Enhancing the Efficiency of Soybean Inoculant for Nodulation under Multi-Environmental Stress Conditions

JENJIRA WONGDEE, WATCHARIN YUTTAVANICHAKUL, APHAKORN LONGTHONGLANG,
KAMONLUCK TEAMTISONG, NANTAKORN BOONKERD, NEUNG TEAUMROONG
and PANLADA TITTABUTR*

School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology,
Nakhon Ratchasima, Thailand

A b s t r a c t

The development of rhizobial inoculants with increased resistance to abiotic stress is critical to mitigating the challenges related to climate change. This study aims at developing a soybean stress-tolerant *Bradyrhizobium* inoculant to be used under the mixed stress conditions of acidity, high temperature, and drought. Six isolates of *Bradyrhizobium* with high symbiotic performance on soybean were tested to determine their growth or survival abilities under *in vitro* conditions. The representative stress-tolerant *Bradyrhizobium* isolates 184, 188, and 194 were selected to test their ability to promote soybean growth under stress conditions compared to the type strain *Bradyrhizobium diazoefficiens* USDA110. The plant experiment indicated that isolate 194 performed better in symbiosis with soybean than other *Bradyrhizobium* strains under stress conditions. Based on the stress tolerance index, soybeans inoculated with isolate 194 showed a high growth performance and significantly better nodulation competition ability than USDA110 under several stress conditions. Interestingly, supplementation of sucrose in the culture medium significantly enhances the survival of the isolate and leads to improved plant biomass under various stress conditions. Analysis of the intra-cellular sugars of isolate 194 supplemented with sucrose showed the accumulation of compatible solutes, such as trehalose and glycerol, that may act as osmoprotectants. This study indicates that inoculation of stress-tolerant *Bradyrhizobium* together with sucrose supplementation in a medium could enhance bacterial survival and symbiosis efficiency under stress conditions. Although it can be applied for inoculant production, this strategy requires validation of its performance in field conditions before adopting this technology.

K e y w o r d s : *Bradyrhizobium*, nodulation competition, stress conditions, compatible solutes, osmoprotectant

Introduction

Bradyrhizobium is used as a soybean inoculant, because it reduces atmospheric nitrogen gas (N_2) into a nitrogenous compound that can be utilized directly by the plant. Application of a *Bradyrhizobium* inoculant, an environment-friendly biofertilizer, is, therefore, an essential factor that can increase the soybean yield and reduce the utilization of chemical N fertilizer (Suyal et al. 2016; Ntambo et al. 2017; Jalloh 2020). Although the effective nitrogen-fixing *Bradyrhizobium* is used as an inoculant, several abiotic factors have been reported to interfere with successful nodulation. Abiotic factors, as defined here, such as salinity, unfavorable soil pH,

nutrient deficiency, mineral toxicity, extreme temperature, and soil moisture, are environmental stress factors that can affect the efficiency of symbiosis. For example, in severe drought stress, the total dry weight of soybean inoculated with a commercial liquid inoculant of *Bradyrhizobium* (Simbiose Nod Soja®) was decreased; even co-inoculation with *Azospirillum brasilens* had this effect when compared to the normal condition (Silva et al. 2019). Wang et al. (2016) also showed that salt stress negatively affects alfalfa (*Medicago sativa* L.) production and biological nitrogen fixation. However, inoculation with an effective rhizobial inoculant had a positive effect on alfalfa's salt tolerance by improving antioxidant enzymes and osmotic adjustment capacity.

Thus, it is necessary to develop an inoculant that can support plant growth under abiotic conditions.

In the field, stress conditions cause rapid death of the *Bradyrhizobium* inoculum and reduce its capability to compete for nodulation and fix nitrogen (Sindhu et al. 2010; Gopalakrishnan et al. 2015). The most critical factors, potentially limiting the rhizobium-legume symbiosis, are pH, drought, and high temperature (Dimkpa et al. 2009; Zhang et al. 2020), and several times these stress factors were found in combination as a multi-environmental stress condition. However, the ability of the legume hosts to grow and survive in stress conditions is improved when they are inoculated with stress-tolerant strains of rhizobia (Wei et al. 2008; Kajić et al. 2019). Therefore, inoculation with multi-stress-tolerant *Bradyrhizobium* strains may enhance the field performance of soybean production.

Moreover, efforts to develop a rhizobial inoculant could be made by increasing nodule occupancy under stress conditions. Iturralde et al. (2019) suggested that improving nodule occupation should also focus on optimizing the inoculant formulation and inoculation technology. Amendment of some sugars in the inoculant formulation is one strategy to improve bacterial survival under stress conditions (Singh et al. 2014). The ability of rhizobia to tolerate stress could be increased by maintaining the osmotic equilibrium across membranes by accumulating compatible solutes, mainly organic osmolytes (Saxena et al. 2013; Maryani et al. 2018). Many of the best-characterized osmoregulatory mechanisms are designed to adjust compatible solute levels by modulating their biosynthesis, catabolism, uptake, and efflux (Kajić et al. 2019). However, the composition of endogenous compatible solutes accumulated by rhizobia varies at the species level. Therefore, the accumulation of compatible solutes would be another mechanism to improve the stress tolerance and survival of rhizobia, finally supporting the nodulation and nitrogen fixation ability of inoculated *Bradyrhizobium* under stress conditions.

Thus, the objectives of the present work were to select stress-tolerant strains of *Bradyrhizobium* and search for an appropriate sugar that contributes to the accumulation of compatible solutes in their cells to improve stress tolerance of *Bradyrhizobium* inoculant under several environmental stress conditions. The plant growth experiments were performed in both single and mixed stress conditions. The effect of supplemented sugar on the accumulation of compatible solutes in *Bradyrhizobium* cells and its effect on cell survival and soybean growth under stress conditions were also investigated. Then, the symbiosis efficiency of the developed *Bradyrhizobium* inoculant on soybean was determined by testing in the soil collected from different locations in Thailand.

Experimental

Materials and Methods

***Bradyrhizobium* strains and culture conditions.** The six isolates of soybean *Bradyrhizobium*, including isolates 184, 188, 193, 194, 197, and 199, were obtained from the Department of Agriculture (DOA), Ministry of Agriculture and Cooperatives, Thailand, and used in this study based on their symbiotic performance in soybean under normal conditions. Box-PCR (Schneider and De Bruijn 1996) and dendrogram analysis (Quantity One® Version 4.6.3 for Windows and Macintosh) were performed to investigate the bacterial DNA fingerprint profiles in order to avoid repetitive isolates. *Bradyrhizobium* strains were grown at 28°C on yeast extract-mannitol (YM) broth or agar containing congo red (pH 6.8) (Somasegaran and Hoben 1994) as basal growth condition. The soybean *Bradyrhizobium* strain used in this experiment was *B. diazoefficiens* USDA110 as type strain, while *Bradyrhizobium* sp. strain CB1809 was used as the stress-tolerant strain under *in vitro* conditions (Botha et al. 2004).

Determination of growth and survival of *Bradyrhizobium* strains under *in vitro* stress conditions. To observe the stress tolerance of *Bradyrhizobium* strains under *in vitro* conditions, the cell cultures were washed with normal saline twice before adjusting to 10^8 CFU/ml. Then, 10 μl of bacterial cells were dropped on a YM agar medium prepared to determine their ability to grow in different stress conditions. For acid stress, YM agar media were prepared at pH 4, 5, 6, and 6.8 using acetic acid (CH_3COOH; as a representative organic acid found in the natural soil), and 0.5 ml/l of 8 mM bromothymol blue was added as a pH-indicating colorant. Then, plates were incubated at 28°C. For high-temperature stress, YM agar media were prepared at pH 6.8 and incubated in the adjusted incubator at 28, 35, 40, and 45°C. Seven days after incubation, the ability of *Bradyrhizobium* strains to grow on acid-formulated medium and at high-temperature conditions was determined using the growth score as indicated in Table SI. For drought stress, 1 ml of the same bacterial cell concentration was overlaid on 0.2 μm filter membrane and incubated in the adjusted desiccator chamber containing silica gel, saturated $CH_3COOK \cdot 1.5\,H_2O$, and KI solutions to give relative humidity (RH) values of 3, 22, and 67.8%, respectively (Boumahdi et al. 1999). After seven days of incubation, the percentage of cell survival in the drought condition in comparison with the initial cell number was determined using the dilution plate count technique.

