RESEARCH ARTICLE

Identification of stress tolerant rhizobial strains inhabiting *Gliricidia sepium* in *Polonnaruwa* district, Sri Lanka

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Abstract: Rhizobia are well-reputed biological nitrogen fixing organisms due to their proficiency in symbiotic root nodule formation with leguminous plants. *Gliricidia sepium* is a widely distributed legume which hosts a wide range of rhizobia. Few studies have been carried out to identify the stress tolerant rhizobial populations inhabiting *G. sepium* in Sri Lanka. This study was focused on the characterization and identification of stress tolerant rhizobial strains residing in the root nodules of *G. sepium* growing in seven selected locations of *Polonnaruwa* district, Sri Lanka. Isolated rhizobial strains were separately subjected to stress conditions (pH, temperature, salinity, and drought) and to a combination of given stress conditions. Most of the strains were tolerant to 25 - 45 ºC temperature range, extreme drought conditions and 1.5 % and 2.0 % salinity levels. However all the strains were adversely affected by acidic pH. Among the fifteen strains that were selected from the statistical analysis, fourteen displayed higher survival capacity when the combinations of physiological conditions were applied. There was great genetic diversity within these isolates and they belonged to 10 clusters. Cross inoculation of these isolated stress tolerant strains into crop legumes can be used as an ideal alternative for chemical nitrogen fertilizers.

Keywords: ERIC 1R, ERIC 2R, pH tolerance, Biological Nitrogen Fixation, Combination of physiological conditions.

INTRODUCTION

Biological Nitrogen Fixation (BNF) is the conversion of atmospheric dinitrogen to available forms such as NH_4. In the symbiotic association between nitrogen-fixing bacteria and the leguminous plants, the legumes transfer their photosynthetic carbon into the nodules to generate energy through microbial respiration under low oxygen concentrations for the nitrogenase enzyme catalyzed reduction of dinitrogen to ammonia which is incorporated as amino acids (Franche *et al*., 2009). Usually, BNF fulfills the major nitrogen requirement of crop legumes, and the excess amount is released to the soil (Peoples and Craswell, 1992). Thus, rhizobia can contribute to an economically attractive and ecologically beneficial agricultural system through BNF.

G. sepium, which belongs to the family Fabaceae is a multipurpose tree legume that is widely distributed throughout the world, including Sri Lanka. Due to its high biomass production and extensive range of soil and climatic adaptation abilities, *G. sepium* is cultivated as the second most crucial multipurpose tree legume after *Leucaena leucocephala* (Jayasundara *et al*., 1997). In Sri Lanka, *G. sepium* is cultivated in coconut fields to improve soil fertility and enhance the productivity of coconut cultivations by facilitating the fixation of atmospheric nitrogen around 64 % in field conditions (Liyanage *et al*., 1994; Liyanage *et al*., 1988). Moreover, *G. sepium* is used as a nitrogen fertilizer, animal forage, fuelwood, green manure, shade trees for plantation crops, and as a soil stabilizer (Jayasundara *et al*., 1997). In addition to tea, rubber, and coconut, the Sri Lankan government has nominated *G. sepium* as the 4th major plantation crop in Sri Lanka for the generation of dendro power.

G. sepium is widely distributed in the *Polonnaruwa* district, due to its soil fertility enhancement, soil erosion prevention, and soil rehabilitation capacity in the poor-quality soil (Gunathilake *et al*., 2005). According to Udagedara and Sugirtharan, (2018), the atmospheric temperature in *Polonnaruwa* district exceeds 30 ºC (30 ºC-33 ºC) throughout the year and it leads to a high evaporation rate. Although the annual rainfall in *Polonnaruwa* district is 1180-1800 mm, 34.9 % of agro-based activities such as paddy cultivation, homesteads, and croplands are the primary sources of income. Moreover, people abundantly cultivate crop legumes such as cowpea, green gram, black gram, groundnut, and beans for over 120 years and rhizobia had been used as a biofertilizer in agriculture in Sri Lanka perhaps during the past four decades (Kulasooriya *et al.,* 2017). As a general practice, *G. sepium* is purposely cultivated along agricultural lands to increase soil nitrogen fertility through BNF (Ulluwishewa, 1993).

Rhizobia belong to the classes alpha-proteobacteria and beta-proteobacteria, based on the information obtained from *16S rRNA* sequence analyzing (Somasegaran and Hoben, 2012; Sawada *et al*., 2003). Furthermore, rhizobia can be classified into six genera; *Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium,* and *Allorrhizobium* (Brockwell *et al*., 2005). According to the

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host range factor, *Rhizobium* is further classified into six species; *Rhizobium leguminosarum, R. trifolii, R. phaseoli, R. meliloti, R. japonicum,* and *R. lupin* (Peter *et al*., 1996). However, the environmental conditions are strongly related to the physiological state of host legumes such as extreme salinity and temperature, unfavorable soil pH, drought, nutrient deficiency, mineral toxicity, and plant diseases affect the rhizobial growth and efficiency of nitrogen fixation in legumes (Brockwell *et al*., 1995). Among the six genera, *Rhizobium* and *Bradyrhizobium* have a higher tolerance for severe conditions and most of them can be found within the legume-based crops. Although the optimum temperature of most of the *in vitro* cultured rhizobia is 28-31 ºC; some strains like *R. leguminosarum* can survive extreme temperatures up to 35-40 ºC by forming an effective symbiotic association with the host legume (Graham, 1992; Zahran, 2001). Even though the optimum pH range found in growing culture is 6.0-7.0, *R. trifolii* even can tolerate high acidic condition such as pH 4.5 (Martinez-Romero *et al*., 1991). Specific colony morphologies such as round shaped, domed, smooth margined, gummy and soft, glistening and milky to thin and translucent features can be used to isolate the rhizobia in stable physiological states (Somasegaran and Hoben, 2012).

Research studies on the symbiotic nitrogen-fixing efficiency of legume plants in both controlled environmental conditions and field conditions had been conducted in many countries including Sri Lanka . Only few studies have focused on the physiological characterization and genetic diversity of stress tolerant rhizobial strains of certain legume crops and their wild relatives (Hettiarachchi *et al*., 2013a and 2013b), but these research have not studied about *G. sepium*. Thus, the present study was mainly focused on isolating rhizobia from *G. sepium* in selected locations of *Polonnaruwa* district, Sri Lanka and physiologically characterize them under different stress conditions such as pH, salinity, drought, and temperature and to determine the genetic diversity of stress tolerant strains by Enterobacterial Repetitive Intergenic Consensus (*ERIC*) profiling. According to Hettiarachchi, (2015), crop lrgumes cross inoculated by single strain and multi starin inoculants have shown great yield in each trial compared to the chemical nitrogen fertilizer application. Thus, identified stress tolerant rhizobial strains will clearly be a great alternative to commercially used chemical nitrogen fertilizers like urea and further exploiting these strains by cross inoculating to crop legumes can lead to establishing a sustainable agricultural system in the dry zone.

