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Identification and characterization of native rhizobia from three mungbean varieties

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ABSTRACT

Aims: Native rhizobia from root nodules of mungbean could reduce atmospheric nitrogen to ammonia for assimilation. The objective of this study was to find the best native rhizobium from mungbean.
Methodology and results: Three rhizobia isolates from three mungbean varieties (Maejo 3, Khampangsan 2 and Chainat 72) were collected from 10 undamaged fresh nodules at Prince Chakrabandh Pensiri Center for Plant Development, Saraburi Province, Thailand in 2016. 16S rDNA analysis identified the three rhizobia isolates as *Bradyrhizobium* sp. (SB1), *Bradyrhizobium elkanii* (SB2) and *Rhizobium* sp. (SB3). All the isolates could grow well in yeast mannitol agar (YMA) at pH 7, and all isolates could tolerate up to 35 °C, with isolate SB3 tolerate up to 45 °C. Isolate SB2 produced the highest amount of indole acetic acid (IAA; 8.37 mg/L) and had the highest phosphate solubilization index (7.60 SI). In a Leonard jar trial, inoculation with isolate SB2 resulted in the highest shoot fresh and dry biomass of mungbean host. Further, the mungbean inoculated with SB2 had the highest number of root nodules, nodule fresh dry weight, chlorophyll content index, and shoot and root nitrogen contents.
Conclusion, significance and impact of study: This study suggested that the strain SB2 (*B. elkanii*) is a suitable bioinoculant to improve mungbean growth and yield.

Keywords: Identification, characterization, native rhizobium, mung bean

INTRODUCTION

Mungbean or green gram (*Vigna radiata*) is a well-known economic crop in Thailand and other South and Southeastern Asian countries. In central Thailand, mungbean is commonly grown twice during the region's annual rainy season. Moreover, mungbean can provide a large amount of biomass and improve soil organic matter after harvesting time (Ullah *et al*., 2011). This leguminous plant harbors rhizobium species, which form nodules on plant roots and encourage atmospheric nitrogen (N_2) fixation to the plant as part of a symbiotic relationship (Dudeja *et al*., 2012). N_2 fixation by native rhizobium strains influences soil moisture, temperature, and host reaction to the light and nutrients availability (Evans and Russell 1971; Mohammadi *et al*., 2012). It has been previously observed that host plants more readily form relationship with their native rhizobium than with commercially produced rhizobium, and these native relationships can improve overall soil health (Ouma *et al*., 2016). Moreover, inoculation of host plants with native rhizobia have been shown to increase shoot dry weight, nodule dry weight, and the absorption of phosphorus (P) and nitrogen (N) in climbing beans and mungbean (Shutsrirung *et al*., 2002; Yahya-Abadi, 2008; Koskey *et al*., 2017).

Some rhizobia benefit their host plants through mechanisms other than N_2 fixation, including the production of plant growth hormones and phosphorus solubilization (Rodringuez and Fraga, 1999; Whitelaw, 2000). It is thought that the presence of rhizobia can increase mungbean yield through any of these mechanisms. The yield of mungbean could increase from 10 to 37% by rhizobium inoculation (Rao, 1980; Satter and Ahmed, 1992). In this study, we aim to identify and characterize a strain or strains of native rhizobium that can efficiently increase nutrient availability and regulate plant growth hormones to the benefit of its host plant.

MATERIALS AND METHODS

Isolation of rhizobium species

Root nodules were collected from three mungbean varieties (Maejo 3, Khampangsan 2, and Chainat 72) at Prince Chakrabandh Pensiri Center for Plant

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Development, Saraburi Province in 2016. Ten undamaged nodules were detached from the roots of each mungbean variety. Nodules were surface sterilized by soaking in a 3% (v/v) solution of sodium hypochlorite for 2-4 minutes, rinsing by sterile water. The nodules were crushed in a sterile glass rod and streaked on yeast mannitol agar (YMA) (Vincent, 1970; Somasegaran and Hoben, 1994). Fifteen isolates were grown on YMA, and all isolates were evaluated by a plant infection method for rechecking as rhizobium (Somasegaran and Hoben, 1994). Briefly, all the 15 isolates were increased to multiply in yeast mannitol broth (YMB) at 125 rpm/min for 7 days (30 °C). After that, 1 mL of inoculant was added into each mungbean growth pouch. The only 3 isolates which induced nodulation in mungbean plants upon reinfection, hereafter referred to as *Bradyrhizobium* sp. (SB1), *Bradyrhizobium elkanii* (SB2) and *Rhizobium* sp. (SB3) throughout in this study. One pure colony (assumed to be equivalent to one strain) was chosen from each of the three varieties.

