การเพิ่มประสิทธิภาพหัวเชื้อไรโซเบียม ในการเข้าสร้างปมกับถั่วเหลืองภายใต้สภาวะเครียดแบบต่าง ๆ ภายในดิน

นางสาวเจนจิรา วงษ์ดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ENHANCING THE EFFICIENCY OF SOYBEAN INOCULANT FOR NODULATION UNDER MULTI-ENVIRONMENTAL STRESS SOIL CONDITIONS

Jenjira Wongdee

A Thesis Submitted in Partial Fulfillment of the Requirements for the

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ENHANCING THE EFFICIENCY OF SOYBEAN INOCULANT FOR NODULATION UNDER MULTI-ENVIRONMENTAL STRESS SOIL CONDITIONS

Suranaree University of Technology has approved this thesis submitted in partial fulfillments of the requirements for a Master's Degree.

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สภาวะแวดล้อมในธรรมชาติที่ไม่เหมาะสมแบบต่าง ๆ เป็นปัจจัยที่จำกัดการเจริญเติบโตของถั่วเหลือง รวมถึงการเข้าสร้างปม และการตรึงไนโตรเจนของไรโซเบียม ซึ่งสัมพันธ์กับการพัฒนาทางด้านสรีระของพืช ทั้งนี้ การใช้หัวเชื้อไรโซเบียมที่ทนต่อสภาวะเครียด สามารถส่งเสริมให้ถั่วเหลืองเจริญในสภาวะเครียดได้ดีขึ้น งานวิจัยนี้ ได้ศึกษาผลกระทบของสภาวะเครียดแบบต่าง ๆ ต่อการเจริญและการอยู่รอดของไรโซเบียมบนอาหารเลี้ยงเชื้อ รวมถึงพิจารณาผลของการเข้าสร้างปม และการตรึงไนโตรเจน เพื่อส่งเสริมการเจริญของถั่วเหลือง ผลการทดสอบพบว่า จากเชื้อที่คัดแยกได้ 20 ไอโซเลท มี 5 ไอโซเลท ที่สามารถเจริญได้ดีบนอาหารที่ใช้คัดเลือก โดยการทดสอบไอโซเลท 194 กับถั่วเหลืองที่ปลูกในสภาวะเครียดแบบต่าง ๆ ในทราย และในดิน พบว่า มีการตรึงไนโตรเจน และให้น้ำหนักแห้งของถั่วเหลืองสูงสุด อีกทั้งสามารถแข่งขันเพื่อเข้าสร้างปมกับถั่วเหลืองได้ดีกว่าเชื้อทางการค้า *Bradyrhizobium japonicum* USDA110 นอกจากนี้ การเสริมซูโครส (300 มิลลิโมลาร์) ที่เป็นสาร compatible solute ร่วมในอาหารเลี้ยงเชื้อ สามารถช่วยให้เชื้ออยู่รอด และเจริญภายใต้สภาวะเครียดได้ดีกว่าการเติมสารชนิดอื่น เมื่อเลี้ยงเชื้อในสภาวะแห้งแล้งหลังวันที่ 5 และในสภาวะกรดหลังวันที่ 3 พบว่า มีการสะสมทรีฮาโรส และกลีเซอรอลภายในเซลล์ ส่วนในสภาวะอุณหภูมิสูง ทั้งที่เสริมและไม่เสริมซูโครส พบว่า มีการสะสมกลีเซอรอลตลอดช่วงการเจริญของเชื้อ ซึ่งการสะสมน้ำตาลดังกล่าว มีความสัมพันธ์ไปในทิศทางเดียวกันกับอัตราการเจริญและการอยู่รอดของเชื้อ แสดงให้เห็นว่า ไรโซเบียมที่ทนต่อสภาวะเครียด จะอยู่รอดได้ดีขึ้น เมื่อมีการเสริมด้วยซูโครส เพราะการสะสมน้ำตาลกลีเซอรอล และทรีฮาโรสนั้น เกี่ยวข้องกับการป้องกันเซลล์ ส่งผลต่อความสามารถในการส่งเสริมการเจริญของถั่วเหลืองในสภาวะเครียดได้ดีขึ้น

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

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JENJIRA WONGDEE : ENHANCING THE EFFICIENCY OF SOYBEAN INOCULANT FOR NODULATION UNDER MULTI-ENVIRONMENTAL STRESS SOIL CONDITIONS. THESIS ADVISOR : ASST. PROF. PANLADA TITTABUTR, Ph.D., 79 PP.

Bradyrhizobium japonicum/SOYBEAN INOCULANT/SYMBIOTIC EFFICEINCY/ NODULATION/COMPETTITION/ MULTI-ENVIRONMENTALSTRESS CONDITIONS/SUPPLEMENTATION/COMPATIBLE SOLUTE

Several adverse environmental conditions are the limiting factors for soybean growth and symbiosis capability of rhizobia. The process of N_2-fixation by symbiont is strongly related to physiological development of the host plant. *Bradyrhizobium* spp. that can tolerate to environmental stress would increase soybean growth under stress conditions. This study examined the effect of single and mixed stress conditions on the growth and survival of *Bradyrhizobium* spp. in culture media, and the effect on symbiosis with soybean plant grew in the sand and soil conditions. Twenty isolates of bradyrhizobia were isolated from nodules of soybean grown in fields, and five isolates were selected based on their tolerant ability under stress conditions *in vitro* experiments. The efficiency of stress tolerant bradyrhizobia on soybean growth was investigated under various stress conditions. *Bradyrhizobium* sp. isolate 194 could promote high level of nitrogenase activity and plant biomass when plants were grown in sand and soil under stress conditions. *Bradyrhizobium* sp. isolate 194 also showed higher nodulation competition ability than *B. japonicum* USDA110 under stress conditions. Moreover, supplementation with compatible solutes was used to improve

the symbiosis efficiency of bradyrhizobial inoculant under stress conditions. The isolate 194 supplemented with sucrose showed the highest cell survival when it was cultured in medium under various stress conditions. The appropriate concentration of 300 mM sucrose could promote the cell growth under stress conditions. It was found that the bacterial cells in sucrose supplemented medium were able to accumulate trehalose and glycerol after growing under drought condition for 5 days. Trehalose and glycerol were also found to be accumulated in cell grown under acid condition after 3days. The accumulation of glycerol was found in every bacterial growth period under high temperature with and without sucrose supplementation. The accumulation of sugars inside the cell was related to cell growth and survival under stress conditions. Results of this study suggest that inoculation of stress tolerant bradyrhizobia could enhance the symbiosis efficiency and soybean growth under stress conditions and sucrose supplementation in medium could improve their survival by accumulating several types of sugar, especially glycerol and trehalose inside the cells when they were encounter various environmental stresses.

School of Biotechnology

Academic Year 2013

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Jenjira Wongdee

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LIST OF ABBREVIATIONS

°C	=	degree celcius
µm	=	micrometer
µg	=	microgram
µl	=	microlitre
ANOVA	=	Analysis of Variance
ARA	=	Acetylene Reduction Assay
cfu	=	Colony Forming Unite
DOA	=	Department of Agriculture
DAI	=	Day after inoculation
et al.	=	Et alia (and other)
g	=	gram
h	=	hour
GC	=	Gas chromatography
Kg	=	kilogram
M	=	molarity
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
MSM	=	Minimal Salt Medium
N	=	Nitrogen

LIST OF ABBREVIATIONS (Continued)

PCR	=	polymerase chain reaction
SD	=	Standard Deviation
SUT	=	Suranaree University of Technology
USDA	=	United States Department of Agriculture
YEM	=	Yeast Extract Mannitol mannitol Agar

CHAPTER I

INTRODUCTION

Soybean production in Thailand has been applied with symbiotic nitrogen fixing bacteria, *Bradyrhizobium japonicum* as biofertilizer inoculum for over the past decade. This bacterium is able to reduce atmospheric dinitrogen gas (N_2) into nitrogenous compound that can be utilized directly as nitrogen source for plant. Therefore, the application of bradyrhizobial inoculant as biofertilizer is an essential factor that can increase the soybean yield because it is cheap and environmental friendly fertilizer that can be used instead of chemical fertilizer (Kucey et al., 1988). The symbiosis interaction between soybean and *Bradyrhizobium* leads to the formation of nitrogen-fixing organ on the plant or root nodule that act as a factory of nitrogen fertilizer production. In general, inoculation of soybean with bradyrhizobial inoculant would increase the nodulation, nitrogen fixation, and soybean yield. Practically, although the effective nitrogen-fixing strain of Bradyrhizobia is used as inoculant, the plants are often fail of nodulation or produce low soybean yield. The biotic and abiotic factors have been reported in relating to reduce inoculum efficiency. Biotic factors including the amount of inoculant (McDermoti and Graham, 1990), the abundance and diversity of indigenous competitive bradyrhizobia capable of nodulating soybean in the soil, the compatibility between soybean varieties and strain of *Bradyrhizobium* (Wasike et al., 2009), and the efficiency of bradyrhizobail

inoculant (Sadowsky and Triplett, 2000) have been reported to reduce the efficiency of nodulation. On the other hand, abiotic factors involve in the environmental stresses, such as salinity, unfavorable soil pH, nutrient deficiency, mineral toxicity, extreme temperature, and soil moisture are severe factors affecting growth and competitiveness of the inoculated N_2-fixing bradyrhizobia (Dowling and Broughton, 1986; Sadowsky and Triplett, 2000; Wielbo et al., 2012). Moreover, the multi-stress conditions can be occurred especially in the field condition, such as salinity-drought, drought-high temperature, acidity-drought, acidity-high temperature, which are stronger reduces inculum efficiency than single stress conditions. Under such condition, inoculum of bradyrhizobial strain could not be expected to express its full capacity for nitrogen fixation and nodulation competition (Zahran, 1999). The rapid deaths of bradyrhizobial inoculum after appied to seed and soil are the main problem under multi-environmental stress conditions in the field.

In this research, two strategies have been proposed to improve the efficiency of soybean inoculant to be used under multi-environmental stress condition. First, the inoculation of stresses tolerant strain of bradyrhizobia may enhance the nodulation and nitrogen fixation ability of soybean under stress conditions. It has been reported that the ability of legume hosts to grow and survive in stress condition is improved when they are inoculated with stress tolerant strains of rhizobia (Wei et al., 2008). Selection of the effective, efficient, compatible and stresses tolerant rhizobial strains could help in ecological rehabilitation (restoration) of degraded soils and increases soil fertility by improving the growth of associated plants, which also influence on increase crop yield (Ali et al., 2009). Therefore, inoculation of stress tolerant

bradyrhizobium may enhance the nodulation, nitrogen fixation, and competitiveness ability under multi stress conditions.

Secondly, stress tolerant ability of rhizobia could be increased by maintaining the osmotic equilibrium across membrane through the reduction of stress factors and accumulation of compatible solutes, mainly organic osmolytes. (Poolman and Glaasker, 1998; Talibart et al., 1994a). Many of the best-characterized osmoregulatory mechanisms are designed to adjust compatible solute levels by modulating their biosynthesis, catabolism, uptake, and efflux out of cell (Kempf and Bremer, 1998; Natera et al., 2006). However, the composition of the set of endogenous compatible solutes accumulated by rhizobia varies at the species level (Fernandez-Aunión et al., 2010; Le Rudulier, 2005). Therefore, compatible solutes would be an another mechanism to improve the stress tolerance and survival of bradyrhizobial cell and finally supporting the nodulation, nitrogen fixation, and competitivness ability of inoculated bradyrhizobium under multi stress conditions.

1.1 Research objectives

a) To obtain multi–environmental stresses tolerant bradyrhizobial strain.

b) To determine the efficiency of improved bradyrhizobial inoculant on symbiosis with soybean under stress conditions.

c) To investigate the effect of compatible solutes on symbiosis with soybean under stress conditions

CHAPTER II

LITERATURE REVIEWS

2.1 Rhizobium-legume symbiosis and rhizobial inoculant

Rhizobia are gram-negative chemoheterotrophic organotroph bacilli that live freely in the soil. They are easily cultivated on medium containing carbohydrates and a considerable portion of this carbohydrate is converted to extracellular slime, which presumably help organisms for survival under extreme osmotic stress and thermal stress in the environment (Boone et al., 2005). They have symbiotic relationships with legume plants. The bacteria fix nitrogen from the atmosphere into a plant usable form. In return, the plant feeds the bacteria with sugars, proteins, and oxygen. They are capable of forming relationships with a wide variety of legumes such as alfalfa, clover, soybeans, and peas with highly specificity between legume-rhizobium. In nitrogen-poor soils, rhizobia give the advantage to their hosts, allowing them to grow in nitrogen poor soil.

The interaction between a particular strain of rhizobia and the "appropriate" legume is mediated by a "Nod factor" secreted by the rhizobia and transmembrane receptors on the cells of the root hairs of the legume. Different strains of rhizobia produce different Nod factors, and different legumes produce receptors of different specificity (Rao, 1993). If the combination is correct, the bacteria enter an epithelial cell of the root; then migrate into the cortex. Their path runs within an intracellular channel that grows through one cortex cell after another, then the infection threads

developing (Kalita et al., 2006). Meanwhile, the infection threads make their way into the nodule cells and release rhizobia into the cytoplasm of infected cells. The rhizobia, which act as symbiosomes, enlarge and differentiate into nitrogen-fixing bacteroids, and then the nitrogen fixation started. Nowadays, using of rhizobial cultures in the establishment of legumes has been widely recognized. The benefit by the use of *Rhizobium* inoculants show a good deal of money can be saved by the marginal farmers provided when they use quality tested inoculants on the farm.

In natural systems, nodulation competitiveness is most likely due to the summation of many competitive interactions including competition for: nutrients and *nod* gene-inducing flavonoids in the rhizosphere, attachment to plant root surface interaction sites, and space within nodules. Consequently, a *Rhizobium* strain must compete in several ecological areas for its occupy a majority of nodules in a field grown soybean (Sadowsky and Triplett, 2000).

