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Rhizobia inhabiting *Clitoria ternatea* L. in Anuradhapura District, Sri Lanka: An assessment of stress tolerance and genetic diversity

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Abstract

Legume-Rhizobial symbiosis plays a greater agronomical and ecological significance as it provides fixed nitrogen through Biological Nitrogen Fixation. *Clitoria ternatea* is a leguminous plant that hosts a wide range of rhizobial strains. Even though it is a widely distributed plant, comprehensive information and studies conducted on the rhizobial - *C. ternatea* symbiosis is lacking in Sri Lanka. This study aimed to identify different stress-tolerant rhizobial strains inhabiting root nodules of *C. ternatea* growing in seven selected locations of Anuradhapura district of Sri Lanka. Twenty-eight pure rhizobial colonies were isolated and they were separately grown in ½ Lupin broths and were subjected to four different physiological conditions, pH, temperature, salinity, and drought. Most of the isolates were well-grown within the pH range of 5.0-8.0, the temperature range of 30-35 °C and at 0.2% Polyethylene glycol-8000

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(PEG) concentration. There was no observable pattern in the growth of rhizobial strains in different physiological conditions. The twelve rhizobial strains which showed high tolerance to extreme physiological conditions were subjected to a combination of physiological stress conditions of pH 8.0, temperature 36 °C, 3.0 % NaCl and 0.4 % PEG. The maximum growth in combination physiological study was observed in a strain collected at the Palugaswewa site. According to the dendrogram prepared by the Enterobacterial Repetitive Intergenic Consensus (ERIC) profile, twelve strains are genetically diverse, as they belonged to11 clusters at 69.89 % of similarity level. These stress-tolerant rhizobial strains could be used for further studies on cross-inoculation of crop legumes as an alternative to the nitrogenous fertilizers.

Keywords: *C. ternatea*, ERIC, genetic diversity, rhizobium

INTRODUCTION

Nitrogen is an essential element for the growth and development of organisms. The earth's atmosphere contains about 78 % of nitrogen gas (Sur *et al*., 2010) which is generally not reactive towards either oxidation or reduction. Therefore, most organisms are incapable of utilizing this nitrogen gas directly. However, a certain group of prokaryotes is capable to reduce this inert nitrogen into ammonia through a process known as Biological Nitrogen Fixation (BNF) (Santi *et al*., 2013). BNF benefits both agriculture and the environment as it provides the nitrogen requirement of the plant and reduces the application of chemical fertilizers with nitrogen to the crop plants (Olivares *et al*., 2013). BNF helps in reducing costs for farmers, improving soil quality and also mitigation of greenhouse gas emissions (Black *et al*., 2012).

The prokaryotic rhizobia are important bacteria, involving in this process of BNF usually in leguminous plants root nodules. Most of the leguminous plants can fix atmospheric nitrogen by interacting with rhizobium and fulfill the nitrogen requirements. This symbiotic relationship helps the plant to grow well in nitrogen-deficient soils. The excess fixed nitrogen is released into the external environment when they die and that nitrogen can be utilized by other plants. Because of that, leguminous plants are extensively used as green manure in the agricultural fields. Hence, BNF is an important process in agriculture. The bacteria associated with the legumes are collectively known as rhizobia or root nodulating bacteria. Most of the current rhizobia belong to the Phylum proteobacteria which includes mainly classes of Alpha and Beta proteobacteria. Thirteen genera of leguminous root nodulating species have been identified in the Class Alpha-Proteobacteria (Howieson and Dilworth, 2016).

Clitoria ternatea L. belongs to Family Fabaceae which is commonly known as "butterfly pea", "Asian pigeon wings", etc. These plants can be grown in different soil conditions where they can survive in soils with a pH range from 5.5- 8.5, including the calcareous soils. They can survive in prolonged droughts as well as in extended rainy seasons. *C. ternatea* has numerous medicinal values. Moreover, they are also grown as ornamental and fodder plants (Chukwuma *et al*., 2014; Gupta *et al*., 2010). This plant is a naturally growing abundant plant in the Anuradhapura district and it is usually grown as an ornamental plant.

Even though a limited number of studies have been conducted on the symbiosis between *C. ternatea* and rhizobia, several types of research have found that *C. ternatea* has a high potential to fix nitrogen through symbiosis with rhizobia (Oguis *et al*., 2019). Different rhizobial strains have been isolated from *C. ternatea* growing in Thailand. They have been identified as *Bradyrhizobium elkanii,* and *Bradyrhizobium japonicum* inhabiting in the *C. ternatea,* growing in Thailand (Aeron *et al.,* 2015).

This study focuses on the identification of rhizobial strains which are adapted to extreme environmental conditions such as high salinity, high temperature, high drought conditions and extreme pH. These extreme stress-tolerant and genetically diverse rhizobial strains can be used for cross inoculation in crop plants that are grown in stress-full environments. When these stress-tolerant rhizobial strains are successfully cross-inoculated into the crop plants they fix atmospheric nitrogen and fulfill the nitrogen requirement of the crop plant. Some studies showed that cross inoculation of rhizobia to the crops has increased the crop yield and the plant growth than applying chemical fertilizers (Bhardwaj *et al*., 2014). The use of environment-friendly fertilizer is becoming a trend due to many adverse effects caused by chemical fertilizers. If the stress-tolerant rhizobial strains are successfully cross-inoculated into crop plants, the application of chemical fertilizers would no more be required. The main objective of this study is to identify different stress-tolerant rhizobial strains from the root nodules of *C. ternatea* in the Anuradhapura district of Sri Lanka.