DNA sequencing

To confirm the identity of the rhizobium isolates, genomic DNA was extracted via a genomic DNA extraction kit (RBC-YGP50, Taiwan). The partial 16S rDNA gene was selected and amplified by polymerase chain reaction (PCR) with primers 27F(5'-AGAGTTTGATCMTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCRCCGCA -3'). The PCR conditions were initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 1 min, annealing of primers at 55 °C for 1 min and extension at 72 °C for 1 min. Final extension was carried out at 72 °C for 5 min. The PCR products were checked by agarose gel electrophoresis with GEL/PCR DNA fragments extraction kit (70 bp–20 kb DNA fragments, Cat. Number GMB100, Geneaid Biotech Ltd., Taiwan) and nucleotide sequenced (First Base Laboratories, Malaysia) with GenBank nucleotide database via BLAST search.

Morphology of rhizobium

The rhizobium isolates were examined for colony morphology, cell shape (Prescott, 2002) and Gram reaction (Vincent, 1970). On the other hand, the acid production from glucose in the YMA was replaced by an equal amount of glucose and bromothymol blue (25 mg/L). The modified medium was used to observe the changes in color around the colonies (from green to blue or yellow as indicators of alkali or acid). The ability of rhizobium isolates to grow at different pH was tested by streaking on YMA with pH adjusted to 5.5, 6.5, 7.5, 8.5 and 9.5 using 1N HCl and 1N NaOH (Jordan, 1984; Graham *et al*., 1991; Graham, 1992; Bernal and Graham, 2001). Besides, freshly grown isolates were incubated on YMA plates at different temperatures (30, 35, 40 and 45 °C) to screen for temperature tolerance, and growth was confirmed via visual observation after 3 days (Bansal *et al*., 2014).

Screening phosphate solubilizing

Phosphate solubilizing activity of the isolates was screened via Pikovskaya (PVK) agar (Pikovskaya, 1948). Freshly grown cultures were spot inoculated on the center of PVK plates, and plates were incubated at 28 ± 2 °C for 7 days. A clear zone surrounding the colonies is indicative of positive phosphatase activity. Phosphate solubilization index (SI) was determined by using the following formula (Premono *et al*., 1996; Arun and Sridhar, 2005):

$$\text{Solubilization index (SI)} = \frac{\text{Colony diameter (cm)} + \text{Halo zone diameter (cm)}}{\text{Colony diameter (cm)}}$$

Indole-3-acetic acid (IAA) potential activity

All isolates were screened for the indole-3-acetic acid (IAA) production. Rhizobium-isolates were grown on nutrient broth (NB) with tryptophan 0.102 g/L and shaking at 125 rpm/min (30 °C). The broth was centrifuged after 7 days. Supernatant was reserved and 1 mL of supernatant was mixed with 2 mL of Salkowski's reagent (Ehmann, 1977), and then kept in the dark at room temperature for 30 min. The optical density (OD) was recorded at 530 nm after developing color. Pure IAA (Sigma Aldrich, MW= 175.19) was used as a standard (Gamburg, 2017).

Leonard jar test

The ability of native rhizobium to infect host plants and infection impact on host health was confirmed in a Leonard jar test following Somasegaran and Hoben (1994). The experiment was set up with completely randomized design (CRD) with 4 replications. The experiment consisted of five treatments as follows: control (no N source and non-inoculated), applied potassium nitrate (KNO_3: 0.05% w/v) as nitrogen source (non-inoculated), and separate inoculations of rhizobium SB1, rhizobium SB2, and rhizobium SB3 with no nitrogen source. The rhizobium isolates were allowed to multiply in YMB at 125 rpm/min for 7 days at 30 °C, and the final count was estimated as 4×10^8 CFU/mL by dilution method.