The intrinsic biotic factors of *Rhizobium* strains; genetic and physiological characteristics possessed by *Rhizobium* strain; are involved in competition and successful symbiosis. “Inoculant rhizobial stains” are the effective nitrogen fixation strain which have been well screened, characterized and selected for the highest performance in nodulation. The criteria of selection are high N_2 fixation levels, able to adapt to the set of environmental conditions in each specific site and wide host range preference. “Indigenous rhizobial stains” are the native rhizobia that naturally colonize in soil and generally have high competitive ability, but ineffective in nitrogen fixation. The process how an organism becomes indigenous is not presently known. It is though that the primary or early preemptive colonization followed by prolonged periods of stable maintenance in a soil population leads to the establishment of the

"indigenous" state. Several studies suggested that a nonindigenous microbe can become a member of the indigenous, autochthonous, population by prolong and repeat applications of the microbe in the soil as inoculants (McInnes et al., 2004).

Intrinsic factors influencing competition of both inoculant rhizobia and indigenous rhizobia include: *i*) cell surface molecules *ii*) motility and chemotaxis; *iii*) production of antibiotics; *iv*) nodulation efficiency genes; *v*) speed of nodulation; *vi*) number of indigenous rhizobia. There factor could affect directly to successful nodulation of inoculant.

***i)*. Cell surface molecules**: Alterations or deletion in genes controlling cell surface characteristics influence competition for nodulation. In *S. fredii,* nonmucoid mutants of strain USDA208 are more competitive for nodulation of "Peking" soybean roots than the wild-type strain (Spaink, 1995). Moreover, transposon *Tn*5 insertion mutants of *B. japonicum* deficient in exopolysaccharide synthesis were less competitive than the wild-type strain (Chun and Stacey, 2004).

***ii)*. Motility and chemotaxis**: The motility and chemotaxis mutants have also been shown to be impaired in competition for nodulation (Pérez-Giménez et al., 2011). Zdor and Pueppke (1991), who examined nonmotile mutants of *R. meliloti* either flagellated or nonflagellated. Both types of nonmotile mutants were less competitive for nodulation than the wild-type strain, but were identical to the wild-type in growth rate and nodule formation. Althabegoiti et al. (2008) reported that a nonmotile *Tn*7 mutant of *B. japonicum* was decreased relative to the wild-type strains.

***iii)*. Production of antibiotics**: Genes encoding the production of antibiotic factors have also been shown to increase nodulation competitiveness (Sadowsky and Triplett, 2000). For example, trifolitoxin genes in *R. leguminosarum* bv. *trifolii* have

been shown to increase nodulation competitiveness of *R. etli* and presumably other rhizobia, in soil ((Chun and Stacey, 2004).

***iv)*. Nodulation efficiency genes:** In some cases, the ability of a microsymbiont to efficiently and effectively nodulate its legume host has been shown to affect competition for nodulation. Genes influencing the efficiency of nodulation, *nfe* (for nodule formation efficiency), have been identified in *R. meliloti* (Sanjuan and Olivares, 2001) and *B. japonicum* (Chun and Stacey, 2004). In *B. japonicum*, the *nfe*C gene has also been shown to influence competitiveness (Chun and Stacey, 2004). In addition, mutations in the *B. japonicum nodVW* and *R. fredii nolJ* genes have been reported to cause a delay in nodulation (Okamoto et al., 2009). In *S. fredii* (formally *Rhizobium fredii)* strain USDA257, a single, chromosomally located cultivar-specific nodulation gene, *nolC,* has been shown to control nodulation of a commercial soybean cultivar (Durán et al., 2013).

***v)*. Speed of nodulation:** Early rhizobia1 infection of legume roots induces an autoregulatory response in the plant that prevents infection by subsequent inoculations (Kalita et al., 2006). This has also been demonstrated in split-root systems in which two sides of a root are spatially separated and inoculated at different time intervals (Magori et al., 2009). Nodulation is prevented on that side of the split root that is inoculated 24 hours after the first inoculate. Suppression of nodulation increases as the time interval between inoculations of the two sides (Louis and Galinski, 1997). The suppression of late nodulation occurs at the nodule-meristem stage of development prior to nodule growth (McInnes et al., 2004). The split-root system appears to be a useful screening method for determining the competitiveness of strains (Blanco et al., 2010). However, in each of these studies, unrelated strains

were used rather than genetically defined isogenic ones. As a result, no definitive conclusion about the role of speed of nodulation in nodulation competitiveness can be ascertained. Moreover, there are some conflicting reports about the correlation between speed of nodulation and nodulation competitiveness (McInnes et al., 2004). However, these studies also used genetically unrelated strains.

***vi)*. Number of indigenous rhizobia:** Several studies have shown that number of indigenous rhizobia affect the competition of nodulation. percentage of nodules formed by the inoculant strain when indigenous strains occur at levels of only 10 rhizobia g^{-1} soil (Sanging et al., 2001; Thies et al., 2001). Frequently, introduced strains are outnumbered by indigenous soil populations by as much as 250:1. It means that the introduced strains are not evenly distributed throughout the soil (Brockwell et al., 1995), and are not well adapted to general soil conditions. On the other hand, the numbers of inoculant strain are also important in order to overcoming the indigenous rhizobia. Weaver and Frederick (1999) have estimated the nodule occupancy of inoculum strain at 50%, thus an inoculant rate of 1000 times over the indigenous soil population would be required. Singleton et al. (1992) inoculated soybeans with various mixtures of effective and ineffective strains of *B. japonicum*. As anticipated, they found that the proportion of effective nodules formed increased as the ratio of effective to ineffective bacteria in inoculant. However, the total volume of effective nodules tissue remained approximately constant. This was regarded as a 'compensatory mechanism' for keeping the amount of effective nodules tissue constant even as the proportion of effective nodules declined.

2.2 Factor affects rhizobial inoculant efficiency and symbiosis

Nodule formation by effective inoculant often fails when inocula are applied to the area. There are a lot of factors controlling this phenomenon. It can be categorized into 2 major groups.

2.2.1 Biotic factors

Rhizobium-Leguminous symbiosis is a complex biological interaction. Therefore, the success in symbiosis depends upon both sides of organisms. In relating, the successful of nodulation of *Rhizobium* mainly depends upon biotic factors which are connected to living things. It is consisted of i) leguminous plant host; ii) *Rhizobium* strains.

2.2.1.1 Leguminous plant host

In many symbiotic partnerships, the host plant exerts a major influence on initiation of symbiosis (Acuña et al., 1997; Duzan et al., 2004). Numerous studies have shown that the legume host can dramatically influence the prevalence, types and competitiveness of rhizobia in soils (Bottomley, 1992; Lohrke et al., 1995). This is thought to be in part due to host-controlled selective or restrictive nodulation mechanisms (Cregan et al., 2005), physiological differences between soybean genotypes and to differential responses of *Rhizobium* strains to *nod* gene-inducing signal molecules (Bottomley, 1992).

In addition to their ability to select specific strains of bradyrhizobia, there are several examples where the host plant restricts nodulation by specific strains or serogroups of *B. japonicum* strains. Preempting nodulation by ineffective or inefficient indigenous strains of bradyrhizobia has been proposed as a means to control competition for nodulation (Cregan et al., 2005).

Strain by cultivar (or genotype) interactions have been demonstrated in the *B. japonicum*-soybean symbiosis and this host-controlled restriction of nodulation occurs at the strain or serogroup level (Sadowsky and Graham, 1998), Lohrke et al. (1996) showed that single recessive soybean allele interact with a single *B. japonicum* gene, *noe*D interact to control selective nodulation specificity.

2.2.2 Abiotic factors

Abiotic factors are involved in several substances or environment conditions which affect the Rhizobium-legume symbiosis. The process of N_2 fixation is strongly related to the physiological state of the host plant. Therefore, rhizobial strain is not expected to express its full capacity for nitrogen fixation if limiting factors impose limitations on the vigor of the host legume (Brockwell et al., 1995; Peoples et al., 1995). Some of these factors might directly affect competitiveness, many most likely act by altering the persistence and survival of inoculated. Abiotic factors have reported to influence the competition including: *i)* temperature; *ii)* plant and microbial nutrient limitations and requirements; *iii)* soil moisture; *iv)* soil pH; and *v)* soil salinity.

2.2.2.1 Temperature

High soil temperature in tropical regions is one of the major constrains for biological nitrogen fixation in legume crops. Temperatures in these regions average above 40ºC (Marsh et al., 2006; Yakubu et al., 2010) may affect symbiotic relationships, nitrogen content and plant production For example, Stefan et al. (2010) tested the beneficial effects of various bacterial strains on soybean growth and physiology under suboptimal root zone temperatures, and found that bacterial stimulation is interactively dependent on temperature. It has often been claimed that

growth-promoting effects are caused by the bacterial nitrogen-fixing activities, but in this case, positive effects on the plant's physiology were detected before start of nitrogen fixation, indicating that mechanisms independent of nitrogen status are involved. Soil temperature has been shown to greatly influence the growth and survival of rhizobia in soil and competition for nodulation (Sadowsky and Triplett, 2000). Kennedy and Wollum (1988) reported that population levels of *B. japonicum* decreased in soils that were exposed to elevated temperature, as well as Montanez et al. (1995) reported the alter competitiveness of *B. japonicum* in response to soil temperature. High temperatures above 35◦C decrease nodule weight and number, nitrogenase activity, and shoot-dry matter production in soybean(Munévar and Wollum, 1981), pigeon pea, cow pea (Marsh et al., 2006) and leucena (Hashem et al., 1998).

2.2.2.2 Plant and microbial nutrient limitations and requirements

A variety of nutritional factors are influence the growth of rhizobia in the rhizosphere, in some instances directly affect competitive interactions. Brockwell et al. (1995) have reviewed the nutritional factors influencing the ecology of rhizobia in soil. *Bradyrhizobium* is fairly metabolically diverse and has been shown to use a variety of plant-derived compounds for growth. Some compounds have been shown to be chemotactic and induce *nod* genes in *B. japonicum* (Graham et al., 1994; Sadowsky and Triplett, 2000) Metabolic engineering of rhizobia using specific host-derived nutritional factors in the rhizosphere, e.g. rhizopines produced in a "biased rhizosphere, has been proposed as one means to alter competitiveness (Mansouri et al., 2002).

2.2.2.3 Soil moisture

Symbiotic N_2 fixation of legumes is also highly sensitive to soil water deficiency. A number of temperate and tropical legumes, e.g., *Medicago sativa*, *Pisum, Arachis hypogaea, Vicia faba* (Devries et al., 1989) exhibit a reduction in nitrogen fixation when subject to soil moisture deficit. Athar and Johnson (1996) reported that two mutant strains of *R. meliloti* were competitive with naturalized alfalfa rhizobia and were symbiotically effective under drought stress. These results suggest that nodulation, growth, and N_2 fixation in alfalfa can be improved by inoculating plants with competitive and drought-tolerant rhizobia (Russelle, 2004). Mild water stress reduces the number of nodules formed on the roots of soybean, while moderate and severe water stress reduces both the number and size of the nodules (Ramos et al., 1999). In general the increase in drought condition of PEG concentration from 0-30% proportionally decreased the nodulation, nodule ARA and nodule N content of soybean plants among the four isolates tested Bradyrhizobium strain, SBJ-14, SBJ-2, SBJ-23 and SBJ-10 (Uma et al., 2013).

2.2.2.4 Soil pH

For bradyrhizobia and rhizobia, competitive interactions have been shown to be influenced by soil pH (Sadowsky, 2000; Graham, 1997), due to *Rhizobium* strains are unable to grew in culture media at pH 5.0, while *Bradyrhizobium* sp. are able to tolerate pH4.5 (Brockwell et al., 1991). Neelawan and Achara (2007) noted that nodulation and nitrogen fixation by some strains of *Bradyrhizobium* at acidic pH differ with the cultivar of mung bean used. Vargas and Graham (1989) examined the cultivar and pH affects on competition for nodule sites among isolates of *Rhizobium* in beans (*P. vulgaris*) under acidic conditions. They

found a significant effect of host cultivar, ratio of inoculation, and pH on the percentage of nodule occupancy by each strain. Low soil pH is estimated that about 30% of the world's land surface is acidic (pH , 5.5), including an extensive 40% of arable land (Meghvansi et al., 2005).These poor growth conditions lead to reductions in root development and nodulation and compromise nutrient transport (Zahran, 1999). This results in yield losses of more than 50% in grain crops, such as wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), and in many legume crops including common bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*), and pea *Pisum sativu* (Vejsadova et al., 1993).

2.2.2.5 Soil salinity

Salinity affects the competition due to the reduction of survival rate of inoculum. Unsuccessful symbiosis under salt-stress may be due to failure in the infection process because of the effect of salinity on the establishment of rhizobia (Elsheikh and Wood, 1990b). However, Chien et al. (1992) have shown that high salt tolerant rhizobial strains are could symbiosis more efficient than salt sensitive ones under salt stress.

2.3 Compatible solutes as osmoprotectants for cell protection and survival of rhizobia under abiotic stress conditions.

2.3.1 Compatible solute

Normally, compatible solutes are polar small organic osmolytes and highly soluble molecules that usually do not carry a net electrical charge at physiological pH, including sugar (trehalose and sucrose), polyols (mannitol), amino acids (glutamate), quaternary ammonium compounds (glycine bataine, proline

bataine, choline, and choline-O-sulphate), sulphonuim compounds (dimethyl-sulphoniopropionate, DMSP), ectoine, and a small peptide (N-acetylglutaminyl-glutamine amide, NAGGN) (Fig. 1) (Kurz, 2008).

These compatible solute molecules are compatible with cellular metabolism to increase the intracellular osmotic pressure, restore the turgor, and protect some macromolecular structure against denaturation (Chen et al., 2007). Compatible solutes are mainly found in microorganism, such as *Archaea*, *Bacteria*, and *Eucarya*, and also found in higher organism, and are used in a broad of applications (Le Rudulier, 2005). Previously, most studies were initiated with the compatible solutes accumulation by halophillic/osmophillic bacteria and yeast, their bioengenetics and relevance for bio-remediation. Recently, it was found that the accumulation of compatible solutes act as a natural component of food traditionally processed by microorganism. However, remarkable feature of some rhizobia is the ability to use a large rang of compatible solutes not only as osmoregulate but also as carbon and nitrogen sources (Talibart et al., 1994b). A number of these compounds found in the cytoplasm under stress conditions could support the function agent of osmotic balancing, which is main function of compatible solutes as along with the compound theoretical in osmotic protection (Le Rudulier, 2005).