Soybean growth and planting conditions. Seeds of surface-sterilized soybean (*Glycine max* (L.) Merr.) variety "Chiang mai 60" were germinated and transferred

to Leonard jars containing 0.35 kg of the sterilized sand (autoclaved at 121°C for 90 min). The *Bradyrhizobium* cells washed with normal saline were inoculated at 10^8 cells per soybean seedling grown under normal and stress conditions. Plants were watered with N-free plant nutrient solution (Somasegaran and Hoben 1994) and grown at 25°C at a 12/12 day/night cycle with a light intensity of 639 $\mu E/m^2/s$ as a normal condition, while other stress conditions were adjusted as follows: (i) acid condition – N-free solution was adjusted to pH 4.5; (ii) high-temperature condition – plants were incubated in growth chambers (Contherm's Biosyn Series of Tissue and Plant Growth Chambers-620RHS P6 Models, Wellington, New Zealand) at 40°C; and (iii) drought condition – the sand was desiccated at −3.20 bars using polyethylene glycol (PEG) 8000 solution (Michel 1983). For the mixed stress conditions, two stress factors as indicated were combined (the stress condition of three factors was not performed here due to the drastic effect on plant growth). Data on nitrogen fixation, number of nodules, plant biomass, and nodule dry weight were collected at 30 DAI (days after inoculation) as an appropriate time for determining the symbiotic efficiency.

Determination of stress tolerance index (STI). The stress tolerance index (STI) was determined according to Shetty et al. (1995) following equations (1) and (2):

$$STI = DWS/DWC \qquad (1)$$
$$STI = DWH/DWC \qquad (2)$$

where, DWS – dry weight of plants grown under stress, DWH – dry weight of plants grown under stress with inoculation of bacteria, and DWC – dry weight of plants grown in the control condition (without stress and inoculation of bacteria).

Nodulation competition test. The plasmid pCAM120 containing Tn5 fusion with the β-glucuronidase (GUS) gene (Wilson et al. 1995) was transformed into USDA110 as a reporter gene for monitoring the nodule occupancy compared with the selected stress-tolerant strain under stress conditions. This GUS-tagged USDA110 was obtained from the Applied Soil Microbiology Laboratory, School of Biotechnology, the Suranaree University of Technology, Thailand, and cultured in a YM medium with an appropriate antibiotic. In Leonard's jar experiment, surface-sterilized soybean seeds were co-inoculated with stress-tolerant *Bradyrhizobium* and GUS-tagged USDA110 in normal saline using a ratio of 1 : 1 at 10^8 cells/seed. Plant growth conditions were adjusted in single stress and mixed stress conditions as described above. After one month, soybean nodules were collected, cut in half, and stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) as a substrate according to the method of Krause et al. (2002). The blue and white-colored nodules were observed, and the percentage of nodule occupancy was

determined using a method described by Payakapong et al. (2004) and Talbi et al. (2013).

Characterization of plant growth-promoting (PGP) properties of *Bradyrhizobium*. Some PGP properties of the selected *Bradyrhizobium* were characterized in comparison with that of *Bradyrhizobium* sp. USDA110. The PGP traits were determined as described below.

P-solubilization. The 10 µl of bacterial culture (10^8 CFU/ml) was dropped on Pikovskaya's (PVK) medium plates containing 5 g/l of $Ca_3(PO_4)_2$ as a sole source of phosphorus. The plates were incubated at 30°C for 7 days. The ability of bacteria to solubilize the insoluble P was observed based on the clearing zone in the PVK agar plates (Nautiyal 1999).

Exopolysaccharide (EPS) production. The 100 ml of 7 day-olds bacterial culture in 100 ml YM broth was centrifuged at 4,000 rpm for 20 min to remove bacterial cells. Then, the supernatant was transferred to a new centrifuge tube and mixed with final 35% (v/v) ethanol, and incubated overnight at 4°C. The EPS pellet was precipitated by centrifugation and dried at 30°C for a day. The EPS was measured as dry weight (mg) per 100 ml culture (Castellane et al. 2017).

Indole acetic acid (IAA) production. The ability of rhizobial strains to produce IAA was determined in YM broth medium added with tryptophan (0.1 g/l). This broth medium was inoculated with standard inoculum 1.0×10^8 CFU/ml. The broth cultures were incubated in dark at 30°C for 7 days and then centrifuged at 4,000 rpm for 15 min. The supernatant was collected for 1 ml to mix with 2 ml of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% of $HCLO_4$ solution) and kept in the dark. The optical density (OD) was recorded at 530 nm after 15 min (Sarwar et al. 1992). The IAA production of tested bacterial strains was also determined when they grew under stress conditions as described above.

1-aminocyclopropane-1-carboxylic acid* (*ACC*) *deaminase activity. The cells from the early stationary phase were washed twice with minimal medium and induced for the ACC deaminase production by inoculation in 15 ml of YM-supplemented minimal medium containing 1 mM ACC, then shaking at 200 rpm for 40 hr. The α-ketobutyrate released from ACC during the culture supernatant incubation with ACC was measured for the ACC deaminase activity as described previously by Mayak et al. (2004).

Bacterial growth assay using sugar as an osmoprotectant under stress conditions. *Bradyrhizobium* strains were aerobically grown at 28°C in YM broth for seven days as a starter. Then, 1% (v/v) (containing 10^8 CFU/ml) of starter was inoculated into minimal broth medium (MSM) (Miller 1996) containing sucrose at different concentrations ranging from 0 to

500 mM (Gouffi et al. 1999; Le Rudulier 2005). Then, the stress conditions were applied by adjusting the conditions as described for the determination of cell survival and adaptation under stress. The number of surviving cells was determined at 10 DAI (like during the stationary phase). The selected sugar was used for further experiments to determine the appropriate concentrations that support the specific growth rate in each stress condition.

Determination of cell survival in the sand under stress conditions. Ten grams of sterilized sand were added into a test tube (50 ml); then, the pH was adjusted to 7.0 with 2 N NaOH and the sand was incubated at 28°C as a normal condition, while other stress conditions were adjusted explicitly as follows. For the acidic condition, the sand pH was adjusted to 4.5 with acetic acid. For the high temperature, the sand was incubated at 40°C. For drought, the sand was desiccated to −3.02 bars using PEG8000. The mixed stress conditions were also arranged as described above. To determine the bacterial cells' response to stress conditions, 10^8 *Bradyrhizobium* cells were inoculated into the prepared sand tubes in each condition and incubated for two days (as an approximate time for soybean germination after sowing). The survival of cells under the stress conditions was investigated by serial dilution and total plate count. The number of living bacteria was determined as CFU/g of the sand (Idris et al. 2007) and the percentage survival of cells in stress conditions was calculated in relation to the initial cell number.

Analysis of the accumulation of sugars in bacterial cells by HPLC. The cell pellets were precipitated from 20 ml of the cultured medium at 10^8 CFU/ml and washed twice with sterilized 0.85% NaCl solution. The intracellular compatible solutes were extracted twice by incubating at 65°C for 5 min in 1 ml of 70% (v/v) ethanol. Crude extracts were centrifuged at 5,000 g for 5 min (Lai et al. 1991), and ethanol was evaporated using a rotary evaporator (Buechi R-142, Nordrhein-Westfalen, Germany) at 45°C. The cell extracts were dissolved in 1 ml deionized water and filtered through a 0.2 μm hydrophobic membrane, and immediately injected into the chromatograph. The sugars were determined using HPLC with an ion-exchange column (Aminex HPX-87H, 7.8 × 300 mm, Bio-Rad) at 45°C and a refractive index detector (RI-150, Thermo Spectra System, USA). The mobile phase was 4 mM sulfuric acid at a flow rate of 0.4 ml/min (Sangproo et al. 2012). A flow rate of 0.3 ml/min and a column temperature of 60°C were used for sugar analyses.