Mungbean seeds from the Maejo 3 variety were sterilized by soaking in a 3% (v/v) solution of sodium hypochlorite for 2-4 min. Three seeds were planted in each jar, and the seedlings were each inoculated with 1 mL of their native rhizobium culture. The Leonard jars were packed medium with sterilized sand and 500 mL of N-free nutrient solution (Somasegaran and Hoben, 1994). The plants were harvested after 30 days, and fresh above- and below-ground biomass was recorded. Dry biomass was also obtained after oven-drying at 65 °C for 48 h. The number of nodules, and the mass of the nodules in fresh and dry matter were evaluated. Nitrogen content in shoots and roots were determined by Kjeldahl method (Jackson, 1958). Briefly, from each jar, 3 fully-expanded leaves at the third node were selected at 30

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days to determine the chlorophyll contents. The SPAD value (index of relative chlorophyll content) was obtained, using a chlorophyll meter (SPAD-502, Minolta Corp. Ltd, Osaka, Japan).

Statistical analysis

All data were analyzed with analysis of variance (ANOVA) by using Statistix 10, followed by the least significant difference (LSD).

RESULTS AND DISCUSSION

Morphology and characterization of isolated rhizobium

Based on 16S rDNA result, three rhizobium isolates were identified, which are *Bradyrhizobium* sp. (SB1) (Genbank accession number AY961984), *Bradyrhizobium elkanii* (SB2) (Genbank accession number AB110484) and *Rhizobium* sp. (SB3) (Genbank accession number CP041204) with 99.99, 100 and 99.74% identity matching respectively from three mungbean varieties (Maejo 3, Khampangsan 2 and Chainat 72). The result of 16S rDNA similar to Zhang *et al.* (2008) that mentioned 90% of *Vigna* rhizobia strains in China were related to *Bradyrhizobium japonicum*, *Bradyrhizobium liaoningense*, *Bradyrhizobium yuanmingense and Bradyrhizobium elkanii*. Moreover, Yokoyama *et al.* (2006) showed that nucleotide sequences of 16S rRNA of *Bradyrhizobium* strains isolated from Thai *Vigna* plants, and they found that 4 strains of *Bradyrhizobium* sp. present in the nodules of *Vigna radiata.* All the isolates were Gram-negative and motile rod shape (Figure 1) as described by Somasegaran and Hoben (1994). The bacterial isolates were grown in YMA, and colony morphology was documented after 2-3 days of growth. It was found that the *Rhizobium* sp. (SB3) isolate grew quickly, and colonies showed convex elevation, punctiform morphology, and were pale pink in color. The other two isolates (SB1 and SB2) were grew slower. Colonies from these isolates showed convex elevation, circular form (SB1), punctiform morphology (SB2) and were milky white in color (Table 1) as was observed by Kaur *et al.* (2012) found that colonies were white colored, entire and circular shape of *Bradyrhizobium* spp.

All isolates grew at all pH values tested, except for SB2 which did not display growth at pH 5.5 (Table 2 and Figure 2). Moreover, all isolates grew well at pH 7.5. Singh *et al.* (2008) and Bhatt *et al.* (2013) report similar findings. Deora and Singhal (2010) have proposed that rhizobium isolates may be highly sensitive to low pH (Zahran, 1999), as was observed in our study with the limited growth of SB2 at pH 5.5.

From Table 2, it shows that all three isolates able to grow at 30 °C and 35 °C. For isolate SB2 and SB3, they were able grew at 40 °C; and only isolate SB3 able grew at temperatures of up to 45 °C. Our results agree with Fentahun *et al.* (2013) who characterized *Rhizobium* spp. isolate from haricot bean growing in Ethiopia. They found