2.3.2 Function of compatible solute under salinity

General responses of rhizobia to NaCl differ in their ability to respond to an increase in osmotic pressure and salt stress. A model can be derived from several studies and is similar to the response of enteric bacteria (Miller and Wood, 1996; Wei et al., 2008). Generally, the metabolism of bacteria is slow down after an osmotic upshift, (Domínguez-Ferreras et al., 2006). This was shown by the findings of

Domínguez-Ferreras et al. (2006) reported that genes involved in the tricarboxylic acid cycle, in the uptake of a carbon source (they used mannitol), and in respiratory chains and ribosomal genes were repressed. Interestingly, 25% of all genes specifically down regulated by NaCl, encoded ribosomal proteins. On the other hand,

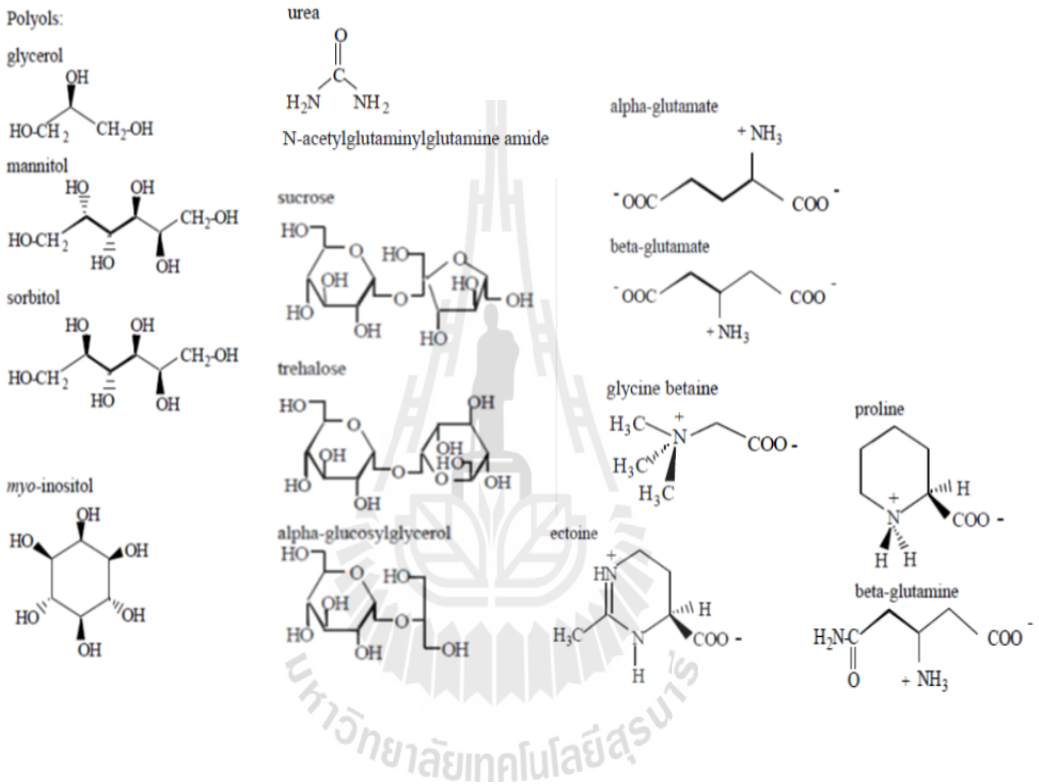


Figure 1 Example of compatible solutes that can be accumulated in rhizobia under stresses condition.

It has been reported that rhizobia accumulate potassium ions without new protein synthesis is required under salt stress condition (Botsford and Lewis, 1990). This proposes that K uptake is biochemically regulated and used as a secondary messenger. Nogales et al. (2002) reported a high-affinity K uptake (Kup) system in *R. tropici* that has a homolog to *S. meliloti* 1021 (SMa1798), while a second, low-affinity

Kup system (SMc00873), as well as the osmosensitive Kdp system (SMa2329, SMa2331, and SMa2333) could be identified. BetS is a betaine/proline transporter which also involved in the early response to osmotic stress. As with K uptake activity, BetS is biochemically regulated (Boscari et al., 2002; Oteras et al., 1998; Pocard et al., 2000).

The accumulation of compatible solutes in bacteria mostly can be accumulated to high level by *de novo* synthesis or transport from the environment. These solutes are often accumulated in the cytoplasm of stressed cell. However, the solutes were added to the medium that decrease the inhibitory effect of osmotic stress on bacteria when they add to the culture medium, are called osmoprotectants (Poolman and Glaasker, 1998). On the other hand, compatible solutes can be accumulated for alleviate the inhibitory stressed such as temperature, salinity and desiccated in some bacteria at high level when culture with the media properly. In 1998, Pedro and colleagues have examined the effects of temperature, salinity, and medium composition on compatible solute accumulation by *Thermococcus* spp. The bacteria growth phase had a strong influence on the type and level of compatible solutes. Mannosyl glycerate and aspartate were the major solutes during exponential growth, while di-myo-inositol-1,1'(3,3')-phosphate was the predominant organic solutes during the stationary phase when cultured on medium supplemented with 4% NaCl at 85˚C. (Deaker et al., 2007) have studied the influence of polymers on desiccation tolerance of rhizobia. Polymer is one of compatible solutes that are commonly used to improve the adhesion of carrier with rhizobial inoculants and seed surface.

2.3.3 Function of compatible solute under nutrient starvation

Under growth-limiting conditions, C sources accumulate in the form of glycogen, which may assist in restoring cell volume after osmotic shock (Han et al., 2005) This is supported by the finding of *glgA2*, *glgB2*, and *glgX*, genes which involved in glycogen metabolism (SMb20704, SMb21447, and SMb21446, respectively), are expressed at higher levels during exposure to osmotic stress. It could be indicated that glycogen was accumulated during osmotic stress.

However, the accumulation of glycogen may also be a response to prevent starvation (Domínguez-Ferreras et al., 2006). After these initial the accumulated reaction, stressed cells accumulate compatible solutes, and uptake of carbohydrates, disaccharides were preferred over synthesis. Not all compounds are taken up from the medium when available, but some are synthesized *de novo*, for example, sucrose, and trehalose (Gouffi et al., 1998; Miller and Wood, 1996); However, a conflict that trehalose and glycine betaine are accumulated to prevent starvation rather than to function as osmotic stabilizers (Vriezen et al., 2007).

2.3.4 Function of compatible solute under desiccation

The accumulation of osmoprotectants and compatible solutes may also increase cell survival during desiccation. For example, betaine increased survival of *Rhizobium* during desiccation in peat cultures and reduces the negative effects of NaCl that were observed under certain conditions (Pocard et al., 1989; Rasanen et al., 2004). Sixty percentages of the betaine accumulation during osmotic stress through the betaine transporter BetS (Boscari et al., 2002), making this locus especially interesting in understanding the early responses to NaCl and desiccation survival. Genetic mechanisms that support the accumulation of betaines have been identified as

betaine/choline uptake or synthesis operon (SMc00093 to 00095 and SMc00127) as well as *betP* (SMb20333) and *betB2* (SMa1731).

The accumulation of compatible solutes by synthesis and catabolism in most bacteria is rare appearance during growth in normal conditions. However, stress conditions are specific factors for bacteria to induce in various solute productions in order to survive and protected the cell from environment stresses. Thus, accumulation of compatible solutes would be the protection mechanisms that can promote bacteria grow under stresses condition and enhance the efficiency of rhizobial inoculant.

CHAPTER III

MATERIALS AND METHODS

3.1 Collected locations and soil properties

The acidic soil sample was collected from Nong Sua District, Phathum Thani Province (14°8′6″N and 100°49′27″E), while the neutral soil sample used as control condition was collected from Suranaree University of Technology organic farm, Muang District, Nakhon Ratchasima Province (14° 52′N and 102° 01′E), which has no history of leguminous cultivation. Finally, the selected bradyrhizobium was investigated its symbiotic efficiency in the representative soils collected from some parts of Thailand; Suphan Buri Province (14°24′8″N 100°9′16″E), Phetchaburi Province (12°47′59″N 99°58′1″E), and Yasothon Province (15°47′41″N 104°8′26″E). The soil characteristics properties were reported in Table 1.

Table 1 Data of soil example analyses collected from five locations of Thailand.

Soil samples (Province)	**pH**	**% OM**	**EC (mS/cm)**	**P (ppm)**	**K (ppm)**	**Ca (ppm)**	**SO_2^{-4}(S/kg)**
Phathum Thani	4.40	1.83	3.30	21.96	418.56	3,878.00	725.00
Nakhon ratchasima	6.33	1.34	0.12	27.80	92.00	1340.00	51.22
Suphan Buri	5.22	3.56	1.91	40.70	26.00	1635.80	-
Phetchaburi	6.53	1.74	0.70	4.25	146.50	1040.00	-
Yasothon	6.95	3.34	0.29	12.40	41.50	1416.30	-

3.2 Bradyrhizobial strains and culture

Bradyrhizobial strain used for soybean inoculation including twenty isolates and the reference strains of *Bradyrhizobium japonicum* USDA110, *Bradyrhizobium* sp. DASA1014, and *Bradyrhizobium* sp. CB1809 that were obtained from Department of Agriculture (DOA), Thailand. Those bacteria were extracted the DNA to determine that closely related strain with the commercial strains. The Box-PCR (Schneider and De Bruijn, 1996), and dendrogram analysis (Quantity One® Version 4.6.3 Windows and Macintosh) was performed to investigate the DNA fingerprint profile and the unique profiles were selected for further test. Bradyrhizobia were grown at 28˚C on the yeast extract-mannitol (YM) agar plates containing congo red (pH 6.8) (Somasegaran and Hoben, 1994) and maintained at 4˚C.

3.3 Screening stress tolerant strains

To screen the stress tolerant bradyrhizobial strains *in vitro* condition, the cell cultures were washed by normal saline twice and 10 µl cultures were dropped onto YM agar medium which was adjusted in different stress conditions. For acid stress; YM agar medium was prepared at pH 4, 5, 6 and 6.8 and the 0.5 ml/L of 8 mM bromthymol blue buffer was added as pH indicator. Then, plates were incubated at 28˚C until 1 week. For the high temperature stress; YM agar medium was prepared at pH 6.8, and plates were incubated at 28, 35, 40, and 45˚C until 1 week. Drought stress; the 10^8 cfu/ml bacterial cells were overlaid on 0.2 µM filter membrane were incubated in desiccator chamber adjusted to have different desiccated condition by using silica gel, saturated $CH_3COOK.5H_2O$, $K_2CO_3.2H_2O$, and KI solutions to give the R.H. values (at 30 °C) of 3, 22, 43.6, and 67.8%, respectively (Boumahdi et al., 1999). The level of growth abilities were determined in acid and high temperature

stress conditions, while the % survival of cell was determined under drought stress condition.

3.4 Plant growth conditions

3.4.1 Experiment in sand

Surface sterilized soybean (*Glycine max* (L.) Merr.) variety Chiengmai 60 seeds were germinated and transferred to Leonard's jars containing 0.35 kg sterilized sand. Bradyrhizobial isolates were washed and inoculated with 10^8 cells/seed. Plants were watered with N-free solution (Somasegaran and Hoben, 1994) and grown at 25°C on 12/12 day/night cycle with light intensity 639 $\mu E/m^2/s$ as normal condition, while other stress conditions were adjusted as followed. Acid stress condition; N-free solution was adjusted to pH 4.5. High temperature stress condition; plants were grown in growth chamber (Contherm's Biosyn Series of Tissue and Plant Growth Chambers-620RHS P6 Models, Wellington, New Zealand) at 40˚C. Drought stress condition; sand was desiccated at -0.32 bars by using polyethylene glycol (PEG) 8000 solution. Acid-high temperature stress condition; N-free solution was adjusted to pH 4.5 and plants were grown at 40°C. Acid-drought stress condition; N-free solution was adjusted to pH 4.5 and plants were grown in sand which was desiccated at -0.32 bars by using polyethylene glycol (PEG) 8000 solution. Data of nitrogen fixation, number of nodule, plant biomass, and nodule dry weight were collected at 30 DAI.

3.4.2 Experiment in soil

Surface sterilized soybean seeds were placed into the pot containing 2 kg sterilized soil collected from 2 locations as indicated above. Seed was inoculated with bradyrhizobial strains at 10^8 cells/seed. Soybeans were grown in the same condition as

described in the experiment in sand excepted watering with sterilized tap water. The neutral soil from SUT organic farm was used for normal, high temperature stress, and drought stress conditions, while the acidic soil from Patum tani province was used for mixed stress of acid-high temperature and acid-drought conditions. Data of nitrogen fixation, number of nodule, plant biomass, and nodule dry weight were collected at 30 DAI.

3.5 Determination of stress tolerance index (STI)

Stress tolerance indices (STI) of inoculated and non-inoculated plants were determined according to Shetty et al. (1995) as: STI = DWS or DWH/DWC (where, DWS = dry weight of plant grown under stress, DWH = dry weight of plant grown under stress with inoculation of bacteria, and DWC = dry weight of plant grown in control condition (without stress and inoculation of bacteria).

3.6 Cell survival under stresses conditions

Ten grams of sterilzed sand were added into test tube (50 ml) and pH was adjusted to 7.0, while other stress conditions were adjusted as followed. Acid stress condition; sand pH was adjusted to 4.5. High temperature stress condition; sand was incubated at 40°C. Drought stress condition; sand was desiccated to -3.02 bars by using PEG8000. Acid-high temperature condition; sand pH was adjusted to 4.5 and incubated at 40°C. Acid-drought stress condition; sand pH was adjusted to 4.5 and desiccated to -3.02 bars by using PEG8000. The 10^8 cells of bradyrhizobium were inoculated into prepared sand tube in each condition and incubated for 2 days. The survival of cell under stress condition was investigated by serial dilution and total

plate count. The number of living cell was determined as colony forming unit/g sand (Idris et al., 2007), and the % survival of cell in stresses condition were calculated related to the initial cell number.

3.7 Nodulation competition test

The pCAM120, Tn5 fusion with *gus* gene, encoded the enzyme β-glucuronidase (GUS) (Wilson et al., 1995), was transformed into *B. japonicum* USDA 110. In Leonard's jar experiment, surface sterilized soybean seeds were co-inoculated with stresses tolerant bradyrhizobia and GUS-marked USDA110 in ratio of 1:1 at 10^8 cells/seed and watered with N-free medium. Plant growth conditions were adjusted in single stress and mixed stresses conditions as described above. Each condition was conducted in triplicates. After 1 month, soybean nodules were collected and cut in half followed by staining with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as a substrate according to method of Krause et al. (2002). The blue and white color nodules were observed and the percentage of nodule occupancy was determined as described by Payakapong et al. (2004).