Testing the symbiosis efficiency of *Bradyrhizobium* inoculant supplemented with the selected sugar. The symbiosis efficiency tests were performed both in an experiment with the sterilized sand and in the soils collected from 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). These representative soils had been used in a crop rotation system of legume and rice (Table SII). All *Bradyrhizobium* strains were grown in YM medium with and without supplementation of an appropriate concentration of the selected sugar. The bacterial inoculant was prepared as previously described. The plant experiment was performed under normal and different stress conditions using the same strategy as described above. The symbiosis efficiency of *Bradyrhizobium* inoculant supplemented with the selected sugar was also tested in soil samples collected from different locations. In this case, plants were watered with sterilized water and grown at 25°C on a 12/12 day/night cycle with a light intensity 639 μE/m²/s. At 30 DAI, data on nodule number, plant biomass, and nodule dry weight were collected.

Statistical analysis. Mean values and standard deviations of the data in all experiments were determined with SPSS software (SPSS versions 19.0 Windows; SPSS Inc., Chicago, IL, USA) and the significance of the values determined by Tukey's HSD (Honestly Significant Difference) test (Tukey 1949). Student's *t*-test was also used to determine the significant difference of the means between two sets of data.

Results

Growth, properties, and survival ability of *Bradyrhizobium* isolates *in vitro* under stress conditions. Six *Bradyrhizobium* isolates and the type strains were tested on an agar medium adjusted to different stress conditions. The strain CB1809 and isolates 188, 194, and 197 grew very well in the acid condition of pH 5, while a poor growth of most strains was observed in strong acid of pH 4. Every strain, except isolate 199 and USDA110, could grow on the medium plate at high temperatures, even at 45°C, while CB1809 and 188 showed a better growth ability than other strains. Under drought conditions, isolate 194 exhibited the highest percentage of survival (Table SI). Thus, several *Bradyrhizobium* isolates can resist various stress conditions, while it seems that isolate 194 was able to grow under several stress conditions. Since these *Bradyrhizobium* isolates will be inoculated on plant grown under stress conditions, it is interesting to investigate their plant growth-promoting (PGP) properties. Thus, isolate 194 was selected to preliminary determine its PGP properties compared to the reference strain of USDA110 (Table I). The EPS production by isolate 194 and USDA110 was 9 and 6 mg/100 ml culture, respectively. Likewise, the ACC deaminase activity of isolate 194 was higher than that of USDA110 (1.100 and 0.736 μmol α-ketobutyrate/mg

Table I
Characterization of the plant growth-promoting (PGP) properties of USDA110 and isolate 194.

PGP properties	USDA110	Isolate 194
EPS production (mg/100 ml)	$6^b \pm 1.25$	$9^a \pm 1.45$
ACC deaminase (μmol α-ketobutyrate/mg protein/h)	$0.74^b \pm 0.05$	$1.10^a \pm 0.05$
P-solubilization	no	no
IAA production (μg/ml)		
Normal condition	$0.015^b \pm 0.002$	$0.030^a \pm 0.004$
Acidity	$0.007^b \pm 0.000$	$0.010^a \pm 0.001$
Drought	$0.009^b \pm 0.001$	$0.020^a \pm 0.002$
High temperature	$0.006^b \pm 0.000$	$0.013^a \pm 0.000$

Means (n = 3) in the same PGP activity followed by different letters in the same row are significantly different at $p \leq 0.05$, ± standard deviation

protein/h, respectively). Unfortunately, the P-solubilization ability of both isolate 194 and USDA110 could not be detected. Furthermore, the ability of IAA production by isolate 194 and USDA110, which might be involved in supporting plant growth under stress conditions, was determined exclusively under normal and stress conditions. It was found that although isolate 194 was able to produce IAA higher than that of USDA110 under all tested conditions, the level of IAA production tended to reduce when encountering stresses (Table I).

To further investigating these *Bradyrhizobium* isolates on plant growth promotion under stress conditions, DNA polymorphism using Box-PCR and dendrogram analyses were used to select the representative *Bradyrhizobium* strains to avoid the repetitive isolate selection. The result indicated that there are two clades of *Bradyrhizobium*. The first large clade contained the closely related strains CB1809, USDA110, DASA1014, and isolates 184 and 197, while isolates 193, 188, 194, and 199 were separated to form the second clade (Fig. S1). Based on these data, isolates 184, 188, and 194 were selected for further experiments.

Plant growth promotion by selected *Bradyrhizobium* isolates under single and mixed stress conditions. All *Bradyrhizobium* strains promoted soybean growth well under normal conditions when compared with non-inoculated plants. However, the symbiotic efficiency of these bacteria in soybean was reduced when plants were grown under stress conditions, especially under mixed stresses (Fig. 1). Among the isolated strains, isolate 194 provided the highest plant biomass when tested under all single stress conditions, and the plant biomass was significantly different from that of USDA110 under the single stress of drought and in high-temperature conditions. However, the nodule dry weight and nodule number of soybean inoculated with isolate 194 were not significantly different from those of plants inoculated with USDA110. Isolate 194 displayed the highest nitrogenase activity on

soybean grown under every stress condition except in the drought condition, where isolate 184 exhibited the highest nitrogenase activity. However, isolate 184 had the lowest level of nitrogenase activity when plants were grown under other single stress conditions, while isolate 188 was in the middle range. Therefore, symbiosis with soybean was improved for isolate 194 compared to other isolated strains under both single and mixed stress conditions. However, the performance was not significantly different from that of USDA110 (Fig. 1). The STI value indicated that isolate 194 was the strain that best-facilitated plant growth under single and mixed stress conditions, while isolate 188 could also promote soybean growth under drought and mixed acid-drought conditions (Table SIII). Thus, isolate 194 was selected for comparison with USDA110.

Soybean nodulation competitiveness of isolate 194 and the type strain USDA110 under stress conditions. To investigate the competitive ability of isolate 194 with USDA110, which is usually used as the soybean inoculant, both single and dual nodule occupancies were observed in soybean co-inoculated with isolate 194 and the GUS tagged strain of USDA110 under normal and stress conditions. The nodulation occupancy of isolate 194 under normal, drought, and high-temperature stress conditions was significantly higher than that of USDA110, while there was no significant difference in nodule occupancy of these two strains under acid stress conditions (Fig. 2). Similarly, the nodulation competitiveness of isolate 194 was significantly better than that of USDA110 under mixed stress conditions. Some nodules were occupied by both bacteria and called dual occupied nodules. However, the percentage of dual occupied nodules was low in all conditions. This result indicated that isolate 194 has the potential to compete for nodulation under several stress conditions.

Improved growth rate of *Bradyrhizobium* under stress conditions by supplementation of the culture medium with sugar. Optimization of the inoculant
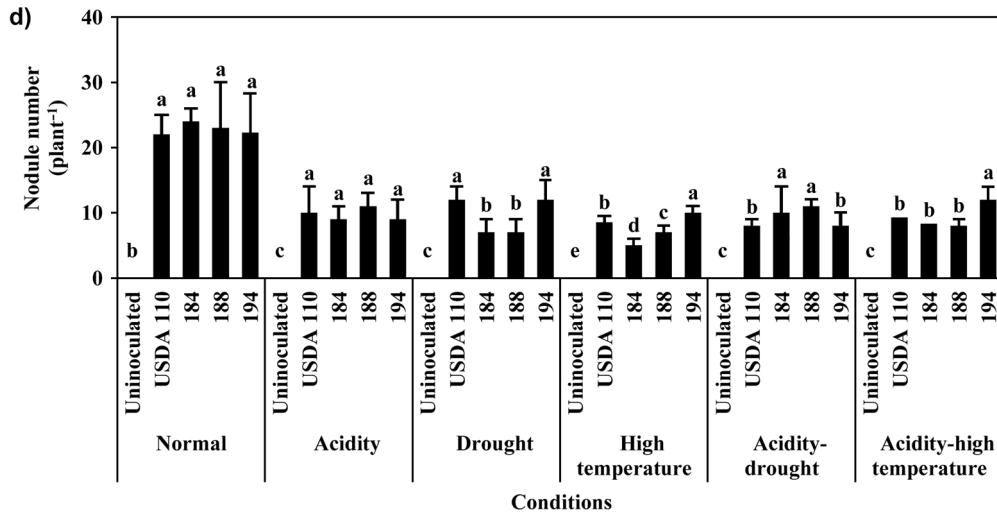
a)

