants recovered
Drought resistance score	0	Highly resistant: No symptoms
	1	Resistant: Light tip drying
	3	Moderately resistant: Tip drying to 1/4 length in most leaves
	5	Moderately susceptible: 1/4 to 1/2 of leaves fully dried
	7	Susceptible: More than 2/3 of all leaves fully dried
	9	Highly susceptible: All plants apparently dead

4.3.9 Rice growth and yield

Under laboratory condition, the growth parameters including plant dry weight and plant fresh weight were recorded (five replicates of each treatment) at 14 DAI at a well-watered condition. For dry weight estimations, the plant samples were dried at 70˚C for 3 days. Moreover, the plant dry weights of the rice in field condition were also investigated in both stages of seedling and tillering stages as well as their tiller numbers. After rice plants were harvested, their seed number, infertile grain, grain yield, and 100-grain weight were measured. Ten replicates were provided for each treatment.

4.4 Results

4.4.1 Effects of ACC deaminase improvement on plant growth under laboratory condition in the well-watered experiment

The fresh weight of rice seedlings (14 DAI) was significantly greater in the bacterial inoculations than un-inoculated seedling (control) as well as the results of shoot and root fresh weights (Figure 4.1A-C), and plant dry weights of shoot and root (Figure 1D-F). However, the results of the rice inoculated with SUTN9-2:pMG103::*acdRS* showed higher significant improvement in the plant dry weight and shoot dry weight than those of SUTN9-2 WT and SUTN9-2 (ACCDadap) (Figure 4.1D-F).

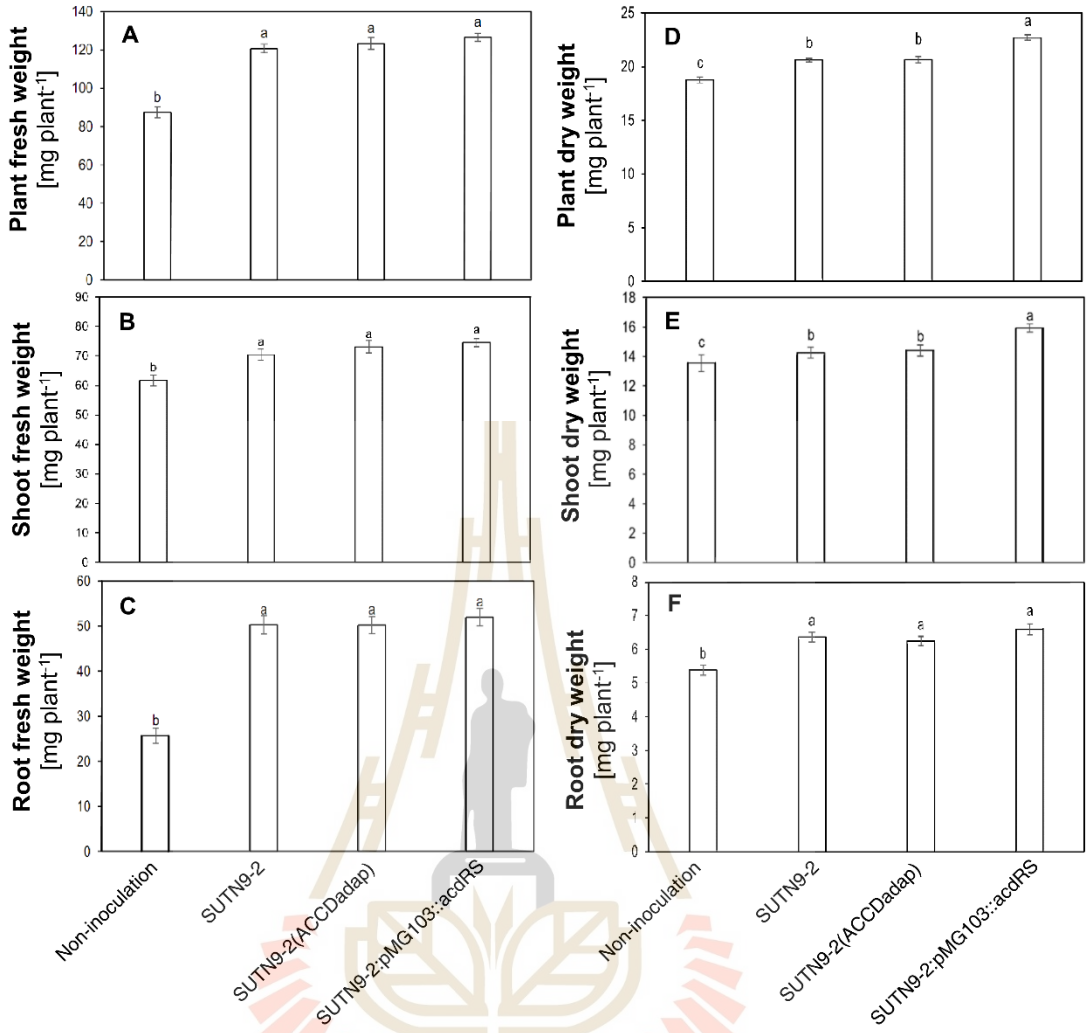

Figure 4.1 The averages of plant fresh weight (A), shoot fresh weight (B), root fresh weight (C), plant dry weight (D), shoot dry weight (E) and root dry weight (F) of rice seedlings at 14 DAI inoculated with difference bacterial strains including SUTN9-2 WT, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* compared with non-inoculation under well-watered condition in laboratory. Significant differences were showed with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests. The data are presented as the mean of five replicates and the vertical bars indicate standard error.

4.4.2 Effects of the improved ACC deaminase activity on ethylene production and MDA contents under laboratory condition

In laboratory experiment, the ethylene emissions were observed in a small amount with no statistically significant differences when compared with non-inoculation under well-watered condition (Figure 4.2A). The water stress condition induced by PEG significantly increased ethylene production in rice. However, ethylene emission were reduced around 0.05-fold in rice seedling inoculated with SUTN9-2 WT and SUTN9-2 (ACCDadap), while SUTN9-2:pMG103::*acdRS* decreased the ethylene emission around 1.0-fold or 0.85-fold when compared with non-inoculation and SUTN9-2 WT strain, respectively (Figure 4.2A).

The amount of MDA content was used to estimate the lipid peroxidation in leaves of the rice seedlings which exposed to water stress condition. The results showed that the MDA content was significantly higher in water deficit condition than well-watered condition (Figure 4.2B). Under well-watered condition, there were no statistically significant differences in the MDA contents neither bacterial inoculations nor non-inoculation, but the rice inoculated with bacteria showed the tendency of the MDA decreased in both the improved ACC deaminase activity strains when compared with the non-inoculated plant. In addition, under stress condition showed that the amount of MDA contents of rice leaves was reduced by SUTN9-2:pMG103::*acdRS* inoculation around 1.3-fold when compared with the non-inoculated plants (Figure 4.2B).