MATERIALS AND METHODS

Sample collection

Root nodules of *G. sepium* were collected from randomly selected seven locations (*Moragaswewa* [Mw], *Bakamuna* [B], *Jayanthipura* [J], *Aralaganwila* [A], *Monarathanna* [Mn], *Sinhapura* [Si], and *Medirigiriya* [Mg]) in the *Polonnaruwa* district, Sri Lanka. Five nodules from each location were selected from the collected samples.

Preparation of culture medium

For the preparation of ½ Lupine Agar (1/2 LA) medium, 5 g of mannitol, 5 g of D-glucose and 1.25 g yeast extract were weighed using a micro-scale. The components were dissolved in 100 ml of deionized distilled water, and 0.8 g of $MgSO_4$, 0.2 g of NaCl and 0.2 g of $CaCl_2.2H_2O$ were dissolved in another 100 ml of deionized distilled water. Both solutions were mixed, and the precipitation was checked. After that, 10 ml of KH_2PO_4 10 ml of K_2HPO_4, 5 ml of $Fe_2(SO_4)_3$ and 1 ml of trace elements were added to the mixture, if there is no precipitation found. Then 10 ml of Congo red solution (0.25 g/100 ml) was added as the indicator, and the pH was adjusted to 6.8. The volume of the final solution was adjusted to 1000 ml, and 18 g of agar was added to the final mixture. After the preparation, the medium was covered by cotton wool and aluminum foil and autoclaved at 15 lbs. pressure (121 ºC) for 15 minutes.

Isolation of rhizobia from root nodules

The roots were washed carefully to remove soil particles using running tap water, and undamaged five nodules from each location (total nodules 35) were selected. These nodules were surface sterilized under a laminar floor, by washing with 70 % ethanol for 30 seconds followed by 3% (v/v) Sodium hypochlorite for 2 minutes and five times with sterilized distilled water. In applying the crush method, nodules were crushed using a blunt end of a sterilized toothpick and the nodule suspension was streaked on ½ LA medium using the blunt end of the toothpick under sterile conditions. After culturing the selected 35 nodules, all the culture plates were sealed and labeled. Culture plates were incubated at room temperature for 4-5 days in dark conditions. After the incubation period, sub-culturing was done using colonies that have the typical morphology of *Rhizobium* sp. Successive sub-culturing was done for more than five times to obtain pure cultures, and Congo Red ½LA stock cultures were stored in the refrigerator.

Characterization of the tolerance of *Rhizobium* for physiological conditions (pH, Drought, Salinity, and Temperature)

The ½ Lupine broth (1/2 LB) medium (without Congo red) having a range of pH from 3-9 was prepared for pH tolerance, Polyethylene glycol-8000 (PEG 8000) concentration as 0.1 %, 0.2 %, 0.3 %, and 0.4 % for drought tolerance and NaCl concentration as 0.1 %, 1 %, 1.5 %, 2 %, 2.5 % and 3 % for salinity tolerance. Then, 5 ml of ½ LB medium was added to each test tube including the control sample. All the test tubes were wrapped in aluminum foil and autoclaved. After that, samples were inoculated for each parameter separately using a loop full of isolated rhizobia excluding the control. Duplicate test tubes were set up per treatment. The samples for pH tolerance, drought tolerance, and salinity tolerance were incubated at room temperature while the samples for testing temperature tolerance were incubated at 25 ºC, 30 ºC, 35 ºC, 40 ºC, and 45 ºC separately for three days in dark condition. After the incubation period, the absorbance of all the cultures under different treatments was measured at 600 nm and 570 nm wavelengths respectively using a Spectrophotometer. This procedure was done in duplicate.

Characterization of the tolerance of rhizobial strains for a combination of different physiological conditions

The rhizobial strains that have a tolerance to different stresses were selected. Four physiological parameters were selected according to the existing natural soil conditions in *Polonnaruwa* district. The selected strains were subjected to a combination of conditions such as 6.8 pH, 0.4 % PEG concentration, 3.0 % salinity level, and 40 ºC temperature. All the treated samples were incubated for three days, and the absorbance was measured at 600 nm wavelength using a spectrophotometer.

Genomic DNA extraction and *ERIC*- PCR fingerprinting

The selected 15 rhizobial strains that have the tolerance to different stresses were cultured in ½ LA medium and incubated in dark conditions for three days. After the incubation period, genomic DNA of 15 selected strains was extracted using the phenol/chloroform method. DNA fingerprinting was carried out using *ERIC 1R* primer (5'ATGTAAGCTCCTGGGGAATCAC3') and *ERIC 2R* primer (5'AAGTAAGTGACTGGGGTGAGCG3') (Versalovic *et al*., 1991) for the selected strains. The 15 µl PCR reaction mixture was prepared by adding 1 µl of genomic DNA, 7.5 µl of Go Taq green master mixture, 0.5 µl of primer *1R*, 0.5 µl of primer *2R* and 5.5 µl of nuclease-free water. The PCR amplification was carried out by holding the reaction mixture at 94 ºC for 5 min followed by 35 cycles at 90 ºC for 30 sec, 46 ºC for 1 min and a final extension at 70 ºC for 10 min in the thermal cycler (Takara Otsushiga, Japan). The amplified DNA fragments from the selected rhizobial strains were visualized using Agarose Gel electrophoresis in a 3.5 % agarose gel.

Data analysis and representation

The bar charts were prepared using Microsoft Office Excel to compare the absorbance values for all physiological conditions from all seven sites. The strains with higher growth under extreme conditions were selected by subjecting absorbance values to General Linear Model (GLM) procedure and LS means pdiff mean separation procedure using a statistical package, SAS 9.3.1 (SAS Institute, NC Cary, USA). A dendrogram was constructed for the selected 15 rhizobial strains that exhibited tolerance to adverse conditions by Complete Linkage, Euclidean Distance method using the software MINITAB 17.1.0.

RESULTS

pH tolerance

All 35 samples from the seven sites have a common growth pattern between 3 to 5 pH range. All strains show poor growth at pH 3.0 and 4.0 and better growth at pH 6.0 to 8.0. When the pH is changed from 4.0 to 5.0, the growth of most rhizobial strains increased drastically. At the alkaline pH, strains MW-e, B-d, A-c, MN-e, and Si-e showed a higher tolerance compared to others. Although some strains tolerate alkaline pH, isolates MW-b, A-c, J-c, J-d, A-e, MW-c, MN-c, MN-d, and Mg-d showed poor tolerance for pH 9.0 (Figure 1).