b)

c)
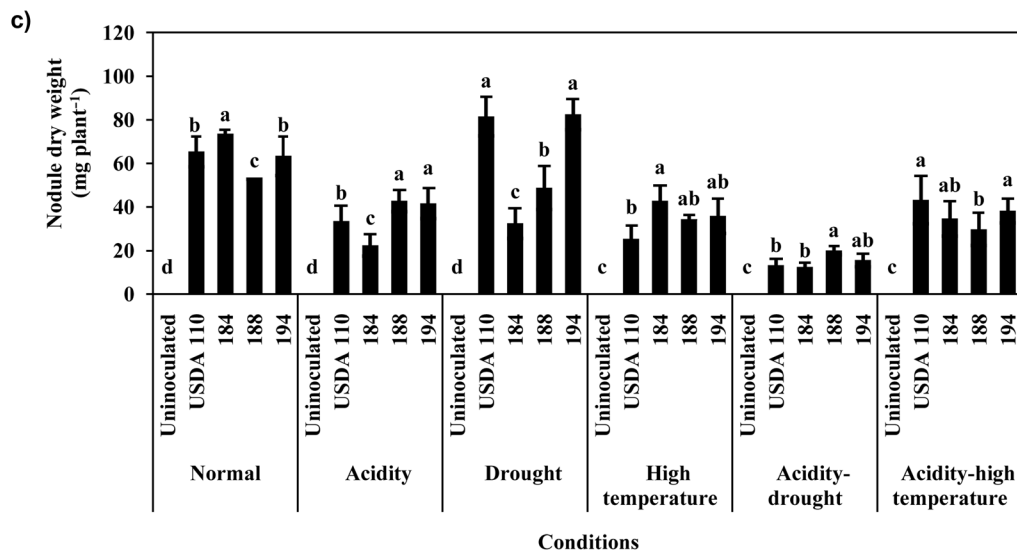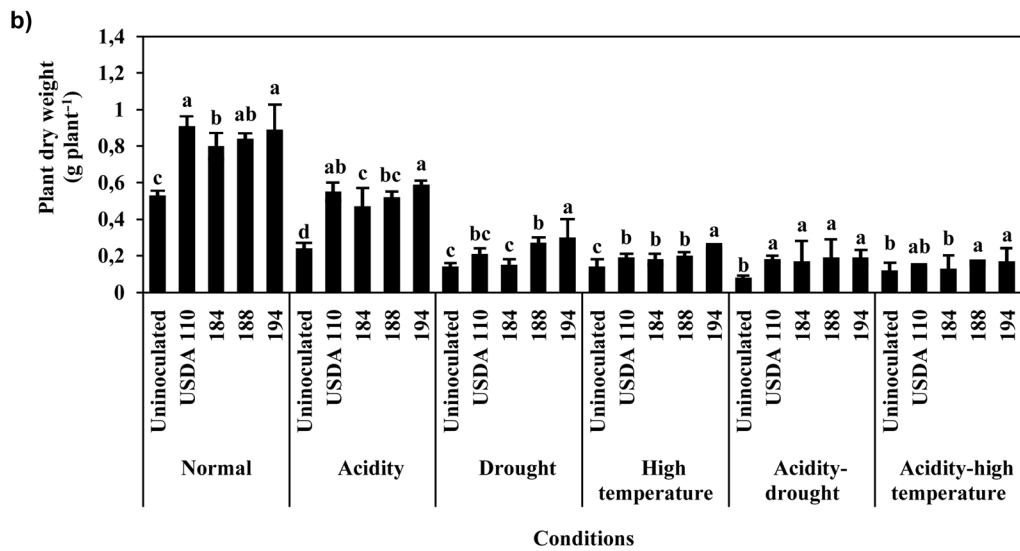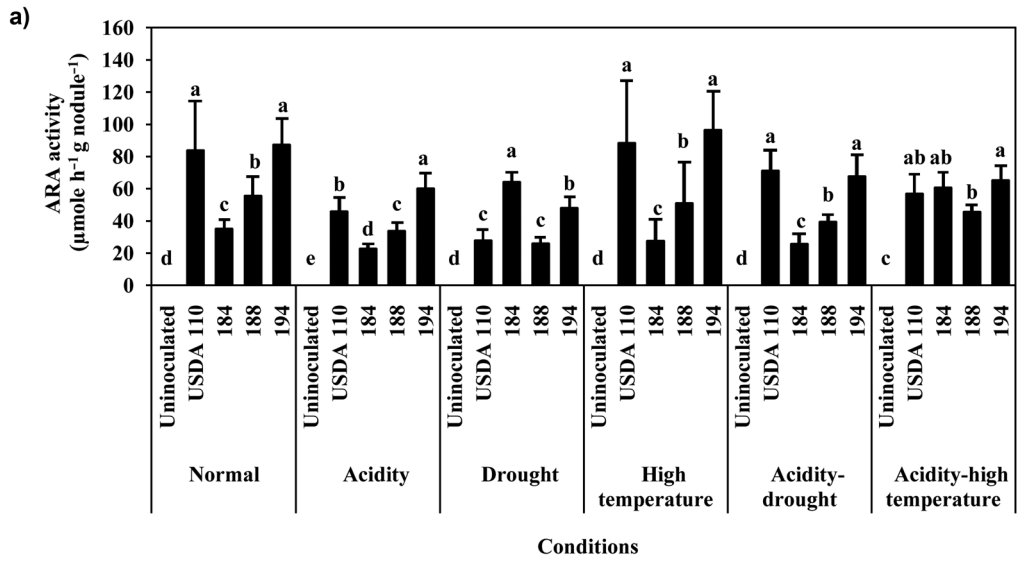
Fig. 1. Symbiotic efficiency of stress-tolerant *Bradyrhizobium* strains grown in soybean in Leonard's jars containing the sand under stress conditions. The Acetylene Reduction Assay (ARA activity) was applied to determine the nitrogenase activity of plants inoculated with different bacterial strains under normal and stress conditions (a). The plant dry weight of non-inoculated and inoculated plants (b) and nodule dry weight per plant (c) were measured, and the nodule numbers were counted per plant (d). Means and standard deviations were calculated from three replicates; for each parameter under the same condition, values with different letters were significantly different at $p \leq 0.05$.
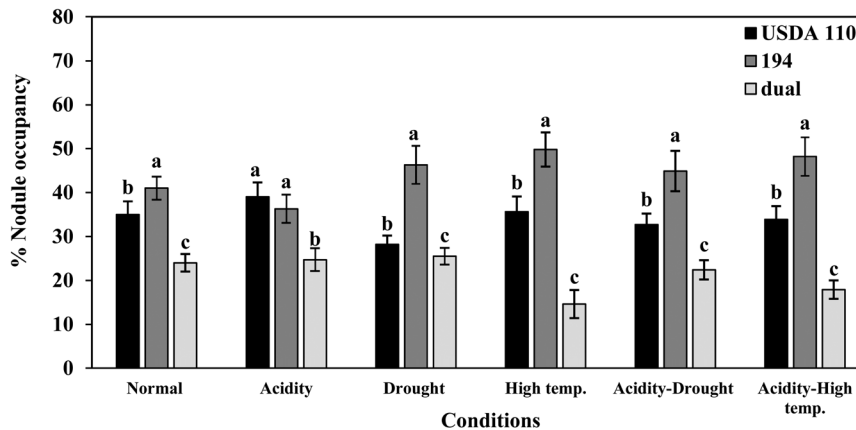