MATERIALS AND METHODS

Root nodules of *C. ternatea* were collected from randomly selected seven locations, Anuradhapura Urban area (AP), Thalawa (TH), Mihintale (MH), Medawachchiya (MW), Kahatagasdigiliya (KH), Palugaswewa (PG) and Thanthirimale (TM) of the Anuradhapura district, Sri Lanka. Four plants from each location were selected and one nodule from each plant was used to conduct the study. The root nodules were surface sterilized and cultured in ½ Lupin agar medium (Somasegaran and Hoben, 2012) using the crush method. Then the culture plates were kept in dark for 3-7 days at room temperature and twenty-eight pure rhizobial colonies were obtained after 3-5 times of subculturing. Naming of each isolated rhiziobial colony was done based on the respective abbreviations of each site (e.g. AP-4 is the 4th sample obtained from the Anuradhapura urban site). Finally, the obtained pure cultures were used for the physiological characterizations.

Physiological tolerance

The isolated pure rhizobial colonies were grown in ½ lupin broths under different physiological conditions. The salt tolerance was assessed by growing the isolated 28 rhizobial strains in different NaCl concentrations (0.1, 1.0, 1.5, 2.0, 2.5 and 3.0 %). The pH tolerance was assessed by culturing the isolated pure rhizobial strains in ½ Lupin broths in different pH values, ranging from 3.0- 9.0 and the isolated 28 rhizobial colonies were grown in different Polyethylene-glycol (PEG-8000) concentrations (0.1, 0.2, 0.3 and 0.4 %) to assess the drought tolerance. The cultures were incubated in dark condition for 3 days at room temperature. The temperature tolerance was assessed by incubating all the isolated rhizobial strains under five different temperatures (25, 30, 35, 40 and 45 °C) in dark condition for three days. After three days, the absorbance was measured at 600 nm using spectrophotometer (Shimadzu-1800UV double-beam UV/ visible).

Combinational physiological test

The twelve-extreme stress-tolerant rhizobial strains were subjected for the combination physiological test where they were inoculated in ½ Lupin broths with, 3.0 % NaCl concentration, 0.4 % PEG concentration, and pH 8.0. These samples were incubated at 36 °C for 3 days in the dark. Then the absorbance was measured at 600 nm using a spectrophotometer. The obtained data was analyzed using Microsoft Excel and SAS 9.1.3 version.

DNA fingerprinting using ERIC sequences as primers

The twelve Rhizobial strains that showed high absorbance at extreme physiological conditions were used for the genomic DNA extraction. Genomic DNA of these stress-tolerant rhizobial strains used for the PCR amplification (Thermal cycler: Takara, Japan), using two primers of ERIC 1R and ERIC 2R. Initially, the PCR mixture was set at 94 °C for 5 minutes, followed by 35 cycles at 90 °C for 30 seconds. Then at 46 °C for one minute and the final extension was carried out at 70 °C for 10 minutes. The PCR amplicons visualized using Agarose gel electrophoresis. The obtained data were analyzed using MINITAB 14.0 software.

RESULTS AND DISCUSSION

pH tolerance of the rhizobial strains

The Figure 1 shows the growth of 28 rhizobial isolates at different pH values (3.0-9.0) in Anuradhapura urban area, Kahatagasdigiliya, Mihintale, Medawachchiya, Palugaswewa, Thalawa and Thanthirimale sites, respectively.

Generally, the optimum pH range for the growth of rhizobial strains found between 6.0-7.0 (Somasegaran and Hoben, 2012). However, most of the isolates in this study showed an optimum growth in the pH range of 5 to 8. pH of the soil in the Anuradhapura district varies between 4.5-8.0 (Renuka and Senavirathna, 2017). This proves that the isolated rhizobial strains from the root nodules of *C. ternatea* well adapted for the existing pH conditions of the soil in the Anuradhapura district. However, in this study, the tolerance of rhizobial strains tested at pH 9.0 and most of the isolates showed high tolerance even at pH 9.0. The AP-2 showed the highest growth at pH 9.0 among all the other isolates.

The AP-2, AP-3 and AP-4 of the Anuradhapura urban site showed a similar growth pattern from pH 5.0-9.0. The isolate AP-2 showed the highest tolerance to pH-9.0 in this site and it also showed a significant growth at pH 3.0 as well. The overall maximum growth at the site Kahatagasdigiliya was shown by the strain KH-2 at pH 6.0. However, almost all the strains of the Kahatagasdigiliya site showed lower growth at the pH range of 3.0-4.0. Moreover, the strain KH-4 showed the highest growth at pH 9.0. At the site of Mihintale, the strains MH-2 and MH-1 showed a significant highest growth at pH 6.0 and pH 7.0 respectively. Even though the strain MH-4 showed considerably lower growth from pH 6.0-9.0, it showed the highest growth at pH 3.0. The isolate MW-2 of the Medawachchiya site showed a significant plunge in its growth at pH 7.0 when comparing its growth at pH 6.0 and 8.0. The isolate MW-4 showed overall-higher growth in pH range of 3.0 to 8.0, except pH 7.0 and its growth has significantly dropped at pH 9.0. In the site Palugaswewa, the strain PG-2 showed the overall-highest growth at this site at pH 8.0. The isolates in the Thalawa site showed a differential growth pattern with the changing pH. All these results prove that there is no observable pattern in growth in rhizobial isolated with changing pH conditions. The sensitivity of rhizobia to different factors is different from rhizobium species to species (Dludlu *et al.,* 2017). Therefore, the differential growth pattern is observed with changing pH values. Moreover, rhizobium species such as *Mesorhizobium* can survive in a wide range of pH (pH 3.0- 10.0) (Dludlu *et al.,* 2017).