3.8 Growth assay with compatible solute

For bacterial growth assay in medium containing compatible solutes, bradyrhizobia were aerobically grown at 28˚C in mannitol salts yeast extract medium (MSY rich medium) (Gouffi et al., 1999) for 24 h as starter. Then, 1% (v/v) of starter culture was separately inoculated into minimal broth medium (MSM) (Talibart et al., 1994a) containing each tested compatible solutes, including glucose, trehalose, mannitol, sucrose, glycerol, and PVA at final concentration of 0, 5, 10, 100, 300, 400

and 500 mM (Gouffi et al., 1999; Le Rudulier, 2005; Talibart et al., 1994a). The pH of culture was adjusted to 6.8 and incubated at 28°C for normal condition growth, while other different stress conditions were applied as follow; Acid stress; medium was adjusted to pH 4 and incubated at 28°C. High temperature stress; medium was adjusted to pH 6.8 and incubated at 40°C. Drought stress; medium was adjusted osmotic pressure to -3.02 bars by using PEG8000 and incubated at 25°C. The number of cell and the bacterial growth rate were evaluated. The appropriate compatible solute was selected and the concentration was optimized to promote the growth of bradyrhizobia under stress conditions.

3.9 Extraction of compatible solute

The cell pellets were precipitated by centrifugation at 4,000 rpm for 5 min. The intracellular compatible solutes of bacterial cell were extracted twice by incubating at 65°C for 5 min in 1 ml of 70% (v/v) ethanol-water. Crude extracts were centrifuged at 5,000×g for 5 min (Lai et al., 1991), ethanol was evaporated by rotary evaporator (Buechi R-142, Germany - Nordrhein-Westfalen) at 45 °C.

3.10 Analysis of compatible solutes by High Performance Liquid Chromatography (HPLC)

The bacterial cell extracted were dissolved in 1ml deionized water and filtered through a 0.2 µm hydrophobic membrane syringe filter and immediately injected into the HPLC. The sugars were determined by an ion exchange column (Aminex HPX-87H, 7.8x300 mm, Bio-Rad, Germany) at 45˚C and a refractive index detector (RI-150, Thermo Spectra System, USA). Mobile phase was 4 mM sulfuric acid at a flow

rate of 0.4 ml/min (Sangproo et al., 2012) for sugar analyses, while the condition of 0.3 ml/min flow rate and column temperature at 60˚C were used for sucrose analyses.

3.11 Testing symbiosis efficiency of bradyrhizobial inoculant that supplemented with compatible solute

The symbiosis efficiency tests were performed both in sterilized sand experiment and in the representative soils collected from some parts of Thailand. Selected bradyrhizobium isolate 194 was grown in YM medium with and without supplementary of appropriate concentration of selected compatible solute (300 mM sucrose) before inoculating to soybean under normal and stress conditions as described above for sand and soil pot experiment. After 30 DAI, the number of nodule, plant biomass, and nodule dry weight were evaluated.

3.12 Statistical analysis

Data in all experiments were resolved into elements as mean values and standard deviations with SPSS software (SPSS versions 17.0 Windows; SPSS Inc., Chicago, IL) by Duncan's multiple range test (Duncan, 1955).

CHAPTER IV

RESULTS

4.1 Growth of isolated bradyrhizobia under stress conditions

Among the total of 20 bradyrhizobial isolates, seven isolates could perform well nodulation with soybean and be able to fix nitrogen (data not shown). These strains were used for testing their growth performance on agar medium adjusted to acid, drought, and high temperature stress conditions. The investigation of acid condition showed that the reference strain CB1809 and ding isolates 188 and 197 had highest average growth score when compared to the commercial strain *Bradyrhizobium japonicum* USDA110. In high temperature stress condition, every strain except isolate 199 showed higher growth score when compared to USDA110, while CB1809 and isolate 188 had highest average growth score at this condition. Under drought condition, CB1809 and isolate 194 exhibited the highest survival and significant difference when compared to the other strains (Table 2).

However, the result of DNA polymorphism by using BOX-PCR and dendrogram analysis indicated that the reference strains CB1809, DASA1014, isolates 184, 193 were closely related to each other (Figure. 2). Thus, isolates 184, 188, and 194 were selected for the further experiments. However, isolate 199 performed less stress tolerance than other. There, isolate 199 was also selected as negative control throughout experiments.

Table 2 Growth and survival ability of isolated bradyrhizobia under *in vitro* stress conditions.

Isolates	Growth score*								% Survival of bacteria under drought stress			
	Acidity				High temperature							
	pH4	pH5	pH6.8	**Average****	30˚C	40˚C	45˚C	**Average**	3%RH	20%RH	67%RH	**Average**
U110	1	1	3	1.67[c]	3	1	0	1.33[d]	7	23	100	43[a]
CB1809	1	3	3	2.33[a]	3	2	2	2.33[a]	11	27	100	46[a]
184	1	1	3	1.67[c]	3	1	1	1.67[c]	7	11	85	34[b]
188	1	3	3	2.33[a]	3	2	2	2.33[a]	10	15	100	42[a]
193	1	2	3	2[b]	3	1	1	1.67[c]	10	20	100	43[a]
194	1	2	3	2[b]	3	2	0	1.67[c]	15	29	100	48[a]
197	1	3	3	2.33[a]	3	2	1	2[b]	9	13	94	39[b]
199	0	0	3	1[d]	2	1	0	1[d]	1	5	56	21[e]

*Growth was scored using a numerical rating of 0 = no growth, 1 = poor growth, 2 = good growth and 3 = very good growth.

**Means are calculated from three replicates, and values with different letters in the same column are significant difference at $P \leq 0.05$.

A. B.

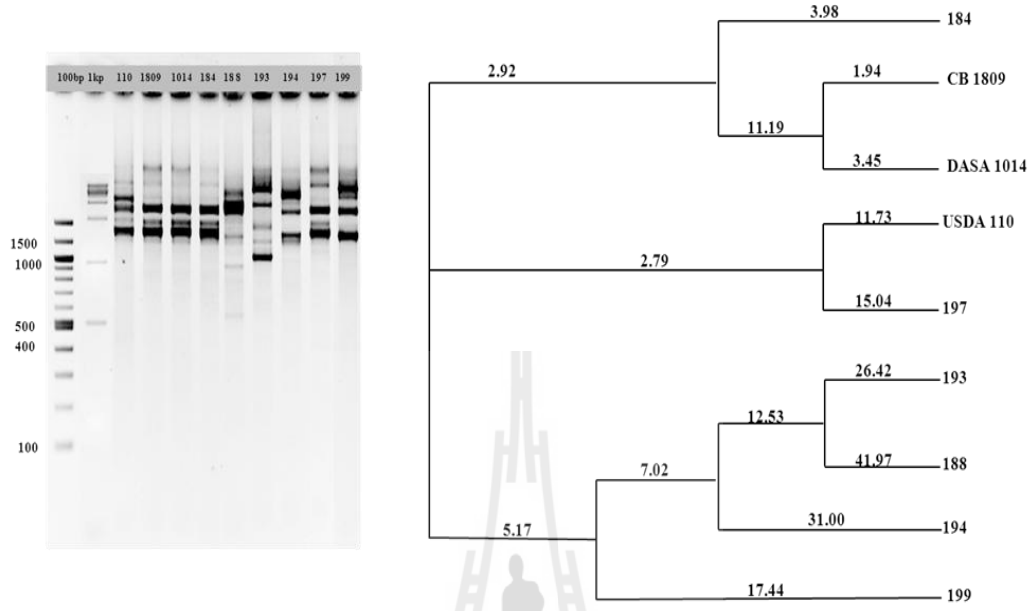


Figure 2 DNA polymorphism of isolated bradyrhizobia (isolates 184, 188, 193, 194, 197) and reference strains (CB1809, DASA 1014, USDA 110) by using Box-PCR. (A), dendrogram analysis (B).

4.2 Plant growth promotion under single and mixed stress conditions

4.2.1 Experiment in Leonard's jar containing sterilized sand: Soybean inoculated with USDA110, isolates 184, 188, and 194 had no statistical significant difference in nitrogenase activity, nodule number, and biomass dry weight when grown under normal condition (Table 3). In acid condition (pH 4.5), the overall nodulation ability, and plant growth were reduced when compared to normal condition (pH 7). The nitrogenase activity of plants inoculated with isolate 194 was higher than other strains under acid stress condition. However, the nitrogenase activities as well as biomass, nodule dry weight, and number of nodule were not

significantly different from plants inoculated with USDA110. Under drought stress conditions, plants inoculated with isolates 184 and 194 promoted high level of nitrogenase activity which correlated to high biomass dry weight under -3.02 bars which were significantly different from USDA110. However, nodule dry weight and nodule number produced by isolate 194 were not different from those of UADA110 inoculations. The experiment under high temperature stress condition showed higher nitrogenase activities and biomass dry weight when plant inoculated with isolate 194 but not significant difference from USDA110. Under the mixed stresses condition of acidity and drought, isolate 194 and USDA110 showed high level of nitrogenase activity and significantly different from isolates 184 and 188 although these isolates provided higher nodule number than isolate 194 and USDA110. However, the plant biomass and nodule dry weight were not significantly different among the strains in this condition. Under the mixed stresses condition of acidity and high temperature, although the plant inoculated with isolate 194 showed highest performance in symbiosis, these values were not significantly different from those inoculated with USDA110 (Table 3). The effectiveness of each bradyrhizobium was calculated as the stress tolerance index (STI) by using biomass of inoculated soybean plant in relation to with the nitrogenase activity, nodule number and nodulation ability. STI of bradyrhizobial inoculated plants were significant difference from uninoculated soybean plant (Table 4). It was indicated that soybean plants biomass could be increased under normal or stress conditions by using bradyrhizobial inoculation.

Under single and mixed stress conditions, plant inoculated with isolate 194 gave highest STI values in every treatment. Under drought and mixed-stress of acidity and drought condition, the STI of plant inoculated with isolate 194 were 2.09 and

2.26, respectively, which were significantly different when compared to the plant inoculated with USDA110. However, there was no significant difference of STI among plants inoculated with all bradyrhizobial isolates under high temperature stress condition, while isolate 194 produced highest STI under mixed stress of acidity and high temperature but it was not significantly different from USDA110inoculation (Table 4). These results indicated the influence of bradyrhizobial strain on overall efficiency of inoculant when apply under different stress conditions.

Table 3 Symbiotic efficiency of isolated stress tolerant bradyrhizobia with soybean growing in sand pot under stress conditions.

Conditions*	**Isolates**	**Acetylene reduction (μmole h^{-1} g $nodule^{-1}$)**	**Biomass dry weight (g $plant^{-1}$)**	**Nodule dry weight (g $plant^{-1}$)**	**Nodule number ($plant^{-1}$)**
Normal	Uninoculated	-	0.53±0.15 [b]	-	-
	USDA 110	**70.01±20.51 [ab]	0.91±0.20 [a]	0.06±0.02	19±3
	184	35.01±6.00 [b]	0.83±0.11 [a]	0.07±0.01	24±2
	188	55.45±12.22 [ab]	0.80±0.15 [ab]	0.05±0.01	23±7
	194	89.21±12.43 [a]	0.89±0.137 [a]	0.06±0.01	20±6
%CV		20.49	18.80	20.80	20.90
Single stress					
Acidity	Uninoculated	-	0.24±0.03 [c]	-	-
	USDA 110	45.76±8.85 [ab]	0.55±0.1 [ab]	0.03±0.01	10±2 [a]
	184	22.59±2.93 [b]	0.47±0.11 [b]	0.02±0.01	9±2 [ab]
	188	33.69±5.08 [b]	0.51±0.05 [ab]	0.04±0.00	11±2 [a]
	194	60.17±9.42 [a]	0.59±0.10 [a]	0.04±0.01	9±3 [ab]
Drought	Uninoculated	-	0.14±0.02 [c]	-	-
	USDA 110	27.79±6.88 [b]	0.17±0.03 [b]	0.08±0.01 [a]	12±2 [a]
	184	64.22±6.15 [a]	0.27±0.03 [a]	0.03±0.02 [b]	7±2 [b]
	188	22.91±3.90 [b]	0.15±0.03 [c]	0.02±0.03 [b]	7±2 [b]
	194	48.10±6.74 [ab]	0.30±0.10 [a]	0.08±0.05 [a]	12±3 [a]

* The plant growth conditions, Normal; pH 7 at 25D/25N°C, Acidity; pH 4.5 at 25D/25N°C, Drought; pH 7 and -3.02 bars at 25D/25N°C, High temperature; pH 7 at 40D/28N°C, Acidity + Drought; pH4.5 and -3.02 bars at 25D/25N°C, and Acidity + High Temperature; pH4.5 at 40D/28N°C.

**Means and standard deviations are calculated from three replicates, and values with different letters in the same column in each condition are significant difference at $P \leq 0.05$.

