The Journal of Agricultural Sciences - Sri Lanka
Vol. 14, No. 1, January 2019. Pp 34-48
http://dx.doi.org/10.4038/jas.v14i1.8455

Physiological Characterization and Genetic Diversity Assessment of the Rhizobial Populations Inhabiting *Gliricidia sepium* in selected Locations of *Ampara* District, Sri Lanka

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Received: 6th April 2018 / Accepted: 24th July 2018

ABSTRACT

Purpose: *Gliricidia sepium is a wide spread multipurpose legume plant which is effective in nitrogen fixation and host to many Rhizobial strains. However, few studies have been carried out to identify and characterize the Rhizobial populations inhabiting G. sepium in Sri Lanka. The main objective of this study was to isolate and identify the stress tolerant Rhizobial strains in G. sepium.*

Research Method: *Root nodules of G. sepium were collected from seven locations in Ampara district, which belongs to dry zone in Sri Lanka. Total of 35 isolates were screened for the tolerance for different pH, salinity, drought conditions and temperatures separately as well as in combinations. Genetic diversity of stress tolerant isolates was assessed using ERIC fingerprinting.*

Findings: *All isolates were grown under wide range of stress conditions. The Rhizobial strains isolated from the site closer to the coast showed a high tolerance for all salinity levels as they are adapted to the high salt stress experienced in their natural habitat. The growth response to drought conditions and temperature was variable. Moreover, 14 isolates showed a high tolerance for more than two extreme stress conditions. When extreme conditions were combined, 12 isolates among 14 were survived. These 14 isolates grouped in to ten clusters at 69% similarity coefficient that make them genetically diverse.*

Research Limitation: *The stress tolerance was observed under laboratory conditions and it does not provide enough evidence for the effectiveness of selected isolates in field.*

Original Value: *The 14 stress tolerant Rhizobial isolates can be used to cross inoculate crop legumes to identify possible cross inoculation groups in order to reduce nitrogen fertilizer usage.*

Keywords: *Biological Nitrogen fixation, ERIC DNA fingerprinting, Rhizobium isolates, Stress tolerance, Legume-Rhizobium symbiosis*

INTRODUCTION

Biological Nitrogen fixation plays an important role as a major natural source of exogenous nitrogen on earth (Sullivan *et al*., 2014) and it improves the soil fertility (Wani *et al*., 1995) which is carried out through the symbiotic relationship between nitrogen fixing bacteria and legume plants. About 20 % of the legumes identified in the world have found to fix nitrogen. Peoples and Craswell (1992) explained that the majority of the nitrogen requirement of legumes is obtained from the biological nitrogen fixation and the excess fixed nitrogen is released into the soil. These factors have made the legume plants to be widely used in tropical agricultural systems as green manure, to prevent soil erosion and for reforestation (Acosta-Duran and Martinez-Romero, 2002).

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Rhizobia have found to be the major and most effective nitrogen fixing microsymbionts. They are gram negative soil bacteria belong to family Rhizobiaceae that consists of different genera including *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium* (Young and Haukka, 1996) and *Allorhizobium* (de Lajudie *et al*., 1998). The optimal growth conditions for most Rhizobial strains are, temperature at 25-30 °C and pH of 6.0-7.0. However, some species such as *R. tropici* has found to be tolerant to highly acidic pH (pH 4.5) and high temperatures (Martinez-Romero *et al*., 1991). In addition to these conditions, soil salinity and water stress have also found to affect the growth and distribution of Rhizobia in soil. In general, *Bradyrhizobium* has found to be the least salinity tolerant genus compared to *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* where *Rhizobium* and *Sinorhizobium* show the highest tolerance (Laranjo and Oliveira, 2011; Brígido *et al*., 2012).

Many members of Rhizobia have the ability to form mutualistic interaction with legumes where the bacteria get photosynthetic products (carbon) and protection from the host legume (Lodwig *et al*., 2003) and the host legume gets fixed nitrogen in return (Peoples and Craswell, 1992). Host specificity of different Rhizobial strains vary widely where some strains successfully nodulate more than one legume and some legumes are successfully inoculated with more than one Rhizobial strains (Denison, 2000; Bala and Giller, 2001). However, successful infection occurs when the legume and Rhizobial strains are compatible (Denison, 2000). It has been found that *Gliricidia sepium* is a host to several Rhizobial strains including *R. tropici*, *R. etli* (Acosta-Duran and Martinez-Romero, 2002), *R. mongolense* and other fast growing Rhizobia (Bala and Giller, 2001) providing evidence to the observation by Denison, (2000).

G. sepium is a leguminous plant belongs to subfamily Papilionoideae that is being widely used as a fuel wood, shade plant, for animal fodder and this is growing in coconut fields and other cultivations in Sri Lanka as a source of nitrogen fertilizer because of its ability to fix atmospheric nitrogen efficiently (Jayasundara *et al*., 1997). The nitrogen fixing efficiency of *G. sepium* has found to be around 75 % under greenhouse conditions (Awonaike *et al*., 1992) and around 64 % in field conditions (Liyanage *et al*., 1994). The available literature has revealed that the cultivation of these legumes as supporting plants for non-legume crops, can reduce the use of nitrogen fertilizers (Yanni *et al*., 1997). However, the ability and the efficiency of nitrogen fixation is determined by the Rhizobial strain, the genotype of *G. sepium*, biotic and abiotic factors that affect the nitrogen fixation and the mode of interaction between the two organisms (Awonaike *et al*., 1992).

G. sepium is a widely distributed plant in Sri Lanka throughout all climatic zones and this is adapted to a wide range of soil conditions. This is an abundant plant in *Ampara* district, which belongs to dry zone. *Ampara* district consists of three main Agro-ecological regions which have average rainfalls of >775 mm, >900 mm and >1150 mm and the soil pH in the region generally varies from slightly acidic to slightly alkaline (Eastern Development Plan, Eastern Provincial council, 2012). *Ampara* district is one of the major producers of maize and legume crops such as cowpea, green gram, ground nut and black gram in Sri Lanka. *G. sepium* is being cultivated in many areas of *Ampara* district including all three agro-ecological regions and in many agricultural lands as well as in domestic lands as a supporting plant, shade plant and as a living fence.