Although MW-e strain had a high tolerance at alkaline pH, the growth of the other strains in *Moragaswewa* site reduced with the increase of pH. Moreover, MW-b and MW-a strains show a relatively low growth rate at each pH level (<0.6840 mean absorbance value) (Figure 1A). Strain B-d of *Bakamuna* site has the highest growth rate at pH 5.0, 6.0 and 9.0. The B-d strain shows the highest fluctuation in growth rates at different pH. (Figure 1B). Except for the acidic pH, the J-a, J-b, and J-e strains have survival ability at other pH levels than J-c and J-d (Figure 1C). In *Aralaganiwila* site, A-a and A-c rhizobial strains have high growth at pH 7.0 and 8.0 (above 1.2000). When the pH gradually increases up to pH 9.0, the growth of A-b strain decreases while the growth of A-d strain increases (Figure 1D). Although MN-b, MN-d, and MN-e show tolerance for pH 7.0 and 8.0, only MN-e strain has tolerance at pH 9.0. However, MN-d shows highest tolerance (>1.2000 mean absorbance) at neutral pH than other strains in *Monarathanna* site. When the pH increases gradually from 7.0 to 9.0, the tolerance of MN-a strain is increased (Figure 1E). All strains in *Sinhapura* site, show exponential growth in 4.0-8.0 pH range. However, only Si-e strain shows high growth at pH 9.0 while the growth of other strains decreases drastically (Figure 1F). In *Madirigiriya* site, the Mg-b and Mg-e strains show high growth at alkaline pH while showing less growth at acidic pH (Figure 1G).

Drought tolerance

A clear pattern of the growth cannot be observed in 35 rhizobial strains at different PEG concentration (0.10 % - 0.40 % PEG). Because most of the strains exhibit more than 0.5000 of mean absorbance value and A-c strain has the highest growth at 0.20 % PEG concentration. However, most of the rhizobial strains survive under high PEG concentration (Figure 2).

In *Moragaswewa* site, the MW-a strain shows the highest growth (0.9980 mean absorbance) at 0.4 % PEG concentration (Figure 2A). Although the B-e strain shows great survival ability at the 4.0 % drought level, the growth of other strains in *Bakamuna* site is decreased (Figure 2B). The J-c strain that isolated from *Jayanthipura* site shows the highest growth at high drought condition. The growth of all strains from *Jayanthipura* is moderately tolerant to high drought conditions except for the J-b strain (Figure 2C). In *Aralaganwila* site, all strains are capable of surviving at high drought conditions except for the A-a strain. The A-c strain exhibits the highest growth at 0.20 % PEG concentration (Figure 2D). All strains in *Monarathanna* site have a moderate ability to survive at each drought level (> 0.7000). At the 0.40 % concentration, MN-a and MN-e strains show relatively higher growth than other strains (Figure 2E). As the drought stress rises, only Si-c strain shows a gradual increase in growth while the growth of all the other strains from *Sinhapura* site decreases when the PEG concentration changes from 0.3 % to 0.4 %. In *Madirigiriya* site, the growth of Mg-c strain is exponentially decreased with the increase in drought. But Mg-e strain has great survival ability even at high drought level (Figure 2G).
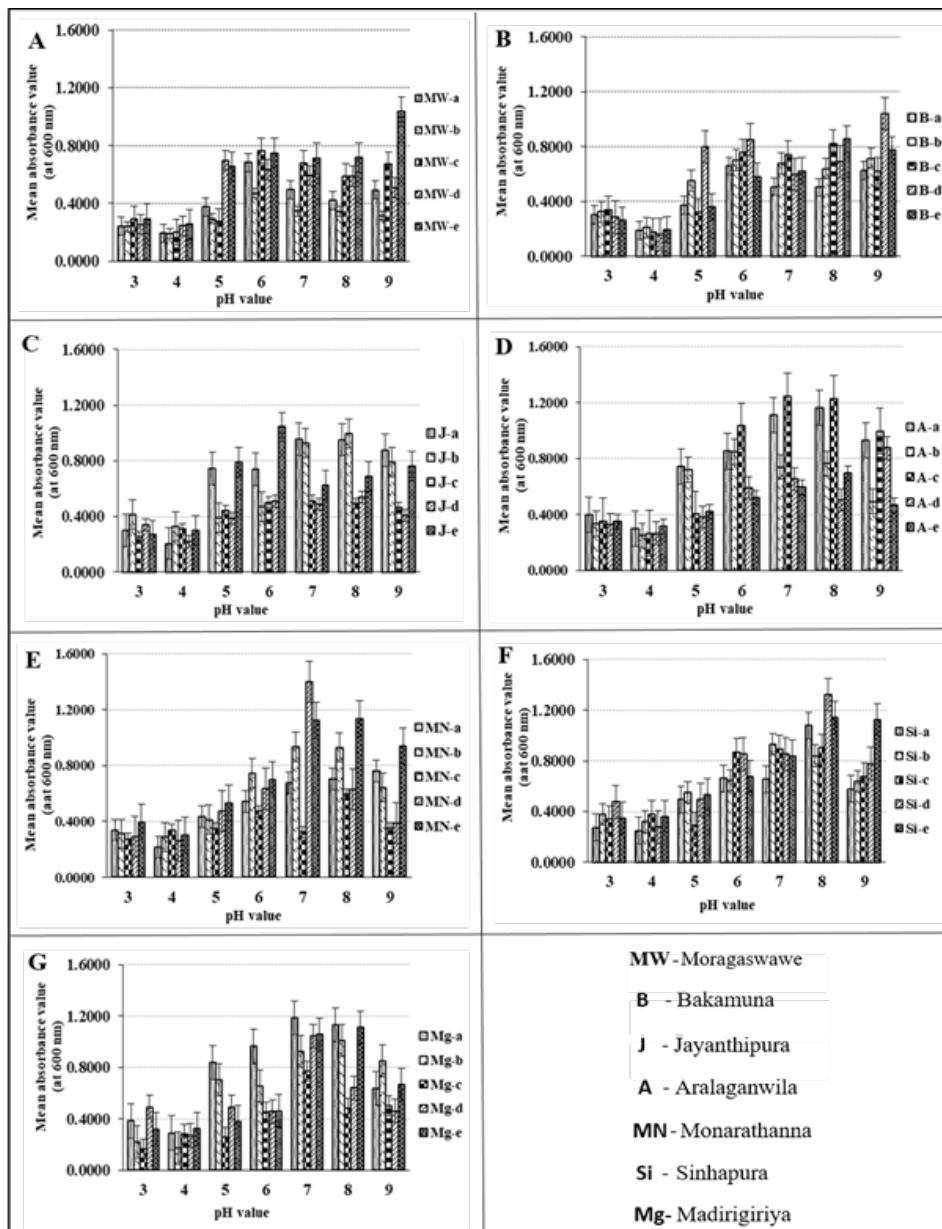
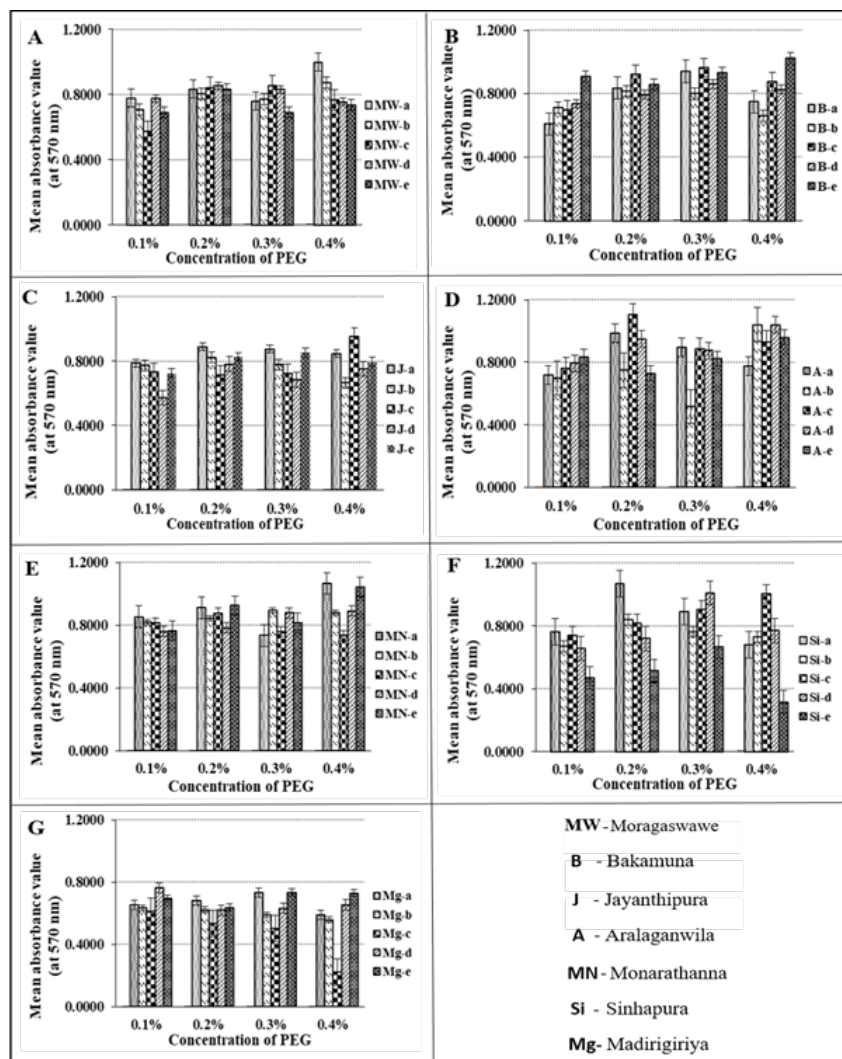*Error bar (+T/- ⊥) indicates the standard error of the sample