Coefficient of variation =CV

Table 3 Continued

Conditions*	Isolates	Acetylene reduction (µmole h^{-1} $g\ nodule^{-1}$)	Biomass dry weight (g $plant^{-1}$)	Nodule dry weight (g $plant^{-1}$)	Nodule number ($plant^{-1}$)
High temperature	Uninoculated	-	0.14±0.04 ^{c}	-	-
	USDA 110	88.37±16.06 ^{a}	0.19±0.02 ^{ab}	0.02±0.00 ^{ab}	8±1 ^{a}
	184	27.51±10.41 ^{bc}	0.18±0.03 ^{b}	0.04±0.01 ^{a}	5±1 ^{b}
	188	50.94±14.91 ^{b}	0.20±0.02 ^{a}	0.03±0.00 ^{ab}	7±1 ^{ab}
	194	96.36±18.16 ^{a}	0.25±0.01 ^{a}	0.03±0.01 ^{ab}	10±1 ^{a}
%CV		17.70	10.30	16.90	19.90
Mixed stress					
Acidity+Drought	Uninoculated	-	0.08±0.01 ^{b}	-	-
	USDA 110	71.03±12.87 ^{a}	0.14±0.02 ^{a}	0.01±0.00 ^{ab}	8±1 ^{b}
	184	25.64±6.22 ^{b}	0.17±0.01 ^{a}	0.01±0.00 ^{a}	10±2 ^{a}
	188	39.43±4.32 ^{b}	0.19±0.01 ^{a}	0.02±0.00 ^{a}	11±1 ^{a}
	194	67.65±8.38 ^{a}	0.19±0.04 ^{a}	0.01±0.00 ^{a}	8±2 ^{b}
Acidity+High temp.	Uninoculated	-	0.12±0.00 ^{ab}	-	-
	USDA 110	54.81±12.30 ^{ab}	0.14±0.01 ^{ab}	0.04±0.01 ^{a}	9±0 ^{ab}
	184	60.66±9.42 ^{a}	0.16±0.07 ^{a}	0.03±0.00 ^{b}	8±0 ^{ab}
	188	40.48±4.32 ^{ab}	0.13±0.01 ^{ab}	0.028±0.00 ^{b}	8±1 ^{ab}
	194	65.21±8.99 ^{a}	0.17±0.01 ^{a}	0.038±0.00 ^{a}	12±2 ^{a}
%CV		15.70	14.80	5.45	12.10

* The plant growth conditions, Normal; pH 7 at 25D/25N°C, Acidity; pH 4.5 at 25D/25N°C, Drought; pH 7 and -3.02 bars at 25D/25N°C, High temperature; pH 7 at 40D/28N°C, Acidity + Drought; pH4.5 and -3.02 bars at 25D/25N°C, and Acidity + High Temperature; pH4.5 at 40D/28N°C.

**Means and standard deviations are calculated from three replicates, and values with different letters in the same column in each condition are significant difference at $P \leq 0.05$.

Coefficient of variation =CV

Table 4 Stress tolerance index (STI) of stressed soybean plant inoculated with and without bradyrhizobia growing in sand pot under different stress conditions.

Conditions	Stress Tolerance Index (STI)				
	Uninoculated	**USDA 110**	**184**	**188**	**194**
Normal	1.00±0.00^{b}	1.70±0.23^{a}	1.55±0.40^{ab}	1.52±0.06^{ab}	1.66±0.04^{a}
Single stress					
Acidity	1.00±0.00^{c}	2.31±0.39^{a}	1.97±0.20^{b}	2.13±0.08^{b}	2.46±0.33^{a}
Drought	0.99±0.05^{c}	1.23±0.11^{bc}	1.93±0.41^{ab}	1.05±0.06^{c}	2.09±0.10^{a}
High temperature	0.99±0.02^{b}	1.43±0.14^{a}	1.46±0.11^{a}	1.22±0.11^{a}	1.80±0.53^{a}
Mixed stress					
Acidity+Drought	1.00±0.00^{c}	1.67 ±0.31^{b}	2.01±0.21^{ab}	2.21±0.54^{a}	2.26 ±0.23^{a}
Acidity+High temp.	1.00±0.00^{c}	1.23 ±0.22^{ab}	1.38 ±0.05^{ab}	1.12±0.09^{b}	1.45±0.25^{a}

* Means and standard deviations are calculated from three replicates of shoot dye weight in the same row followed by different letters are significant difference at $P \leq 0.05$.

4.2.2 Experiment in pot containing sterilized soil: Bradyrhizobia isolates 188 and 194 were selected for further test upon the performance on legume symbiosis compared with USDA110 in sterile soil under different stress conditions. High nitrogenase activity was obtained from plant inoculated with isolate 194 in all tested conditions and its nitrogenase activity 194 was significantly different from nitrogenase activity of USDA110 and isolate 188 under drought stress condition (Table 5). However, the nitrogenase activity, plant biomass, nodule number, and nodule dry weight were not significantly different when compared among the inoculated plants grown in soil pot under other stress conditions. The STI of plant grown in soil experiment indicated significant difference in plant inoculated with bradyrhizobia and uninoculated plant under drought and mixed stress of acidity-drought and acidity-high temperature stresses conditions (Table 6). There were no

statistically significant differences of STI between plant inoculated with isolates 194 and USDA110 in all stress conditions. However, the STI of plant inoculated with isolate 188 was significantly lower than that of isolate 194 under drought and mixed stress of acidity-drought conditions. From the result of overall experiments, isolate 194 was the best candidate strain and was selected for further experiment.

Table 5 Symbiotic efficiency of isolated stress tolerant bradyrhizobia with soybean growing in soil pot under stress conditions.

Conditions*	**Isolates**	**Acetylene reduction (μmole h^{-1} $gnodule^{-1}$)**	**Biomass dry weight (g $plant^{-1}$)**	**Nodule dry weight (g $plant^{-1}$)**	**Nodule number ($plant^{-1}$)**
Normal	Uninoculated	-	0.46±0.00^{b}	-	-
	USDA 110	107.50±6.97^{ab}	0.52±0.05^{a}	0.024±0.00	21±10^{a}
	188	66.51±20.79^{b}	0.63±0.04^{a}	0.036±0.01	17±2^{b}
	194	118.19±20.06^{a}	0.56±0.12^{a}	0.022±0.00	21±7^{a}
%CV		16.3	9	10.9	32.2
Single stress					
Acidity	Uninoculated	-	0.49±0.01^{b}	-	-
	USDA 110	143.19±23.38^{ab}	0.57±0.11^{a}	0.01±0.00^{b}	9±2^{b}
	188	98.31±20.45^{b}	0.66±0.15^{a}	0.02±0.00^{a}	13±4^{a}
	194	165.98±27.60^{a}	0.62±0.01^{a}	0.02±0.00^{a}	9±1^{b}
Drought	Uninoculated	-	0.41±0.09^{b}	-	-
	USDA 110	70.18±20.87^{b}	0.51±0.07^{ab}	0.03±0.00	14.5±6
	188	70.83±35.98^{b}	0.47±0.05^{ab}	0.02±0.00	15±5
	194	100.04±21.43^{a}	0.58±0.04^{a}	0.02±0.00	15±4

* The plant growth conditions, Normal; pH 7 at 25D/25N°C, Acidity; pH 4.5 at 25D/25N°C, Drought; pH 7 and -3.02 bars at 25D/25N°C, High temperature; pH 7 at 40D/28N°C, Acidity + Drought; pH4.5 and -3.02 bars at 25D/25N°C, and Acidity + High Temperature; pH4.5 at 40D/28N°C.

** Means and standard deviations are calculated from three replicates, and values with different letters in the sane column in each condition are significant difference at $P \leq 0.05$.

Coefficient of variation =CV

Table 5 Continued

Conditions*	Isolates	Acetylene reduction (µmole h^{-1} $gnodule^{-1}$)	Biomass dry weight (g $plant^{-1}$)	Nodule dry weight (g $plant^{-1}$)	Nodule number ($plant^{-1}$)
High temperature	Uninoculated	-	0.35±0.01[b]	-	-
	USDA 110	88.37±39.06[a]	0.50±0.03[a]	0.01±0.01	8±2
	188	50.94±25.91[b]	0.50±0.03[a]	0.01±0.00	9 ±3
	194	96.36±24.16[a]	0.50±0.02[a]	0.01±0.00	10±2
%CV		17.5	10.2	6.6	28.2
Mixed stress					
Acidity+Drought	Uninoculated	-	0.12±0.01[c]	-	-
	USDA 110	51.82±9.24[ab]	0.47±0.03[ab]	0.02±0.01	10±2
	188	42.56±3.48[ab]	0.21±0.06[b]	0.02±0.00	13±2
	194	67.41±10.76[a]	0.57±0.13[a]	0.02±0.01	14±2
Acidity+High temp.	Uninoculated	-	0.16±0.07[b]	-	-
	USDA 110	50.68±2.46	0.24±0.06[ab]	0.02±0.00[a]	6±2
	188	59.25±10.39	0.30±0.03[a]	0.01±0.00[ab]	7±2
	194	51.26±5.42	0.27±0.03[ab]	0.02±0.00[a]	8±4
%CV		12.9	17.9	18.1	27.1

* The plant growth conditions, Normal; pH 7 at 25D/25N°C, Acidity; pH 4.5 at 25D/25N°C, Drought; pH 7 and -3.02 bars at 25D/25N°C, High temperature; pH 7 at 40D/28N°C, Acidity + Drought; pH4.5 and -3.02 bars at 25D/25N°C, and Acidity + High Temperature; pH4.5 at 40D/28N°C.

** Means and standard deviations are calculated from three replicates, and values with different letters in the sane column in each condition are significant difference at $P \leq 0.05$.

Coefficient of variation =CV

Table 6 Stress tolerance index (STI) of stressed soybean plant inoculated with and without bradyrhizobia growing in sand pot under different stress conditions.

	Stress Tolerance Index (STI)			
Conditions	**Uninoculated**	**USDA 110**	**188**	**194**
Normal	1.00±0.56^{b}	1.14±0.11^{ab}	1.38±0.09^{ab}	1.22±0.26^{ab}
Acidity	0.81±0.18^{a}	1.51±0.29^{a}	1.73± 0.39^{a}	1.59± 0.02^{a}
drought	1.00±0.17^{c}	1.38± 0.14^{ab}	1.22± 0.13^{b}	1.66 ± 0.39^{a}
High temperature	1.00± 0.02^{ab}	1.13± 0.06^{a}	1.16± 0.08^{a}	1.14 ± 0.02^{a}
Mixed stress				
Acidity+Drought	1.00±0.05^{c}	4.03±0.27^{ab}	1.83±0.48^{c}	4.85±1.08^{a}
Acidity+High temp.	1.00±0.64^{c}	1.50±0.36^{ab}	2.15±0.18^{a}	1.67±0.16^{ab}

* Means and standard deviations are calculated from three replicates of shoot dye weights in the same row followed by different letters are significant difference at $P \leq 0.05$.

4.3 Nodulation competitiveness of isolate 194 and USDA110 under stress conditions.

Both single and dual nodule occupancies were observed in soybean co-inoculated with *Bradyrhizobium* sp. isolate 194 and USDA110 in all conditions. The nodulation occupancy of isolate 194 under normal, drought, and high temperature stress conditions was significant higher than that of USDA110, while there was no significant difference of nodule occupancy of these two strains under acid stress condition (Fig. 3). Similarly, the nodulation competitiveness of isolate 194 was significant better than USDA110 under mixed stress conditions. However, plant co-inoculated of isolate 194 with USDA110 produced some nodules which contain blue and white colors after nodule staining. This type of nodule was called dual occupancy. The dual nodule occupancy was found in range of 14.6 and 25.5 % under normal and stress conditions.

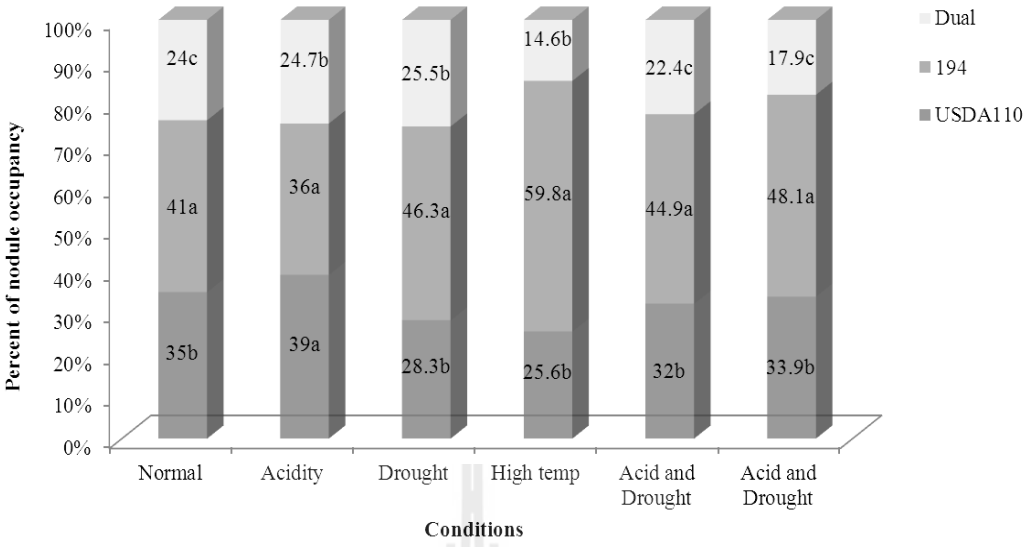


Figure 3 Nodulation competition of bradyrhizobia USDA 110 and isolate 194 on soybean growing under different stress conditions.

4.4 Improve growth of bradyrhizobia under stress conditions by using compatible solute

To improve the efficiency of bradyrhizobial inoculant, the second strategy of using compatible solute to support the growth and survival of bradyrhizoobium was focused in this part. Five sugars including glucose (GLU), glycerol (GLY), mannitol (MAN), sucrose (SU), trehalose (Tre), and polyvinyl alcohol (PVA) were tested. Under normal condition, MSM medium supplemented with all sugars, except PVA could significantly improve the specific growth rate (μ) of bradyrhizobia when compared to non-supplemented medium. The specific growth rate (μ) of isolate 194 was higher than that of USDA110, while the medium supplemented with sucrose could obviously improve the growth rate of isolate 194 under normal condition (Figure. 4). The growth rate of both USDA110 and 194 was reduced when grew under all stress conditions. However, the supplementation of some sugar could improve the growth rate of

bradyrhizobia under stress conditions. The growth rate (μ) of USDA110 could be improved when grew in the medium supplemented with all sugars, except PVA. However, all sugars could improve the growth rate (μ) of isolate 194 under acid stress condition, especially when medium was supplemented with sucrose. Interestingly, there was no any sugars could significantly improve the growth rate (μ) of USDA110 under drought condition, while only glycerol, sucrose and trehalose could support the growth of isolate 194 under drought stress. For high temperature stress condition, medium supplemented with glycerol, mannitol, sucrose and trehalose could support the growth of both USDA110 and 194 strains, while medium supplemented with PVA could improve only the growth of isolate 194. Therefore, sucrose was selected due to its capability to improve bradyrhizobial growth rate under several stress conditions.

The sucrose concentration was optimized in order to investigate the effect on cell growth. It was found that sucrose concentration affected the growth of isolate 194 under different stress conditions. In all conditions, the medium supplemented with sucrose at concentration more than 300 mM decreased the growth rate (μ) of isolate 194. Under normal condition, supplementation of sucrose did not affect cell growth when compared to non-sucrose supplemented cell. However, sucrose at concentration in range of 50-300 mM enhanced the growth rate of cell in all stress conditions. The result showed that sucrose at 300 mM provided the highest growth rate (μ) under stress conditions (Figure 5). Thus, sucrose at 300 mM was selected for testing in further experiments.