Generally, almost all of the rhizobial isolates showed poor growth in the pH range of 3-4. However, few strains showed a higher tolerance even at pH 3.0 (AP-2, MH-4, MW-4 and TM-3). Moreover, the AP-2 showed the highest tolerance at pH 3.0. This further proves that the rhizobial strains can survive in a wide range of pH values. The acid-tolerant rhizobial strains have several mechanisms to increase the internal pH. They increase their internal pH through the proton pump systems, glutamic acid decarboxylase, etc. (Lei *et al*., 2011). Highly alkaline soils above pH 8.0 tend to be high in sodium chloride, bicarbonate, and borate levels, and are often associated with high salinity which diminishes nitrogen fixation (Nandanwar *et al*., 2020). Highly acidic pH (1.0-2.0) was not considered as these conditions are usually not prevailing in natural soils in Sri Lanka (Moormann and Panabokke, 1961). Most of the strains have grown well at higher pH values than the lower pH values of 3.0 and 4.0.

Highly acidic pH affects the nodulation process in which the signal exchange between the host plant and the microsymbiont is disrupted and secretion of plant flavonoids is also decreased. These effects decrease the *Rhizobium nod* gene induction, restricts nod factors and nod metabolite excretion. The restriction of nod factor signaling affects the root hair deformation and root curling. The low pH also affects the attachment of *Rhizobium* on to root and colonization and finally, root nodule formation (Ferguson *et al*., 2013).

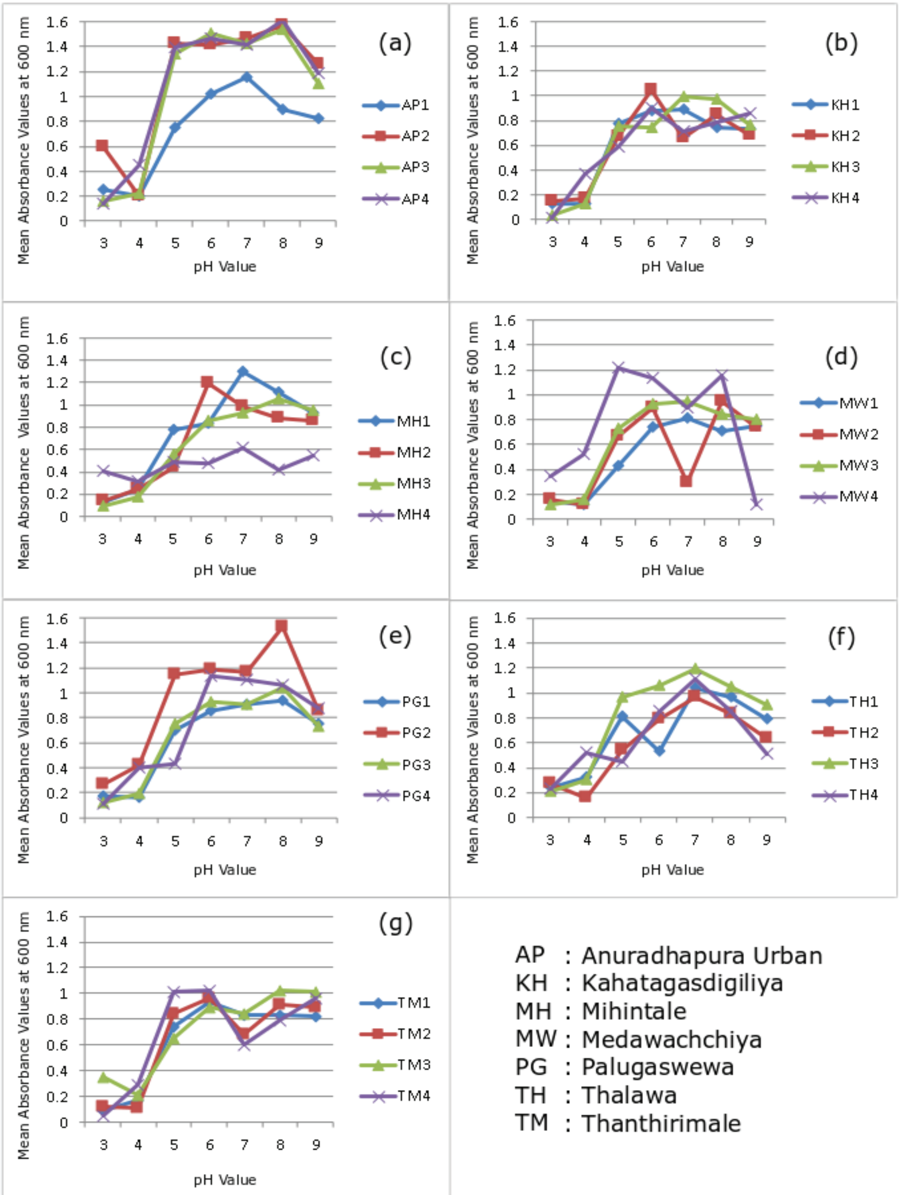

Figure 1: Graphs of (a) AP: Anuradhapura Urban (b) KH: Kahatagasdigiliya (c) MH: Mihintale (d) MW: Medawachchiya (e) PG: Palugaswewa (f) TH: Thalawa and (g) TM: Thanthirimale showing the growth of 28 rhizobial strains at different pH values (3.0 -9.0).

The rhizobial isolates that showed higher tolerance to pH 9.0 includes AP-1, AP-2, AP-3, AP-4, KH-4, MH-1, MH-2, MH-3, MW-3, PG-2, PG-4, TH-3, TM-4, TM-2 and TM-3.

Salinity tolerance of the rhizobial strains

The growth of the 28 rhizobial strains at different NaCl concentrations in Anuradhapura urban area, Kahatagasdigiliya, Mihintale, Medawachchiya, Palugaswewa, Thalawa and Thanthirimale sites are shown in the Figure 2. The graphs of the growth of twenty-eight rhizobial strains at different NaCl concentrations did not highlight a common pattern. The highest mean absorbance (~1.60 nm) value was observed in PG-2 at 1.0 % NaCl concentration while the lowest was observed in KH-3 at 1.5 % NaCl concentration. The rhizobial strains isolated from the Medawachchiya and Thanthirimale sites showed comparatively lower growth at different NaCl concentrations.

