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Research Paper

Rhizobia inhabiting *Gliricidia sepium* in Puttalam district of Sri Lanka: Assessment of stress tolerance and genetic diversity

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Abstract: Legume-rhizobia symbiosis has received higher attention as it enhances soil nutrition through the biological nitrogen fixation. However, stress factors such as excess salts, drought, acidity, alkalinity, and temperature etc. suppress the growth and symbiotic characteristics of rhizobia. Identification of stress tolerant rhizobial strains and their

genetic diversity which influence the efficiency of nitrogen fixation in *Gliricidia sepium* is poorly studied. This study mainly focused on the characterization and identification of stress-tolerant rhizobial strains that were isolated from five root nodules of *G. sepium* from seven selected locations (*Anamaduwa*, *Chilaw*, *Vanathawilluwa*, *Saliyawewa*, *Etiyawala*, *Palaviya*, *Kurinchanpitiya*) of Puttalam district in Sri Lanka. Isolates were separately subjected to four different stress conditions, namely, pH (3.0 - 9.0), temperature (25 °C - 45 °C), salinity induced by NaCl concentrations ranging from 0.1% to 3.0%, and drought induced by polyethylene glycol 8000 (PEG-8000) concentrations ranging from 0.1% to 0.4%. Rhizobial strains isolated from *Anamaduwa*, *Chilaw* and *Kurinchanpitiya* such as An-3, An-4, An-5, Ch-1, Ch-4, Ch-5, Ku-2 and Ku-5 showed tolerance for alkaline pH (8.0 and 9.0) and extreme drought conditions (3.0% and 4.0% of PEG-8000). But their growth was adversely affected by acidic pH conditions (pH 3.0 and 4.0). Most of the rhizobial strains except strains in *Etiyawala* were moderately tolerant to extreme salt concentrations (2.5% - 3.0% of NaCl) and extreme temperature levels (40- 45 °C). In the graphical interpretation, no growth pattern was observed with respect to four physiological conditions. Thirteen strains that were selected from the statistical analysis displayed higher survival capacity when the combination of different stress conditions was applied. As 13 rhizobial strains showed different banding patterns in the Enterobacterial Repetitive Intergenic Consensus (ERIC) profile, they belonged to 09 clusters at the 66.67% similarity level. Furthermore, these stress-tolerant rhizobial strains can be used to further studies on cross-inoculation of crop legumes as an alternative substitution for the vast usage of chemical nitrogen fertilizers.

Keywords: ERIC 1R, ERIC 2R, Salinity tolerance, Biological nitrogen fixation, Drought tolerance, Cross-inoculation

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Introduction

Nitrogen, which is sometimes called as the most common factor that limits the growth and productivity of plants (Wagner, 2011) is most abundant in dinitrogen (N₂) form in the atmosphere. Atmospheric nitrogen is transformed into different forms in the nitrogen cycle. It has been estimated that some 3 x 10⁹ tons of atmospheric N₂ is transformed per year globally (Mabrouk and Belhadj, 2012). This amount is not solely biological as 10 % of the total global fixed nitrogen is coming

from lightning and chemically fixed nitrogen from industries (Vojvodic *et al.*, 2014). The industrial nitrogen fixation process is called Haber Bosch process, and was highly beneficial at the beginning of the twentieth century. But, due to several negative impacts such as water eutrophication, soil acidification, and nitrogen oxide emission related to this process (Bohlool *et al.*, 1992), effective use of Biological Nitrogen Fixation (BNF) in agriculture, has become crucial (Ishizuka, 1992). The amount of

nitrogen fixed through biological fixation is estimated to be 17.2×10^7 tons per year, which is three times higher than that of industrially fixed. These data demonstrate the importance of biological nitrogen fixation in the agricultural field and for the consistency of the natural N cycle (Wani *et al.*, 1995).