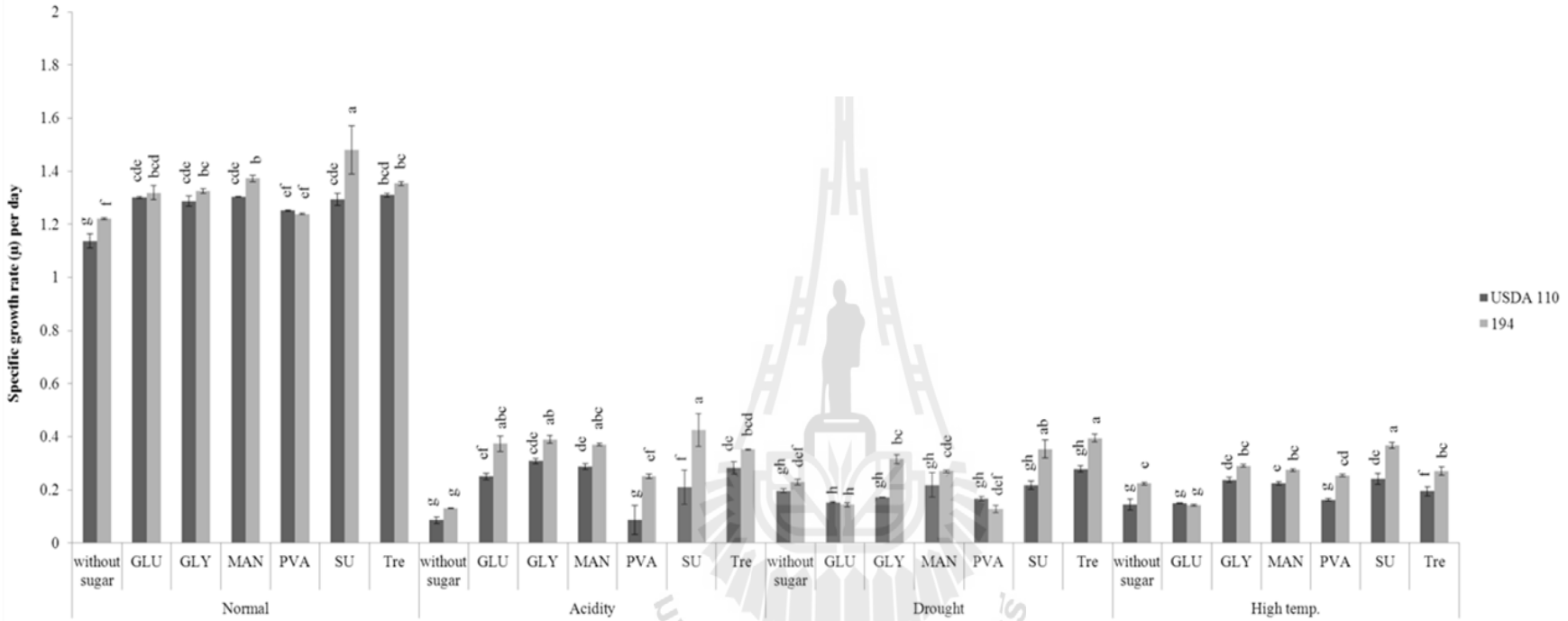
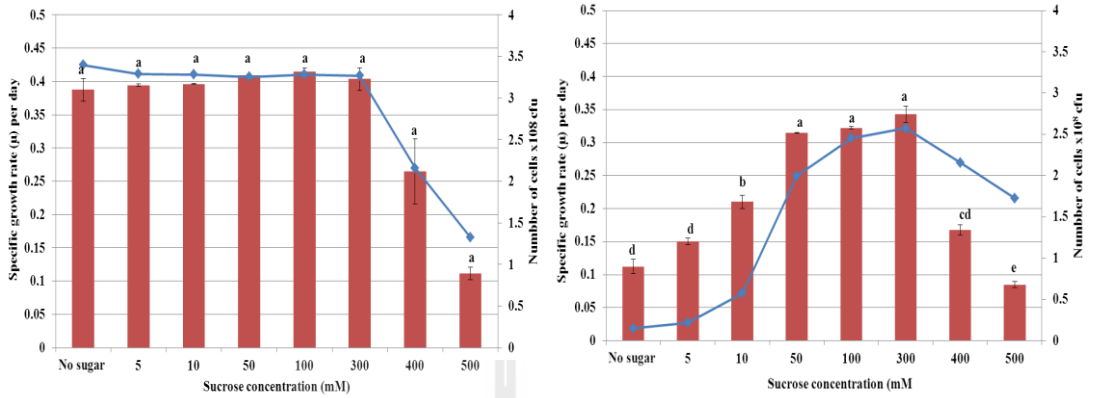


Figure 4 Screening of compatible solute as an osmoprotectant to improve bacterial cell growth under different constructed stress media conditions.

(a) Normal condition

(b) Acid condition



(c) Drought condition

(d) High temperature condition

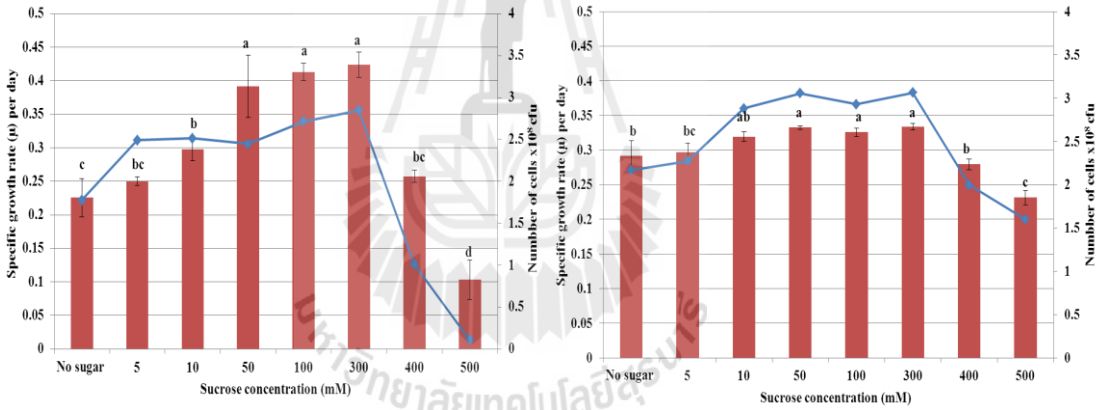


Figure 5 Specific growth rate and number of cells of selected bradyrhizobium isolatev194 in MSM supplemented with various concentrations of sucrose under different conditions.

4.5 Survival of bradyrhizobia supplemented with compatible solute in sand

The percent of bacterial survival when supplemented with and without sucrose after 4 days inoculation into sand under several stress conditions were summarized in Table 7. The percent of isolate 194 survivals was higher than that of USDA110 when tested under normal condition without sucrose supplement, while the survival of both strains was not significantly different when sucrose was supplied in the medium before inoculation into sand under normal condition. However, the percentage of cell survival was sequential decreased when tested under single stress and mixed stress conditions. Acidity stress resulted in decrease survival of USDA 110 and isolate 194 to 0.9 and 1%, respectively, which was equal to 10^4-10^5 cell/g sand when sucrose was not supplied to cell. However, the survival of USDA110 and 194 was significantly increased to 15 and 21 %, respectively, when supplemented cell with sucrose. The sucrose supplementary of isolate 194 was also significantly increased more than 80 % under drought and high temperature conditions, whilst the survival of USDA110 supplemented with sucrose was in range of 71-72 % under these conditions. In addition, under mixed stress conditions of acidity and drought condition, the survival of isolate 194 and USDA110 increased up to 67 and 54 %, respectively when supplemented with sucrose. Whereas the survival of both 194 and USDA110 under mixed acidity and high temperature was less than 1% although sucrose was supplemented into medium. This result revealed the strong effect of mixed acid-high temperature stress condition on cell survival.

Table 7 Survival of bradyrhizobium isolate 194 supplemented with and without 300 mM sucrose in sand under different stress conditions at 4 DAI.

	% Survival of bacteria after 4 day of inoculation into 10g sand			
Conditions	**194**	**194+Su**	**USDA 110**	**USDA 110+Su**
Normal	80 ± 4^{bc}	100 ± 8^{a}	75 ± 2^{c}	94 ± 3^{ab}
Single stress				
Acidity	1 ± 0^{c}	21 ± 2^{a}	0.9 ± 0^{c}	15 ± 3^{b}
Drought	68 ± 3^{b}	87 ± 2^{a}	64 ± 9^{b}	71 ± 0^{b}
High temp	56 ± 1^{c}	83 ± 2^{a}	56 ± 0^{c}	72 ± 4^{b}
Mixed stress				
Acid+Drought	54 ± 4^{b}	67 ± 4^{a}	27 ± 5^{c}	54 ± 10^{b}
Acid+High temp.	0	0.8±0.2	0	0.9±0

* Means and standard deviations are calculated from three replicates, and values with different letters are significant difference at $P \leq 0.05$.

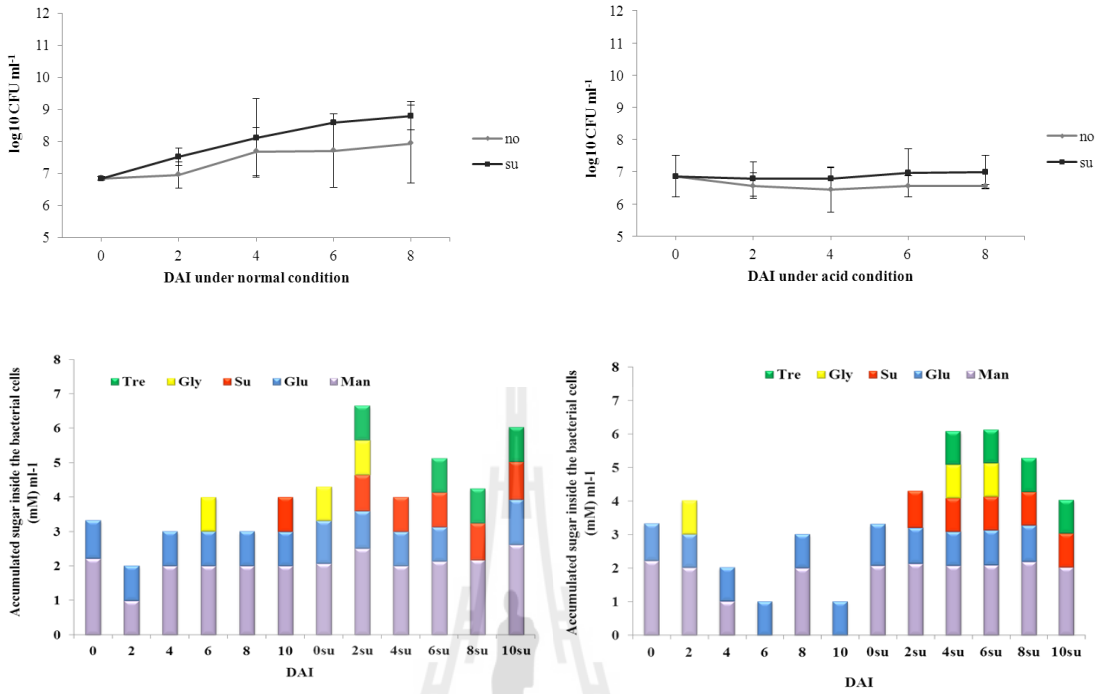
4.6 Compatible solute analysis in cell under stress conditions

In order to understand the mechanism of how sucrose could support cell growth and survival under several stress conditions, the accumulation of sugar inside the cell was investigated along with the cell growth under different stress condition (Figure 5.). Under normal condition, the number of cell increased from 10^7 CFU/ml to 10^9 and 10^8 CFU/ml when grew in medium supplemented with and without sucrose, respectively. Mannitol and glucrose were accumulated in both sucrose and non-sucrose supplemented cell at 0 till 10 DAL, while sucrose was found in the cell supplemented with sucrose at 2 to 10 DAI, but not found in cell grew without sucrose except at 10 DAI. Trehalose and glycerol were found only in cell supplemented with sucrose at 2-10 DAI and 2-4 DAI, respective. The accumulation of several sugars related with the increase of growth under normal condition (Figure 5. (a)). Under acid condition, the number of cell in medium supplemented with sucrose remained at 10^7 CFU/ml at 8 DAI, whereas the number of cells were reduced to 10^6 CFU/ml at 8 DAI

when grew in medium without sucrose (Figure 5. (b)). Investigation of cell extract revealed the lower level of sugar accumulation inside the cell than that of sucrose supplemented cell. Compatible solutes including glycerol, mannitol, and glucose were found in non-sucrose supplemented cell at 2 DAI, while only glucose remained in the cell at 10 DAI, which related to the reduction of all number under acid condition. Interestingly, several types of sugar were accumulated at higher level in sucrose supplemented cell than non-sucrose supplemented cell. Trehalose was found to be accumulated inside the cell since 2 to 10 DAI, while glycerol and sucrose were found at 4-6 DAI and 2-10 DAI respectively. The accumulation of sugars may support the survival of cell under acid condition.

Under drought condition, the cell number in medium supplemented with sucrose was higher than that of non-sucrose supplemented cell. Sugar analysis revealed only mannitol and glucose accumulated at low level in the non-sucrose supplemented cell, while several sugars were accumulated at high level in sucrose supplemented cell especially during 6-10 DAI. Trehalose was found to be accumulated in the cell at 2-10 DAI, whereas glycerol and sucrose were found at 6-10 DAI (Figure 4 (c)). Under high temperature condition, the number of cell increased from 10^7 to 10^8 CFU/ml at 8 DAI in sucrose supplementation, while the number of cell in non-sucrose supplemented cells was lower than 10^8 CFU/ml and tends to reduced at 8 DAI. Sugar analysis showed glycerol accumulation in both sucrose and non-sucrose supplemented cell at 2-10 DAI except at 10 DAI of non-sucrose supplemented cell, which related to the reducing of cell number in this condition. Moreover, trehalose was also accumulated only in sucrose supplemented cell since 2 DAI till 10 DAI. The accumulation of these sugars may involve in support the stress tolerance in bradyrhizobia.