Figure 1: Growth of isolated rhizobial strains from 7 sites at different pH values. Five strains from each site grow in the 3.0-9.0 pH range. The optical absorbance measured at 600 nm to determine growth at 600 nm. All rhizobial strains except *Moragaswewa* and *Sinhapura* strains show a radical increase in their growth when the pH changed from 4.0 to 5.0. All 35 strains exhibit greater survival under alkaline pH conditions than acidic pH conditions because the strains in seven sampling sites show high growth at alkaline pH.

Salinity tolerance

A considerable growth of 35 isolates can be observed at different concentrations of NaCl (0.1 %, 1.0 %, 1.5 %, 2.0 %, 2.5 % and 3.0 %) which is used to induce the salt stress in culture medium. Although most strains show high growth at 1.0 % or 1.5 % concentration, the A-b and B-c strains show a higher growth at 1.5 % than the other strains. Tolerance of most strains is relatively low at high salinity levels. However, there is no visible pattern of growth in response to different salt stresses (Figure 3).

MW-c strain shows relatively high tolerance at the extreme salt level than others. However, the growth of all strains gradually reduces with the change of NaCl concentration from 1.5 % to 3.0 % (Figure 3A). The B-c strain shows high growth at 1.5 % concentration level while the B-d strain shows relatively high growth at 3.0 % concentration. When salt stress is increased, the growth of all the strains of *Bakamuna* site is reduced except for B-d strain (Figure 3B). In *Jayanthipura* site, all strains show moderate tolerance at the highest salt level except J-c strain. Moreover, they have the highest survival ability at 1.5 % NaCl level (Figure 3C). Although the A-b strain exhibited the highest growth at 1.5 %, the growth is reduced with the increase of salt stress. Relatively, the growth of all the strains sensitive to high salt stress (Figure 3D). In *Monarathanna* site, MN-c strain shows the highest growth at 3.0% salt stress level. When the NaCl concentration

*Error bar (+T/- ⊥) indicates the standard error of the sample.

Figure 2: The growth of isolated *Rhizobium* strains from 7 sites at different drought levels. The Polyethylene Glycol (PEG) use in different concentrations (0.1 %, 0.2 %, 0.3 %, and 0.4 %) to induced drought stress in the culture medium. The optical absorbance measured at 570 nm to determine the growth. The growth of most samples in 7 sites except for Si-e and Mg-c show tolerance to high drought stress. A-b, A-d, MN-a, MN-e, and Si-c *Rhizobium* strains show the highest growth with more than 1.0000 mean absorbance value at 0.40% concentration. However, the visible pattern of growth cannot be observed in response to increases in PEG concentration from 0.10 % to 0.40 %.

changes from 1.0 % to 3.0 %, the growth of some strains is exponentially reduced (Figure 3E). The growth of all strains in *Sinhapura* site increases in the range of 2.0 % to 3.0 %. However, Si-e strain shows greate survival ability at low salinity levels (Figure 3F). Mg-b and Mg-e strains show slightly similar growth at 3.0 % concentration. But, the growth of Mg-a, Mg-c, and Mg-d strains are reduced when the NaCl concentration changes from 1.5 % to 3.0 % (Figure 3G).