The strains at Anuradhapura urban site showed their maximum growth at 0.1 % NaCl concentration. The overall-highest growth at this site was shown by AP-2 at 0.1 % NaCl concentration. AP-2 showed maximum tolerance at 3.0 % NaCl concentration as well. The strains KH-2 and KH-3 of the Kahatagasdigiliya site showed their maximum growth at 1.0 % NaCl concentration. The strains KH-1 and KH-4 showed their maximum growth at 2.0 % and 0.1 % NaCl concentrations respectively. All the four strains in this site showed the lowest growth at 1.5 % NaCl concentration. MH-4 of the Mihintale site showed the highest growth at 1.0 % NaCl concentration in this site. In the site Medawachchiya, growth of all the strains has decreased gradually with the increasing salinity.

However, all the strains in this site showed their optimum growth at 0.1 % NaCl concentration. The strains PG-1, PG-3 and PG-4 of the Palugaswewa site showed their maximum growth at 0.1 % NaCl concentration while the PG-2 showed its maximum growth at 1.0 % NaCl concentration. The strain TH-4 of the Thalawa site showed the maximum growth at all salinity levels, except 0.1 % NaCl concentration. TH-3 of this site showed a significant plunge in its growth at 2.5 % NaCl concentration. But, its growth has considerably increased at 3.0 % NaCl concentration. In this site all the strains except TH-3 was well grown in 1.0 % NaCl concentration. TH-3 showed its highest growth at 1.5 % NaCl concentration. In the site Thanthirimale, the highest growth was shown by TM-2 at 1.0 % NaCl concentration. TM-1 and TM-3 showed their highest growth at 0.1 % NaCl concentration. But, TM-2 and TM-4 showed their highest growth at 1.0 % NaCl concentration. Finally, these results suggest that, most of the strains grow well within the salinity range between 0.1 – 1.0 % but some prefer high salinity level as well.

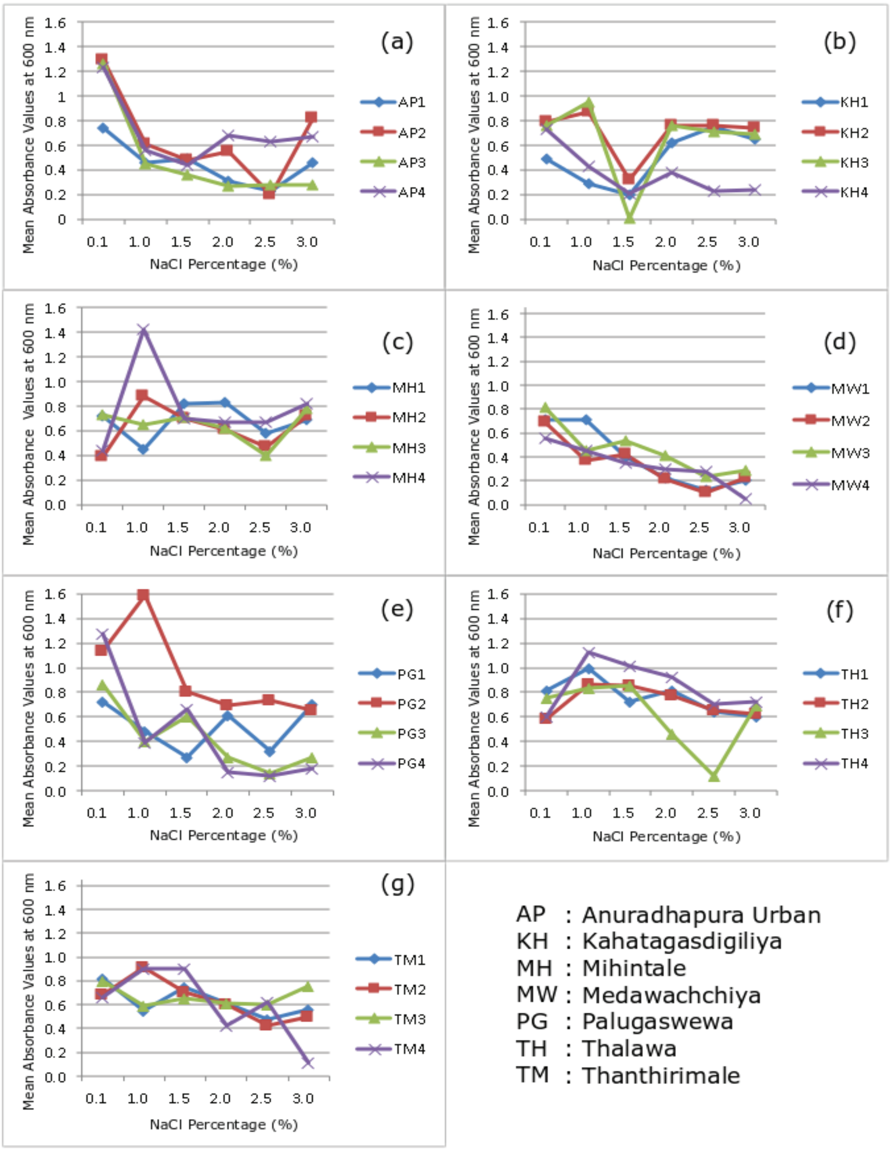

Figure 2: Graphs of (a) AP: Anuradhapura Urban (b) KH: Kahatagasdigiliya (c) MH: Mihintale (d) MW: Medawachchiya (e) PG: Palugaswewa (f) TH: Thalawa and (g) TM: Thanthirimale showing the growth of 28 rhizobial strains at different NaCl concentrations.