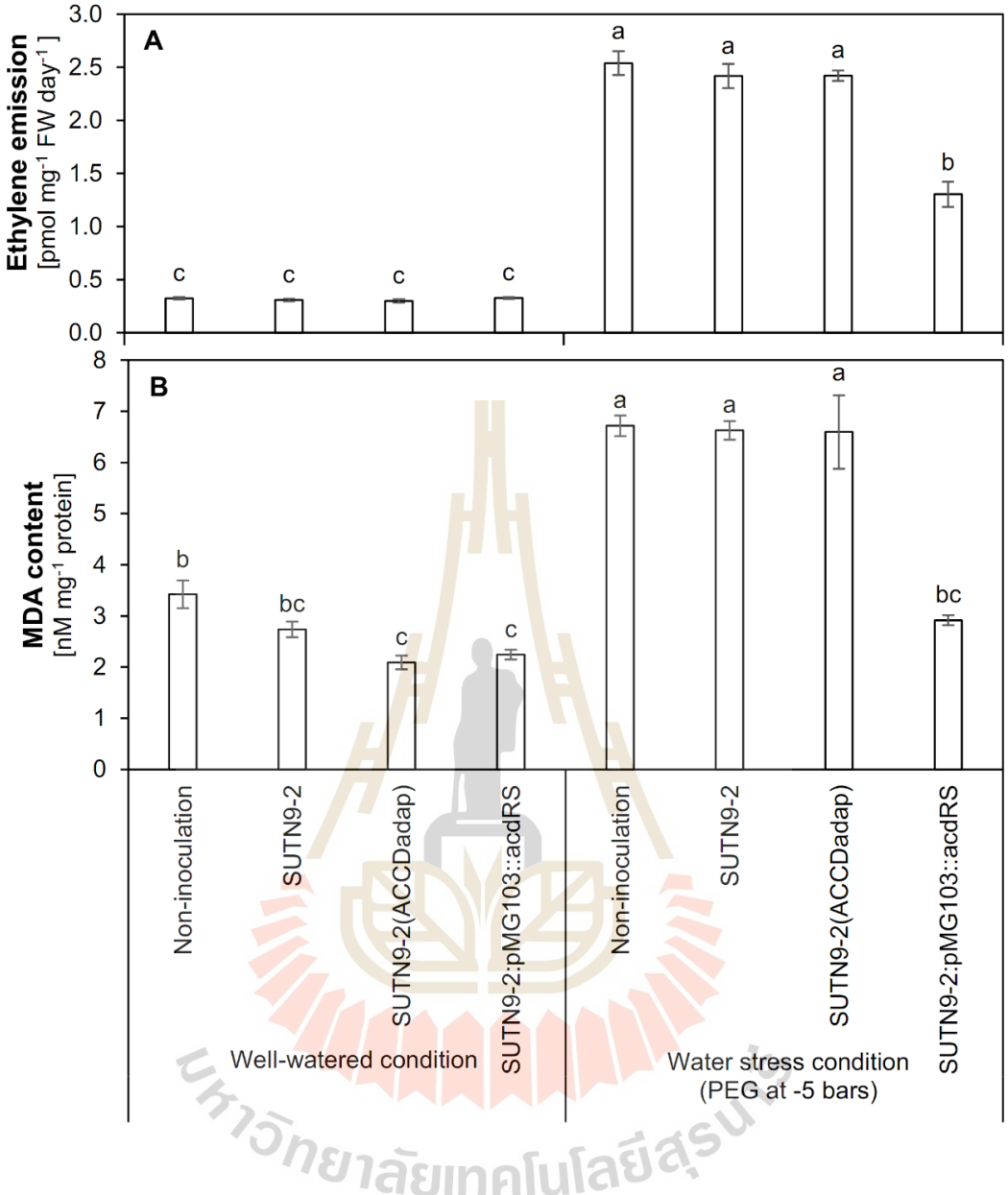

Figure 4.2 Effects of polyethylene glycol (PEG-8000) on ethylene emission (A) and malondialdehyde (MDA) content (B) in rice seedling inoculated with bacteria and non-inoculation at 19 DAI under laboratory condition. The water stress conditions were compared with the well-watered condition. Significant differences were showed with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests. The data are presented as the mean of five replicates and the vertical bars indicate standard error.

4.4.3 Effects of the improved ACC deaminase activity on recovery efficiency, survival rate (%) and relative water content (RWC) under laboratory condition

After 5 days without adding the nutrient solution into rice seedlings, the wilting symptom of rice leaves in all treatments were observed, especially in un-inoculated control (Figure 4.3A). However, after rewatering for 3 days, rice could be recovered, which could be observed from plant morphology and/or their survival rates (Figure 4.3A and B). The rice seedling inoculated with SUTN9-2 WT, SUTN9-2 (ACCDadap), and SUTN9-2:pMG103::*acdRS* increased significantly in plant survival rate around 0.3 to 0.4-fold when compared with non-inoculated plant. Moreover, the results showed that the water deficit affected the leaf RWC after 4 days of the draining, especially the un-inoculated plant (Figure 4.3C). While the rice seedlings inoculated with those endophytes could maintain the percentage of leaf RWC during the soil drying. The water deficit severely affected the leaf RWC by reducing the leaf RWC at 5 days after the draining in all treatments around 25.6% in un-inoculated plant, 42.5% in SUTN9-2 WT, 54.0% in SUTN9-2 (ACCDadap), and 55.4% in SUTN9-2:pMG103::*acdRS* (Figure 4.3C), which correspond to their phenotype (Figure 4.3A). The RWC of leaves was also increased in rice inoculated with SUTN9-2, SUTN9-2 (ACCDadap), and SUTN9-2:pMG103::*acdRS* by 70.2%, 76.0%, and 76.2% after 24 hours, respectively, when compared with non-inoculation (Figure 4.3C).