Diazotrophs or the nitrogen-fixing microorganisms are involved in BNF through converting atmospheric nitrogen into ammonia with the aid of the nitrogenase enzyme complex. It is known to be sole natural system that reduces N_2 to NH_3 (Seefeldt *et al.*, 2009). Among all the nitrogen-fixing microsymbiont, rhizobia, a gram-negative motile soil bacterium is considered as the major and the most effective organism. According to Giller *et al.* (2016), the bacteria associated with legumes and which produce the enzymatic mechanisms that reduce atmospheric nitrogen to ammonia are collectively termed rhizobia or root-nodulating bacteria. Their optimal growth occurs at a temperature range of 25 °C – 30 °C and at a pH level of 6.0 - 7.0 (Somasegaran and Hoben, 2012). In general, rhizobia are aerobic and heterotrophic bacteria. Nevertheless, certain anaerobic and photosynthetic *Bradyrhizobium* strains have also been reported (Laranjo *et al.*, 2014). They can survive as large populations for decades in the absence of host legumes (Giller, 2001). However, the symbiosis with a legume plant helps them in the protection against environmental stresses (Andrade *et al.*, 2002).

Gliricidia sepium is a leguminous, angiosperm tree species that belongs to the family Fabaceae and subfamily Faboideae. It is a semi-deciduous medium-sized tree that grows best in tropical, seasonally dry climates, growing up to a height of 10-15 meters (33-50 feet) with a broad light canopy. It is a fast-growing tree that is easy to propagate by seed, seedlings and cuttings (Simons and Stewart, 1994). As a result of the symbiotic association formed with soil rhizobial strains such as *Rhizobium tropici* type A and B, *Sinorhizobium* spp., and *Rhizobium etli* bv. *Phaseoli* (Acosta-Duran and Martinez-Romero, 2002) *G. sepium* could obtain 46-64% of atmospheric nitrogen (Jayasundara *et al.*, 1997). Hence, apart from being used as fuelwood,

shade plant, livestock fodder and fencing materials (Atapattu *et al.*, 2017), it is grown in coconut fields and other cultivations in Sri Lanka as a source of nitrogen fertilizer (Jayasundara *et al.*, 1997) and the *G. sepium* leaves are used as green manures due to the high nitrogen content (Liyanage, 1987).

Gliricidia sepium is distributed in major ecological zones of Sri Lanka, except in elevations over 750 m and mainly below 500 m and extensively grown in the wet zone and the intermediate zone (Atapattu *et al.*, 2017). In the Puttalam district, having an annual rainfall of 1000 - 1250 mm (Ginigaddara *et al.*, 2016) and 83% of the land comes under Dry Zone and 17% of the land is covered by intermediate zone (Weerasinghe, 2008). The major agricultural products in Puttalam district are coconut but it is one of the major producers of legume crops such as cowpea, peanut and green gram in Sri Lanka. *Gliricidia sepium* can be found in all parts of the Puttalam district and is also cultivated mainly as a supporting plant, shade plant and as a living fence in the agricultural field and domestic lands. With the expectation of increasing nitrogen fixation and crop yield, Agricultural legumes are commonly inoculated with selected strains of rhizobia (Singleton and Tavares, 1986). Kloepper *et al.*, (1986) reports that inoculation of rhizobial strains to the crop field results in the increment of the yield and plant growth of legume crops when compared to the application of chemical fertilizers. Due to the high potential of biological nitrogen fixation possessed by *G. Sepium* and its ability to withstand environmental stress factors and wide distribution, symbiotic rhizobial strains of *G. sepium* could also possess significant characteristics. Their identification would be highly beneficial in cross inoculation of selected crop legumes growing in arid regions and in nitrogen-depleted soil. However, limited research is available in the characterization of the nitrogen-fixing rhizobia inhabiting *G. sepium* in Sri Lanka. Therefore, this study was conducted to characterize the rhizobial strains from *G. sepium* in the Puttalam district to determine the tolerance of them to pH, salinity, temperature and drought and to determine the genetic diversity of stress tolerant rhizobia isolates using the Enterobacterial Repetitive Intergenic Consensus (ERIC) fingerprinting (Versalovic *et al.*, 1991).

Materials and Methods

Collection of root nodules from *G. sepium*

Based on the geography in the Puttalam district, root nodule samples were collected from randomly

selected seven locations (Anamaduwa, Chilaw, Vanathawilluwa, Saliyawewa, Etiyawala, Palaviya, Kurinchanpitiya). Care was taken not to damage or

to separate them from roots. Using a spade, a circle with a radius of about 15 - 25 cm was described around the plant and this section was cut out to a depth of around 10 cm. Roots with nodules were carefully placed in a plastic bag. Undamaged five nodules from each location (total nodules of 35) were collected. A single separate plastic bag was maintained for each location and they were labelled as An, Ch, Vn, Sa, Et, Pa and Ku, respectively. The sample containing bags were placed in ice contained icebox. Fresh nodules were stored in the refrigerator overnight. The samples were collected between May and June of the year. Puttalam district which belongs to dry zone of Sri Lanka and that was selected as the sampling location of this study is being one of the major producer of maize and legume crops such as cowpea, green gram, ground nut and black gram.