Temperature tolerance

The temperature tolerance is evaluated by incubating isolated rhizobial strains cultured in ½ LB at different temperatures (25 ℃, 30 ℃, 35 ℃, 40 ℃, and 45 ℃). Most strains in each site show high growth at 25 ℃ and 30 ℃, but few strains like MW-a, MW-b, and A-c show the highest growth at 45 ℃. There is no any clear observable pattern

of growth since, the strains in *Moragaswewa*, *Sinhapura*, *Monarathanna*, and *Medirigiriya* sites show less growth at high temperatures while strains in *Aralaganwila* and *Jayanthipura* sites show little increase in growth at high temperature (Figure 4).

With increasing temperature, the growth of MW-d strain decreases exponentially, while the growth of MW-c and MW-e strains increased. However, MW-a and MW-b strains had a greater survival ability at high temperatures than other strains in *Moragaswewa* site (Figure 4A). In *Bakamuna* site, B-d strain shows the highest growth in 35 ℃ to 45 ℃ temperature range while the survival ability of the other four strains decrease when the temperature is increased (Figure 4B). Among the well-grown J-c, J-d and J-e strains, J-e strain has the highest tolerance at high temperatures than others. Moreover, the growth of J-a and J-b strains decrease at high temperature (Figure 4C).
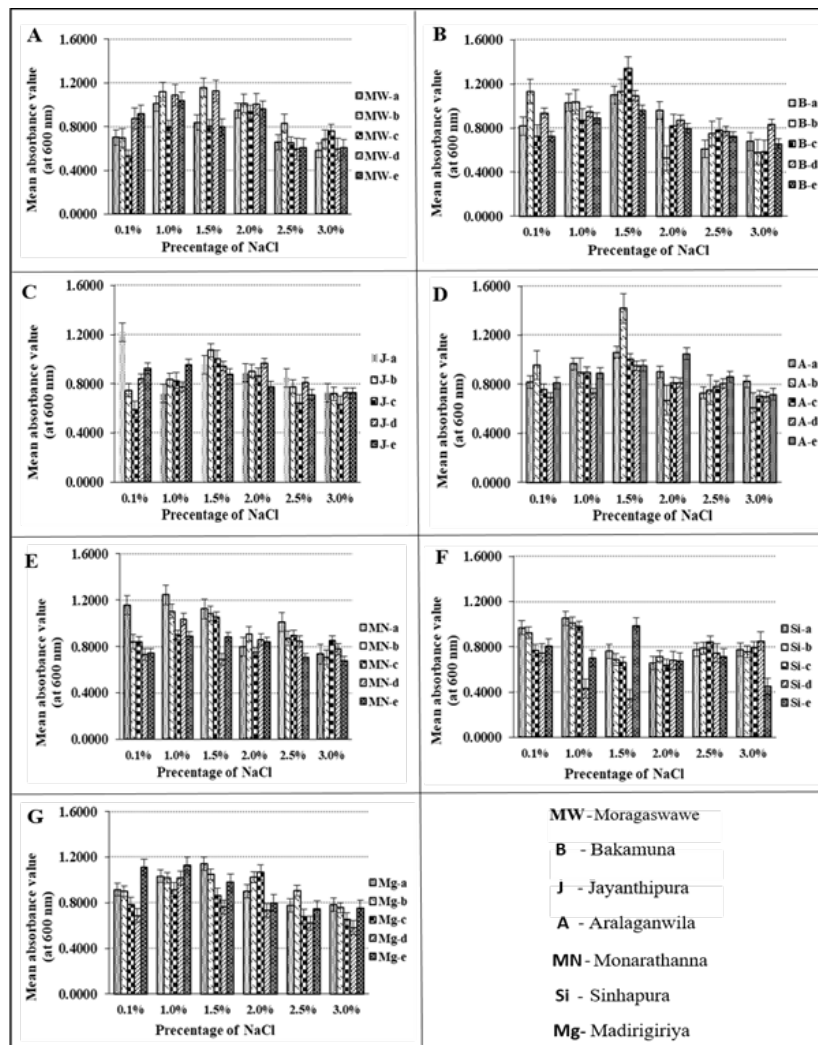
*Error bar (+T/- ⊥) indicates the standard error of the sample.

Figure 3: The growth of isolated *Rhizobium* strains from 7 sites at different salinity levels. NaCl was used at different concentrations (0.1 %, 1.0 %, 1.5 %, 2.0%, 2.5% and 0.4 %) to induce salt stress in the culture medium. The optical absorbance determined the growth at 600 nm. The growth of strains from all the 7 sites decreased at 2.5 % and 3.0 % while higher growth was shown at 1.0 % or 1.5 % concentration level. At high salinity level, A-b, and B-c rhizobial strains showed the highest growth. However, there is no visible pattern of growth in response to salt stresses.

The growth of A-a, A-c, and A-d strains increase while the A-b and A-e strains show less growth within 35 ºC to 45 ºC temperature range. However, A-c strain shows the highest tolerance even at 45 ºC (Figure 4D). The growth of MN-a strain exhibits a higher tolerance at 40 ºC and 45 ºC temperatures than other strains. The MN-d strain maintains its growth in a relatively constant rate within the 35 ºC -45 ºC temperature range while showing a high growth at 25 ºC (Figure 4E). When increasing the temperature from 35 ºC to 45 ºC, the growth of Si-a, Si-c, Si-d and Si-e strains in *Sinhapura* site is reduced. However, Si-b strain shows higher survival ability at 45 ºC level (Figure 4F). When temperature change from 30 ºC to 45 ºC, the overall growth of strains in *Madirigiriya* site is reduced. However, Mg-e strain shows a higher growth rate at 45 ºC than other strains (Figure 4G).

Tolerance for a combination of physiological conditions.

The 15 rhizobial strains that had a higher tolerance to individual physiological conditions exhibit survival ability under laboratory conditions. Instead of extreme temperature and extream pH like 45 ºC and 9.0 respectively, combination experiment conducts using the conditions which exist in the soil of *Polonnaruwa* district. In the combination conditions, MW-a strain shows less growth than in the individual physiological conditions. And Si-b, Si-c, and Mg-e strains show the highest growth at combination condition. However, 14 strains show great tolerance to adverse physiological conditions except for MW-a strain (Figure 5).