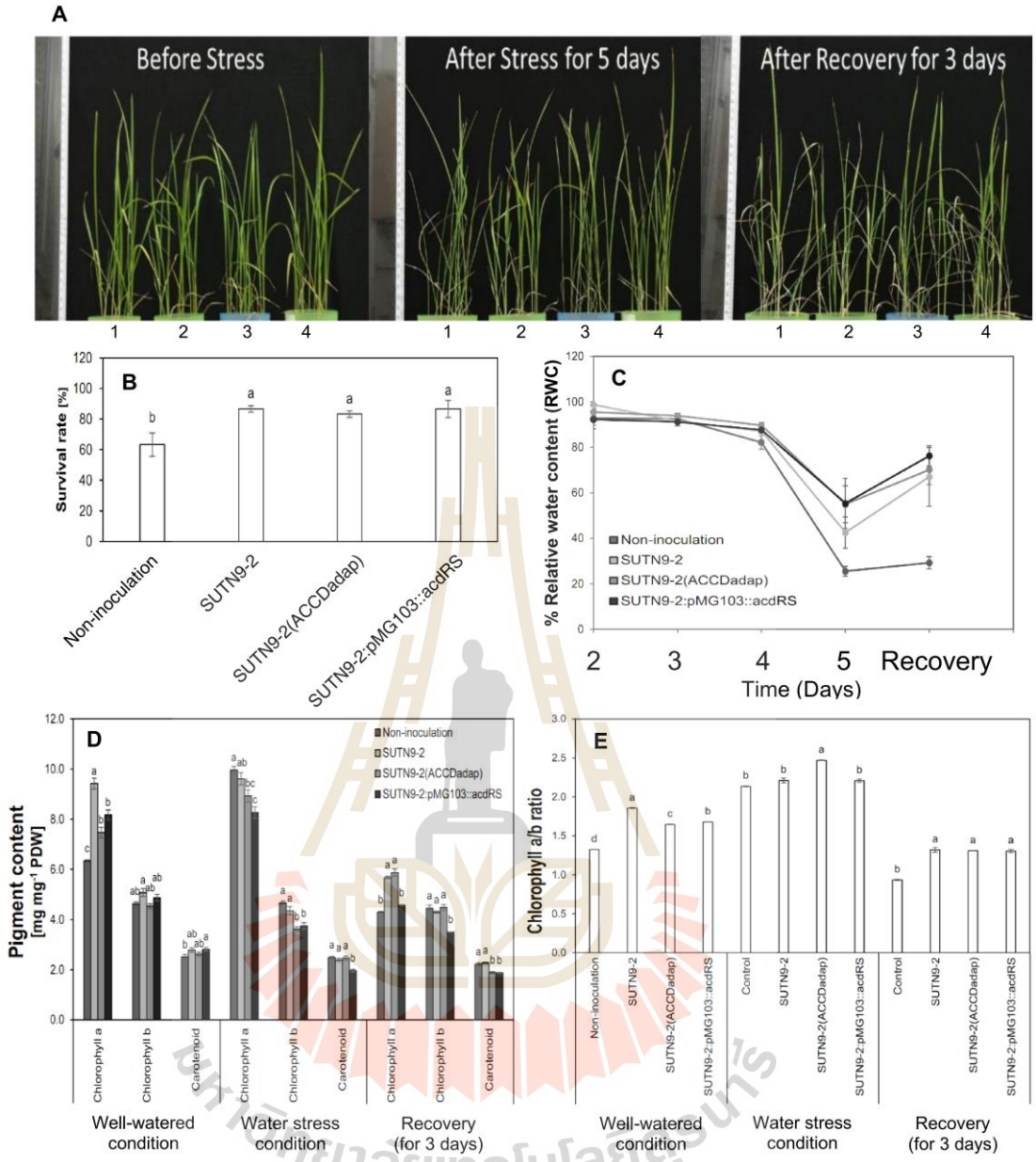

Figure 4.3 Effects of water deficit stress and rewatering on rice seedling morphology (A), survival rate (%) (B), and relative water content (RWC) of leaves (%) (C), chlorophyll a, b and carotenoid contents (D), and chlorophyll a/b ratio (E) in rice seedling inoculated with bacteria and non-inoculation were grown in the mixing of soil and sand (1:1) material under laboratory condition. This water deficit stress was compared with the well-watered condition.

Significant differences were showed with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests. The data are presented as the mean of three replicates and the vertical bars indicate standard error and the statistic comparison was performed separately in the well-watered and water stress conditions.

4.4.4 Effects of the improved ACC deaminase activity on photosynthetic pigment production under laboratory condition

The effects of water deficit stress on photosynthetic pigments of rice seedlings (14 DAI), which were inoculated with SUTN9-2 and its improved strains were depicted in Figure 4.3D-E. The results demonstrated that the amounts of chlorophyll a in rice inoculated with bacteria were significantly greater than that of non-inoculation. While there were no statistically significant differences in the amounts of chlorophyll b and carotenoid when compared with non-inoculation in well-watered condition (Figure 4.3D). Under water deficit condition, the bacterial inoculations showed a decreasing tendency in the chlorophyll a, b, and carotenoid productions, but there were no statistically significant differences when compared with non-inoculation, except the chlorophyll b content in inoculation of SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* were significantly lower than the non-inoculation. The amount of carotenoid was also significant lower than those of other treatments when rice inoculated with SUTN9-2:pMG103::*acdRS*. After 3 days of recovery, the amounts of the chlorophyll a in rice inoculated with SUTN9-2 and SUTN9-2 (ACCDadap) showed significant differences when compared with non-inoculation, while chlorophyll b was no statistically significant differences, except SUTN9-2:pMG103::*acdRS*, and a

significantly decrease was found in carotenoid content when rice plants were inoculated with SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* when compared with non-inoculation (Figure 4.3D). In the chlorophyll a/b ratios, which is an indicator to explain in the change of photosynthetic system for light harvesting, were significantly higher in rice inoculated with bacteria than un-inoculated control under the well-watered condition. However, under water stress situation, the inoculation with SUTN9-2 (ACCDadap) showed the highest chlorophyll a/b when compared with other treatments. While the ratios of chlorophyll a/b after recovery were statistically significant different in the rice with bacterial inoculations when compared with non-inoculation (Figure 4.3E).

4.4.5 Effects of water deficit on rice inoculated with the improved ACC deaminase activity under greenhouse condition

After 20 days without adding water, the rice plants displayed the wilting symptom acting a cause of the lowering of soil moisture in pot, and ethylene emission was also determined. The results displayed a significant increase in the ethylene emission when compared with the well-watered condition (Figure 4.4). However, the ethylene production from rice plants inoculated with bacteria was significantly lower than non-inoculation around 0.26-, 0.78-, and 0.35-folds in SUTN9-2 WT, SUTN9-2 (ACCDadap), and SUTN9-2:pMG103::*acdRS*, respectively. Moreover, the ethylene production of each treatment also performed the same tendency with well-watered condition (Figure 4.4).

In addition, efficiency of SUTN9-2 and its derivative strains were observed in the rice morphology during the lowering of soil moisture in pot as well as recovery condition, which were assessed with scores. The results displayed that the drying of soil

induced the wilting symptom to rice plants after draining for 20 days. The un-inoculated plant showed more severe leaf rolling than the rice inoculated with SUTN9- 2 (ACCDadap), SUTN9-2:pMG103::*acdRS*, and SUTN9-2, respectively. Furthermore, the inoculated plants also showed the tendency of drought resistance more than the un-inoculated plant which assessed with leaf rolling, drought recovery, and drought resistance scores (Figure 4.5A and B).