Fig. 2. Nodulation competition of *Bradyrhizobium* strains USDA 110 and isolate 194 in soybean grown in Leonard jars containing the sterilized sand under different stress conditions. Means and standard deviations were calculated from three replicates, and values in each condition with different letters were significantly different at $p \leq 0.05$.

formulation is one of the strategies to improve *Bradyrhizobium* inoculant efficiency. Our preliminary result of sugar supplementation in culture medium implied that of the six supplemented sugars (mannitol, glucose, trehalose, sucrose, glycerol, and polyvinyl alcohol), sucrose was the most effective sugar for improving the growth of isolate 194 under stress conditions (data not shown). Although a low concentration of sucrose (1 mM sucrose) was added in the culture medium, it could enhance the growth rate of isolate 194 better than other tested sugars. Therefore, sucrose was selected as a supplement to the medium, and the suitable concentration was further determined for improving the growth rate of isolate 194 in different stress conditions. Under normal conditions, compared with non-

sugar supplementation, there was no significant difference in specific growth rate per day (μ, in the range 0.40–0.42) when sucrose was supplemented in the range 5–300 mM. At concentrations of 400 and 500 mM, sucrose significantly reduced the growth of isolate 194 under normal conditions (Fig. S2a). The growth rate of isolate 194 in the medium without sucrose supplementation was obviously reduced when cultured under stress conditions. However, it was found that sucrose supplementation in the range of 50–300 mM could improve μ of the isolate 194 under acid (μ 0.31–0.34), drought (μ 0.38–0.42), and high-temperature stress (μ 0.32–0.34) conditions (Fig. S2b, S2c, and S2d). Since the best μ was obtained under several stress conditions when sucrose was supplemented at 300 mM, this

concentration level was selected to study further how sucrose could maintain the growth of isolate 194 under stress conditions based on the generation of other sugars that may act as compatible solutes inside the cell.

Sugars accumulation inside the bacterial cell may contribute to its stress tolerance ability. Since the supplementation of sucrose in culture medium leads to an increased bacterial ability to tolerate stress, it was hypothesized that after being taken up by bacterial cells, sucrose could be transformed to other sugars that might act as compatible solutes inside the cells and resulted in supporting cell growth under several stress conditions. Therefore, the number of viable cells and the concentration of sugars (including trehalose, glycerol, sucrose, glucose, and mannitol) accumulated inside the cell were determined in sucrose and non-sucrose-supplemented cultures under normal and stress conditions. Under normal and stress conditions of acid and high temperature, the number of viable cells at 10 DAI in the culture supplemented with sucrose remained higher than that in the non-sucrose-supplemented medium. With sucrose supplementation, the numbers of viable cells were (in log10 CFU/ml) 10.07, 7.93, and 9.55, while without sucrose supplementation, the numbers of viable cells were 8.68, 7.24, and 7.70 under normal, acid, and high-temperature stress conditions, respectively. However, the numbers of viable cells of isolate 194 supplemented with and without sucrose were similar when tested under drought stress: the cell numbers were (in log10 CFU/ml) 9.39 and 9.64, respectively. It was clearly shown that supplementation of the medium with sucrose tended to promote higher levels of sugar accumulation inside the cell than in non-sucrose-supplemented cells. Mannitol and glucose were the main sugars accumulated in the cells when cultured for 0–10 days under normal and stress conditions of acid and high temperature. However, mannitol, sucrose, trehalose, and glycerol, which are classified as compatible solutes, could also be detected inside the cell when cultured with sucrose supplementation (Fig. 3). The concentration and the type of sugar accumulation fluctuated per day depending on culture conditions. However, it was noticed that high accumulation of these compatible solutes at 8–10 DAI could reduce the loss of viable cells as shown under drought and high-temperature stress conditions (Fig. 3c and 3d). This result suggested that these compatible solutes may functionally interchange and protect the cells from stress. Therefore, supplementing the culture medium with sucrose could be used as a strategy to prepare a *Bradyrhizobium* inoculum for further application in the field under stress conditions.

Sucrose-supplemented inoculum could improve cell survival under stress conditions. To investigate whether *Bradyrhizobium* inoculum prepared from sucrose-supplemented culture could improve its ability to tolerate stress, the survival of cells after inoculation into the sand at 2 DAI was determined under different stress conditions. The survival of isolate 194 was improved under normal conditions when cells were derived from the sucrose-supplemented inoculum. However, the percentage of cells surviving was obviously decreased when tested under single stress and mixed stress conditions (Fig. 4). Acidity stress adversely affected the cell survival of isolate 194 to remain only 1.5%, which was equal to 10^4–10^5 cells/g of the sand at 2 DAI when sucrose was not supplied in the inoculum. The survival of isolate 194 was significantly improved to 21% when the inoculum was supplemented with sucrose.

Interestingly, the cell survival of the sucrose-supplemented inoculum of isolate 194 was significantly increased to more than 80% under drought and high-temperature stress conditions. In addition, under the mixed stress of acid-drought conditions, the survival of isolate 194 increased up to 54% when supplemented with sucrose. However, the survival of this strain under the mixed stress of acid-high-temperature condition was less than 1% even when sucrose was supplemented into medium (Fig. 4).

Plant growth promotion by sucrose-supplemented *Bradyrhizobium* inoculum under single and mixed stress conditions. The experiment was performed in a Leonard jar containing the sterilized sand under normal and stress conditions. The plant biomass was highest when inoculated with a sucrose-supplemented inoculum of the isolate 194 under normal and all stress conditions, and the biomass was significantly different from that of other treatments in all conditions, except under mixed acid-drought stress (Fig. 5). In terms of nodule number and nodule dry weight, the stress conditions affected the symbiosis efficiency by reducing the nodule formation on soybean. In most cases, although the sucrose-supplemented inocula of isolate 194 tended to increase nodule number, this treatment was not significantly different from that with non-sucrose-supplemented inoculum. However, the dry nodule weight produced from soybean inoculated with a sucrose-supplemented inoculum of isolate 194 was significantly increased in all single stress and the mixed acid-drought conditions (Fig. 5c). From these results, it could be concluded that supplementing the medium with sucrose would be suitable for improving plant growth under stress conditions with isolate 194. Therefore, soil pot experiments were performed with inocula of isolate 194 with and without sucrose supplemented to test the performance.

Performance of the developed *Bradyrhizobium* inoculum on soybean symbiosis. The performance of isolate 194 inoculums supplemented with and without sucrose on plant symbiosis was determined in pots