The Anuradhapura district is in the dry zone where the temperature is high and the annual rainfall is low. This leads to elevated evaporation tending to accumulate high salt concentrations in the soil (Udupamunuwa *et al*., 2020). Therefore, the soil of some areas of the Anuradhapura district experience high salinity level. Almost all the rhizobial strains isolated in this study showed a considerable growth even at 3.0 % salt conditions showing that the rhizobial strains are well adapted for the prevailing saline conditions of the soil in the studied areas of Anuradhapura district. Moreover, out of 28 isolated rhizobial strains seventeen strains (AP-2, AP-4, KH-1, KH-2, KH-3, MH-1, MH-2, MH-3, MH-4, PG-1, PG-2, TH-1, TH-2, TH-3, TH-4, and TM-3) showed highest tolerance to the extreme saline (3.0 %) conditions.

Temperature tolerance of the rhizobial strains

The growth of the 28 rhizobial strains at different temperatures in Anuradhapura urban area, Kahatagasdigiliya, Mihintale, Medawachchiya, Palugaswewa, Thalawa and Thanthirimale sites, are illustrated by the Figure 3.

In the Anuradhapura urban site, all four strains showed a comparatively higher growth from temperature 25 to 35 °C. However, their growth has drastically dropped from 40- 45 °C. But, the strains AP-1 and AP-2 showed a higher growth at 45 °C, than 40 °C. The overall-highest growth in this site was shown by AP-4 at 30 °C. The strain KH-1 of Kahatagasdigiliya site showed the overall highest growth at 25 °C. The other strains of this site showed their highest growth at temperatures of 30 °C and 35 °C. In the site Mihintale, MH-1, MH-2 and MH-3 showed their maximum growth at 30 °C while MH-4 showed their maximum growth at 35 °C. In the site Medawachchiya, MW-4 showed considerable higher growth from temperature 25- 35 °C. However, its growth has significantly dropped at temperatures of 40 °C – 45 °C, when compared to the other strains of this site. In the site Palugaswewa, PG-2 and PG-4 showed their highest growth at 35 °C while PG-1 and PG-3 showed their highest growth at 40 °C. In the sites of Thalawa and Thanthirimale, the overall-maximum growth was shown by TH-3 and TM-3 at temperatures of 35 °C and 30 °C respectively. Furthermore, the temperature sensitivity also varies between rhizobial strains and there is no common pattern in the growth of rhizobium strains with changing temperature.

The average annual temperature of the north-central province of Sri Lanka is 27.3 °C whilst the mean maximum is ranging from 26.2 to 33.9 °C. In some regions, the weekly maximum temperature reaches up to high values such as 39°C (Udupamunuwa *et al.*, 2020). The optimum

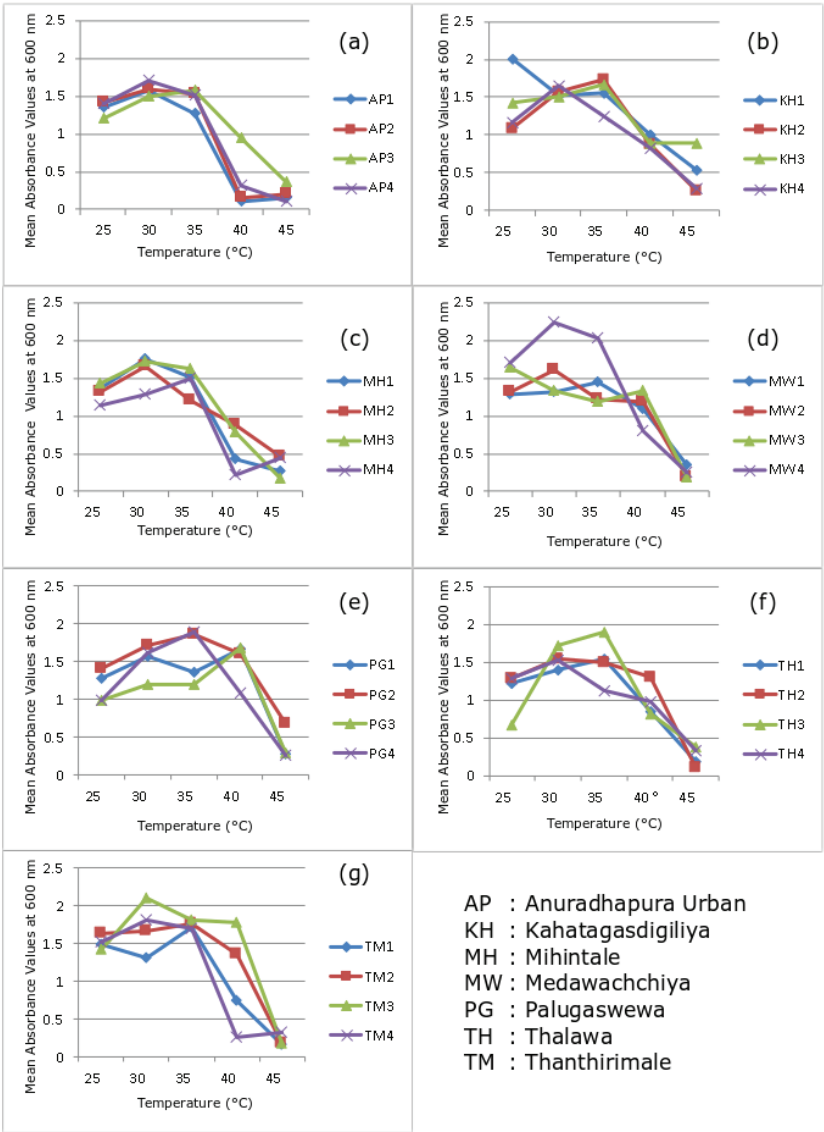

Figure 3: Graphs of (a) AP: Anuradhapura Urban (b) KH: Kahatagasdigiliya (c) MH: Mihintale (d) MW: Medawachchiya (e) PG: Palugaswewa (f) TH: Thalawa and (g) TM: Thanthirimale showing the growth of 28 rhizobial strains at different temperatures.

temperatures for the growth of most rhizobial strains are between 25 °C- 30 °C (Somasegaran and Hoben, 2012). The isolated strains also grew well in temperature between 25- 30 °C hence proving that evidence. Nevertheless, the highest growth was observed in 30 °C and 35 °C as well. However, most of the strains isolated in the Anuradhapura district grew well even at 40 and 45 °C temperatures. This is because the Anuradhapura district usually experience high temperature around 39 °C in some regions and the district is located in the dry zone which is prone to high drought conditions frequently (Kaleel and Nijamir, 2017).