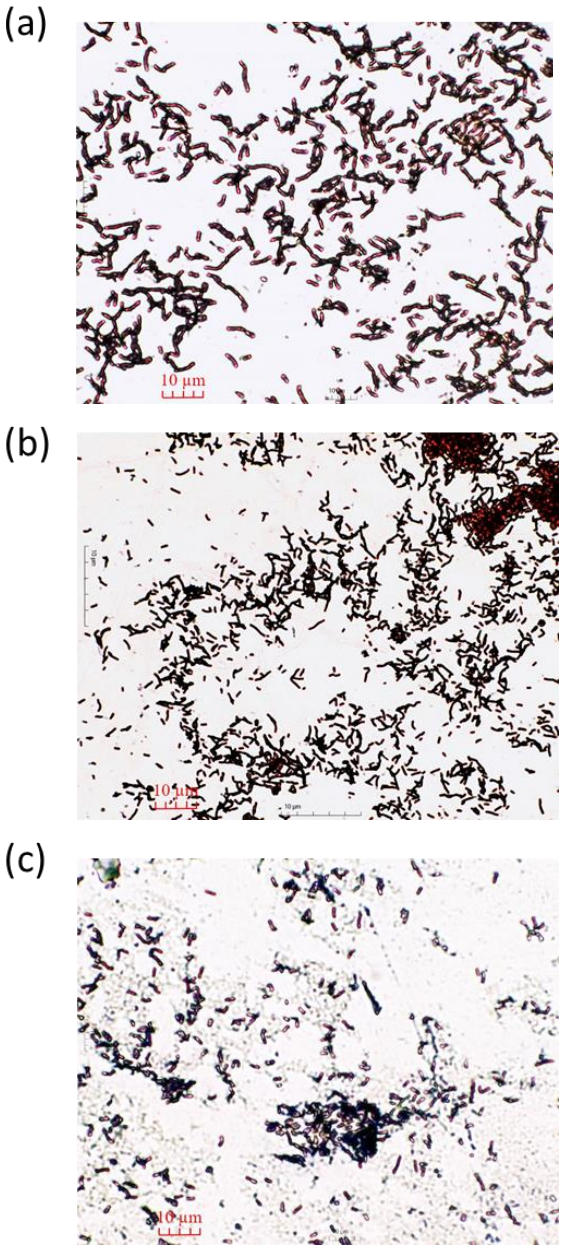

Figure 1: Morphology of isolates under the microscope (100 ×) after Gram staining. (a) *Bradyrhizobium* sp. (SB1), (b) *Bradyrhizobium elkanii* (SB2), (c) *Rhizobium* sp. (SB3).

that most of the isolates were able to grow at 37 °C to 40 °C, but a few isolates were able to grow at temperature as 50 °C. Zahran *et al.* (1994) also recovered some rhizobium strains with the ability to grow at 44 °C. Zahran (2001) further concluded that natural symbiotic rhizobia from wild legumes were also tolerant of other ecological stressors such as salt, elevated temperatures or drought conditions. However, the survival of rhizobium in high soil

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Table 1: Morphological and cultural characteristics of the native rhizobium isolates.

Strains characters	Form	Elevation	Margins	Color	Gram nature	Shape	Acid from glucose
Bradyrhizobium sp.(SB1)	Circular	Convex	Entire	Milky white	Gram negative	Rod shaped	Positive color changes from green to yellow
Bradyrhizobium elkanii (SB2)	Punctiform	Convex	Entire	Milky white	Gram negative	Rod shaped	Positive color changes from green to yellow
Rhizobium sp.(SB3)	Punctiform	Convex	Entire	Whitish pink	Gram negative	Rod shaped	Positive color changes from green to yellow

Table 2: Survival of the native rhizobium isolated at various pH and temperature.

Rhizobium	pH					Temperature			
	5.5	6.5	7.5	8.5	9.5	30 °C	35 °C	40 °C	45 °C
SB 1	+	++	++	++	++	++	++	-	-
SB 2	-	+	++	+	+	++	++	++	-
SB 3	+	+	++	++	++	++	++	++	++

Colonies observed on YMA by visual observation (++): High growth; (+): Moderate; (-): No growth.

temperature conditions does not necessarily indicate an efficient in N_2 fixation (Michiels *et al*., 1994).

Screening phosphate solubilizing

Three isolates were all screened for their ability to solubilize phosphate via PVK agar assay. All isolates effectively presented phosphate solubilization ability after a 3-7 day incubation on the basis of halo zones around their colonies (3.74, 6.60 and 3.20 cm in SB1, SB2, and SB3, respectively). Isolate SB2 created the highest phosphate solubilization index (SI = 7.60) (Table 3). Halder *et al.* (1999) report similar results; they found that *Rhizobium* sp. and *Bradyrhizobium* sp. strains release soluble phosphate from a variety of rock phosphate sources after a three-day incubation. This is important because some species of rhizobium can perform phosphate solubilization during the process of phytohormone production, leading to increased growth of their hosts (Zahran, 2001; Deshwal *et al*., 2003; Sridevi *et al*., 2007). Moreover, Nautiyal (1999) summarized that the rhizobium strains isolated from alkaline soil have the potential to solubilize phosphates at high salt, high pH and high temperature conditions. Uma Maheswar and Sathiyavani (2012) found that rhizobium strains optimally solubilized phosphate at temperatures between 30 °C and 45 °C, which overlaps the range of temperatures found suitable for the growth of isolates SB1, SB2, and SB3.