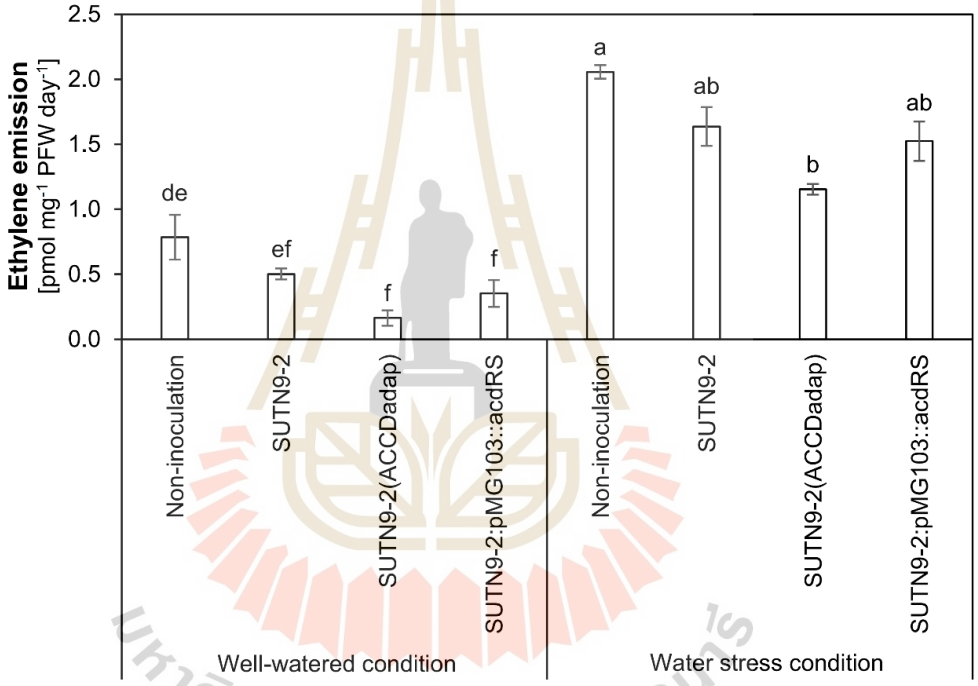

Figure 4.4 Effects of soil drying (withholding water for 20 days) on ethylene emission in rice inoculated with bacteria and non-inoculation at 34 DAI under greenhouse condition. The water stress conditions were compared with the well-watered condition. Significant differences were showed with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests. The data are presented as the mean of five replicates and the vertical bars indicate standard error.

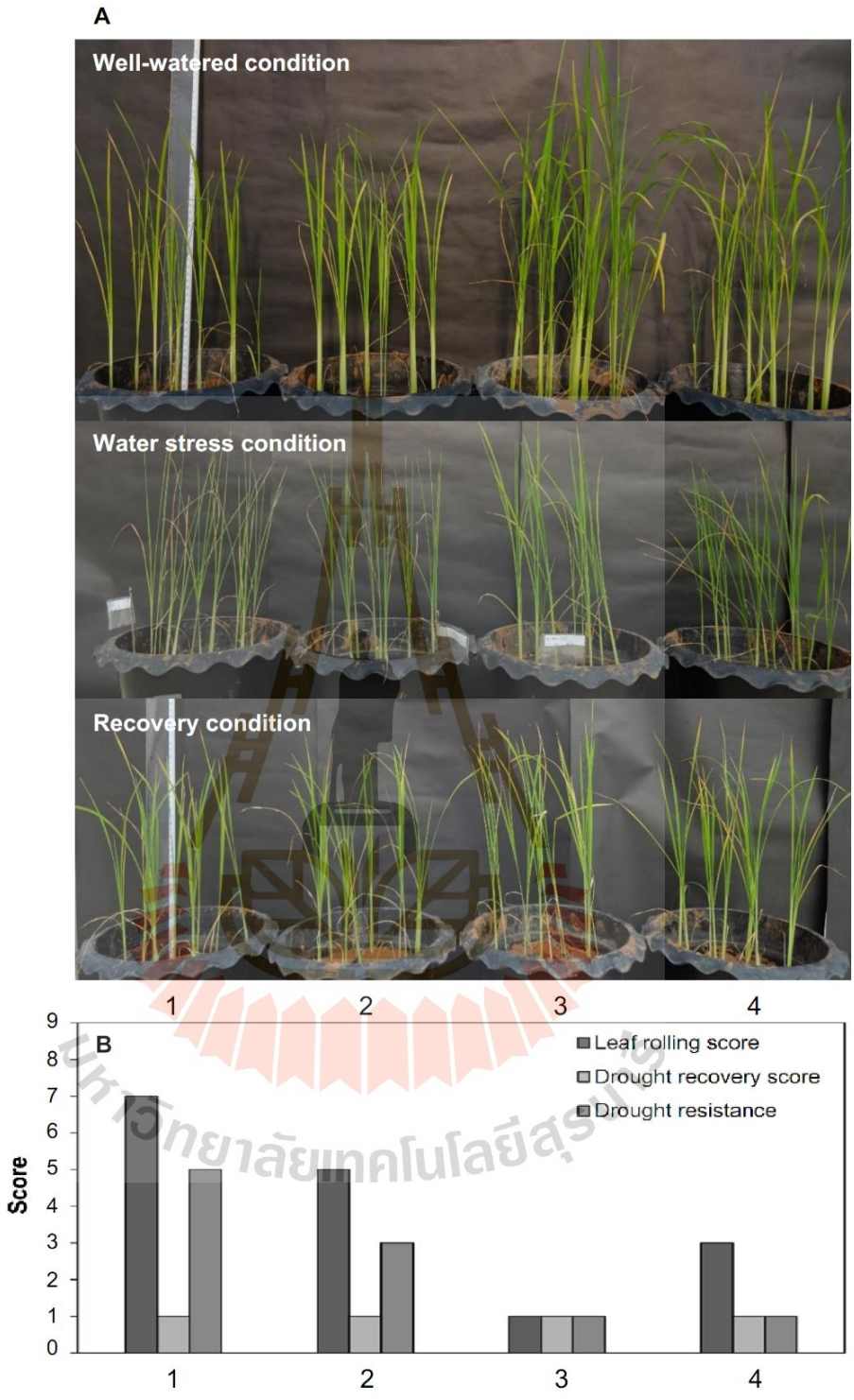

Figure 4.5 Effects of soil drying (withholding water for 20 days) on rice water stress resistance, rice rewatering, leaf rolling during water deficit scores (A), and rice morphology after rewatering for 1 day (B) of rice plant inoculated with

bacterial and non-inoculation were grown in soil under greenhouse condition. The Hindu-Arabic numerals including 1, 2, 3 and 4 represented non-inoculation, SUTN9-2, SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS* inoculations, respectively. Significant differences were showed with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests. The data are presented as the mean of five and ten replicates in laboratory and greenhouse experiments, respectively, and the vertical bars indicate standard error.

4.4.6 Effect of water deficit stress on rice inoculated with bacteria and its yield under field condition

Since SUTN9-2:pMG103::*acdRS* is a mutant derivative strain of SUTN9-2, which was generated by genetic engineering technique, this strain is restricted to apply in all open field experiments. The results showed significant difference in the rice inoculated with SUTN9-2 (ACCDadap) under well-watered condition when compared with SUTN9-2 and non-inoculation, while no statistically significant difference was found under drought condition. This demonstrated that the water deficiency affected the plant growth in seedling stage (Figure 4.6A). At tillering stage, the results displayed that the plant dry weight did not show significant different under well-watered condition, but in the rice inoculated with SUTN9-2 (ACCDadap) showed statistically significant differences in comparison with un-inoculated plant (Figure 4.6A). However, a significant increase were found in rice inoculated with bacterial strains, SUTN9-2 WT and SUTN9-2 (ACCDadap), in both well-watered and water deficiency conditions (Figure 4.6B). Moreover, the other parameters, including the seed number, the infertile

grain, the 100-grain weight data, and grain yield showed that no significant differences were observed between non-inoculation and rice inoculated with bacteria under the well-watered condition (Figure 4.6C-F). While these parameters were statistically significant different in rice inoculated with bacteria, especially, SUTN9-2 (ACCDadap) when compared with non-inoculation (Figure 4.6C-F) according to its morphology when exposed to drought situation (Figure 4.7).