Genetic diversity and correlation between selected *Rhizobium* strains

When visualizing the PCR amplicons amplified using *ERIC* primers of the selected 15 *Rhizobium* strains using gel electrophoresis, a highly polymorphic *ERIC-PCR* profile was obtained. Here, some bands are common and prominent in each strain while other bands are specific for a strain. And 15 strains have two dominant bands with close band length.
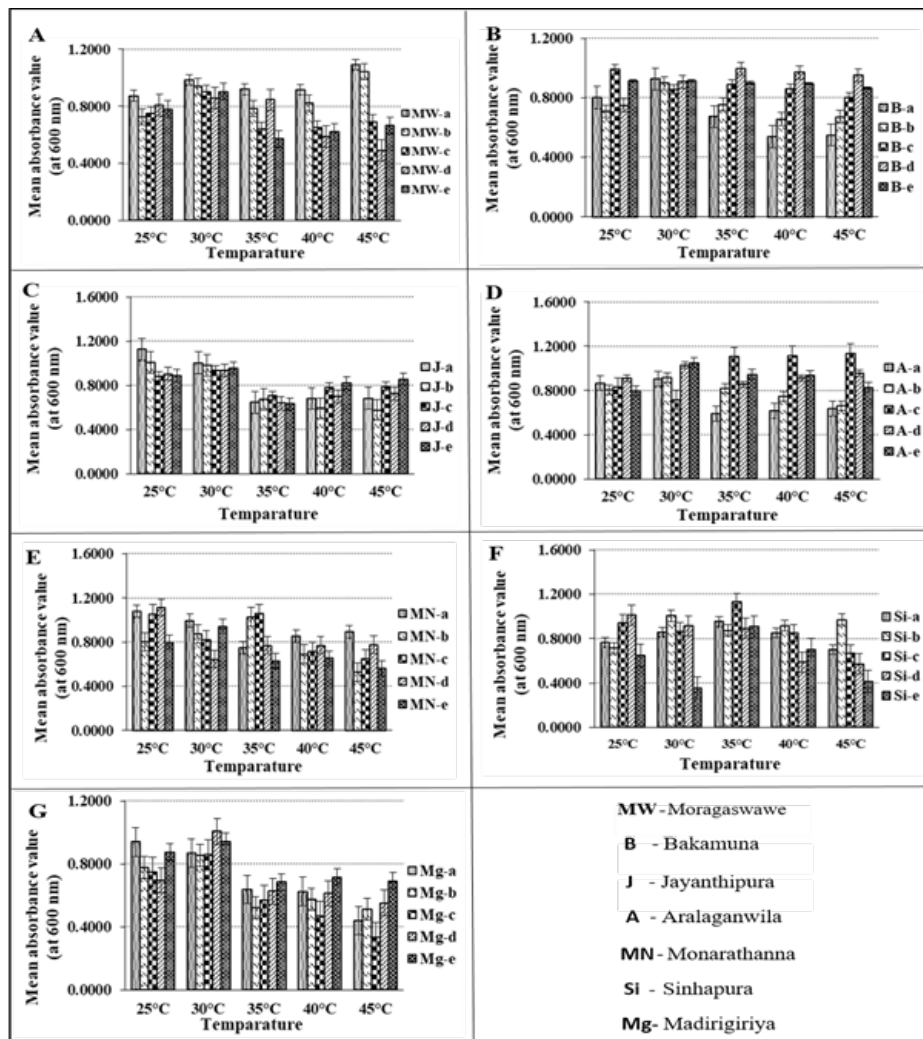
*Error bar (+T/- ⊥) indicates the standard error of the sample.

Figure 4: The growth of isolated *Rhizobium* strains from 7 sites at different temperature levels. The growth medium incubates at different temperatures (25 ºC, 30 ºC,35 ºC,40 ºC, and 45 ºC) to induce the stress and the optical absorbance measured at 600 nm to determine the growth. Most strains show a higher growth at 25 ºC and 30 ºC temperature. However, MW-a, MW-b, and A-c strains show the highest growth at 45 ºC. However, there is no any noticeable pattern of growth in response to the temperature difference since the growth of few strains such as A-C, J-e, and Si-b increase while the growth of other strains reduces with the changing of temperature.
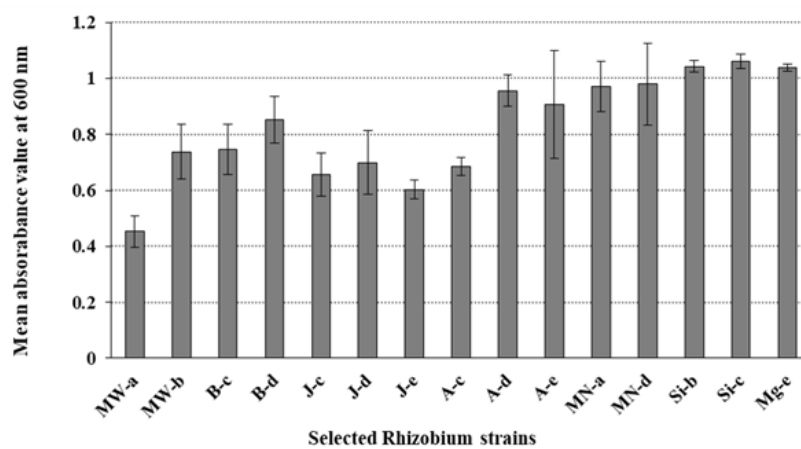
*Error bar (+T/- ⊥) indicates the standard error of the sample

Figure 5: Growth of 15 selected *Rhizobium* strains under a combination of different physiological conditions. These strains were cultured in a medium with 3.0 % NaCl, 0.4 % PEG and pH 6.8 and incubated at 40 °C. The growth was evaluated by measuring the absorbance at 600 nm. The MW-a strain showed less growth while Si-c, Si-b and Mg-e strains showed higher growth under these combined conditions.

MW-a and MW-b strains show specific banding pattern than other strains. Moreover, Mg-e strain shows a different pattern while j-c, J-b and B-c strains have common banding pattern (Figure 6). Based on the *ERIC* profile of selected 15 rhizobial strains, the dendrogram is constructed to assess the genetic diversification and the relationship between strains. The B-c, J-c, and J-d strains cluster together at 75 % molecular similarity coefficient. The 15 rhizobial strains fall into 10 clusters at 65 % molecular similarity coefficient. At 50 % molecular similarity coefficient, the B-d strain cluster together with B-c, J-c, J-d, and J-e strains. And also, MN-a and MN-d strains belong to one cluster and A-e, Mg-e, Si-b, and Si-c belong to another cluster at

50 % molecular similarity coefficient level. The A-c and A-d strains belong to different clusters at 40 % and 38.7 % molecular similarity coefficient respectively. The MW-a and MW-b strains cluster together at 44 % molecular similarity coefficient as an outgroup. (Figure 7).