Indole-3-acetic acid (IAA) potential activity

From Table 3, it presents isolate SB2 produced the highest IAA concentration (8.37 mg/L). Similarly, Amjad Qureshi *et al.* (2017) reported the production of IAA equivalents by *Rhizobium* sp. at 7.52 to 8.69 mg/L. Isolates SB1 and SB3 produced lesser IAA, only 5.35 and 5.21 mg/L, respectively. However, it has previously been found that fast-growing *Rhizobium* sp. from *Cajanus cajan* produced 99.7 µg IAA/mL when grown in basal medium supplemented with L-tryptophan (Datta and Basu, 2000). Furthermore, Santi *et al.* (2007) mentioned that *Rhizobuim* sp. from the root nodule of *Vigna mungo* (L.) Hepper could produce more IAA (11 to 28 µg/mL) in growth medium with L-tryptophan compared with growth in a control medium (8 µg/mL). In addition to L-tryptophan, pH, carbon source and phase of growth have also been reported to affect IAA production (Yurekli *et al*., 2003; Ahmad *et al*., 2005).

Leonard jar test

Plant height of 30 day after planting (DAP) was not significantly different between treatments (Table 4). The shoot fresh and shoot dry matter of mungbean were the highest in SB2 treatment, 7.76 and 1.15 g/plant, respectively compared with control ($p < 0.01$). The root fresh and root dry weights were lowest under the control treatment (2.46 g/plant and 0.19 g/ plant, respectively). Inoculation with isolate SB2 resulted in host plants with the highest root fresh weight (6.68 g/plant; $p < 0.05$). The highest of root dry matter was found in treatment KNO_3 with 0.35 g/plant, but this value was not significantly greater than the dry root weight of plants inoculated with rhizobium isolates. Plants treated with strain SB2 expressed the highest number of nodules (128/plant) and highest nodule fresh and dry weights (0.51 g/plant and 0.118 g/plant, respectively) ($p < 0.01$). Anjum *et al.* (2011) also reported that inoculation with certain *Rhizobium* sp. isolates increased the root and shoot lengths and fresh and dry shoot weights of mungbean when compared with control. Similarly, Bansal *et al.* (2014) recorded that the native rhizobium strains of mungbean from Haryana, India