(a) Normal condition (b) Acid condition



(c) Drought condition (d) High temperature condition

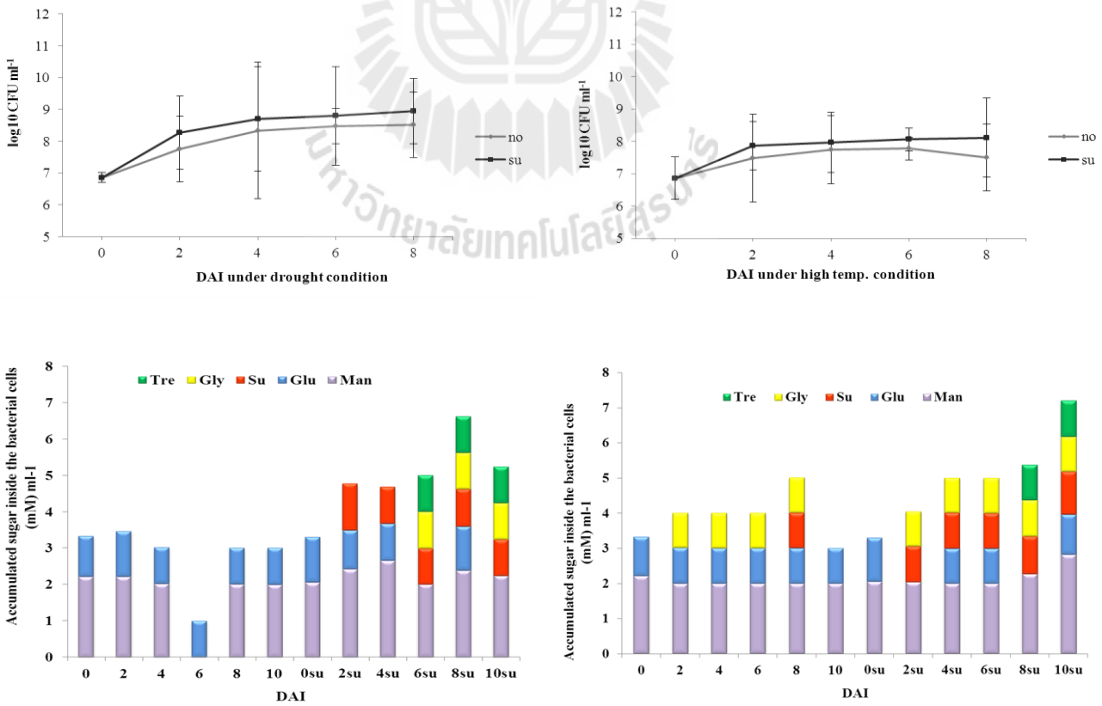


Figure 6 Growth and amount of accumulated intracellular sugars of bradyrhizobium isolate 194 in MSM supplemented with and without 300 mM sucrose under different conditions.

4.7 Performance of bradyrhizobia supplemented with compatible solute on plant symbiosis

4.7.1 Leonard's jar experiment: The performance of sucrose supplementing inoculum on symbiosis was investigated under different conditions. Under normal condition, there were no significant difference in biomass, nodule dry weight, and nodule member among all treatments of sucrose and non-sucrose supplementing inocula. Under acid and drought conditions, the performance of inoculum decreased in all treatments when compared with the performance under normal condition. However, soybean inoculated with sucrose supplementing inocula especially with isolate 194 tended to perform better biomass than non-sucrose supplementing inocula. The biomass dry weight of soybean inoculated with isolate 194 supplemented with sucrose was significantly higher than that of plant inoculated with other treatments under high temperature stress condition. Nevertheless, there was no significant difference in nodule dry weight and nodule number among all treatments, Interestingly under mixed stress condition of acid drought and acid high temperature, soybean inoculated with isolate 194 supplemented with sucrose produced the highest plant biomass and nodule dry weight, which were significantly improved from those of soybean inoculated with non-sucrose supplementing inoculum. However, sucrose supplementation most likely did not improve symbiosis performance of USDA110 under drought, high temperature stress as well as in mixed stress of acid-drought and acid-high temperature stress conditions.

Table 8 Symbiotic efficiency of bradyrhizobial inoculants supplemented with and without 300 mM sucrose on soybean growing under different stress conditions**.**

Conditions	Biomass dry weight (g plant^{-1})					nodule dry weight (mg plant^{-1})				Nodule number (plant^{-1})			
	Uninoculated	**U110**	**U110+Su**	**194**	**194+Su**	**U110**	**U110+Su**	**194**	**194+Su**	**U110**	**U110+Su**	**194**	**194+Su**
Normal	0.45±0.03^{c}	0.64±0.06^{ab}	0.58±0.04^{b}	0.59±0.02^{b}	0.68±0.05^{a}	21.33±1.52^{a}	21±1.0^{a}	23.17±2.7^{a}	25.33±3.9^{a}	20±5.0	16±4.0	20±2.0	21±3.0
%CV	15	10.6	14.5	29.5	13.6	14.22	4.7	11.6	15.3	25	25	10	14.2
Single stress													
Acidity	0.37±0.04^{b}	0.41±0.02^{b}	0.46±0.01^{b}	0.41±0.03^{b}	0.51±0.09^{a}	11.83±2.75^{a}	12.67±2.52^{a}	14.17±1.76^{a}	17±3.46^{a}	11±1.5	10±3.0	11±1.0	12±1.0
Drought	0.27±0.02^{d}	0.44±0.03^{bc}	0.37±0.01^{c}	0.46±0.03^{ab}	0.52±0.02^{a}	15.07±1.08^{a}	10.6±0.87^{b}	16.1±2.76^{a}	19.3±3.58^{a}	14±3.5	13±1.0	13±3.0	15±1.5
High temperature	0.32±0.03^{c}	0.43±0.04^{b}	0.38±0.04^{bc}	0.42±0.01^{b}	0.60±0.04^{a}	11±1.53	11.33±1	13.17±2.75	15.33±3.79	12±2.0	10±1.5	12±3.0	13±2.0
%CV	9.3	21.0	4.9	5.4	9.0	13.7	12.6	16.4	20.8	18.9	16.6	18.9	11.2
Mixed stress													
Acid and Drought	0.27±0.01^{d}	0.37±0.032^{bc}	0.33±0.02^{c}	0.40±0.02^{b}	0.48±0.0^{a}	15±3^{b}	12.±1.53^{c}	14±2.08^{b}	17.67±2.53^{a}	11±1.0	10±1.0	12±1.0	14±3.0
Acid and High temp.	0.28±0.01^{d}	0.41±0.07^{bc}	0.38±0.012^{c}	0.43±0.03^{b}	0.50±0.03^{a}	15±2^{b}	12.33±1.53^{c}	14±1.12^{b}	18±2.0^{a}	9±1.0^{ab}	8.6±1.0^{b}	9±1.0^{ab}	11±1.0^{a}
%CV	3.6	12.8	4.2	6.0	3.0	16.6	12.4	11.4	12.6	1	9.3	9	10.6

*Means and standard deviations are calculated from three replicates, and values with different letters in the same row are significant difference at $P \leq 0.05$.

4.7.2 Soil pot experiment: The performance of isolate 194 supplemented with sucrose on plant symbiosis was also determined in pot containing soil collected from three different locations (Table 1). Soil physicochemical characterization showed that soil from Suphan Buri Province was clay loam in texture, and slightly acidic with high organic matter (OM) and low level available P and exchangeable K. Whereas, soil from Phetchaburi Province was silt clay loam and also slightly acidic with low OM and available P but high in exchangeable K contents. Yasothon Province soils was clay loam in texture, with neutral pH and contain high level of OM, but low level of available P and exchangeable K. The result of plant growth and symbiosis were shown in Table 9. Soybean inoculated with sucrose supplemented inoculum of isolate 194 significantly provided high biomass dry weight when grew in all soil samples.The nodule number obtained form plant using this inoculum was significantly different form non-sucrose supplemented strain when grew in soil collected from Suphan Buri and Petchaburi provinces. These preliminary data revealed the good performance of sucrose supplementing inoculum under soil conditions. However, further field experiments are needed to ensure the efficiency of developed inoculum.

Table 9 Symbiotic efficiency of bradyrhizobium isolate 194 inoculant supplemented with and without 300 mM sucrose on soybean growing under soil conditions.

Soil samples	Biomass dry weight (g plant^{-1})			nodule dry weight (g plant $^{-1}$)		Nodule number (plant $^{-1}$)	
	Uninoculated	194	194+Su	194	194+Su	194	194+Su
Suphan Buri	0.84±0.22^{b}	0.87±0.12^{b}	1.04±0.15^{a}	0.01±0.00	0.02±0.00	8±1^{b}	12±3^{a}
Phetchaburi	0.33±0.12^{c}	0.63±0.15^{b}	1.05±0.27^{a}	0.01±0.00	0.02±0.00	13±3^{b}	28±4^{a}
Yasothon	0.29±0.05^{c}	0.47±0.10^{b}	0.97±0.21^{a}	0.02±0.00	0.03±0.00	11±2	10±3

* Means and standard deviations are calculated from three replicates, and values with different letters in the same row are significant difference at $P \leq 0.05$.

CHAPTER V

DISCUSSION

Abiotic stress factors are normally presented in several environment conditions which affect the legume rhizobium symbiosis. Several environmental conditions are limiting factors to the growth of bacteria and plant which leads to reduce the activity of the N_2-fixation. In the rhizobium-legume symbiosis, the process of N_2 fixation is strongly related to the physiological state of the host plant and the effect of other environmental factors (Brockwell et al., 1991; Peoples et al., 1995). Abiotic factors including: i) temperature; ii) plant and microbial nutrient limitations and requirements; iii) soil moisture; iv) soil pH; and v) soil salinity have been reported influence the symbiosis and nodulation competition. Recently, there have been reported that acid, drought, and high temperature soil environments are important factors for symbiotic sustainable and survival bacteria (Marinković et al., 2013; Raza et al., 2001; Uma et al., 2013). Plant growth under environmental condition usually contains more than one abiotic stress factors that affect legume-rhizobium symbiosis and plant development. Thus, the condition of mixed or multi stresses can be occurred in the natural environment. However, not many researches have been focused on multi stresses effect on symbiosis. In this study, a single stress factors including acid, drought and high temperature and mixed acid-drought and acid-high temperature were applied to plant growth condition in order to improve the efficiency of soybean-bradyrhizobium symbiosis.

For the screening experiment, the isolated bradyrhizobia were compared with reference strains such as, USDA110, DASA1014, and CB1809. *B. japonicum* USDA110 and DASA1014 were used for soybean inoculum in Thailand, while *B. japonicum* CB1809 was the commercial strain for soybean cultivation in Australia. The strain CB1809 has been reported for stress tolerance in several conditions such as acid soil, alkali soil (Botha et al., 2004; Indrasumunar et al., 2011; Lin et al., 2012), and water stress (Ramos, 1996) conditions. This strain could adapt to survive in Brazilian soil under high temperature (>40˚C) (Santos et al., 1999). However, it has been reported that the strain USDA110 grew slowly in acid agar medium at pH 4.5 (Graham et al., 1994). Manassila et al. (2012) was also reported *B. japonicum* strain USDA110, grew slowly at pH4.5, In this research, isolate 184, 188, 193, 194, and 197 could tolerate and survive well under stress condition. The growth score and survival of these isolates under acidity, high temperature and drought were not different with strain CB1809 and other reference strains. It could be possible that these isolated strains were collected from stress soil condition, and in which cell could adapt them selves to tolerate against several stress conditions in the field.

Stress environments usually influence the symbiosis through its effects on the growth and survival of bradyrhizobial inoculant by restriction on the first stage of root colonization as well as inhibition of processes of infection and nodule development. Moreover, stress environment likewise additional affects to development of soybean host plant that may possibly mediated to impairment of active nodule functioning (Dimkpa et al., 2009). The extreme pH of acid condition is one of limiting factors for symbiotic rhizobia and legume plant growth. It was found in this study that the plant biomass, nodule number and nodule dry weight were reduced under acid condition.

The strong acidity such as at pH 4.5 can to decelerate cell division in soybean root elongation and was resulting in decreased plant biomass. Since the plant root system are important modes for nutrient element absorption and symbiotic responses of bacterial colonization, soybean roots which loosed function in strong acid condition would also affected to number of bacterial nodulation (Dimkpa et al., 2009; Lin et al., 2012). Consideration in soil condition, acidic soil could be occurred as acid-sulfate which was used in this experiment. Acid-sulfate soil is low pH environmental soil ascends mainly from the acids produced after oxidation of pyrites (FeS_2) in existence of water and oxygen, in which the production of acid from equation as sulfate (SO_4^{2-}) that actually affects in a drop of pH (Rassam and Cook, 2002). These many cause by the plant developmental problems such as decrease the efficiency of plant nutrient absorption for nitrogen and calcium or phosphorus deficient in plant because its change to unutilized form. Moreover, deficiency of magnesium and manganese in soils, and the percentage of a number of soluble compounds that can toxic to the plant cells can be occurred under low ph.

The reduction in plant biomass was also found in drought stress condition. Water deficiency is certainly affected to soybean plant developmental processes. In many studies indicated that the osmotic potential generated by PEG decreased the root elongation (Whalley et al., 1998) then lead to reduce the plant growth and development. Amooaghaie (2011) reported that PEG concentrations in range from -1.0 to -7.0 bars decreased the soybean yields. Soni et al. (2011) also described the effect of PEG on germination and caused wilting in seedlings of moth bean. In this study, although isolate 184 and 194 performed better than USDA110 in term of plant growth under drought stress condition, the number of nodule, and biomass dry weight of inoculated soybean plant were reduced when compared to normal condition.

The effect of high temperature on biomass production was also reported by Marsh et al. (2006), which indicated plant dry weight of soybean, pigeon pea, and cowpea inoculated with bradyrhizobia was decreased when plants were grown 38˚C, while the temperature of 30˚C was the optimum temperature for bacteria growth and increase the plant yields. These data were similar to our results, in which high temperature stress condition was significantly reduced in plant growth and symbiosis. It could be possible that high temperature may affect bacterial cell survival and damage some biological pathways in plant development.