DISCUSSION

The isolated rhizobial strains that inhabit the root nodules of *G. sepium* in randomly selected seven locations (*Moragaswewa*, *Bakamuna*, *Jayanthipura*, *Aralaganwila*, *Monarathanna*, *Sinhapura*, and *Madirigiriya*) of *Polonnaruwa* district showed a great survival ability for adverse physiological conditions. *G. sepium* is widely
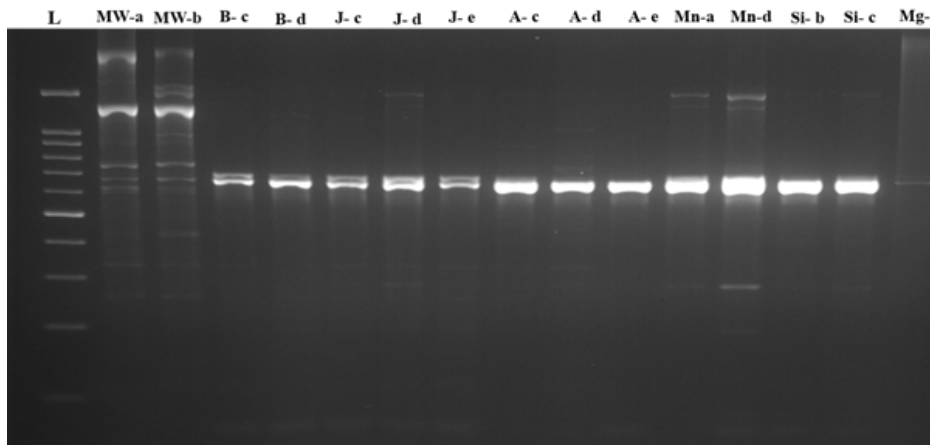

Figure 6: The *ERIC* profiling of 15 rhizobial strains. *ERIC* profile for 15 selected rhizobial strains performed using *ERIC* 1R and *ERIC* 2R primers. DNA banding patterns highly polymorphic which some bands prominent and observe in each strain. The similar banding pattern observes in MW-a and MW-b strains and j-c, J-b and B-c strains
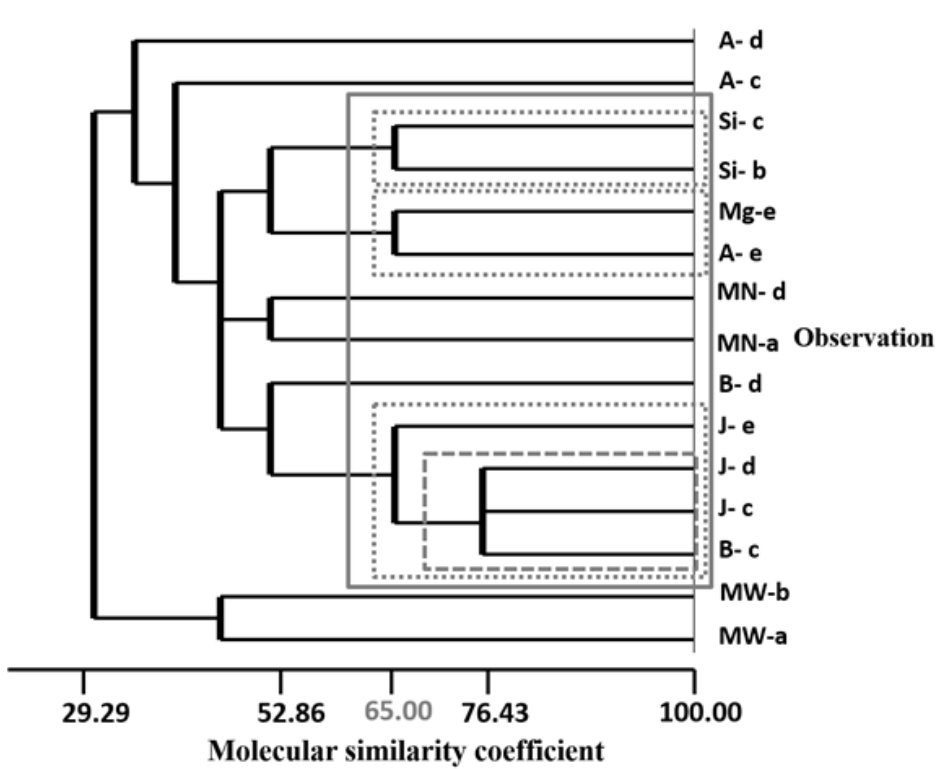

Figure 7: Dendrogram showing the genetic relationship between selected 15 rhizobial strains. The dendrogram was constructed by the complete linkage Euclidean distance method using the statistical software MINITAB 16.1.0. The J-c, J-d, and B-c strains cluster together at 75 % molecular similarity coefficient while MW-a and MW-b belong to out-group cluster at 44 % molecular similarity coefficient. The 15 strains belong to 10 clusters at 65 % molecular similarity coefficient.

distributed in this district and frequently used as a shade for crops, live fences, crop supporters, and green manure in rice fields, other cultivated lands, and home gardens. Usually, a large number of root nodules found in the lateral roots which are located near the surface soil. The rhizobial population showed optimum growth at temperatures 37 ºC and 38 ºC and at the highly aerobic conditions of the surface soil in *Polonnaruwa* district (Rathnayake *et al*., 2009). Most nodules collected from the seven sites had a spherical shape while some nodules in *Medirigiriya*, *Moragaswewa*, and *Bakamuna* locations had coralloid shape. Unlike other nodules, *Monarathanna* sampling site had elongated nodules that was inhabited by a heterogeneous population of rhizobia.

According to the results of pH tolerance, all samples have shown a higher growth at neutral pH. Somasegaran and Hoben, (2012) stated that optimum growth of rhizobia is at the soil pH of 6.0-7.0. But some strains like MW-a, B-d, J-a, J-b, A-c, A-d, MN-e, Si-e, and Mg-b have a higher tolerance to high alkaline pH as the varying soil pH from 5.7 to 7.1 in *Polonnaruwa* district could be the predominant cause (Rathnayake *et al*., 2009). Thus, the pH range of 3.0-9.0 selected to analyze the tolerance of rhizobial strains. Moreover, Degefu *et al*., (2018) have demonstrated that rhizobia nodulating pigeon pea could survive a broad pH range from pH 5.0- 11.0. The highly acidic pH (1.0 and 2.0) or highly alkaline pH (10.0-14.0) was not considered since such conditions do not exist in the natural soil of Sri Lanka. Hungriaa and Vargas, (2000) explained that the highly acidic soil pH severely affects the growth of rhizobial strains and its infection cycle during nodule formation. Hence, the results of the present study correlate with the above findings by showing less growth of rhizobial strains at acidic pH (3.0 and 4.0).