Isolation of rhizobia from root nodules of *G. sepium*

The collected 35 root nodules were washed carefully under a gentle stream of tap water. Under a biosafety cabinet, the nodules were surface sterilized by washing with 70% ethanol for 30 seconds, followed by 3 % (v/v) Sodium hypochlorite for 2 minutes and finally the nodules were washed five times with sterilized distilled water and the nodules were crushed using the blunt end of a sterilized toothpick and the suspension was streaked on ½ Lupine Agar (1/2 LA with Congo red) medium using an inoculating loop. As previous, collected 35 nodules were separately cultured on ½ LA medium. Once the isolation streaking method was done the 35 agar plates were labelled and incubated at room temperature for 4-5 days in dark conditions. Pure cultures were obtained by doing 4-5 sub-culturing.

Characterization of the tolerance of rhizobia for physiological conditions

The pH tolerance was assessed by culturing the isolated 35 in sterile ½ Lupine broth (½ LB) with two replicates at pH ranging from 3.0-9.0. Similarly, the tolerability of the isolated 35 rhizobial strains against salinity, drought and temperature were tested. Salinity tolerance was tested for six NaCl concentrations (0.1%, 1%, 1.5%, 2%, 2.5% and 3.0%) and the drought stress was induced by adding Polyethylene Glycol-8000 (PEG-8000) at different concentrations (0.1%, 0.2%, 0.3% and 0.4%) to ½ Lupin broth. The cultures were incubated at room temperature for three days in the dark. The tolerance for the temperature was assessed by incubating the cultures at five different temperatures (25 °C, 30 °C, 35 °C, 40 °C and 45 °C).

After three days of the incubation period, the growth of rhizobial strains was determined by measuring the optical density at 600 nm using a spectrophotometer.

Combination of different physiological conditions

The rhizobial strains that showed high tolerance to different stress conditions were selected and they were subjected to a combination of selected pH (6.8), drought (0.40%), salinity (3.0%) and temperature (40.0 °C) and were incubated for three days. After three days of incubation, the optical density of the selected strains was measured at 600 nm wavelength using a spectrophotometer.

DNA fingerprinting with *ERIC* primers

The selected rhizobial strains that showed high tolerance against different stress conditions were grown on ½ LA plates and kept for incubation for 3 days. After the incubation period, 3-4 loops of rhizobia were collected from the plate and were washed with TE buffer [10mM Tris-HCl (pH 7.5)/25mM EDTA] and suspended in TE buffer (600 µl). 20 µl of lysozyme (50 mg/ml) was added to the suspension, and the mixture was incubated at room temperature for 30 minutes. Then Proteinase K (10 mg/ml, 20 µl) and 10% SDS (60 µl) were added. The mixture was mixed with a gentle pipetting, and kept for incubation at 50- 55 °C for 1 hour. After incubation, phenol/chloroform (600 µl) was added and centrifuged at 13,000 rpm for 15 minutes. Following the centrifugation, the aqueous supernatant was separated into a new eppendorf tube and 5M NaCl (30 µl) and phenol/chloroform (500 µl) were added. Then mixture was centrifuged at 13,000 rpm for 10 minutes and the aqueous supernatant was separated. After that supernatant was mixed with two volumes of absolute ethanol and it was incubated at -18 °C for 5 minutes. Then mixture was centrifuged for 10 minutes (10000 rpm, 4 °C) and the supernatant was discarded. The pellet was rinsed with 70% ethanol and centrifuged for 10 minutes (10,000 rpm, 4 °C). Once the supernatant was discarded the pellet was air dried and dissolved with TE buffer (50 µl). The extracted genomic DNA was subjected to DNA fingerprinting using *ERIC 1R* and *ERIC 2R* primers. The amplified DNA fragments were visualized using Agarose Gel electrophoresis.