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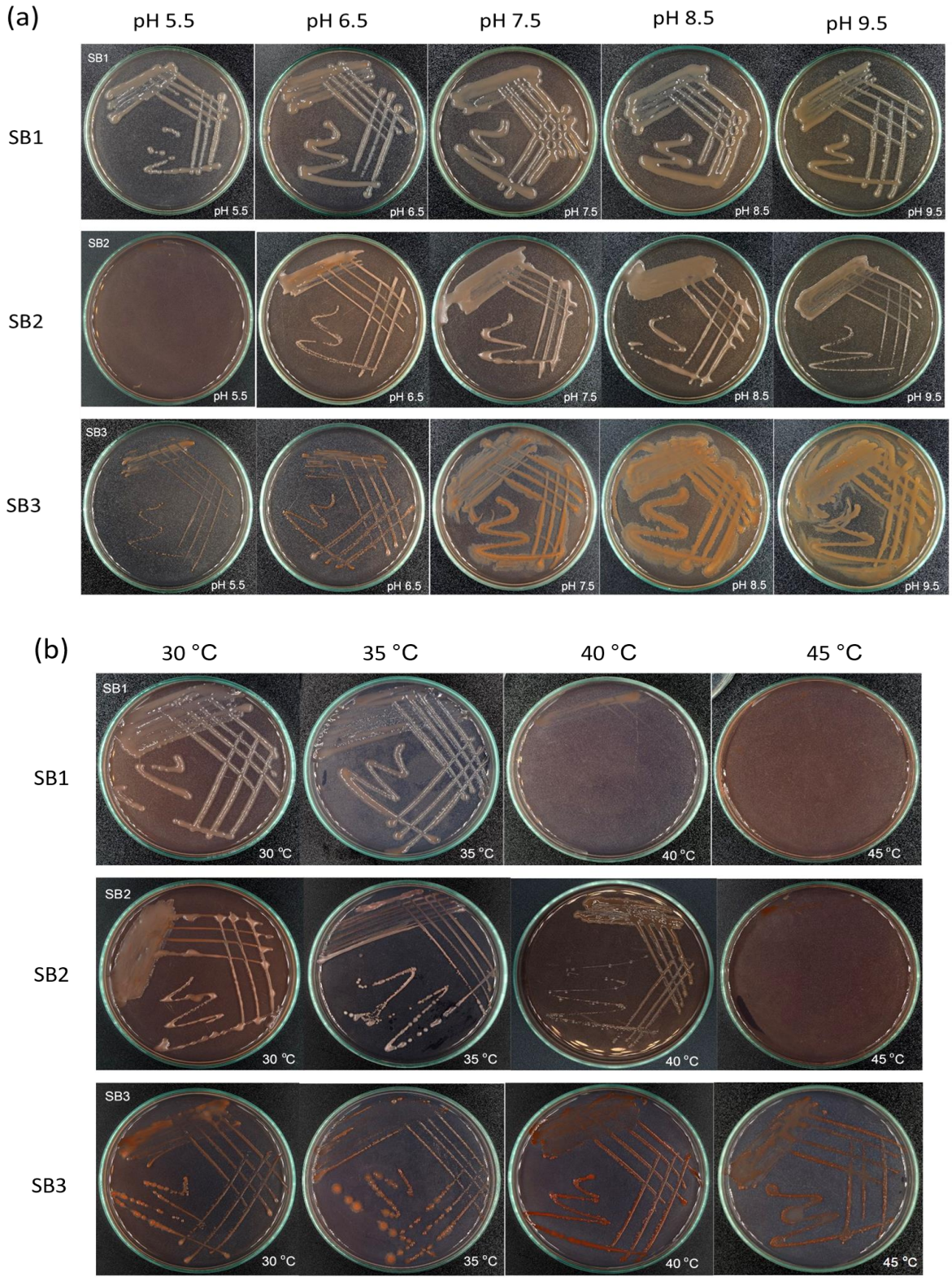

Figure 2: Visual observation of the native rhizobium isolated at various (a) pH and (b) temperature.

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Table 3: Screening phosphate solubilizing and indole-3-acetic acid (IAA) potential activity.

Rhizobium	Colony diameter (cm)	Halo zone diameter (cm)*	Solubilization index (SI)*	IAA (mg/L)*
SB 1	1	3.74 ± 0.3	4.74 ± 0.3	5.35 ± 0.4
SB 2	1	6.60 ± 0.5	7.60 ± 0.5	8.37 ± 0.2
SB 3	1	3.20 ± 0.6	4.20 ± 0.6	5.21 ± 0.4

* The data represented mean of 4 replicates with standard deviation.

Table 4: Leonard jar test of plant height, shoot fresh matter (SFM), shoot dry matter (SDM), root fresh matter (RFM), root dry matter (RDM), nodule fresh matter (NFM), nodule dry matter (NDM) and number of nodules (NN).

Treatment	Plant height (cm)	SFM (g)	SDM (g)	RFM (g)	RDM (g)	NFM (g)	NDM (g)	NN (plant)
Control	17.30 ± 4.0	1.90 ± 0.17^{c}	0.29 ± 0.04^{b}	2.46 ± 0.5^{b}	0.19 ± 0.1^{b}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}
KNO_3	19.50 ± 1.3	4.84 ± 1.51^{b}	0.92 ± 0.31^{a}	6.36 ± 2.1^{ab}	0.35 ± 0.1^{a}	0 ± 0^{b}	0 ± 0^{c}	0 ± 0^{b}
SB 1	15.13 ± 1.8	6.97 ± 1.88^{a}	0.98 ± 0.26^{a}	6.29 ± 1.7^{ab}	0.30 ± 0.1^{a}	0.39 ± 0.1^{a}	0.093 ± 0.02^{ab}	127.5 ± 31^{a}
SB 2	15.88 ± 2.3	7.76 ± 0.84^{a}	1.15 ± 0.12^{a}	6.68 ± 2.3^{a}	0.30 ± 0.1^{a}	0.51 ± 0.1^{a}	0.118 ± 0.02^{a}	128.0 ± 22^{a}
SB 3	14.50 ± 0.7	6.50 ± 1.75^{ab}	0.80 ± 0.27^{a}	6.23 ± 2.4^{ab}	0.26 ± 0.0^{ab}	0.38 ± 0.1^{a}	0.085 ± 0.01^{b}	110.0 ± 27^{a}
Grand mean	16.46	5.59	0.83	5.60	0.28	0.26	0.059	42.12
CV(%)	14.05	17.99	24.37	34.60	23.45	29.99	24.68	73.10
F-test	ns	**	**	*	*	**	**	**