Due to the limited two short rainy seasons and high evaporation rate in *Polonnaruwa* district, the moisture content of the soil is reduced increasing drought stress. Generally, moisture deficiency decreases the growth of the plant and the root nodulation process. However, *G. sepium* that grew in the dry zone naturally has the ability to survive and distribute well under drought conditions. Thus, it leads to the enhancement of the symbiotic relationship with stress tolerant rhizobial strains and the nodulation process. Here, PEG was used *in vitro* to induce water stress in the culture medium. In the drought tolerant test, 570 nm was used to normalize the effect of added PEG. According to the results of drought stress, most strains like MW-a, B-e, J-c, A-b, A-d, A-e, MN-a, MN-e, and Si-c, show a higher tolerance even at the highest PEG concentration (0.40%). However, the moisture percentage in the soil of *Polonnaruwa* district ranged from 7.6 % to 11.5 % between March and April of 2018 (Unpublished data from Division of Soil science, Institute of Regional Agricultural Research and Development Center, *Aralaganwila*, 2018). Due to the adaptation of *G. sepium* for moisture deficiencies found in *Polonnaruwa* district, the inhabiting rhizobial strains also show tolerance for drought stress.

The salinity level of the dry zone soil is relatively higher than that of the wet zone. This is mainly due to the accumulation of high concentration of salt in the soil which occurs through high evaporation and irrigation of salt content water to cultivation (Lauchli and Epstein, 1990). Moreover, the growth of rhizobia and their symbiotic relationship are adversely affected by high salinity level. Thus, most rhizobial strains isolated from *Polonnaruwa* district show adaptation for high salinity levels. Although most strains show high tolerance at 1.0 % and 1.5 % NaCl concentration, the growth of the isolates is reduced at high salt concentrations. The soil salinity in *Polonnaruwa* district is varying from 0.07 to 0.300 ds/m and this could be a major factor for moderate salinity conditions (Unpublished data from the Division of Soil science, Institute of Regional Agricultural Research and Development Center, *Aralaganwila*, 2018). Some strains like MW-c, B-d, A-a, MN-c, Si-d, Si-a, Si-c, and Mg-a show moderate tolerance (>0.7000 mean absorbance value) at 3.0 % of NaCl concentration because 3.0 % of tested salinity level gives 0.495 ds/m electron conductivity. Usually, the *R. etli,* and *R. meliloti* are highly tolerant to high salt concentration (Graham and Parker, 1964). Thus, the tested NaCl concentration ranging from 0.1 % to 3.0 % is reliable as more than 3.0 % of salinity level does not exist in the soil condition of *Polonnaruwa* district. The soil temperature in *Polonnaruwa* distinct is varying from 37 ºC to 38 ºC which causes water loss from the soil (Rathnayake *et al*., 2009). The high soil temperature can affect the plasmid deletion, chromosomal rearrangement, and survival of the rhizobial strains (Moawad and Beck, 1991). Thus, the high temperature negatively affects the growth of most strains as their best growth is shown at 25 ºC and 30 ºC. On the contrary, some strains such as MW-a, MW-b, B-d, B-e, A-c, A-d, MN-a, and Si-b show adaptations even at 45 ºC.

The combination of physiological conditions was selected by considering the natural conditions in the *Polonnaruwa* district. The incubation temperature of 40 ºC was selected because the soil temperature of the *Polonnaruwa* district is close to 40 ºC. Since the rhizobia shows optimum growth at 6.0 - 7.0 range, the pH 6.8 is used for combination conditions. The 3.0 % of salt concentration and 0.40 % of drought conditions were selected because the salinity level spontaneously increases with the increase of drought condition in the soil. Although, MW-a strain shows high growth for some individual conditions like pH, temperature, and drought, the extreme salinity in the combination medium might have become a limiting factor for MW-a strain as it has shown poor growth at 3.0 % NaCl concentration. Other selected strains are highly adapted to extreme conditions as they show a considerable tolerance for combined conditions while Si-b, Si-c, and Mg-e strains show high tolerance as expected. Stress factors such as extreme temperature, high salinity level and extreme pH lead to reduction of nodulation and thus low rate of nirogen fixation resulting in reduced crop yield. Thus, stress tolerant rhizobial strains can be used to as inoculants to improve biological nitrogen fixation in sustainable agricultural systems (Atieno and Lesueur, 2019).

The genetic diversity determined using the *ERIC* profiling showed high polymorphism among the 15

selected strains. The MW-a and MW-b strains belong to an out-group cluster indicating their higher relatedness in the dendrogram analysis. The three strains of *Jayanthipura*, two strains of *Monarathanna* and two strains of *Sinhapura* separately cluster together by representing their geographical relationship. And the relationship among *Sinhapura* strains was also confirmed in the combined physiological condition. But there is no relationship between the sampling site and rhizobial strains. Nawanjana and Rajapakse, (2018) have reported that there is a high genetic diversity of rhizobia in *G. sepium* from *Ampara* district, Sri Lanka. Similarly, the present study provides further evidence for the fact that a diverse group of stress tolerant rhizobia inhabits *G. sepium* in dry zone soils, Sri Lanka. Rhizobial inoculants both single strain and multistrain have the capability of replacing urea application to black gram, soybean, and mung bean crop cultivations in Sri Lanka (Hettiarachchi, 2015). Therefore there is a potential to use the stress tolerant rhizobial isolates identified in this study to cross inoculate crop legumes such as cowpea, mung bean, black gram which are important grain legumes in rain fed farming systems in dry and intermediate zones of Sri Lanka including the districts of *Polonnaruwa*, *Anuradhapura*, and *Puttalam*.

CONCLUSIONS

The 35 samples of rhizobia isolated from root nodules of *G. sepium* in *Polonnaruwa* district showed substantial tolerance to a wide range of pH, salinity, drought, and temperature conditions. Among the selected 15 rhizobial strains, 14 strains have great tolerance except for MW-a strain. All 15 isolates exhibit high adaptations towards the considered extreme physiological conditions similar to their host, *G. sepium* in *Polonnaruwa* district. According to the dendrogram analysis prepared using *ERIC-PCR* fingerprinting, the selected 15 rhizobial strains have a wide genetic diversity.

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DECLARATION OF CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest.

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