Data analysis and representation

The comparison of the absorbance results gained for the tested individual stress conditions (pH, drought, salinity, temperature) was done by plotting bar charts. The selection of the most tolerant rhizobia

strains under combined extreme physiological conditions was carried out through statistical analysis using General Linear Model (GLM) procedure and LS means pdiff mean separation

Results

The pH tolerance of rhizobial strains

No common pattern was exhibited by the 35 rhizobia samples of the 7 sites for the tested pH range (3.0-9.0). All strains in seven sites showed less growth at pH 3.0 and 4.0. However, the growth of rhizobial strains in some sites drastically increased when the pH 4.0 changed to 5.0. Most strains showed high growth at a pH range of 5.0 to 7.0. In site *Anamaduwa*, An-5 strain was shown significantly higher mean absorbance value at pH 9.0 than others. The highest mean absorbance value was shown by An-5 at pH 5.0 (Figure 1A). Strain Ch-2 from site *Chilaw* showed a higher growth rate at pH 5.0, 6.0 and 7.0. Ch-4 showed a gradual increase in its growth when pH value increases from 5 to 8 but showed a sudden drop at pH 9.0.

The mean absorbance value of Ch-5 was relatively lower at pH 3.0 and 4.0 than the rest of the strains, but a rapid increase in the growth was seen at pH 5.0, since then, a fluctuation in the mean absorbance value was visible (Figure 1B). In the *Vanathavilluwa* site, except Vn-2, all the strains exhibited the lowest mean absorbance value at pH 4.0. The highest growth rate at pH 9.0 was shown by Vn-5, while others expressed a slight decline in their growth when pH was increased from 8.0 to 9.0. The highest mean absorbance value was observed from Vn-1 at pH 5.0 (Figure 1C). All the rhizobial strains from site *Saliyawewa* expressed the highest growth rate at pH 5.0 and Sa-4 showed the highest mean absorbance value at pH 5.0. From pH 4.0 to 8.0, all five strains of site *Saliyawewa* exhibited a similar growth pattern (Figure 1D). With the increase of pH from 6.0 to 9.0, Strain Et-4 from the site *Etiyawala* expressed the lowest growth rate. Both Et-2 and Et-5 strains expressed their highest growth rate at pH 6.0, but a significant drop was observed at acidic and alkaline pH values (Figure 1E).

The highest mean absorbance value from site *Palaviya* rhizobial strains was observed from Pa-3, at pH 5.0, which was also the highest value in comparison to all the other 34 strains (Figure 1F). The growth rates at pH 3.0 and 4.0 of all five strains from site *Kurinchanpitiya* were comparatively lower but rapidly increased at pH 5.0, followed by a slight fluctuation with the increase of the pH value (Figure 1G).

procedure using statistical package, SAS 9.3.1 (SAS Institute, NC, Cary, USA).

Drought tolerance of rhizobial strains

Significant growth was shown by all 35 rhizobial strains under PEG-induced different drought conditions which varying the PEG concentration of growth medium from 0.10% to 0.40%. However, no clear pattern of growth was observed in response to the variation of PEG concentrations in the medium. Most rhizobial strains survived under high PEG concentration (Figure 2). When considering the site, *Anamaduwa*, it is clear that An-4 showed the highest growth whereas An-1 showed the lowest growth at the highest PEG concentration (0.4%).

Except for An-2 and An-3, all other strains expressed a better growth with the increasing drought condition (Figure 2A). In site *Chilaw*, Ch-4 strain showed great survival ability whereas other strains had less growth at 0.40% of PEG concentration and there is no visible growth pattern with varying PEG concentration (Figure 2B). The In-1, Vn-2, Vn-4 and Vn-5 expressed higher growth levels at the lowest and the highest drought conditions, and the growth rate of Vn-3 was nearly stable from 0.1% to 0.3% of PEG concentration but the growth drastically dropped at the highest drought condition (Figure 2C). The Sa-5 strain from site *Saliyawewa* exhibited the highest growth rate at all four drought conditions tested.

The growth rate of Sa-1 was nearly in a plateau but dropped considerably at the highest PEG level (Figure 2D). In comparison to the rest of the site *Etiyawala* strains, Et-1, Et-4 and Et-5 strains expressed higher survival ability as their highest growth was observed at 0.3% PEG concentration, and they shared the same growth pattern against the increase of tested drought conditions (Figure 2E). The growth of Pa-3 and Pa-4 from site *Palaviya* decreased gradually when the PEG concentration was increased from 0.1% to 0.4%, whereas Pa-5 showed an elevation in the growth, reaching a peak at 0.3% PEG but dropped at 0.4% PEG (Figure 2F). Strains Ku-1, Ku-2 and Ku-4 showed higher growth at 0.4% PEG, while Ku-2 expressed higher survival ability as it gained a mean absorbance of more than 0.300 in all four drought conditions (Figure 2G).