Available literature reveals that *G. sepium* has a great potential of biological nitrogen fixation (Jayasundara *et al*., 1997; Liyanage *et al*., 1994) and therefore cultivation of this plant adds additional advantage to improve the quality of food crops. Moreover, the wide distribution and the ability to withstand in a wide range of environmental conditions suggest that the Rhizobia in *G. sepium* might possess the similar characteristics as same as the host. Even though there are research that have been carried out to estimate the nitrogen fixing

efficiency, there are very few research that have done to characterize the nitrogen fixing bacteria (*Rhizobium* sp.) inhabiting *G. sepium* in Sri Lanka. Moreover, the identification of stress tolerant Rhizobial strains in *G. sepium* allow those strains to be used for cross inoculation of selected crop legumes growing in arid regions and in nitrogen-depleted soil.

Therefore, the present study was done to characterize the Rhizobial strains from *G. sepium* in *Ampara* district to determine the tolerance of them to pH, salinity, temperature and drought and to determine the genetic diversity of stress tolerant Rhizobial isolates using *ERIC* (Enterobacterial Repetitive Intergenic Consensus) fingerprinting (Versalovic *et al*., 1991).

MATERIALS AND METHODS

Collection of root nodules and Isolation of Rhizobia

Root nodules were collected from seven different locations (*Deegavapiya*, *Karativu*, *Uhana*, *Paragahakele*, *Keviliyamadu*, *Padiyathalawa*, *Ampara*) in *Ampara* district that belongs to dry zone in Sri Lanka. One plant of *G. sepium* was selected from each location and five nodules were collected from each plant to have a total of 35. The nodules were surface sterilized and isolation was done using crush method (Somasegaran and Hoben, 1994) followed by streaking on ½ Lupin agar plates. The cultures were incubated at room temperature in dark for 3-5 days. Pure cultures of Rhizobia was obtained by doing 4-5 subcultures.

Tolerance of Rhizobia for physiological conditions

pH tolerance was assessed by culturing the isolated 35 strains in ½ Lupin broth at pH ranging from 3.0-10.0. Salinity tolerance was tested for six NaCl concentrations (0.1 %, 1 %, 1.5 %, 2 %, 2.5 % and 3 %) in ½ Lupin broth 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 %). The cultures were incubated at room temperature for three days in 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). The growth of Rhizobial isolates under these conditions were assessed by measuring the optical absorbance at 600 nm wave length.

Tolerance for combination of different conditions

The Rhizobial strains that showed high tolerance to the different conditions were selected and those samples were treated with combinations of selected pH (7.5), salinity (3.0 % NaCl) and drought conditions (0.4 % PEG) and were incubated at 37 °C for three days. The absorbance at 600 nm was measured to determine the growth of Rhizobial strains.

DNA fingerprinting with ERIC primers

Genomic DNA of 14 selected isolates were extracted using phenol chloroform method and DNA fingerprinting was carried out for the isolated Rhizobial strains using *ERIC 1R* and *ERIC 2R* primers. The amplified DNA fragments were visualized using Agarose Gel electrophoresis.

Data analysis and representation

The growth of Rhizobial strains for all conditions in all the seven sites were compared by plotting bar charts. Absorbance values were subjected to statistical analysis using General Linear Model (GLM) procedure and LS means-pdiff mean separation procedure using statistical package, SAS 9.3.1 (SAS Institute, NC, Cary, USA) to select the strains with higher growth under extreme conditions. A dendogram was prepared for the Rhizobial strains that showed tolerance to extreme conditions by Complete Linkage, Euclidean Distance using the software MINITAB 17.1.0.

RESULTS

pH tolerance of Rhizobial strains

All the 35 strains from all 7 sampling sites showed the highest growth in pH 6.0-8.0 range. The growth was very poor in highly acidic pH (pH 3.0 and 4.0) and the growth in highly alkaline pH was higher than that of acidic pH. A drastic increase of growth was observed in all 35 strains when pH changes from 4.0 to 5.0. Even though there was a reduction of growth collectively when pH increase from 8.0 to 10.0, there was no clearly observable pattern since some strains such as KR-a, UH-b, UH-e, PK-b, AM-b and AM-d showed a higher growth at pH 9.0 and 10.0 than that of pH 7.0 and 8.0 (Figure 01).

The highest growth was observed in strain DV-a at pH 8.0 and all strains showed a minimum growth (absorbance <0.075) in acidic pH (pH 3.0 and 4.0) in site *Deegavapiya*. DV-b and DV-c showed a considerable high growth in highly alkaline pH 10.0 (absorbance >0.125) (Fig. 01A). A similar growth pattern was observed in the site *Karativu*. The growth at pH 8.0, 9.0 and 10.0 were almost similar in all 5 Rhizobial strains (Figure. 01B). UH-e was the only strain from the strains tested from site *Uhana* that could grow well under high pH (Fig. 01C). In the site *Paragahakele*, PK-d strain showed the highest growth from all strains and only PK-b showed a considerably higher growth than others in pH 10.0 (Figure 01D). KM-a and KM-e from *Keviliyamadu* were the strains that showed a higher growth than others in all pH levels (Fig. 01E). The growth of KM-c and KM-d were extremely low in all pH levels. PD-a and PD-e were able to grow well under pH 10.0 than other strains from *Padiyathalawa* (Fig. 01F). AM-b and AM-d showed a higher growth than others under both pH 9.0 and 10.0. AM-e was the least grown strain from the Rhizobial strains isolated from site *Ampara* (Figure 01G).

Tolerance of Rhizobial strains to salinity

Salinity tolerance of the isolated Rhizobial strains were assessed by growing them in the medium with varying NaCl concentrations ranging from 0.1 % to 3.0 %. All the strains except PK-e, KM-b, KM-c and KM-d have shown a high growth at 0.1 % salt concentration. KR-d showed the highest growth among all 35 strains at all salt levels. KM-b and KM-c showed a very low growth even at 0.1 % as well as in higher salt levels (Figure 02).