In this study, *Bradyrhizobium* sp. isolate 194 was selected as stress tolerant strain and has potential to develop as soybean inoculant to be used under multi-stress conditions. Although isolate194 did not have significantly higher nitrogen fixation ability than USDA110, this strain performed better symbiosis with soybean than USDA110 under stress conditions. Mubarik et al. (2012) reported that three strains Bj(wt), Bj11(5), and Bj(19) could be the best inoculant for soybean plant on acid soil pH 4.5 even not significantly influent in nitogenase activity from the reference strain, USDA 110. Appunu et al. (2008) also reported that the variation in symbiotic efficiency of *B. japonicum* strains and soybean cultivars could be occurred under field conditions. The plant dry matter appeared as paramount principle for selection of the most effective legume-rhizobium associations for specified soil environment.

From our results, isolate 194 was the best stress tolerant strain, which had different colony formation and growth rate from USDA110 when grew in YEM medium. Isolate 194 produced high amount of exopolysaccharide and grew a bit faster than USDA110. These data may described why isolate 194 survive and grow well in stress conditions. However, isolate 194 could not promote plant growth as good as normal condition. These

data were correlated in many studies which suggested the efficiency of stress tolerant bradyrhizobial strain under adverse condition that could promote plant health and growth but not equally to plant cultivated under common condition (Botha et al., 2004; Busse and Bottomley, 1989; Idris et al., 2007; Kantolic and Slafer, 2007).

The second strategy to improve the survival of bradyrhizobium under stress condition was the application of compatible solute. The advantage of compatible solute is widespread for application to bacterial cell protection. As use an osmoprotectants supplementing formula into the culture medium that exposed to unfavorable growth conditions. On the other hand, the stressed cell bacteria can be self synthesized by using biosynthetic pathway of *de novo* that automatically generate inside the stressed cells. Most of compounds could accumulate as intracytoplasmic organic solutes to balancing the osmotic pressure between intracellular bacteria and extracellular environment, increase the stability of enzymes, and reservation the completeness of biological membranes. Several compatible solutes (sugar, polymers, polyols, protein, and derivatives) have been observed their function as osmotic balancers with Rhizobiaceae, such as *R. gallicum*, *R. leguminosarum*, *R. etli*, *R. meliloti*, *S. meliloti*, and Bradyrhizobiaceae including *Bradyrhizobium* sp. and *B. japonicum* (Deaker et al., 2007; Elsheikh and Wood, 1990a; Fernandez-Aunión et al., 2010; Ghalamboran and Ramsden, 2010; Hoelzle and Streeter, 1990; McIntyre et al., 2007; Miller and Wood, 1996; Natera et al., 2006; Smith et al., 1988; Streeter, 2003).

The specific growth rate (μ) of isolate 194 and USDA110 could be increased in the presence of sugar supplementation, in which the growth rate of isolate 194 was more than USDA110. It is possible that isolate 194 could grow faster in MSM medium and be able to use and uptake several sugars into cell. In this research four sugars, such as

sucrose, trehalose, glycerol, and mannitol were found to support the growth and survival of cell in all stress conditions. McIntyre et al. (2007) suggested 1 mM trehalose cloud increase percent of cell survival of *R. leguminosarum* bv. *trifolii* strain NZP561 when compared with lactose and water treatment. However, Gouffi et al. (1999) was reported that 0.5 mM sucrose did not significantly improve cell growth when compared to the no sugar addition. It was also emphasized that sucrose is nonaccumulated osmoprotectant in *Sinorhizobium meliloti* under hyperosmotic pressure (0.5 M NaCl, KCl and 0.8 M Mannitol, Glycerol). On the other hand, mannitol has been used as osmoprotectant and enhanced survival of *Lactococcus lactis* (Efiuvwevwere et al., 1999), *R. tropici* CIAT 899 and *R. gallicum* bv. *phaseoli* 8a3 (Fernandez-Aunión et al., 2010). In this research, 300 mM sucrose was used as the appropriate concentration that enhance survival of stress bacterial cells, although the growth rate of isolate 194 was not significant different when compared with 100 mM sucrose. However, medium supplemented with 300 mM sucrose produced higher number of bacterial cell (cfu/ml) than in 100 mM sucrose.

Sucrose is nonreducing disaccharides that similarly with trehalose, both of them may act as osmoprotection against several osmotic stresses environment in rhizobia and bradyrhizobia (Elsheikh and Wood, 1990a) by maintaining membrane integrity during drying and rewetting (Leslie et al., 1995). Sucrose was also reported to increase survival of cell during the stationary phase and when the cell is exposed to NaCl. (McIntyre et al., 2007). In this study, isolate 194 could grow or survive in the medium supplemented sucrose under such single stress and mixed acid-drought stress conditions, but not in mixed acid-high temperature condition. The double strong effects caused by mixed stresses of acid and high temperature may cause the rapid dead of bacteria via dehydrated water cells and resulting in inhibition the functional proteins or some enzymes (Hiratsu et al. 1995).

Interestingly, isolate 194 supplemented with sucrose enhanced plant growth in mixed stress conditions. These data indicated sucrose could protect some of symbiotic bacteria and indirect alleviate stress effects. Moreover, sucrose may be used as environmental carbon source for metabolic plant pathway which also indirectly facilitates the plant survival under stress condition. (Koch 2004). Sucrose and various disaccharide osmoprotectants are primary environmental concern for rhizobia, since several of those compounds are certainly present in the soil and the rhizosphere which is abundant in plant root exudates (Miller and Wood 1996). Thus, sucrose could participate in bacterial osmoprotection and could be an energy supply as the natural carbon source, which is mainly entered through active dicarboxylic acid systems and catabolism. This sucrose is key compatible solute that can be supplied to medium in order to improve bacteria cell resistance to single and mixed stress occurred to environment.

Analysis of sugar accumulation in the cell extract along the time was performed to observe the mechanism of sucrose in protecting cell from stress condition. The extracellular sucrose enters the periplasm of gram negative bacteria via porin channels in the outer membrane, and is then transported through the inner (cell) membrane (Postma et al., 1993). This pathway involves the cell membrane-associated phosphotransferase system (PTS) that brings about both sugar transport and phosphorylation, resulting in an intracellular pool of phosphorylated sugar. The PTS is composed of general energy-coupling proteins (EI and HPr) that catalyze the transfer of a phosphoryl group for all PTS sugars and sugar-specific protein(s) (EII) to the translocated sugar to yield the 6-phosphate derivative of the sugar subsequent hydrolysis of the phosphorylated disaccharide in the cytosol is catalyzed by sugar specific enzymes, such as sucrose hydrolase. The product β-

D-glucose-6-phosphate enters glycolysis, and D-fructose-6-phosphate is a glycolytic intermediate, which can be transformed to other sugars by several pathway links (Lee et al., 2010). These information explain why difference of sugars could be detected in isolate 194 extracts although it was supplemented with or without sucrose in culture. Moreover, catalyzed D-glucose-6-phosphate and D-fructose-6-phosphate could be used as supplied molecules for biosynthetic *de no vo* pathway of stressed cell bacteria to produce alleviated stress compounds. Thus, the accumulation of soluble sugar inside the cell may be derived from both produced and uptake into bacterial cell.

Glucose and mannitol were detected in every samples indicating both sugars may presence as the progression of living bacterial cell in the culture. Mannitol is an important carbon source and able to be transformed to glucose molecule by D-mannitol dehydrogenase activity presence in rhizobial cell during growth and carbohydrate metabolic processes (Wisselink et al., 2002).

Trehalose was found specifically in stress cell extract from conditions of sucrose supplemented medium. Under acid condition, both trehalose and glycerol were found in period of growth during 4-10 DAI. However, some research reported that no difference in growth of strain *Sinorhizobium meliloti* between supplemented with and without each 1 mM sucrose and trehalose (Gouffi et al., 1999; Gouffi et al., 1998). They suggested that sugars may be involved in bacterial cell survival and induced cells to produce high quantity molecules of glutamate and the dipeptide of N-acetyl glutaminyl glutamine amide (NAGGN) under hyperosmolality from salt stress, while *Bra bium* sp. SEMIA 6144 has been reported to increase of glutamate synthesis as a compatible solute in response to acid stress (Natera et al., 2006).

Similarly, trehalose and glycerol may also involve in drought tolerance of bacteria since both sugars were detected in intracellular cell extracts in the presence of sucrose in the culture. It has been described in Streeter (2003) that the accumulation of trehalose in the cytoplasm is critical to the survival of *B. japonicum* during desiccation. Increasing the periplasmic concentration of trehalose is also beneficial but is not so critical as the concentration of trehalose in the cytoplasm. The effect of drought stress was also reported by Zacarías et al. (2004) suggested that trehalose content increased significantly while acetylene reduction activity (ARA) decreased in the nodules of plants. The pathway of trehalose synthesis in bacterial cell has been reported to involve with two enzymes, OtsA, the trehalose-6-phosphate synthase, catalyses the enzymatic condensation of the precursors glucose-6-phosphate and UDP-glucose, and free trehalose is then generated by trehalose-6-phosphate phosphatase. The accumulation of glycerol in bacterial cell under osmotic stress was not much reported. However, transfer of salt tolerant yeast *Debaryomyces hansenii* to media of higher salinity resulted in an increased production and intracellular accumulation of glycerol, which was relative to the level of the shift in salinity (Louis et al., 1994).

Interestingly, glycerol was found in both supplemented with and without sucrose at high temperature condition of bacterial cell extract. Thus, the responded compounds for high temperature tolerance of isolate 194 could be accomplished by the accumulation of glycerol. On the other hand, trehalose could only be accumulated in cell extracted form sucrose supplemented condition. Both of these sugars have been reported to stabilize proteins at high temperatures (Kempf and Bremer, 1998) and reserved the present form of proteins, resulting in a favored hydration of protein surfaces (Yancey, 2005). In addition, the response to high temperature in *Saccharomyces cerevisiae* is controlled by increase the glycerol level which may have an indirect effect by influencing signaling through the PKC

(protein kinase C) MAP kinase pathway, which plays an important role in maintenance of cellular integrity (Wojda et al., 2003). In this study, different amounts of sucrose, trehalose, and glycerol were also found in extracts under different stress conditions. It could be indicated that these sugars may be involved in survival of isolate 194 under different stress conditions.

Although bradyrhizobial inoculant could tolerate to stress conditions and able to nodulate and fix nitrogen to plant under stress conditions similar to normal ondition, soybean plant needs to improve for its tolerance to such stress to make the highest efficiency of using bradyrhizobial inoculants (Meghvansi et al., 2005). It is indicated that the strategy to improve plant growth development under stress condition must be depend on either with symbiotic bacteria and host plant tolerance to environmental stress conditions.

CHAPTER IV

CONCLUSION

Different bradyrhizobial isolates performed differently under different stress conditions. Isolate 194 performed well for nodule competition and plant growth promotion under single and mixed stress conditions when compared to USDA110. The survival of isolate 194 under stress conditions could be improved when supplemented with 300 mM sucrose into the medium. Sucrose not only enhanced survival of stresses cells but also involved in stimulation the sugar biosynthetic pathway relation in accumulation of several sugar compounds in cell for different stresses protection. Trehalose and glycerol would have an important role in cell protection against drought and high temperature stress, while loss of sugar accumulation inside the cells directly affect to the cell survival under acid condition. Finally sucrose supplemented of isolate 194 could improve the symbiosis efficiency between soybean-bardyrhizobium under single and mixed stress condition.

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APPENDICES

Appendix 1. Yeast Mannitol medium (YM) (Vincent, 1970)

D-mannitol	10.0 g
Yeast extracts	0.4 g
NaCl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
H_2O	1000 ml
pH	6.8

Appendix 2. Composition of N-free Soution (Broughton and Dilworth, 1971)

Stock solution	Element	Form	g liter^{-1}
1	Ca	$CaCL_2 \cdot H_2O$	294.1
2	P	KH_2PO_4	136.1
3	Fe	Fe citrate	6.7
	Mg	$MgSO_4 \cdot 7H_2O$	123.3
	K	K_2SO_4	87.0
	Mn	$MnSO_4 \cdot H_2O$	0.338
4	B	H_3BO_3	0.247
	Zn	$ZnSO_4 \cdot 7H_2O$	0.288
	Cu	$CuSO_4 \cdot 7H_2O$	0.100
	Co	$CoSO_4 \cdot 7H_2O$	0.056
	Mo	$Na_2MoO_2 \cdot 2H_2O$	0.048

Appendix 3. MSMmedium (Minimal salt medium) (adapt form Brown and Dilworth, 1975)

Mannital	5 g
KH_2P0_4	0.36 g
K_2HP0_4	1.4 g
MgSO $7H_20$	0.25 g
CaCl $2H_20$	0.02 g
NaCl	0.2 g
FeCl	6.6 mg
EDTA	15 mg
ZnSO $7H_20$	0.16 mg
Na_2Mo0	0.2 mg
HBO	0.25 mg
$MnSO_{,}.\ 4H_20$	0.2 mg
$CuSO_{,}.\ 5H_2O$	0.02 mg
CoCl, . $6H_20$	1 pg
Yeast extracts	0.1g
Thiamine-HC1	1 mg
Calcium pantothenate	2 mg
Biotin	1pg

BIOGRAPHY

Miss Jenjira Wongdee was born on July 24, 1986 in Nakhonphanom, Thailand. She studied primary school at Ban Nakhae School and in high school at Ban Phang pittayakhom School. She graduated with the Bachelor's degree of Science in Biology, from Mahsaracharm University in 2008. In 2009, she enrolled at School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima and received a scholarship from National Research Council of Thailand (NRCT) and Suranaree University of Technology (SUT) for conducting her master thesis research. She had presented poster in the topic of "enhancing the efficiency of soybean inoculant for nodulation under multi-environmental stresses soil condition" in the 1st Asian Conference on Plant-Microbe Symbiosis and Nitrogen-Fixation, Miyazaki, Japan. 2010, and performed oral presentation in the topic "improving of Bradyrhizobial inoculant for soybean cultivation under environmental stress conditions" in Global Change: Opportunity & Risk, Burapha University International Conference 2012 Pattaya, Thailand, with fullpaper publication in proceeding Moreover, she had presented the poster in the topic of "improving of bradyrhizobial inoculant for soybean nodulation under multi-environmental stress conditions" in the 2nd Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation (2nd APMNF), Phuket, Thailand during October 28-31, 2012.