Figure 1: The growth of isolated rhizobial strains from 7 sites at different pH values. Five strains of each site were grown in the 3.0-9.0 pH range. The optical absorbance determined growth at 600 nm. All rhizobial strains showed a radical increase in their growth when the pH change from 4.0 to 5.0. Most of the isolated Strains were exhibited greater growth under tested extreme alkaline pH levels (8-9) than tested extreme acidic pH levels (3-4). Vertical lines (+T/- $\pm$) indicate the standard error of the sample.

Figure 2: The growth of isolated rhizobial strains from 7 sites at different drought levels. The Polyethylene Glycol (PEG) was used in different concentrations (0.1%, 0.2%, 0.3%, and 0.4%) to induced drought stress in the culture medium. Vertical lines (+T/- -) indicate the standard error of the sample.

Salinity tolerance of rhizobial strains

The growth of all 35 rhizobial strains in 7 sites showed considerable mean absorbance values for different concentrations of NaCl (0.1%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0 %) which induced salt stress in the culture medium. The isolated rhizobial strains from site *Kurinchanpitiya* expressed the highest tolerability at 3.0% of NaCl (Figure 3G). However, there is no visible pattern of growth in response to different salt stresses.

The An-1 from *Anamaduwa* site showed least salinity tolerability in comparison to all other strains of site *Anamaduwa*, whereas An-4 expressed the highest survival rate, although it showed less growth at 1% of NaCl concentration (Figure 3A). Except for the Ch-4 strain, the other four strains from site *Chilaw* showed their highest growth at 2.5% of NaCl, but a significant decline in the growth rate was noticed at 3.0% NaCl (Figure 3B).

Except for Vn-3 and Vn-5, all other isolated rhizobial strains *Vanathavilluwa* showed higher survival ability at higher salinity level, as their highest growth rate was observed at 3.0% of NaCl (Figure 3C). Among all the five rhizobial strains of *Saliyawewa*, Sa-3 strain expressed the highest salinity tolerance at 2.5% and 3.0% of NaCl, whereas Sa-1, Sa-2 and Sa-4 almost maintained a constant growth level at higher NaCl concentrations (Figure 3D). Site *Etiyawala* strains showed the least survival ability against salinity stress factor and all five rhizobial strains of site *Kurinchanpitiya* showed higher growth at 3.0% NaCl (Figures 3E and 3G).

Temperature tolerance of isolated rhizobial strains

The temperature tolerance is evaluated by incubating isolated rhizobial strains cultured in ½ Lupine broth at different temperatures (25 °C, 30 °C, 35 °C, 40 °C, and 45 °C). Most strains in each site showed high growth at 25 °C and 30 °C, but few strains like An-1, Pa-4, Ku-1, and Ku-5 showed the highest growth at 45 °C (Figure 4). There is no pattern of growth since the growth of some strains has been shown a decrease with increases in the temperature while some strains in *Anamaduwa* and *Kurinchanpitiya* sites showed a little increase in growth at high temperatures than low temperature.

The An-1 strain has a greater ability to survive at 45 °C than other strains that isolated from site *Anamaduwa* as it showed its highest growth at the highest temperature condition (Figure 4A). Both Ch-1 and Ch-5 strains from site *Chilaw* possess significant survival ability at 45 °C, and their growth pattern against the increasing temperature

conditions is almost similar (Figure 4B). The Vn-2 and Vn-4 could tolerate a temperature of 45 °C, as their mean absorbance values at 45 °C are relatively higher than that of other strains.

All five rhizobial strains of site *Etiyawala* showed their least growth at 40 °C, even though a significant rise in the growth was noted at 45 °C and the highest mean absorbance was expressed by Et-5 (Figure 4E). The growth pattern of Ku-1 and Ku-5 is almost the same as their growth reduced gradually with the increase of temperature from 25 °C to 30 °C, but from that point significant acceleration in the growth rate was observed, finally reaching the highest growth at 45 °C (Figure 4G).