Therefore, this suggests that most of the rhizobial strains are adapted to grow well in high temperatures. Moreover, most of the strains grow well even at 45 °C which was selected as the extreme temperature in this study. High temperature causes the interruption of signaling between the host plant and the rhizobium and also affects nodule development (Aranjuelo *et al*., 2015).

The strains such as AP-3, KH-1, KH-3, MH-2, MH-4, MW-1, PG-2, TH-3, TH-4 and TM-4 showed the highest tolerance at 45 °C of temperature.

Drought tolerance of the rhizobial strains

The Figure 4 shows the growth of 28 rhizobial strains at different PEG concentrations in Anuradhapura urban area, Kahatagasdigiliya, Mihintale, Medawachchiya, Palugaswewa, Thalawa and Thanthirimale sites.

According to the current study all of the strains have grown well at 0.2 % PEG concentration. However, some strains have grown well even at 0.4 % PEG concentration, among them MH-3 was the best in survival.

In the Anuradhapura urban site, all the four strains showed their maximum growth at 0.2 % PEG concentration among them, AP-4 (~ 1.17 nm) showed the highest growth. The growth of all strains has reduced from 0.3 %- 0.4 % PEG concentration. All the strains of the Kahatagasdigiliya site also showed their maximum growth at 0.2 % PEG concentration. Thereafter, the growth of all the strains has gradually decreased. In the site Mihintale, MH-1 and MH-3 showed a considerable higher growth at 0.2 % PEG concentration, compared to the MH-2 and MH-4. All the strains in Mihintale site showed a gradual decrease in their growth after 0.2 % PEG concentration. However, the strain MH-3 showed a drastic plunge in its growth at 0.3 % PEG concentration and at 0.4 % PEG concentration its growth has increased drastically. MW-3 of Medawachchiya site showed the overall-maximum growth at 0.2 % PEG concentration. PG-2 of the site Palugaswewa, TH-1 of Thalawa site and TM-1 of Thanthirimale sites showed the overall-highest growth in the respective site at 0.2 % PEG concentration. This proves that the 0.2 % PEG concentration is the optimum drought condition for the growth of isolated rhizobial strains in the Anuradhapura district. However, some strains are growing well even at 0.4 % PEG concentration. There was no clear pattern of growth observed in response to variation of PEG concentrations in the medium as in other physiological conditions.

Significant growth was shown by all 28 rhizobial strains under PEG-induced different drought conditions which varying the PEG concentration of growth medium from 0.1 % to 0.4 %. The PEG treatment results in the change in the osmotic potential in cells, there by stimulating the water-deficient conditions. Therefore, drought conditions are artificially induced in rhizobial cells. The water deficiency affects the symbiotic nitrogen fixation as well as the number of rhizobial strains in soil, their development and infection ability. The water deficiency causes the formation of free radicals resulting in protein denaturation and lipid peroxidation (Kibido *et al*., 2019). The mean annual rainfall of the Anuradhapura district is 1368 mm (Climate-data.org). Nevertheless, during *Yala* season which lasts from the end of March to mid-May it receives about 300 mm of rain. As this district is located in the dry zone it experiences high evaporation leading to water scarcity, introducing drought conditions (Kaleel and Nijamir, 2017). The rhizobial growth has decreased with the increasing concentration of PEG which is supported by the study carried out by Udapamunuwa *et al*. (2020).

The strains AP-3, KH-4, MH-2, MH-3, MW-1, MW-2, PG-1, PG-2, PG-4, TH-1, TH-2, TH-3, TM-2 and TM-3 showed the highest survival at 0.4 % PEG concentration.

The tolerance of rhizobial strains under combination of physiological conditions

The growth of the 12 selected rhizobial strains in combination of different physiological conditions (pH 8.0, Salinity 3.0 %, Drought 0.4 %, and incubated at 36 °C) at 600 nm of optical absorbance are shown in the Figure 5. The stress-tolerant 12 rhizobial strains were subjected to the ‘combination physiological conditions’ in which the physiological