a) Normal conditon

b) Acid condition

c) Drought condition

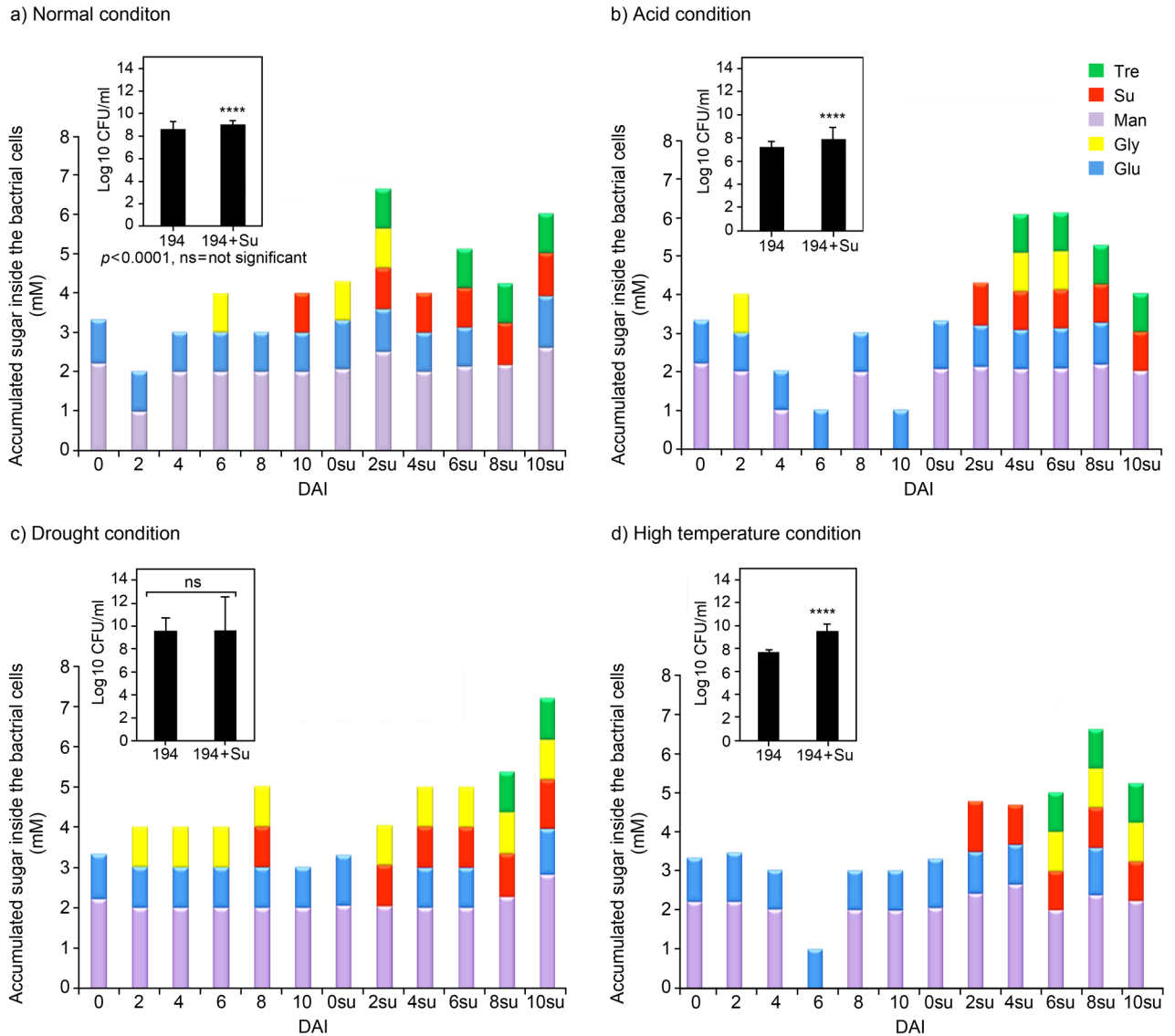### d) High temperature condition

Fig. 3. Accumulation of intracellular sugars in isolate 194 cultured in minimal broth medium (MSM) supplemented with and without 300 mM sucrose under different conditions and the number of viable cells at 10 DAI (days after inoculation). Student's t-test was used to determine the significance of the difference in the means between the two data sets of viable cells in each condition.
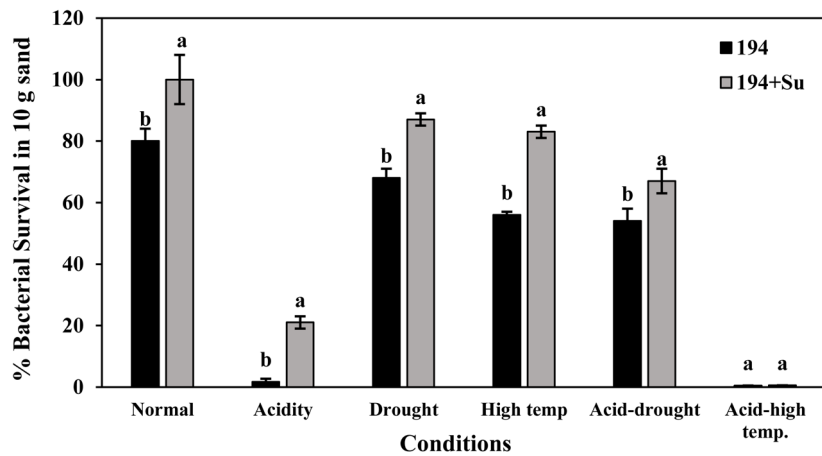