Tolerance of rhizobial strains to a combination of different physiological conditions

All the tested physiological conditions had a significant effect on the growth of rhizobial strains ($P < 0.05$). Based on the mean separation analysis done using the General Linear Model (GLM) procedure and LS means-pdiff mean separation procedure using statistical package, SAS 9.3.1 (SAS Institute, NC, Cary, USA).

Thirteen strains among 35 isolated rhizobial strains which showed significant growth under more than two extreme conditions (Data not shown) were considered as highly tolerant rhizobial strains and were subjected in the combined physiological condition testing. According to the absorbance results, The Et-5 strain showed the least growth while the Vn-2 strain showed the highest growth in the combination condition (Figure 5).

The PCR amplicons, which amplified DNA fragments of the selected 13 rhizobial strains using *ERIC* primers, were visualized using gel electrophoresis. Based on the *ERIC* profile of selected 13 rhizobial strains, the dendrogram was constructed to assess the genetic diversification and the relationship between strains. An-4, Ku-2, and Ku-4 were similar to each other at 100% whereas the Vn-1 and Vn-2 were similar to each other at 100%. Vn-4 and Sa-3 showed a 100% similarity. The next level was observed at 65% where there are four clusters. An-4, Ku-2 and Ku-4 were clustered together as the first cluster, Vn-1, Vn-2, Vn-4 and Sa-3 have together formed a second cluster, Pa-1 and Pa-4 formed the third and Ku-1 and Ku-5 belong to the fourth cluster. The Et-5 clustered together with Pa-1 and Pa-4 at a 50% similarity level. Pa-2 was similar to the cluster with Vn-2 and Sa-3 at a 39% similarity level whereas Et-5 was similar to the cluster with Ku-1 and Ku-5 only at a 30% similarity level (Figure 6).

Figure 3: The growth of isolated rhizobial strains from 7 sites at different salinity levels. The NaCl was used in different concentrations (0.1%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0%) to induce salt stress in the culture medium. The optical absorbance determined the growth at 600 nm. There is no visible pattern of growth in response to salt stress. However, the isolated rhizobial strains from site *Kurinchanpitiya* showed the highest survival ability at 3.0% of NaCl concentration. Site *Etiyawala* strains showed the least tolerability against the salinity stress factor. Vertical lines (+T/-) indicate the standard error of the sample.

Figure 4: The growth of isolated Rhizobium strains from 7 sites at different temperature levels. The growth medium was incubated at different temperatures (25 °C, 30 °C, 35 °C, 40 °C, and 45 °C) to induce the stress and the optical absorbance determined the growth at 600 nm. An-1, Ku-1 and Ku-5 strains were shown the highest growth at 45 °C. Vertical lines (+T/-) indicate the standard error of the sample.

Figure 5: Growth of 13 selected rhizobial 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 evaluated by measuring the absorbance at 600 nm. The Et-5 strain showed less growth while the Vn-2 strain showed high growth in the combination condition. Vertical lines (+T/- $\pm$) indicate the standard error of the sample.

Figure 6: Genetic relationship between selected 13 rhizobial strains. The dendrogram was constructed using complete linkage euclidean distance using the statistical software MINITAB 17.1.0. Strains similar at the 100 % level are indicated in solid line boxes. Strains similar at the 65 % level are indicated in dash line boxes. These 13 strains belong to 9 clusters at 66.67 % molecular similarity coefficient.

Discussion

Gliricidia sepium was relatively abundant in all the sites except in *Kalpitiya*, *Palaviya*. The plant was rare in coastal areas. This was mainly grown as a living fence and as a supporting plant in domestic and agricultural lands. Root nodules were found closer to the surface soil where bacteria have more access to the gases in the atmosphere. However, they were less abundant in sandy soil that could be found mainly in *Palaviya* and *Kalpitiya*. Moreover, the size of the nodules collected from *Palaviya* and *Kalpitiya* was significantly smaller, when compared with the nodules from the rest of the sites. The shapes of the nodules vary widely despite the sampling location. In general, this study showed that there was noticeable variability in the level of stress tolerance of rhizobial isolates obtained from *G. sepium* plants in *Puttalam* district.