The 5 Rhizobial strains from *Deegavapiya*, showed a gradual decrease in growth as the salinity level increase from 0.1 % to 3 %. DV-b had the highest growth at 3.0 % NaCl while DV-e showed the lowest growth (Figure 02A). All 5 strains isolated from *Karativu* collectively showed a higher growth than the strains isolated from other 6 sites. Those strains survived well even at 3.0 % NaCl concentration (Figure 02B). The Rhizobial strains from *Uhana* have not shown a clearly observable growth pattern in response to increasing salt concentration (Figure 02C). A clearly observable relationship between salt concentration and growth was not obtained for *Paragahakele*, *Keviliyamadu*, *Padiyathalawa* and *Ampara* as same as in *Uhana*. The highest growth among the strains isolated from *Paragahakele* at 3.0 % NaCl was observed in PK-c and PK-e while PK-a and PK-b showed the lowest growth (Figure 02D). All the strains from *Keviliyamadu* showed a poor growth at 3.0 % (Fig. 02E). PD-a, PD-b and PD-d have resulted a higher growth at 3.0 % NaCl PD-a and PD-d showed a considerable higher growth at other NaCl concentrations (Fig. 02F). AM-a, AM-c and AM-d were the Rhizobial strains that survived well under high salt concentration (3.0 %) (Figure G).

Drought tolerance of Rhizobial strains

The drought stress was induced by adding PEG to the culture medium at different concentrations. All the isolated Rhizobial strains collectively showed a substantial growth at all PEG concentrations. However, there was no clear pattern of growth in response to varying drought conditions (Figure. 03). KR-c showed the highest growth among all 35 strains at 0.1 % PEG.

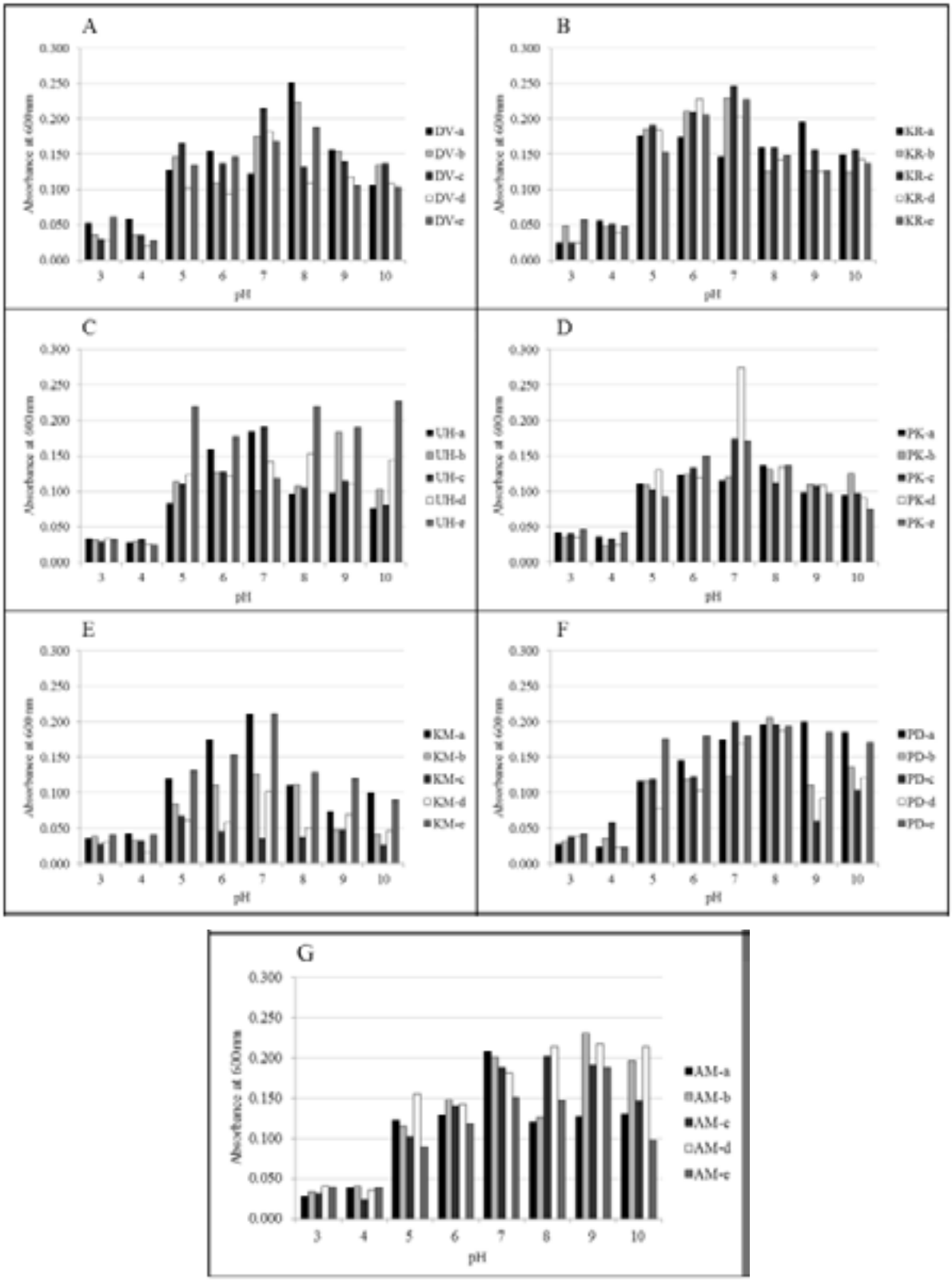

Figure 01: **Growth of isolated Rhizobial strains from 7 sites at different pH values. The growth increase with pH up to 8.0 and growth is decreased beyond pH 8.0. A- Growth of Rhizobial strains from the site *Deegavapiya* (DV). B- Growth of Rhizobial strains from the site *Karativu* (KR). C- Growth of Rhizobial strains from the site *Uhana* (UH). D- Growth of Rhizobial strains from the site *Paragahakele* (PK). E- Growth of Rhizobial strains from the site *Keviliyamadu* (KM). F- Growth of Rhizobial strains from the site *Padiyathalawa* (PD). G- Growth of Rhizobial strains from the site *Ampara* (AM).**