Means (n=4) with standard deviation in the same column followed by different letters were significantly different by LSD, **= 0.01, *= 0.05 and ns = Non-significant.

Table 5: Leonard jar test for chlorophyll content, nitrogen content in the shoot and root.

Treatment	Chlorophyll content	Nitrogen content in the shoot (%)	Nitrogen content in the root (%)
Control	17.83 ± 3.0^{d}	0.98 ± 0.1^{c}	0.89 ± 0.1^{b}
KNO_3	30.88 ± 5.1^{c}	2.57 ± 0.4^{b}	1.94 ± 0.6^{ab}
SB 1	38.45 ± 0.3^{ab}	3.65 ± 0.3^{a}	2.65 ± 0.9^{a}
SB 2	39.58 ± 4.1^{a}	3.91 ± 0.1^{a}	2.91 ± 0.3^{a}
SB 3	31.50 ± 3.0^{bc}	3.58 ± 0.4^{a}	2.50 ± 0.5^{a}
Grand mean	31.65	2.94	2.18
CV(%)	11.00	10.35	25.76
F-test	**	**	**

Means (n = 4) with standard deviation in the same column followed by different letters were significantly different by LSD, ** = 0.01.

could increase nodule number, nodule fresh weight, root fresh weight, shoot height, shoot dry weight and shoot nitrogen when compared with uninoculated plant in Leonard jars experiment.

The chlorophyll content was measured via SPAD-502 Plus at 30 DAP. The mungbean leaves inoculated with isolate SB2 showed the highest chlorophyll content index at 39.58, and plants under the control treatment had the lowest chlorophyll index of 17.83 ($p < 0.01$) (Table 5). Similarly, Ahmad *et al.* (2013) reported that the SPAD chlorophyll value in mungbean was significantly improved by inoculation or co-inoculation with rhizobium.

Nitrogen content in the shoot and root were not significant between rhizobium treatments, but it showed rhizobium strains enhancing nitrogen efficiency better than non-inoculation treatments. Herridge *et al.* (2005) proved that rhizobium inoculation though nitrogen fixation in nodules providing in high shoot and root nitrogen contents in mungbean. However, inoculation with SB2 resulted highest nitrogen content in shoot and root (3.91 and 2.91%, respectively) compared with control ($p < 0.01$) (Table 5).

CONCLUSION

In conclusion, we have identified and characterized native rhizobium isolated from three varieties of mungbean. The isolates were identified as *Bradyrhizobium* sp. (SB1), *Bradyrhizobium elkanii* (SB2) and *Rhizobium* sp. (SB3). All the isolates could grow well in pH 7.5; SB2 in particular did not grow at pH 5.5. Interestingly, isolate SB3 displayed growth at the highest temperatures, both 40 °C and 45 °C. Isolate SB2 produced the maximum IAA

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concentration and had the highest phosphate solubilization index at 8.37 mg/L and 7.60 SI, respectively. In the Leonard jar experiment, the shoot fresh and dry matters of mungbean were highest under the SB2 treatment. Inoculation with the isolate SB2 allowed for the production of the highest number of nodules, highest nodule fresh dry weight, highest chlorophyll content index, and highest nitrogen content in the shoot and root. Therefore, we conclude that SB2 may be a suitable bioinoculant to improve mungbean growth and yield at a neutral pH. However, we suggest that further field experiments need to be conducted to verify our results before extending the use of SB2 to mungbean farmers.

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