The optimum growth of rhizobia showed at 6.0-7.0 soil pH range (Somasegaran and Hoben, 2012). This is clearly explained from the results of the pH tolerance experiment as all samples except Et-3 showed high growth at pH 5.0 to 7.0 (Figure 1). The pH of dry zone soil ranges from slightly acidic to slightly alkaline but mostly lies within the neutral range (Department of Agriculture, 2017). Generally, the soil pH in the *Puttalam* district is varying from 5.5 - 7.5 (Unpublished data from the Regional Agricultural Research & Development Centre at *Makandura*, Sri Lanka - 2019). Due to this reason and based on the criteria that acidic pH (1.0 and 2.0) or highly alkaline pH (10.0 - 14.0) does not exist in the natural soil of Sri Lanka, the 3.0 - 9.0 reliable pH range was selected for analyzing the tolerance of rhizobial strains. Highly acidic soil pH severely affected the growth of rhizobia and its infection cycle during nodule formation (Hungriaa and Vargas, 2000). Therefore, the results revealed the above details by showing less growth at acidic pH (3.0 and 4.0). However, *Sinorhizobium meliloti* is rhizobia which possesses acidic pH tolerability (Draghi *et al.*, 2017). Certain rhizobial strains isolated from root nodules of *Sesbania formosa*, *Acacia farnesiana*, and *Dalbergia sissoo* were well adapted to grow at pH 12.0 (Surange *et al.*, 1997). Arun and Sridhar (2005) also reported the presence of alkaline tolerant rhizobial strains isolated from wild legumes from two dune systems of the southwest coast of India. These findings provide the proof for the existence of highly alkaline tolerant rhizobial strains.

Drought conditions could cause negative effects on Legume-rhizobia symbiosis through enhancing

nodule senescence which affects the leghemoglobin content and reduces nitrogenase activity (Ashraf and Iram, 2005). In the *Puttalam* district, there is no significant fluctuation in average temperature during the year and mainly there is a high temperature compared with the wet zone (Hydrodynamics and Geophysical Survey, 2016). Due to the limited two short rainy seasons and high evaporation rate in *Puttalam* district which belongs to the dry zone, the moisture content of soil reduces. In general, moisture deficiency negatively affects the growth of the plant and the nodulation process. However, *G. sepium* in the dry zone showed adaptations for drought conditions by enhancing the symbiotic relationship with rhizobial strains. All the isolated rhizobial strains collectively showed substantial growth at all PEG concentrations. Even though there was no clear pattern of growth in response to varying drought conditions, strains like An-3, Vn-1, Vn-2, Vn-5, and Sa-5 expressed the highest growth at the extreme drought condition. Strains like An-4, Ch-1, Et-1 and Et-4 showed an increase in their growth with respect to the rise of the drought condition (Figure 2). Therefore, rhizobial strains isolated from *G. sepium* showed a considerable tolerance to drought stress-induced under laboratory conditions.

The accumulation of a high concentration of salt in the soil caused by high evaporation becomes the causative reason for higher salinity levels in dry zone soil than the wet zone (Lauchli and Epstein, 1990). High salinity levels in the soil negatively affect the growth and distribution of rhizobia in the soil (Jenkins *et al.*, 1989). Additionally, the high salt concentration in the soil could also limit the root nodule formation, growth and the function of the root nodules. In *Puttalam* district, both inland and coastal salinity can be observed (Sirisena *et al.*, 2014). Moreover, the salinity of *Puttalam* district could be <0.1 to >2.0 ds/m, and the majority of the inland areas have less than 0.1 ds/m of soil salinity (Sirisena, 2013). This might be the reason for the observation of considerable tolerance of many strains to high salt concentrations in this study. Odee *et al.*, 1997 revealed the presence of rhizobial strains which could tolerate high as 3.0% of NaCl Concentration, which was proven even from the results gained from the current study, as almost all the isolated rhizobial strain survived even at 3.0% NaCl concentration.

The study conducted by Arun and Sridhar (2005) reported that out of 10 rhizobial strains isolated

from Sand Dune legumes of the Southwest Coast of India only certain strains expressed higher growth at 3.0 - 4.5% of NaCl Concentrations and others showed higher growth at lower salinity levels. Ali *et al.* (2009) showed that out of isolated rhizobia from tree legume *Leucaena leucocephala* some isolates were tolerant up to 2.5 - 3.5% salinity and some were expressed their highest growth at lower salinity levels. A similar observation was noticed in this study as in all the sites except in site *Etiyawala*, both strains that showed higher growth at lower salinity levels and strains that showed higher growth at higher salinity levels were observed (Figure 3). This indicates that the soil of these sites become occupied by both higher salinity preferred and lower salinity preferred rhizobia species.