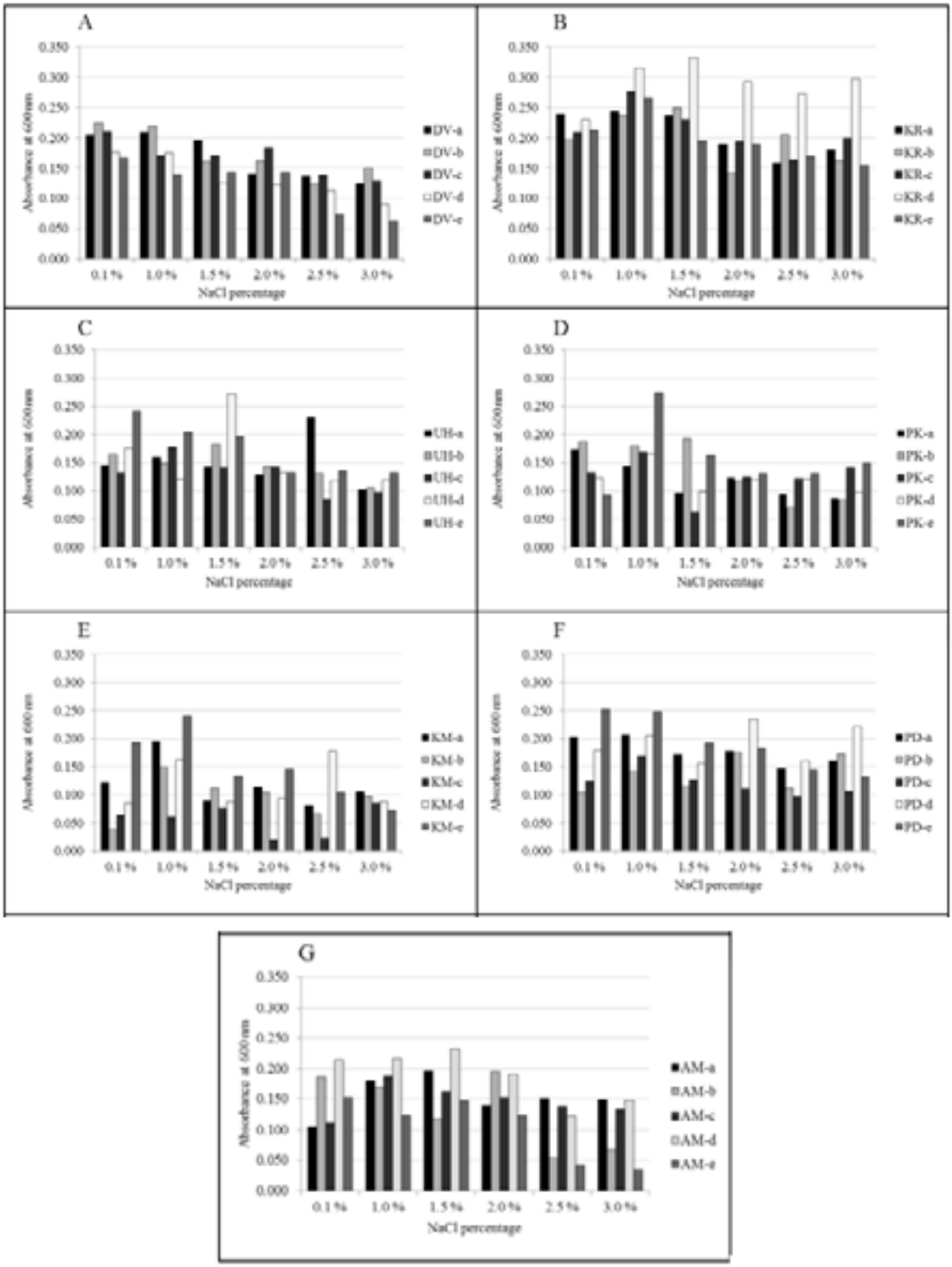

Figure 02: **Growth of isolated Rhizobial strains from 7 sites at different salinity levels ranging from 0.1 % to 3.0 % NaCl concentrations. There was no clearly observable relationship between growth of Rhizobial strains and salt concentration. A- Growth of Rhizobial strains from the site *Deegavapiya* (DV). B- Growth of Rhizobial strains from the site *Karativu* (KR). C- Growth of Rhizobial strains from the site *Uhana* (UH). D- Growth of Rhizobial strains from the site *Paragahakele* (PK). E- Growth of Rhizobial strains from the site *Keviliyamadu* (KM). F- Growth of Rhizobial strains from the site *Padiyathalawa* (PD). G- Growth of Rhizobial strains from the site *Ampara* (AM).**