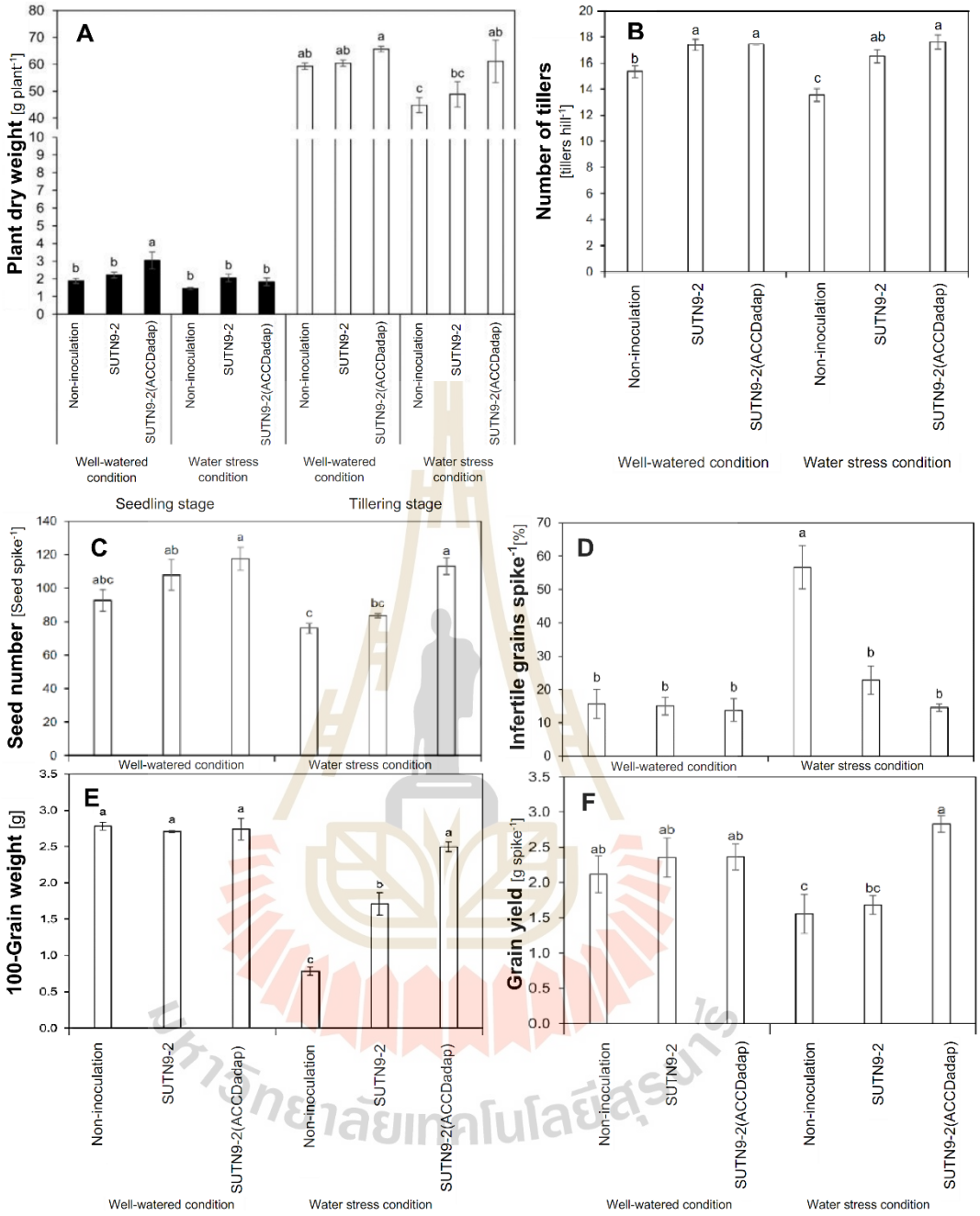

Figure 4.6 The averages of plant dry weight (A), number of tillers of rice plants at seedling and tillering stage (B), seed number (C), infertile grains per spike, 100-grain weight (E), and grain yield (F) of rice plant inoculated with bacteria and non-inoculation were grown in soil under field condition. The water stress was compared with the well-watered condition. Significant

differences were showed with different alphabets at $P \leq 0.05$ according to Duncan's multiple range tests. The data are presented as the mean of ten replicates and the vertical bars indicate standard error and the statistic comparison was performed separately in the well-watered and water stress conditions.