Fig. 4. Survival of isolate 194 supplemented with and without 300 mM sucrose in the sand under different stress conditions at 2 DAI (days after inoculation). Means and standard deviations were calculated from three replicates, and in each condition, values with different letters were significantly different at $p \leq 0.05$.
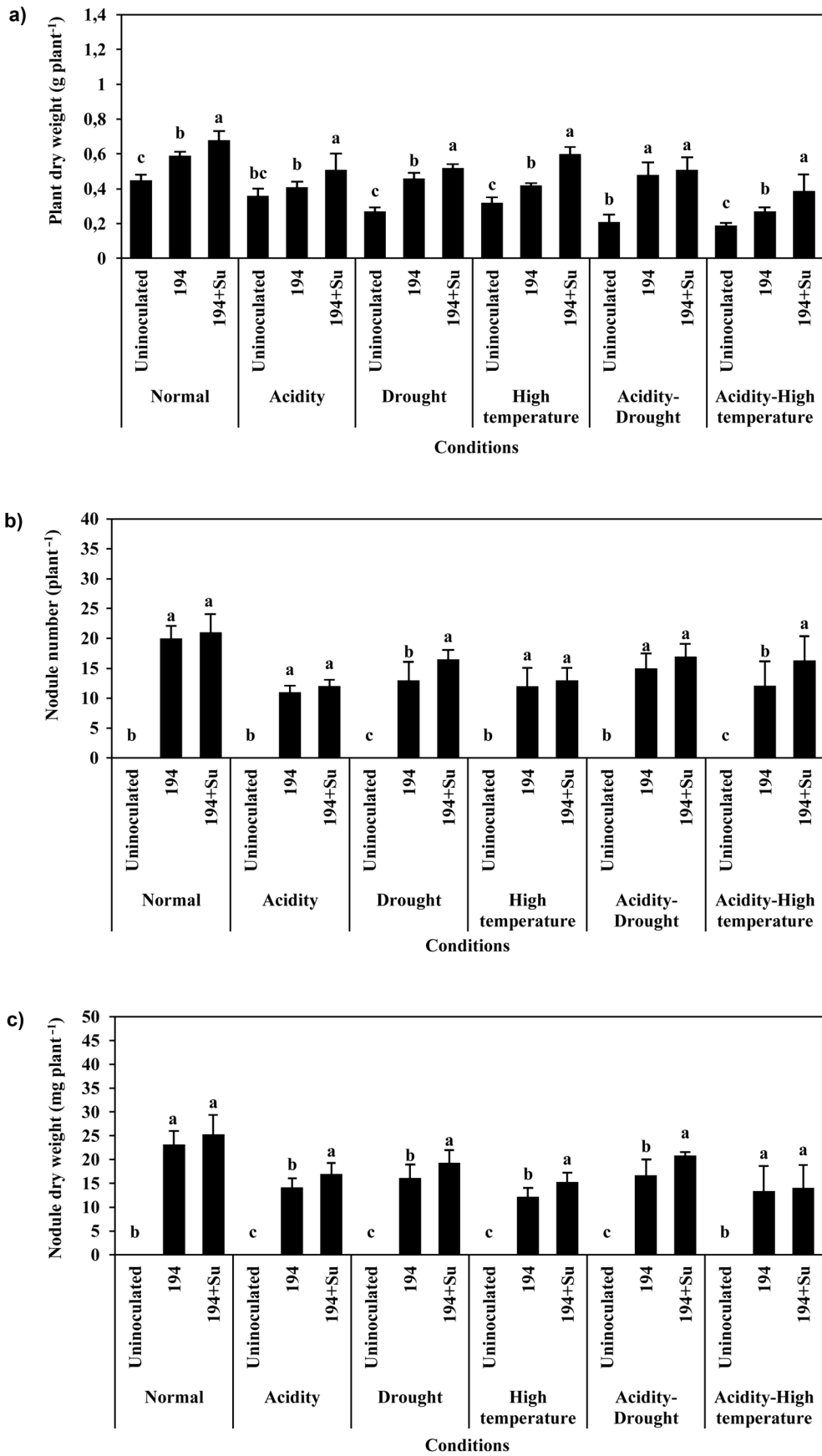
Fig. 5. Symbiotic efficiency of stress-tolerant *Bradyrhizobium* inoculants supplemented with and without 300 mM sucrose on soybean grown in Leonard jars containing the sterilized sand under different stress conditions. Means and standard deviations were calculated from three replicates, and in each condition, values with different letters were significantly different at $p \leq 0.05$.
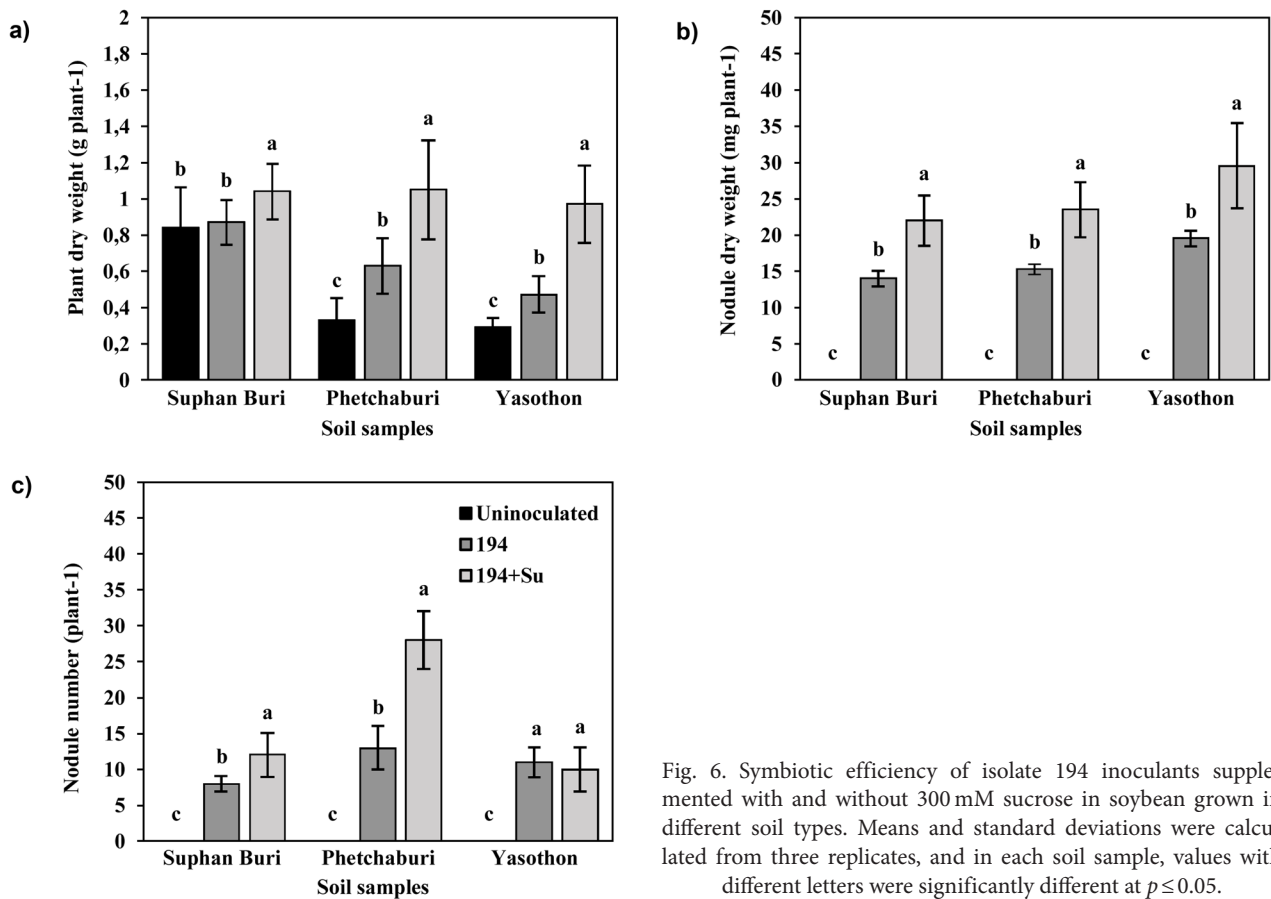
a)

b)

c)

Fig. 6. Symbiotic efficiency of isolate 194 inoculants supplemented with and without 300 mM sucrose in soybean grown in different soil types. Means and standard deviations were calculated from three replicates, and in each soil sample, values with different letters were significantly different at $p \leq 0.05$.

containing soil collected from three different locations in Thailand where soybean had been planted (Table SII). The results of plant growth and symbiosis are shown in Fig. 6. Soybean inoculated with sucrose-supplemented inoculum had a significantly higher biomass and nodule dry weight than non-inoculated plants or plants inoculated with non-sucrose-supplemented inoculum when tested in all soil samples. The nodule number obtained on plants using sucrose-supplemented inoculum was significantly increased when compared with non-sucrose-supplemented inoculum when grown in soil collected from Suphan Buri and Petchaburi provinces. These preliminary data revealed the good performance of inoculum supplemented with sucrose under soil conditions. However, further field experiments are needed to ensure the efficiency of the developed inoculum.

Discussion

In this study, the abiotic stress-tolerant ability of isolated *Bradyrhizobium* strains was compared with that of type strains of *Bradyrhizobium,* including USDA110 and CB1809. USDA110 is normally used as soybean inoculum in Thailand, while the strain CB1809 has been reported as a stress-tolerant *Bradyrhizobium* in several conditions, such as acid soil and alkaline soil

(Botha et al. 2004; Indrasumunar et al. 2011). However, it has been reported that USDA110 is sensitive to acid stress, and it grew slowly in acid agar medium at pH 4.5 (Indrasumunar et al. 2011; Manassila et al. 2012). Our results indicate that although soybean inocula perform well with soybean under normal conditions, their performance could be reduced if the *Bradyrhizobium* strains cannot tolerate conditions of stress (Fig. 1 and Table SI). Soil environmental condition is one of the critical factors that affect the persistence and survival of rhizobial inocula; thus, changes in the rhizosphere environment could influence the competitiveness and persistence of rhizobia (Abd-Alla et al. 2014). Environmental stress conditions, such as acid soil, drought, or high temperature in the field, can occur in terms of mixed stresses. Therefore, mixed stress conditions might have an extreme effect on symbiosis and plant growth. Stress in the environment additionally affects soybean development by impairing the function of active nodules (Dimkpa et al. 2009; Wielbo et al. 2012). This study also found that plant growth was reduced under stress conditions, especially under mixed stress conditions. It has also been reported that the drought stress generated by PEG, in the range from −1.0 to −7.0 bars, decreases root elongation and plant development (Amooaghaie 2011; Uma et al. 2013). Marsh et al. (2006) also reported the effect of high

temperature, who indicated that the yields of soybean, pigeon pea, and cowpea inoculated with *Bradyrhizobium* were decreased when plants were grown at 38°C. These data are similar to our results in that plant growth and symbiotic performance were significantly reduced at high temperatures. High temperatures may affect bacterial cell survival and damage some biological pathways in plant development. These data agree with many studies that suggest the efficiency of tolerant *Bradyrhizobium* strains under adverse conditions could promote plant growth, but that plant growth may not be similar to that of plants cultivated under normal conditions (Wielbo et al. 2012; Atieno and Lesueur 2019).

We identified isolate 194 as a *Bradyrhizobium* strain using the 16S ribosomal RNA gene and recorded it under accession number KF913342.1 in the GenBank. In this present study, this isolate was selected as a tolerant strain that can be developed as a soybean inoculant to be used under multi-stress conditions based on its performance in promoting plant growth in the sand-involving experiments. Although isolate 194 did not provide significantly higher nitrogen fixation ability than USDA110, this strain overall promoted soybean growth under stress conditions better than USDA110. Mubarik et al. (2012) reported that the nitrogenase activity of stress-tolerant *Bradyrhizobium* strains might not significantly differ from that of USDA110. However, their ability to tolerate stress was the important criterion to select the best strain as soybean inoculum for further application under conditions of stress. The stress tolerance in many bacteria is linked to the appropriate composition and membrane structure of the cell. For example, the efficiency in exopolysaccharide (EPS) production of bean rhizobia and *Bradyrhizobium* sp. strains are related to their pH tolerance, leading to increased symbiotic nitrogen fixation in legumes (Donot et al. 2012; Razika et al. 2012).