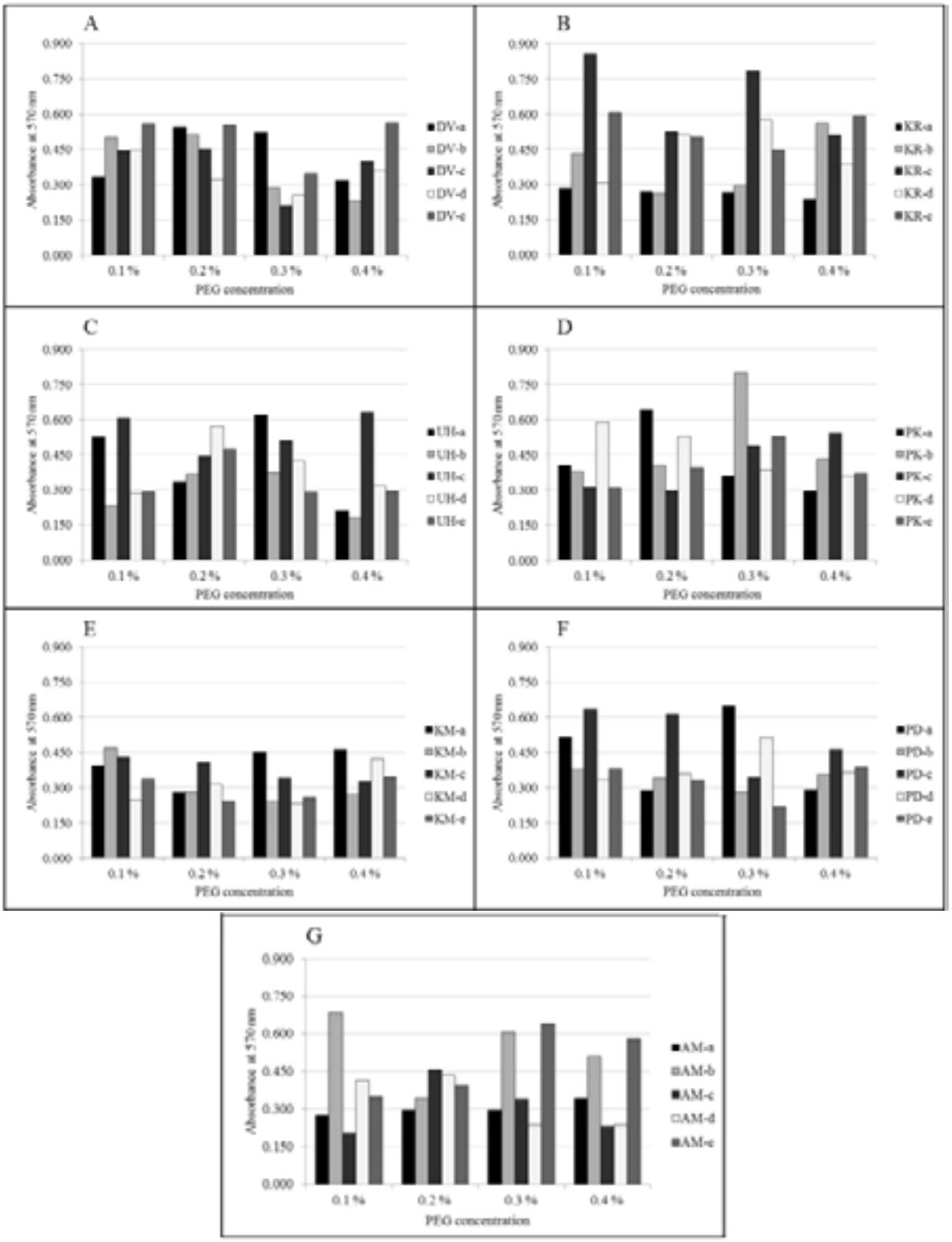

Figure 03: **Growth of isolated Rhizobial strains from 7 sites at different drought levels (0.1 %, 0.2 %, 0.3 % and 0.4 % PEG). The growth response was variable in all strains. A- Growth of Rhizobial strains from the site *Deegavapiya* (DV). B- Growth of Rhizobial strains from the site *Karativu* (KR). C- Growth of Rhizobial strains from the site *Uhana* (UH). D- Growth of Rhizobial strains from the site *Paragahakele* (PK). E- Growth of Rhizobial strains from the site *Keviliyamadu* (KM). F- Growth of Rhizobial strains from the site *Padiyathalawa* (PD). G- Growth of Rhizobial strains from the site *Ampara* (AM).**

DV-e showed the highest growth among strains isolated from *Deegavapiya* at all PEG levels except 0.3 %. It was well grown even under high (0.4 %) drought conditions. The growth of DV-b was reduced gradually as the PEG concentration increased. DV-a, DV-c and DV-d have shown a varying growth at different drought conditions (Fig. 03A). KR-b, KR-c and KR-e were the strains that showed a high growth at 0.4 % PEG concentration. The growth of KR-a was low at all PEG concentrations (Fig. 03B). When considering growth of Rhizobial strains isolated from *Uhana*, UH-c showed the highest growth at 0.4 % PEG. All the other strains showed a lower growth at 0.4 % compared to that of low PEG concentrations (Fig. 03C). PK-c showed the highest growth among the strains isolated form the site *Paragahakele* at 0.4 % PEG concentration (Fig. 03D). KM-a and KM-d strains from *Keviliyamadu* showed a higher growth at 0.4 % PEG than other strains from the same site (Figure 03E). Only PD-c from the site *Padiyathalawa* showed a considerably high growth at high drought condition (0.4 %) (Fig. 03F). AM-b and AM-e were the strains that showed a high growth at 0.4 % PEG concentration while AM-c and AM-d showed the lowest growth (Figure 03G). However, a clear difference of growth among different sites were not observed.

Temperature tolerance of Rhizobial strains

All the Rhizobial strains have grown well under all the temperatures ranging from 25 °C to 45 °C. However, few strains showed a poor growth at 45 °C. DV-d, PD-c and AM-b were the strains whose growth was low at highest temperature used. The growth of all the strains at 25 °C were slightly lower compared to higher temperatures. 35 °C was the temperature at which many strains including DV-a, KR-c, KR-d, UH-b, PK-c, PK-d, KM-d, PD-a, PD-b and AM-c showed their highest growth (Figure 04).

DV-a and DV-b showed the highest growth at 45 °C among the strains isolated from *Deegavapiya* while DV-d showed the lowest growth (Fig. 04A). All the 5 strains from *Karativu* have shown a considerable tolerance to high temperatures where KR-c and KR-d showed the highest growth at 45 °C. The growth of all these 5 strains at 25 °C were lower than that at 45 °C (Fig. 04B). UH-b and UH-c were the strains that showed a higher growth than others at 45 °C. However, the absorbance values that indicate the growth were lower than those of KR strains (Fig. 04C). When considering the growth of strains from *Paragahakele*, PK-c and PK-e showed a higher growth at 45 °C than other strains (Fig. 04D). KM-a and KM-c showed a higher growth at 45 °C and the growth of all 5 strains were low at 25 °C (Fig. 04E). PD-b showed the highest growth at high temperature while the growth of PD-c and PD-e were very low (Fig. 04F). All the strains from *Ampara* showed a poor growth at 45 °C compared to other sites. However, AM-a showed the highest growth among them (Figure 04G).

Selection of strains with high tolerance to extreme physiological conditions

All conditions had a significant effect on the growth of Rhizobial strains ($P<0.05$). Highly tolerant strains were selected based on the mean separation analysis done using General Linear Model (GLM) procedure and LS means-pdiff mean separation procedure using statistical package, SAS 9.3.1 (SAS Institute, NC, Cary, USA). Fourteen strains among 35 showed a significantly high growth under more than two extreme conditions (Data not shown).