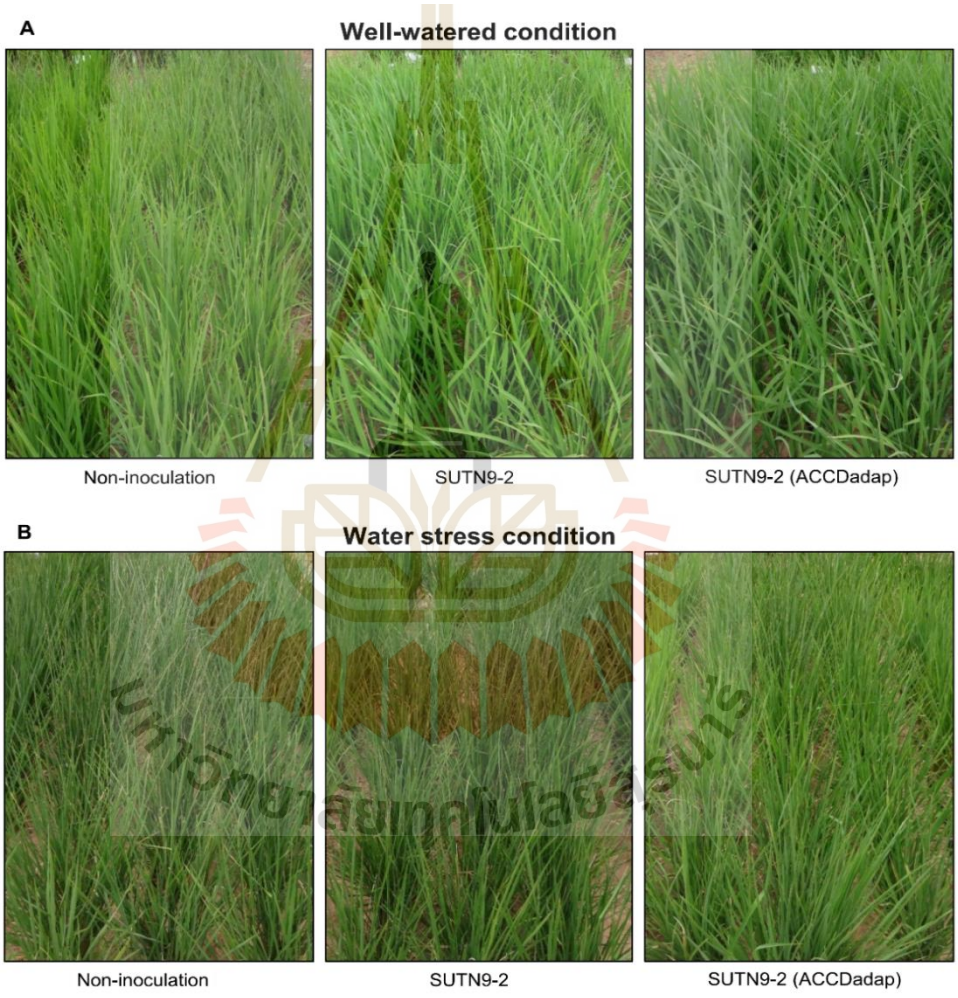

Figure 4.7 The morphology of rice inoculated with bacterial strains, SUTN9-2 WT and SUTN9-2 (ACCDadap), in comparison with non-inoculation under the well-watered condition (A), and drought (withholding water for 7 days) (B) under field condition.

4.5 Discussion

In this study, the rice inoculated with the endophytic bacteria producing ACC deaminase, including SUTN9-2, SUTN9-2 (ACCDadap), and SUTN9-2:pMG103::*acdRS* strains, could enhance the plant growth under laboratory condition, especially rice inoculated with SUTN9-2:pMG103::*acdRS* (Figure 4.1A-F), while SUTN9-2 (ACCDadap) exerted on its host at 34 DAI under well-watered condition in field experiment as well as the results of tillering stage under the same condition (Figure 4.6A). It was suggested that the growth promotion of rice seedling inoculated with bacteria might be due to the plant growth promoting traits from bacteria that supplied to the rice plant. When rice inoculated with SUTN9-2 could promote the rice growth at 7 DAI under NH_4NO_3 as a nitrogen (N) source and under N-free at 14 DAI, SUTN9-2 also enhanced the biomasses of rice seedling (especially, Thai rice cultivars such as *O. sativa* L. var.*indica* cv. Pathumthani 1, *O. sativa* L. cv. KDML 105, and *O. sativa* Leuang Yai 148) at 30 DAI and 28 DAI when supplemented with 0.1 and 1.0 mM NH_4NO_3 when compared with un-inoculated control (Greetatorn et al. 2019; Piromyou et al. 2015a; Piromyou et al. 2015b), and the same trend was observed in 1.0 mM KNO_3, NH_4NO_3 and urea supplementation (Piromyou et al. 2017). Productions of IAA and ACC deaminase were reported as the essential mechanisms to facilitate the rice endophytic bacteria and rhizobacteria for root colonization as well as plant growth promotion (Bal et al. 2013; Etesami et al. 2014a; Etesami et al. 2014b). Both the endogenous IAA (plant production) and exogenous IAA (bacteria produce) are absorbed and accumulated in the plant cell, and then rise to high concentration, resulting in stimulation of the ACC synthase gene expression. The ACC synthase enzyme converts S-adenosyl methionine (SAM) to be ACC in the ethylene biosynthesis

pathway, which is a cause of the high ethylene production acting as cell division or plant growth inhibitions (Glick et al. 1998). However, the phenomenon is interrupted by the bacterial ACC deaminase that degrades the ethylene's substrate (ACC) as a result of decreasing the ethylene production. Therefore, the loop of IAA production does not affect the cell division and/or plant growth, but instead acts as the positive effect with stimulation in shoot/root elongation on its host plant together with lowering ACC by ACC deaminase containing bacteria. In this regard, it could be suggested that the rice inoculation with SUTN9-2 and its derivative strains, may enhance the rice growths via this mechanism. Moreover, the levels of enzymatic activity of ACC deaminase might influenced on plant growths at seedling stage. From previous work proposed the possession of ACC deaminase activity in SUTN9-2:pMG103::*acdRS* at 34.72 μmol of α-ketobutyrate h^{-1} mg^{-1} protein (higher than SUTN9-2 as 8.9-fold), while SUTN9-2 and SUTN9-2 (ACCDadap) presented in 3.88 and 5.58 μmol of α-ketobutyrate h^{-1} mg^{-1} protein, respectively (Sarapat et al. 2020), together with the influence of bacterial ACC deaminase was induced with ACC before planting. Thus, it might suggested that the plant growth promotion trait property could be enhanced by increasing the efficiency of ACC deaminase activity.

The alleviation of stress in plants is a beneficial major of the bacteria producing ACC deaminase activity, which is considered as a mechanism to provide the stress tolerance in host plants. Significantly, under water-deficit condition, the rice seedling inoculated with SUTN9-2:pMG103::*acdRS* could decrease the ethylene emission more than others (Figure 4.2A) as well as the amount of MDA content (Figure 4.2B). This data displayed that the bacterial ACC deaminase could influence on antioxidative defense mechanism in rice plants via a controlling the ethylene synthesis. Under

different water deficit of alfalfa plant, the amounts of MDA content were detected as a parallel with the levels of ethylene evolution rate as well as the H_2O_2 levels (Irigoyen et al. 1992). This indicated that the levels of ethylene emission were directly connected with ROS production, and a large amount of ethylene led to the deterioration of cell membranes which determined by MDA content. Ethylene may act as a plant signaling that not only regulates plant developments in different stages, but also it is a plant stress response signaling. Thus, it could be suggested that SUTN9-2:pMG103::*acdRS* relieved the negative effect of ROS generation (lipid peroxidation), which induced by PEG. Moreover, in well-watered condition, the MDA contents of rice seedling inoculated with bacteria were lower than that of non-inoculation (Figure 4.2B), which was discussed as the influence of the ACC deaminase enzyme. However, it seems that the overproduced ACC deaminase SUTN9-2:pMG103::*acdRS* was not exert when confronted with water deficit stress under greenhouse condition. In this case, it was postulated that the water deficit condition which induced by PEG acting as a osmotic agent to mimic the water shortage that leads to the secondary peak (a large ethylene or stress ethylene) of ethylene biosynthesis in a few hours without the first ethylene peak (Glick et al. 2007). Therefore, the PEG-induced stress could induce ACC to be produced in a large amount, resulting in produce more unit of ACC deaminase in SUTN9-2:pMG103::*acdRS*. This led to the high efficiency to reduce the ethylene emission. On the other hand, SUTN9-2:pMG103::*acdRS* did not exert to facilitate the lowering of ethylene emission in its host plants under greenhouse experiment, while rice seedling inoculated with SUTN9-2 (ACCDadap) showed the lowest ethylene emission (Figure 4.4). In this regard, it was hypothesized that the greenhouse condition, which soil for planting was conducted with a dryness stress of soil. The rice plants,

which confronted with the slow stress condition produced a small amount or the first ethylene peak to stimulate plant defense mechanisms to deal with the stress condition. ACC deaminase gene (*acdS*) in SUTN9-2 (ACCDadap) was mutated at 419 of nucleic acid sequence. The point mutation changed amino acid residue encoding ACC deaminase enzyme at 140 region, which was predicted to locate in its binding site and probably led to a higher affinity of ACC binding than the original SUTN9-2 and a higher ACC deaminase activity (Sarapat et al. 2020), while SUTN9-2:pMG103::*acdRS*, overproduced ACC deaminase strain, was significantly reduced ethylene emission due to the increased amount of the enzyme. Even though, the ACC deaminase activity of SUTN9-2 (ACCDadap) was lower than activity of that of the SUTN9-2:pMG103::*acdRS*, but it is adequate to promote water deficit tolerant in field and greenhouse condition.