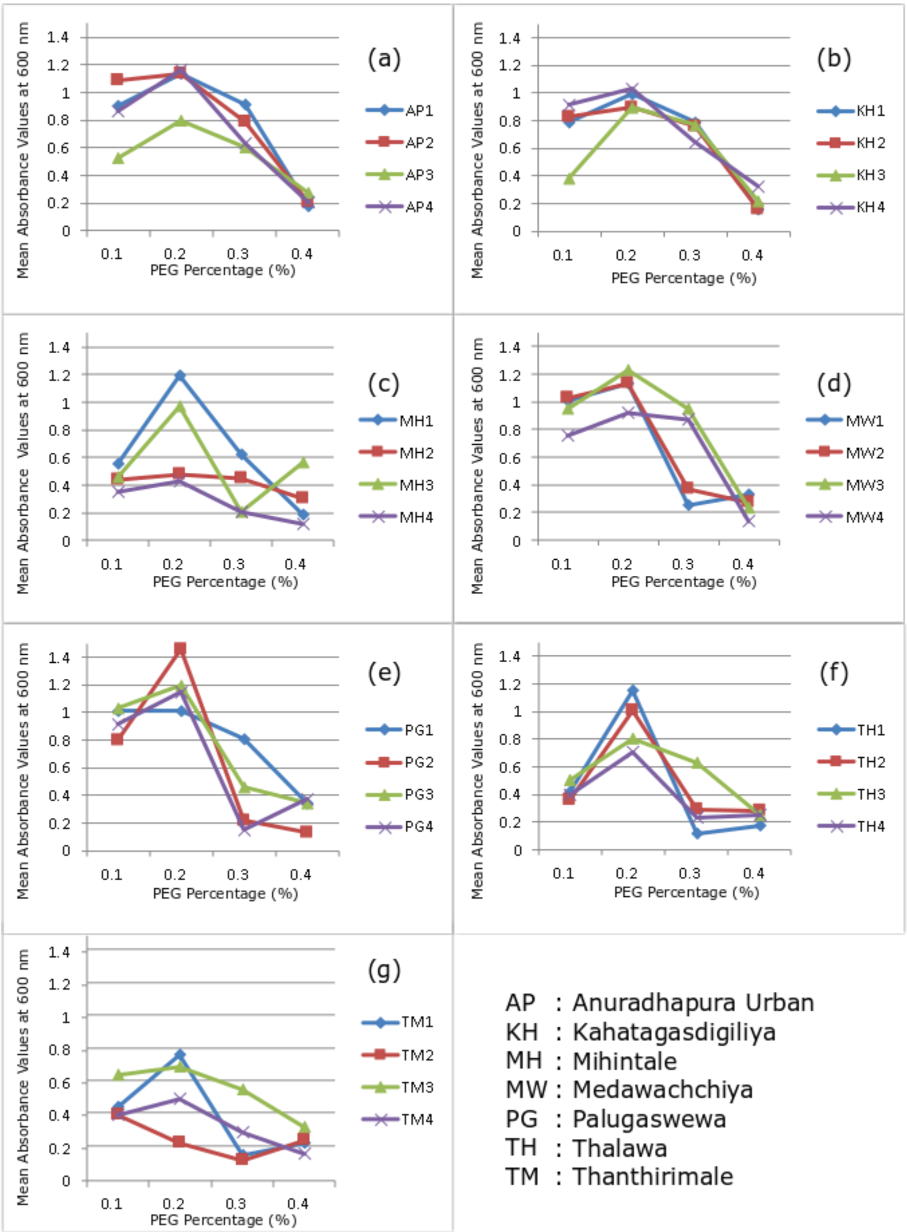

Figure 4: Graphs of (a) AP: Anuradhapura Urban (b) KH: Kahatagasdigiliya (c) MH: Mihintale (d) MW: Medawachchiya (e) PG: Palugaswewa (f) TH: Thalawa and (g) TM: Thanthirimale showing the growth of 28 rhizobial strains at different PEG concentrations.

conditions were selected based on the prevailing natural environmental conditions in the Anuradhapura district. As the pH value of natural soil does not increase up to pH 9.0, pH 8.0 was selected for the combination study. Similarly, the strains were incubated at 36 °C as the natural soil temperature does not generally rise up to 45 °C. Due to the high-water deficiency and saline conditions of the dry zone and due to high evaporation, 0.4 % and 3.0 % PEG and NaCl concentrations were selected respectively. When these isolated strains were grown in a combination with extreme physiological conditions, some of them demonstrated much-reduced growth compared to the growth they have shown when testing for each physiological condition separately. The highest growth was observed in the strain PG-1. In addition to that, strain AP-2, AP-3, KH-1, KH-3, TH-4, and TM-2 showed considerably higher growth at these combined physiological conditions. The strains AP-4, KH-4, MH-3, MW-1, and PG-4 showed poor growth at these combined physiological conditions. Moreover, AP-4 showed the lowest growth among the poorly grown strains. The highest tolerance was observed in PG-1 where it showed maximum tolerance at 0.4 % PEG concentration and 3.0 % NaCl concentration. However, a high tolerance was not observed at 45 °C and pH 9.0. Further, PG-1 showed high tolerance when grown at 35 °C and pH 8.0 separately. Moreover, AP-4, KH-4, MH-3, MW-1 and PG-4 showed low growth at the combination of physiological conditions. The less growth of AP-4 may be due to the intolerability of 0.4 % drought condition as its growth is less at that particular drought condition. The growth of KH-4 and PG-4 is limited due to the intolerability of 3.0 % NaCl concentrations, as they showed poor growth when they were grown separately in that particular NaCl concentration. The poor growth of MH-5 has resulted due to intolerability of both pH and temperature.

Khalid *et al*. (2020) reported that the isolated rhizobial strains from the root nodules of *Arachis hypogaea* growing in an abiotic stress environment were well survived in the pH range of 5-10, salinity level 3.0 % (NaCl) and temperature range of 20 to 37 °C. We have also observed similar results as most of the isolated strains showed better survival at the same physiological conditions. Moreover, most of the strains isolated from *C. terantea* were well grown even in the extreme temperature of 45 °C.

Genetic diversity of isolated stress-tolerant rhizobial strains

The dendrogram with Average Linkage and Euclidean Distance is shown in the Figure 6. The ERIC profile which was used for assessing the genetic