Tolerance of Rhizobial strains to combination of different physiological conditions

When the strains that showed tolerance to individual extreme conditions were grown under combined conditions, 12 strains among 14 selected strains showed a considerable high growth. PD-a showed the highest growth among these strains. PK-e and KM-a were the strains that showed the lowest growth (Figure 05).

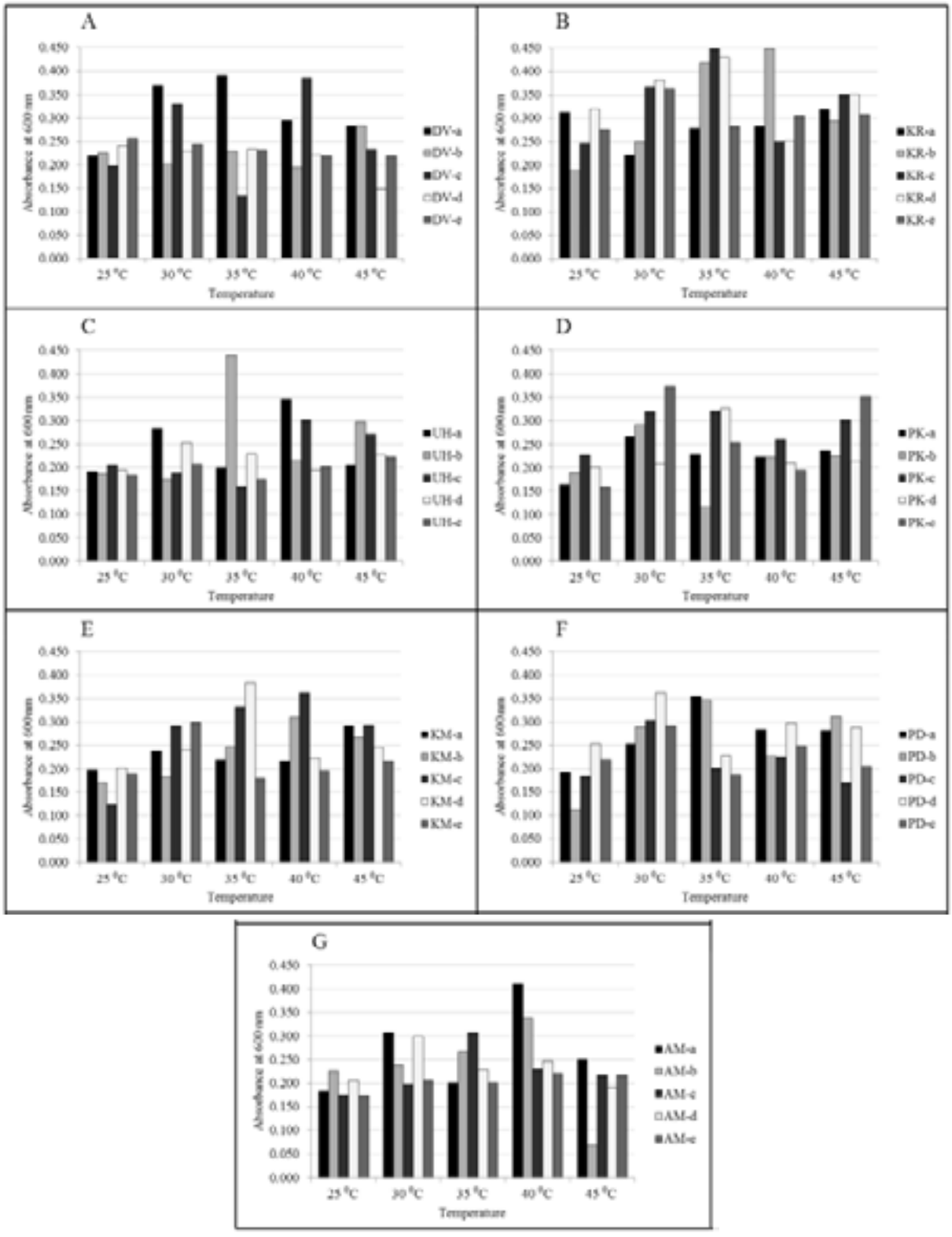

Figure 04: **Growth of isolated Rhizobial strains from 7 sites at different temperatures varying from 25 °C to 45 °C. All the 35 strains generally well grown under all temperatures. A- Growth of Rhizobial strains from the site *Deegavapiya* (DV). B- Growth of Rhizobial strains from the site *Karativu* (KR). C- Growth of Rhizobial strains from the site *Uhana* (UH). D- Growth of Rhizobial strains from the site *Paragahakele* (PK). E- Growth of Rhizobial strains from the site *Keviliyamadu* (KM). F- Growth of Rhizobial strains from the site *Padiyathalawa* (PD). G- Growth of Rhizobial strains from the site *Ampara* (AM).**