After nutrient solution or water was removed, the wilting symptom of leaf rice seedlings was continuously developed as well as the leaf RWC (%) was decreased (Figure 4.3A and C). The wilting is a visual signal of plant responding to the lower turgor pressure (Fang and Xiong 2015; Russell Jones 2013), as well as the RWC values of leaves can be used to explain in the intensity of water potential sensing from root to leaves for photosynthesis. Smirnoff (1993) explained that the reduction of RWC remains approximately 70% as a medium loss of water can affect to carbon dioxide assimilation and generate ROS or oxidative stress as a causing from dark reaction during stomatal closure, while RWC is lower than 30% as a severe loss of water, leading to drying of plant tissues and death which cannot be salvaged. Here, our experiments demonstrated that rice inoculated with bacteria could influence on the water retention in plant tissues with decreasing the water loss via transpiration process during water

deficit Obviously, SUTN9-2 (ACCDadap) and SUTN9-2: pMG103: : *acdRS* could enhance the leaf RWC both the water stress for 5 days and after rewatering (Figure 4.3A and C), while SUTN9-2 (ACCDadap) impacted in the lowering of soil moisture condition (Figure 4.5). This water retention led to the beneficial facilitation in the higher survival rate (Figure 4.3B and Figure 4.5). It was speculated that the water content in plant tissues, which is regulated by stomatal closure, might be controlled by bacterial ACC deaminase activity via the ethylene signaling, which might be produced in different levels depended on ACC deaminase activity. The amount of ethylene emission can affect the signaling pathway ratio between ethylene and abscisic acid, which is a regulator for controlling the stomata aperture with ethylene crosstalk. This ratio can affect the rapid response of guard cells or size of stomata aperture, leading to stomata closed or closed slowly as well as it did not complete (Tanaka et al. 2005). This demonstrated that both improved strains could efficiently facilitate the drought stress tolerance via controlling ethylene to regulate the opening and closing stomatal aperture and then led to prevent the drying of plant tissues and death.

The plants inoculated with bacteria containing ACC deaminase could not only improve the plant growth, germination (%), vigor index, and decrease ethylene production (Karthikeyan et al. 2012) but also enhance the leaf pigment production (chlorophyll a, chlorophyll b and carotenoid). The results indicated that inoculation of rice seedling with bacteria could increase chlorophyll a content. However, both of the improved strains seem to affect the chlorophyll a production in rice seedling with bacterial inoculation under well-watered condition, while non-inoculation displayed a remarkable increasing in the chlorophyll a, when exposed to water deficit condition (Figure 4.3D). Based on these results demonstrated that the levels of bacterial ACC

deaminase activity could influence the photosynthetic pigments, especially chlorophyll a in the opposite direction. The lowering of chlorophyll a might led to change the ratio of photosystem II (PSII) to PSI to decrease oxidative stress which occurs from photosynthetic systems (Ashraf and Harris 2013; Pirzad et al. 2011; Saeidi and Zabihi-e-Mahmoodabad 2009), which confirmed by the higher of the chlorophyll a/b ratio that is the strategy to survive under water deficit stress (Figure 4.3E). Therefore, during plants face to water deficit condition, the reduced or unchanged pigment contents can provide plant survival and this trait is the drought tolerance. In this, the rice inoculated with bacteria seems to affect the pigment contents of rice seedlings (Figure 4.3D and E), we hypothesized that the amount of pigment contents in rice inoculation with the bacteria might be controlled by ethylene levels via bacterial ACC deaminase activity. Ethylene can regulate the chlorophyll content via ethylene response factors (ERFs) which is an abiotic stress-triggered transcription and the ethylene can exert as both beneficial and deleterious aspects in plants, which depended on its level. Therefore, the perception of ERFs is an important to activate as well as the optimum level of ethylene which lead to the benefit signaling (Sharma et al. 2019). Overall, these data were evidence to confirm the rice endophytic bacteria could facilitate plant growth and mechanisms to avoid the cell damage from water deficit stress, especially SUTN9-2 (ACCDadap) strain. This strategy led to improve rice biomass, crop yield as well as drought tolerance.

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CHAPTER V

CONCLUSION

In this study demonstrated that the improvement of ACC deaminase activity in SUTN9-2 using both methods of ALE approach and genetic engineering led to increments of ACC deaminase activity producing SUTN9-2 (ACCDadap) and SUTN9-2:pMG103::*acdRS*, respectively. These improved bacterial strains also facilitated in the plant growth promotion and enhanced mechanism tolerances to water deficit stress, which summarized as following:

1. The improved ACC deaminase activity could improve nodulation process in mung bean plant.

1.2 The high nodule number of mung bean inoculated with the improved ACC deaminase activity led to promotion of nodule dry weight and nitrogen-fixing efficiency at seedling stages of mung bean plant.

2. The negative effect from water deficit against nitrogen fixation could be relieved by increasing the expression levels of *acdS* and *acdR* genes of SUTN9-2:pMG103::*acdRS* strain.

2.1 Plant growth and nitrogen fixation in mung bean plant inoculated with bradyrhizobium containing the improved ACC deaminase activity could be promoted under water deficit condition.

2.2 The high level of ACC deaminase activity can enhance symbiosis interaction, drought tolerance and reactivation of growth

3. The efficiency of ACC deaminase in SUTN9-2 and its derivative strains was conducted with rice. The results demonstrated that those bacterial strains could promote plant growth (plant biomass) in laboratory, greenhouse and field conditions under normal situation.

3.1 The bacteria were facilitated the water deficit tolerance to their hosts via possession of ACC deaminase which could control the levels of ethylene emission.