The temperature in *Puttalam* district varies from 20 °C to 36 °C (Weerasinghe, 2008) which caused water loss from the soil. The high soil temperature can be affected by the plasmid deletion, chromosomal rearrangement, and survival of rhizobial strains (Moawad and Beck, 1991). Optimum growth of rhizobia showed at 25 °C - 30 °C soil temperature range (Somasegaran and Hoben, 2012). Some previous studies conducted on rhizobial strains also confirmed this finding by reporting that optimum temperature for growth of root nodulating bacteria ranged from 25 °C - 30 °C (Ali *et al.*, 2009; Gaur, 1993).

These findings are similar to the results of the temperature tolerance experiment as most of the strains in each site showed considerable growth at 25 °C and 30 °C (Figure 4). However certain strains of peanut rhizobia (*Bradyrhizobium* sp.) were reported to have extreme temperature tolerability (Elsaeid *et al.*, 1990). In all the sites, except site *Etiyawala*, 3/5 of the isolated strains showed their highest growth at either 40 °C or 45 °C, whereas 2/5 of the strains preferred 25 °C - 35 °C of temperature range and similar results were reported in previous studies, emphasizing the presence of rhizobial strains which could tolerate higher temperatures ranging from 40-50 °C (Ali *et al.*, 2009; Arun and Sridhar, 2005). Furthermore, this concludes that the majority of the isolated rhizobial strains from each site, except site *Etiyawala*, possessed a better

adaptation to extreme temperature conditions encountered in their natural habitat.

The combination of physiological condition was selected by considering the natural conditions in the *Puttalam* district. The 40 °C was selected as the incubation temperature because the maximum soil temperature of *Puttalam* district is closer to 40 °C and generally, the natural environment does not have a high temperature like 45 °C. The selected 13 rhizobial strains were grown in the pH 6.8 broth medium as the optimum growth of rhizobia shows between 6.0 and 7.0 range. The 3.0% salinity and 0.4% drought conditions were selected as the salinity level is spontaneously increased with the increase of drought condition of the soil (Mahajan and Tuteja, 2005). Surange *et al.*, (1997) explains that rhizobial strains that could tolerate high pH could also tolerate high salinity and high-temperature conditions. This reflects from the results of the combined physiological condition experiment as all 13 strains showed significant high growth as all the strains expressed a mean absorbance value of higher than 0.2 which concludes that all the selected 13 rhizobial strains have a significant survival ability even at such a combination of stress factors (Figure 5).

The dendrogram was constructed using the *ERIC* profile to analyze the diversity of 13 rhizobial strains. Sa-1 and Sa-2 belong to a single cluster at 100% similarity, indicates the close relationship between them. However, the growth results shown by these two strains at combined physiological conditions were not similar. An-1, Ku-2 and Ku-4 cluster together at 100% similarity and the results obtained for the tolerance of these strains for physiological conditions also confirm their relatedness. The Vn-4 and Sa-3 clustered together at 100% similarity (Figure 6). This indicates that similar rhizobial strains can be found in different locations. When considering the relationship between these strains at 66.67% similarity level, these 13 strains fall into nine different clusters. However, there was no clear evidence provided by the dendrogram for the interconnection between the sampling site and isolated rhizobial strains.

Conclusion

The isolated 35 rhizobia samples from the root nodules of *G. sepium* in selected locations of *Puttalam* district showed considerable tolerance to a wide range of pH, salinity, drought, and

temperature. Among 35 isolates, the selected 13 rhizobial strains showed relatively higher tolerance at least two or more individual abiotic stress conditions and they also have considerable

tolerance against the combined abiotic stress conditions. Based on the dendrogram which was prepared using the *ERIC* profile, the selected 13 rhizobial strains exhibit higher genetic diversity.

Further studies should be conducted for the identification of the selected 13 rhizobial strains at

the genus or species level by using molecular techniques such as *16S rRNA* sequencing. Additionally, stress-tolerant 13 rhizobial strains can be directed in further studies for cross-inoculation of crop legumes as a successful application in a substantial agriculture system.

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