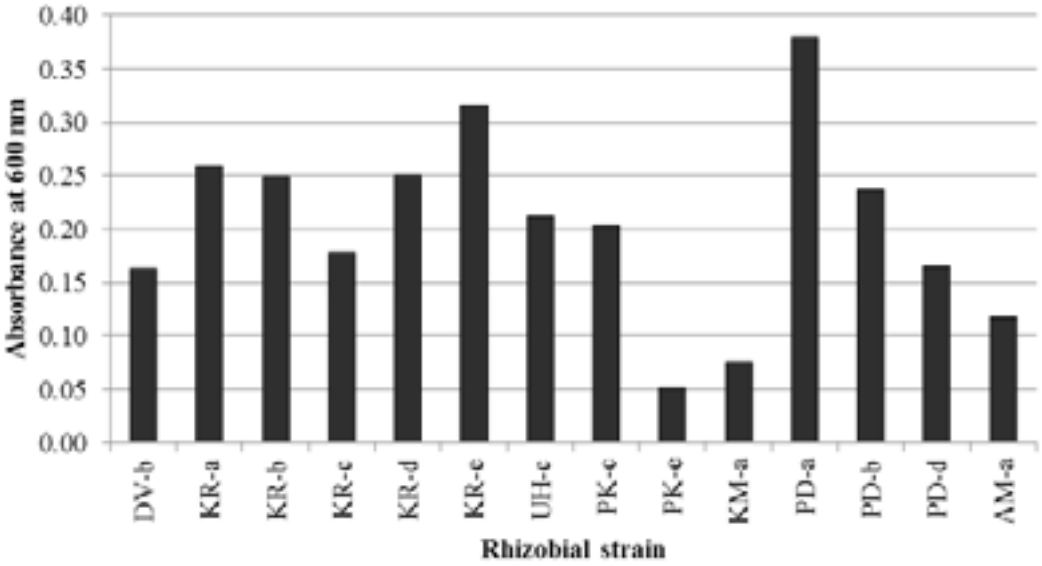

Figure 05: **Growth of 14 selected Rhizobial strains under combination of different physiological conditions. These strains were cultured in a medium with 3.0 % NaCl, 0.4 % PEG and pH 7.5 and incubated at 37 °C. The growth was assessed by measuring the absorbance at 600 nm. PK-e and KM-a showed a very poor growth upon these extreme conditions. PD-a showed the highest growth among these strains.**

Genetic diversity and relationship between selected Rhizobial strains

The banding pattern of *ERIC* profiling was highly polymorphic. KR-a, KR-c and KR-e showed a similar banding pattern (Figure 06) Genetic diversity and the relationship between selected strains were assessed by preparing a dendogram based on the banding patterns obtained for *ERIC* profiling. KR-a, KR-c and KR-e were similar to each other at 100 % similarity level. The next level was observed at 69 % where there were 10 clusters. Four strains from *Karativu* (KR-a, KR-c, KR-d, KR-e) belong to one cluster, UH-c and AM-a belong to one cluster and all the other 8 strains belong to 8 separate clusters. PD-b was similar to UH-c and AM-a and DV-b was similar to PD-a at 57 % similarity level. KM-a was similar to the cluster with UH-c only at 9 % similarity level (Fig. 07).

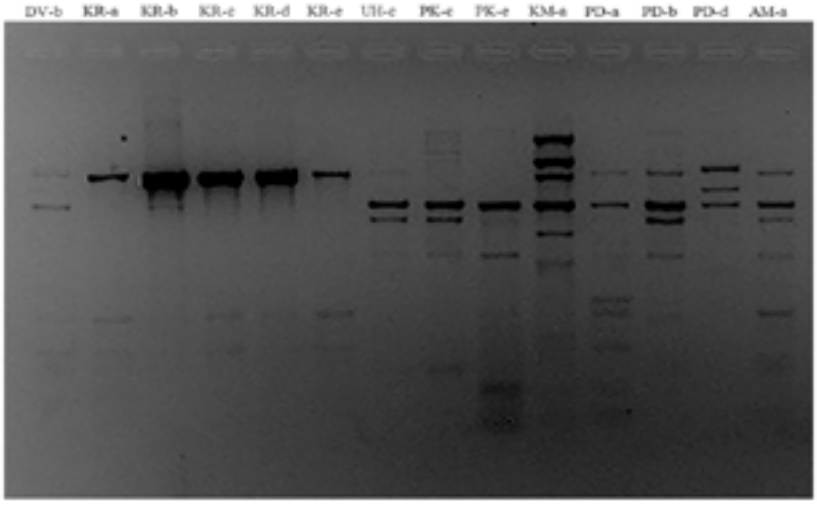

Figure 06: ***ERIC* profiling for 14 selected Rhizobial strains. This was carried out using *ERIC* 1R and *ERIC* 2R primers. The gel electrophoresis for *ERIC* PCR products has shown highly polymorphic banding patterns in these strains.**

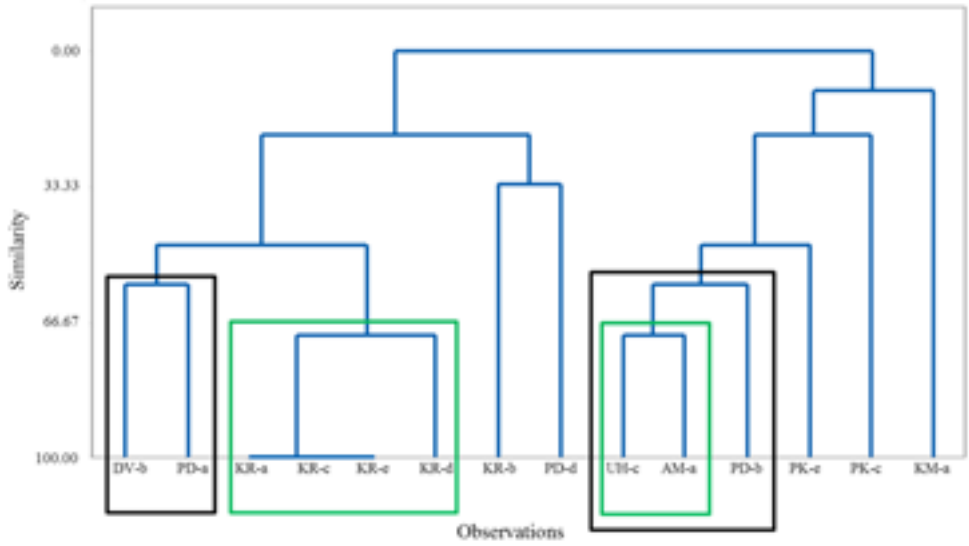

Figure 07: **Genetic relationship between selected 14 Rhizobial strains. The dendogram was constructed using complete linkage euclidean distance using the statistical software MINITAB 17.1.0. KR-a, KR-c and KR-e are similar to each other at 100 % level. Strains similar at 69 % level are indicated in green colour box. Strains similar at 57 % level are indicated in black colour box.**

DISCUSSION

G. sepium was abundant in all the sites except in *Karativu*. The plant was rare in coastal areas. This was mainly growing as living fence and as a supporting plant in domestic and agricultural lands. Root nodules were found in large numbers closer to the surface soil where bacteria have more access to the gases in the atmosphere. However, they were less abundant in hard clay soil that could be found mainly in some regions of *Deegavapiya*. The shape and size of the nodules vary widely despite of the sampling location.