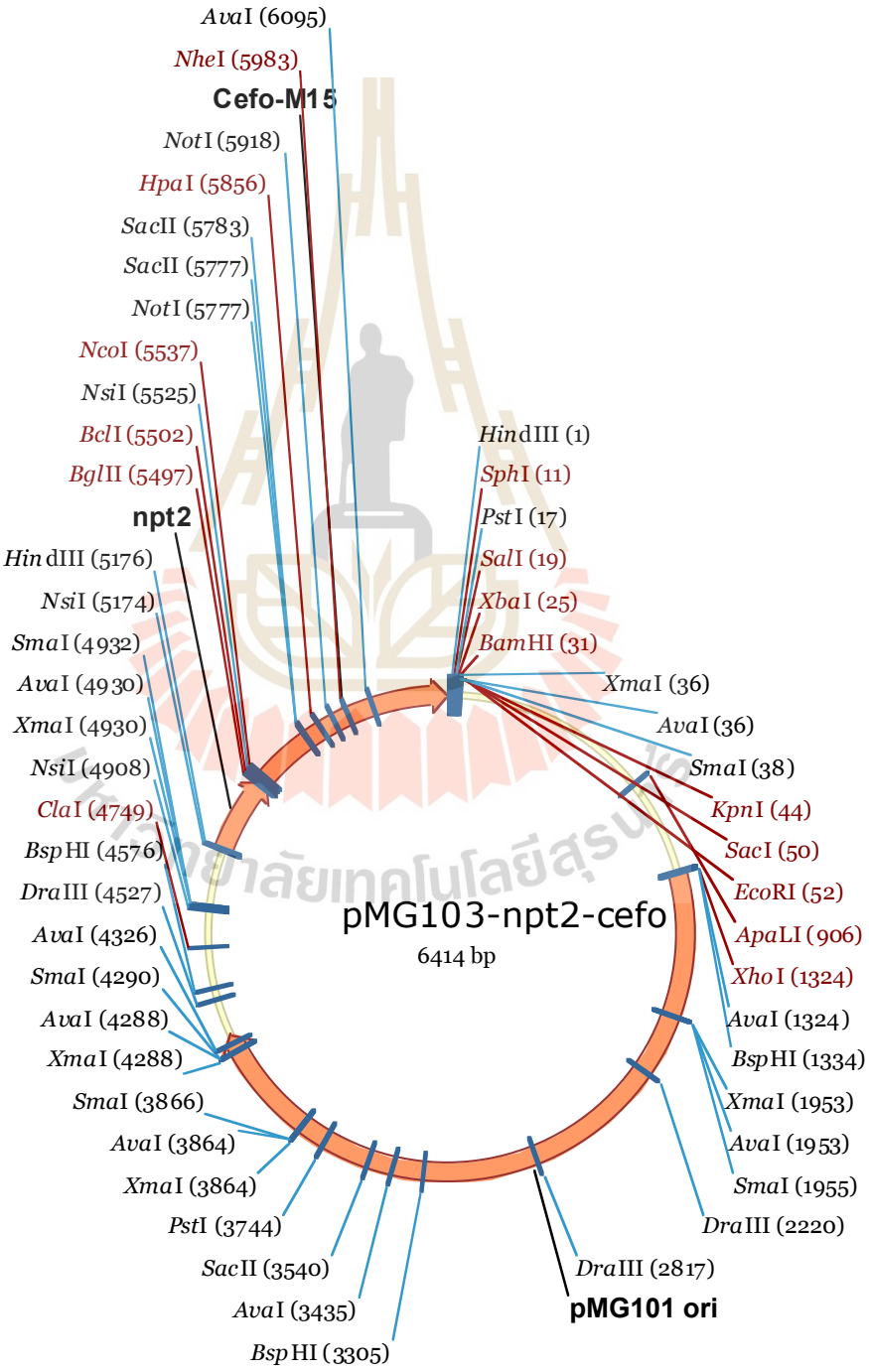
3.2 The lower amount of ethylene leads to decreasing the membrane destruction (MDA content) from reactive oxygen species (ROS) and prevented the oxidative stress from photosynthesis inhibition by decreasing the chlorophyll contents.

3.3 SUTN9-2 and its derivative strains also improved the RWC in leaves, the rate of survival, and plant recovery from water deficit condition as well as the crop yield.

4. SUTN9-2 (ACCDadap), which was an adaptive evolution using ALE approach, is a candidate strategy for application in field condition.

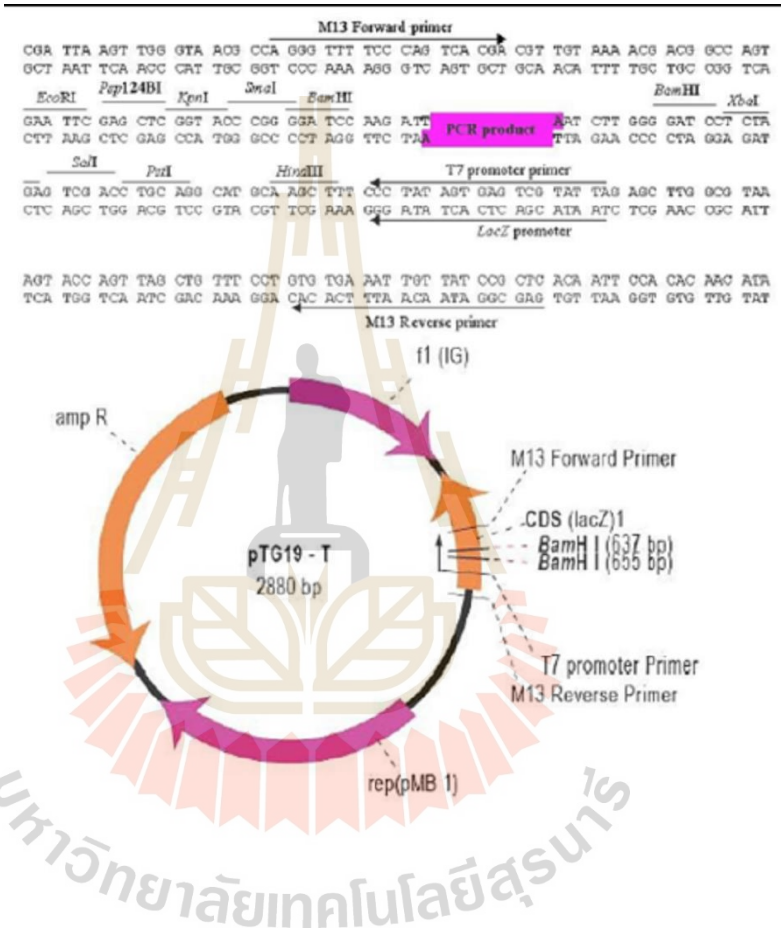
APPENDIX

Plasmid map of pMG103-npt2-Cefo



APPENDIX

Plasmid map of pTG19-T PCR cloning vector



BIOGRAPHY

Sukanlaya Sarapat was born in Nong Bua Lumphu, Thailand on February 26th, 1989 as the first daughter. She studied primary school at Ban Khok Thung noi School and high school at Nong Bua Pittayakarn School. She graduated with the Bechelor' degree of crop production technology from Suranaree University of technology in 2010. In 2013, she has a great opportunity to do Doctoral degree in Biotechnology at Suranaree University of technology. During study, she was support from the Royal Golden Jubilee Ph.D. Program (RGJ-Ph. D. Program). Beside her organizational experiences, she have been worked several times in Application of Soil Microbiology Laboratory of Biotechnology, Suranaree University of Technology Farm (SUT Farm), Crop Production Technology Laboratory and Food Technology Laboratory. She started the publication in the topic of "Effects of Increased 1 - Aminocyclopropane-1 - Carboxylate (ACC) deaminase activity in *Bradyrhizobium* sp. SUTN9-2 on mung bean symbiosis under water deficit conditions" in *Microbes and Environments*. Volume 35 (2020) Issue 3. DOI https://doi.org/10.1264/jsme2.ME20024.