Similarly, stressed bacterial cells have a high lipopolysaccharide production (LPS) and accumulate compound products from secondary metabolites such as polyols, polyamines, proline, trehalose, etc. (Saxena et al. 2013). These compounds could act as osmolytes, which protect the cell from osmotic stresses. Likewise, isolate 194 has a higher EPS production than USDA110 when grown on medium (Table I), which might be one reason why isolate 194 facilitates stress tolerance. Besides, its ability to grow better than USDA110 under various stress conditions may also be caused by the cellular accumulation of appropriate compatible solute compounds, as shown in Fig. 3. Furthermore, isolate 194 was found to contain some plant growth-promoting (PGP) traits. The ACC deaminase activity of isolate 194 was higher than that of USDA110 (Table I), suggesting that isolate 194 also has the potential to alleviate plant stress by reducing the production in the plant of

ethylene, which regulates many processes in response to biotic and abiotic stresses (Gamalero and Glick 2015). Another PGP trait is the production of indole-3-acetic acid (IAA). IAA is also involved in supporting plant growth under stress conditions. It has been reported that a high level of IAA promotes the formation of lateral roots (Gupta and Pandey 2019) and increases root length and surface area (Olanrewaju et al. 2017), which might be essential for plant growth under drought conditions. Isolate 194 and USDA110 also produce IAA. However, the level of IAA production was reduced when bacteria encounter conditions of stress. As shown in Table I, the levels of IAA production by isolate 194 and USDA110 tended to reduce by more than 50% under stress conditions. Sijilmassi et al. (2020) also showed a significant reduction in IAA production by rhizobia when grown under abiotic stress conditions such as drought at −2 to −7.5 bar and concentrated salt at 0.5 to 3% NaCl. Based on this result, other PGP traits of isolate 194 and USDA110 may also be reduced under stress conditions, and this might adversely affect or could not fully facilitate plant growth under stress conditions. Although the level of IAA produced by isolate 194 was significantly higher than that of USDA110 in all stress conditions, the question remains whether this level of IAA is appropriate to promote plant growth under conditions of stress and whether other PGP traits might be involved in this promotion of plant growth under different stress conditions. Therefore, it is interesting to investigate further the mechanisms of this *Bradyrhizobium*, which not only performs nitrogen fixation but also has other PGP abilities that alleviate plant stress and promote plant growth under single- and multi-stress conditions.

The results of nodulation competition between isolate 194 and USDA110 under multi-stress conditions (Fig. 2) indicates that isolate 194 can overcome the stress and competes with USDA110 to nodulate soybean under several stress conditions. Stress-sensitive *Bradyrhizobium* inoculants are directly affected by environmental stress conditions and may lose their nodulation competitiveness. Thus, the indigenous rhizobia, which generally have a low nitrogenase activity can compete with soybean inoculant, resulting in the reduction of the yield when most nodules are occupied by ineffective indigenous rhizobia (Shamseldin and Werner 2004). Therefore, the improved nodulation competition of isolate 194 under multi-stress conditions compared to USDA110 revealed the potential of developing this stress-tolerant *Bradyrhizobium* strain as a soybean inoculant for application in the field.

To improve the stress tolerance efficiency of soybean *Bradyrhizobium* inocula, using compatible solutes to protect the cell from stress was applied in this study. Several compatible solutes (sugars, polymers,

polyols, protein, and derivatives) have been studied for their function as osmotic balancers with *Rhizobium, Sinorhizobium*, and *Bradyrhizobium* (Deaker et al. 2007; Fernandez-Aunión et al. 2010; Ghalamboran and Ramsden 2010). In this experiment, sucrose was supplemented in the medium during the cultivation of *Bradyrhizobium*. Sucrose has been reported to act as an osmoprotection against several environmental osmotic stresses in rhizobia by maintaining the membrane's integrity (Le Rudulier 2005). The presence of sucrose in the culture medium is involved in the high EPS production in *Rhizobium* strains and extends the shelf life of *Rhizobium* biofertilizers (Razika et al. 2012; Singh et al. 2014). Supplementation of 0.5 mM sucrose was reported to increase cell survival of *Sinorhizobium meliloti* and *Rhizobium leguminosarum* strains during the stationary phase under salt stress (Gouffi et al. 1999). In addition, it has been most popular to use varying concentrations of sucrose as a compatible solute to induce stress tolerance in lactic acid bacteria (LABs) such as *Lactococcus lactis* (Kilimann et al. 2006) and *Lactobacillus delbrueckii* (Silva et al. 2004) and both strains could increase their survival under heat and drying conditions by supplementing with 0.06 and 1.5 M of sucrose, respectively. Interestingly, analysis of sugar accumulation inside the *Bradyrhizobium* cell after supplementation with sucrose found many more compatible solutes, such as mannitol, trehalose, and glycerol, inside the cell than in non-sucrose-supplemented cells (Fig. 3). In Gram-negative bacteria, the extracellular sucrose can enter through the inner membrane (Reid and Abratt 2005) and enter to glycolysis as a translocated sugar, which can be transformed to other sugars by several pathway links (Lee et al. 2010). This explains why different sugars could be detected inside cells of isolate 194 after being supplemented with sucrose in the culture.

Moreover, the stressed bacterial cells could synthesize compounds alleviating the stress from the supplied molecules by a biosynthetic *de novo* pathway (Blanco et al. 2010). Thus, the accumulation of soluble sugar inside the bacterial cell may be derived from self-production and uptake. High accumulation of compatible solutes such as glycerol and trehalose during 8–10 DAI could reduce the loss of viable cells, especially under drought and high-temperature stress conditions (Fig. 3c and 3d). The accumulation of trehalose in the cytoplasm is critical to the survival of *Bradyrhizobium japonicum* during desiccation (Streeter 2003). Trehalose and glycerol have been reported to stabilize proteins at high temperatures (Empadinhas and da Costa 2008) and preserve the present form of proteins, resulting in a favored hydration of protein surfaces (Thomas et al. 2013). These sugars may maintain the ability of the cell to cope with stress conditions. The survival of sucrose-supplemented *Bradyrhizobium* isolate 194 was signifi-

cantly increased under most stress conditions (Fig. 4) and may lead to improvements in the biomass of soybean grown under several stress conditions as well as on different soils (Fig. 5 and 6). The good performance of sucrose-supplemented *Bradyrhizobium* inoculant when tested with soybean in soil samples indicates that cell survival was improved successfully, promoting the symbiosis efficiency compared with non-sucrose-supplemented inoculum. The application of trehalose could increase the survival of cells of *R. leguminosarum* bv. *trifolii* strain NZP561 when compared to treatment with lactose and water (McIntyre et al. 2007). Mannitol has also been used as an osmoprotectant and found to enhance the survival of *L. lactis* (Efiuvwevwere et al. 1999), *Rhizobium tropici* CIAT 899, and *Rhizobium gallicum* bv. *phaseoli* 8a3 (Fernandez-Aunión et al. 2010). Moreover, the application of 120 mM sucrose in a liquid medium for *Azotobacter* and *Rhizobium* inoculant production could also increase the seed germination and development of *Trigonella foenum-graecum* L. under *in vitro* condition (Nagananda et al. 2010).

This study suggests that the application of abiotic-stress-tolerant *Bradyrhizobium* strain and supplementing an appropriate sugar in the medium could be a promising strategy for developing a soybean *Bradyrhizobium* inoculant for application under multi-environmental stress conditions. Overcoming the challenges of climate change is very important for soybean inoculant developers. However, further testing of the soybean inoculant developed here under various field conditions is needed to validate its symbiotic efficiency and promote plant growth and soybean yield before adopting this technology.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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