Rhizobia generally show their optimum growth at pH 6.0-7.0 (Somasegaran and Hoben, 1994). When considering the results obtained for pH tolerance of these Rhizobial strains, all the strains showed the highest growth in pH 6.0-8.0 and some strains (DV-b, DV-c, KR-a, KR-c, KR-d, KR-e, UH-e, PK-b, KM-a, PD-a, PD-b, PD-e, AM-a, AM-b, AM-d) showed a high growth even at pH 10.0 (Fig. 01). These results indicate that these strains have the tolerance for broad range of pH. The soil pH in dry zone soils usually varies from slightly acidic to slightly alkaline. However, soil pH mostly lies within neutral pH range (Department of Agriculture, 2017) and therefore the selection of pH range (3.0-10.0) was done based on the criteria that highly acidic pH (1.0, 2.0) do not exist in natural soil. Therefore, these Rhizobial strains isolated from dry zone soils showed the expected results. The strains that could survive even under very high alkaline conditions contained isolates from all seven sites. Thus, the differences among the conditions in sampling sites have not affected the pH tolerance of these Rhizobial strains. Highly acidic soil affects the activity of *Rhizobium* and cause the reduction of root nodule formation process (Hungriaa and Vargas, 2000). This provides the reason for the observation of poor growth of all Rhizobial strains at highly acidic pH (3.0 and 4.0). However, some Rhizobia such as *Sinorhizobium meliloti* have found to gain the tolerance to acidic conditions through the modifications in their metabolic pathways (Draghi *et al*., 2017).

High salt concentrations have shown to have negative effects on growth and distribution of *Rhizobium* sp. in soil (Jenkins *et al*., 1989) through limiting root infection via reducing the number of Rhizobial cells followed by inhibition of nodule growth and damaging the activity of nodules (Laranjo and Oliveira, 2011; Brígido *et al*., 2012; Moussaid *et al*., 2015). However,

several studies have identified salt tolerant Rhizobia. *R. meliloti* and *R. leguminosarum* bv. trifolii are two species of Rhizobia that have been identified to show high tolerance to extreme saline conditions in arid regions (El-Mokadem *et al*., 1991; El-Sheikh and Wood, 1995). According to the results obtained for this study, all 5 strains isolated from *Karativu* showed a higher tolerance to all salinity levels than other strains. The sampling site of *Karativu* was very closer to the seashore (<100 m) where the soil is highly saline. Therefore, the Rhizobial strains inhabiting legumes in these areas have a high tolerance to extreme salinity levels. The strains from *Deegavapiya* showed a gradual sensitivity to increasing salinity. All the other strains showed irregular and lower tolerance than KR strains to increasing salinity (Figure. 02). Dry zone soils encounter a higher amount of evaporation and thus salts get concentrated in soil eventually (Mahajan and Tuteja, 2005). Therefore, the Rhizobia inhabiting dry soils develop a tolerance to high salinity levels. This might be the reason for the observation of considerable tolerance of many strains to high salt concentrations in this study.

Drought conditions have found to cause negative effects on Legume-*Rhizobium* sysmbiosis through enhancing nodule senescence (Ashraf and Iram 2005), affecting leghemoglobin content and reducing nitrogenase activity (Figueiredo *et al*., 2008). Dry zone soils as in *Ampara* district experience a high amount of water loss due to evaporation in many months of the year resulting in a drought stress in the soil. Even though this drought conditions affect the growth and survival of many plants, *G. sepium* has the ability to withstand drought and has a wide distribution throughout the district. Therefore, the *Rhizobium* sp. isolated from *G. sepium* showed a considerable tolerance to drought stress induced under laboratory conditions (Figure. 03). A clear pattern of growth was not observed upon increasing drought stress. The average temperature of *Ampara* district is 30 °C where it rises up to 36 °C during long dry season which consists of 7 months of the year (*Ampara* district secretariat, 2014). Therefore, the Rhizobial strains showed a high tolerance to 35 °C in this study and some strains such as DV-a, DV-b, KR-c, KR-d, UH-b, UH-c, PK-c, PK-e, KM-a, KM-c and PD-b could survive even under 45 °C. All the 35 strains showed a lower growth at 25 °C, because they are adapted for the high temperatures encountered in their natural habitats (Figure 04).

When, the best tolerant strains were subjected to the combination of conditions, the natural conditions exist in the *Ampara* district and Dry zone was considered. The pH was selected as 7.5 because dry zone soils naturally do not exist at pH 10.0 and the temperature was selected as 37 °C because 45 °C is not usually observed in soil. These environmental conditions have interconnections to each other. Soil salinity get increased as drought conditions are increased (Mahajan and Tuteja, 2005). According to the research done by Surange *et al*., (1997), Rhizobial strains that have a tolerance to high pH also has the ability to tolerate high salinity and high temperatures. However, according to the results in Figure 05, PK-e and KM-a showed a poor growth while all the other strains grew well as expected.

The diversity determined using *ERIC* profiling showed a high polymorphism in 14 selected strains (Figure. 06). Four strains from *Karativu* belong to a single cluster indicating the close relationship between them. The results obtained for the tolerance of these strains for physiological conditions also confirm their relatedness. The strains PK-e and KM-a which showed a similar poor growth in response to combined physiological conditions, did not appear to be closely related. KM-a belongs to an individual lineage where it was only related to PK-c at 9 % similarity level. When considering the relationship between these strains at 69 % similarity level, these 14 strains can be considered as genetically diverse group as they fall into 10 different clusters (Figure 07). However, there was no clear evidence provided by the dendogram for the interconnection between the sampling site and the *Rhizobium* sp. diversity except for *Karativu*.

CONCLUSION

The 35 Rhizobial isolates tested in this study showed a considerable tolerance to wide ranges of pH, salinity, drought and temperature conditions because, these strains have adapted to the stress conditions experienced by the host plant in their natural habitats. Among these 35 isolates, 14 isolates were identified as best tolerant strains for many adverse conditions. Two strains among them were not tolerant to combined adverse conditions whereas all the other 12 strains could survive well. These selected strains were genetically diverse based on the dendogram prepared using *ERIC* fingerprinting. These selected strains can be used for cross inoculation studies with selected crop legumes growing in arid regions as well as in nitrogen-depleted soil.

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