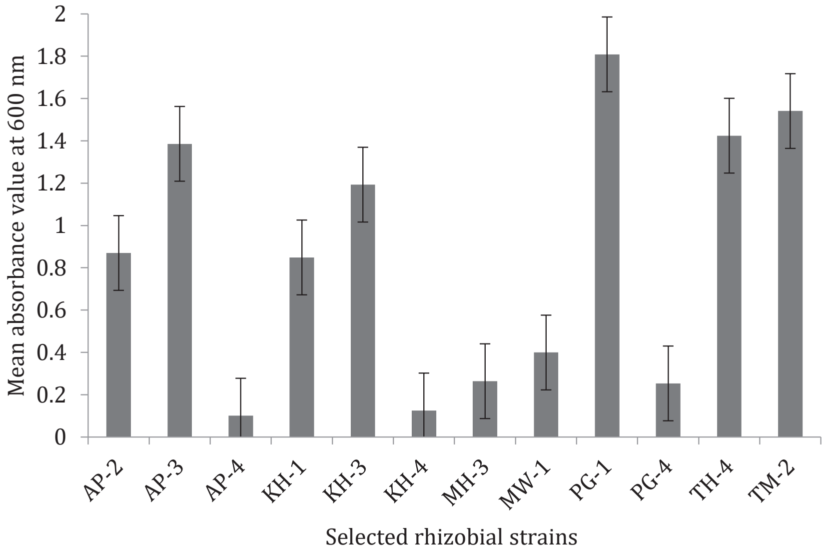

Figure 5: Growth of the 12 stress-tolerant rhizobial strains (PG-1[a], TM-2[a], TH-4[a], AP-3[a], KH-3[a,b], AP-2[a,b,c], KH-1[a,b,c], MW-1[b,c], MH-3[b,c], PG-4[b,c], KH-4[c], AP-4[c]) in combination of different physiological conditions (pH=8.0, Salinity 3.0 %, Drought 0.4 %, and incubated at 36 °C) at 600 nm of optical absorbance (Means denoted by same letters are not significantly different at $p<0.05$).

diversity showed a high polymorphism for the 12 selected rhizobial strains. The dendrogram was constructed from the polymorphic bands using Average linkage Euclidean distance method. The results suggest that strains MW-1 and MH-3 showed a closer relationship at 100 % similarity. Additionally, there was no significant difference between MW-1 and MH-3, which was supported by results of combination of physiological conditions study.

Moreover, Mihintale and Medawachchiya sites have more similar conditions, therefore microclimatic conditions are also similar showing a closer relationship between strains MW-1 and MH-3. At the 69.89 % similarity level eleven clusters were obtained in which two strains AP-2 and AP-3 of Anuradhapura urban site belong to two different clusters. However, the other selected strain (AP-4) in the Anuradhapura site does not belong to any of these clusters and it clusters with the strain (TH-4) isolated from the Thalawa site. The KH-3 and AP-2 clustered together at 69.89 % similarity further this is supported by the combined physiological data which are rather similar. However, though PG-4 and AP-3 clustered together at 69.89 % similarity, their combined physiological data are not correlated. This is also because they are 30.11 % dissimilar to each other. Even though, AP-4 and TH-4 clustering together at 52.86 % similarity, their combined physiological data are totally different. This is because their genetic make-up is different. The strains isolated from the Palugaswewa site (PG-1 and PG-4) belong to different clusters at 52.86 % similarity. KH-1 and KH-4 strains isolated from the Kahatagasdigiliya sites also belong to two distant clusters. Therefore, these results suggest that there is no correlation between the location and the rhizobial strains. Furthermore, similar rhizobial strains can be found in different geographical locations. These results are further supported by the studies carried out by Udupamunuwa *et al*. (2020) and Samarakoon and Rajapakse (2020). Even though some of the strains belong to similar clusters, their

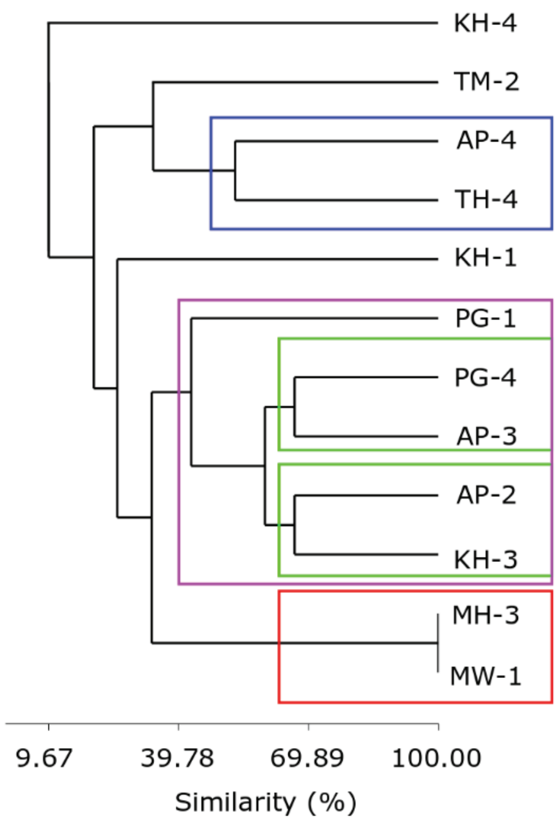

Figure 6: The Dendrogram with Average Linkage and Euclidean Distance.

physiological behaviors are different due to the difference in their genetic makeup. Furthermore, the ERIC profile suggests that all these 12 different strains are genetically diverse.

CONCLUSIONS

In this study, twelve rhizobial strains were identified, which showed high tolerance to extreme physiological conditions of temperature, salinity, drought, and pH. According to the dendrogram studies which was prepared using the ERIC profile, it was found that these twelve strains are highly genetically diverse, and they can withstand the extreme stress environmental conditions. PG-1 strain isolated from the Palugaswewa site showed the highest growth in combination of physiological study. Moreover, it is found that there is no correlation between the location and the rhizobial strains.

FUTURE DIRECTIONS

This study is very significant, as the isolated stress-tolerant rhizobial strains, in combination or as a single strain can be used for the cross inoculation for widely grown leguminous plants in Anuradhapura district. The successful stress-tolerant rhizobial strains can be used for manufacturing of Biofertilizers and the genetic information can be used for other strategies. Further studies can be focused for the identification of these isolated twelve rhizobial strains by sequencing of *16S rRNA* region and there is a high possibility for the identification of new rhizobial strains as well.

DECLARATION OF CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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