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ISOLATION AND IDENTIFICATION OF RHIZOBIUM SPECIES FROM DIFFERENT LEGUME PLANT ROOTS

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ABSTRACT

Rhizobium is a soil habitat, gram negative bacterium which is associated symbiotically with the roots of leguminous plants. The main scope of this study was to isolate Rhizobium species from different leguminous plant root nodules and identify the characteristics of Rhizobium species by conducting biochemical tests. A total number of 22 leguminous plants of Fabaceae family were collected from Colombo (10 plants) and Anuradhapura (10 plants) districts of Sri Lanka. Rhizobium colonies were obtained by streaking samples of crushed root nodules of different plants on yeast mannitol agar medium. All samples which mean 100% of total sample size were found to be nitrogen fixing root nodulating bacteria with similar colony morphological characteristics as Rhizobium species. Microscopic examination revealed that 20 of the sample isolates which means 91% were rod shaped and gram negative in nature indicating the presence of nitrogen fixing Rhizobium species. The 20 sample isolates which revealed gram negative bacteria were further characterized by biochemical including tryptophan test, starch hydrolysis test, oxidase test, catalase test, voges Proskauer test which revealed 17 sample isolates (85%) positive for Rhizobium species. However, 3 samples (15%) needed further investigations in order to confirm the presence and characteristics of Rhizobium species. Antibiotic sensitivity pattern for all isolates against four different antibiotics was evaluated by disk diffusion method. It was observed that most of the Rhizobium isolates were susceptible to chloramphenicol and kanamycin and most were resistant to erythromycin and ampicillin at the amount of antibiotics under observation.

Keywords: Rhizobium, Fabaceae, Leguminous, Antibiotic sensitivity

INTRODUCTION

Interactions between plants and symbiotic soil microorganisms are major determinants of ecosystem productivity and diversity (Thrall et al., 2011). Nitrogen is one of the most essential nutrients required for both growth and development of plants. plants usually depend on combined or fixed forms of nitrogen such as ammonia and nitrate since it unavailable in its most prevalent form as atmospheric nitrogen (Al-Mujahidy et al., 2013). The nitrogen can be provided in the form of biological fixation or industrially produced chemical fertilizers (Patil et al., 2014). Biological nitrogen fixation (BNF) is a process by which atmospheric nitrogen (N2) is converted into ammonia (NH3) by free living soil microbes and by microbes forming symbiotic association with leguminous plants, with the benefit of allowing legumes to grow in nitrogen poor soil conditions. The Leguminosae, (Fabaceae, the legume family) possess a unique ability to establish symbiosis with nitrogen fixing bacteria known as Rhizobia. Rhizobia are a diverse group of alpha and beta proteobacteria, consisting

of more than 100 species under 13 genera currently that form nitrogen-fixing symbiosis with legumes (Andrews and Andrews, 2017; Diagne et al., 2017). However, most of the known legume symbionts belong to mainly six different genera including Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and Bradyrhizobium (Datta, Singh and Tabassum, 2015; Martinez- Romero, 2003; Willems, 2006). The first isolation of bacteria from root nodules of legumes were performed by Beijernick, a Dutch scientist in 1888 and named the bacteria as Bacillus radicicola which is now placed under the genus Rhizobium (Aneja, 2003).

The Rhizobium spp. is short rod shaped, aerobic, gram-negative bacteria that functions as the primary symbiotic fixer of nitrogen. Generally, its cell size is 0.5-0.9 microns in width and 1.2-3.0 microns in length. These bacteria are motile due to the presence of a single polar flagellum or two to six peritrichous flagella. All Rhizobium spp. is non-endospore forming and non-acid fast bacteria. These symbiotic bacteria are difficult to cultivate in ordinary culture media but effectively grows on yeast extract mannitol agar (YEMA) media (Menge, Lichstein and Angeles-Perez, 2014; Somasegaran and Hoben, 1994). The gram-negative cell wall consisting with lipopolysaccharides of the outer membrane of cell envelopes is important and benefits in the attachment of the Rhizobium bacteria to the infection site of the plant. Rhizobium spp. infect the roots of legume plants, leading to the development of small outgrowths known as nodules where the nitrogen fixation takes place (Al-Mujahidy et al., 2013). In root nodules the bacteria occur as endophytes that exhibit pleomorphic forms, termed “Bacteroides”, which fix or reduce gaseous atmospheric N2 into a combined form utilizable by the host plant (Kuykendall et al., 2015). The plant provides nutrients and energy for the activities of the bacterium while the bacterium’s enzyme complex provides a constant source of reduced nitrogen to the host plant (Shetta, Al-Shaharani and Abdel-Aal, 2011). This symbiotic relationship reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops and also enhance soil with nitrogen (Singh et al., 2011). Rhizobium spp. can also be classified as “fast growing and slow growing” bacteria according to the growth characteristics based on the growth rate and effect on the pH of yeast extract mannitol agar (YEMA) medium under standard laboratory conditions. Fast growing rhizobia have a net decrease in the pH of YEMA culture medium with a 2-4 hour mean generation time whereas slow growing bacteria have a 6 hour mean generation times and longer and do not decrease the pH of YEMA medium (Niste et al., 2015).

Rhizobia have been shown to improve legume yields in agriculture. Most of the research conducted on legume-rhizobium symbiosis has focused on legume crops and model legumes, such as bean, soybean, peanuts, butterfly pea, Alfalfa whereas few studies have examined other legumes (Huang et al., 2016). Several species of rhizobia are host-specific. Classification of rhizobia according to the host legumes they nodulate divides them into cross-inoculation groups. Different species of rhizobia have difference legume hosts and different rhizobia isolates in the same species have different BNF efficiencies (Jia et al., 2015). Several environmental conditions are limiting factors to the growth and development of nitrogen fixing plants. The process of nitrogen fixation is strongly related with the physiological state of the host legume plant. Therefore, environment factors such as soil pH, salinity, nutrient deficiency, mineral toxicity, insufficient or excessive soil moisture, temperature extremes, different plant diseases and inadequate photosynthesis by plants may affect the

BNF process and the development of rhizobium-legume symbiosis. The most problematic environments for rhizobia growth and development are marginal lands with low rainfall, acidic soils with low nutrient status, extremes of temperature and also soils with poor water-holding capacity do not allow the rhizobia to survive in adequate numbers in free living state (Deka and Azad, 2006; Kucuk, Kivanc and Kinaci 2006; Zahran, 1999).

Scientists have understood the potential benefits associated with the usage of different antibiotics to evaluate the susceptibility (sensitivity) or resistance level of Rhizobium species. Since the rhizosphere consist with large populations of different microbes, some microbes naturally produce antibiotics which are lethal to susceptible Rhizobium population. Therefore, evaluation of intrinsic resistance to antibiotics is a desirable trait for the Rhizobium species. Intrinsic resistance may increase the chances of growth, multiplication and persistence of Rhizobium population in soil. Great differences in degree of tolerance to different antibiotics among fast and slow growing Rhizobium species have been reported (Cole and Elkan, 1973; Naamala, Jaiswal and Dakora, 2016) Massive application of chemical fertilizers in conventional agriculture systems have been causing serious problems including ground, air and water pollution. Therefore, usage of pure Rhizobium isolates for biofilm fertilizer production is a beneficial process for the growth and development of legumes in agriculture systems.

MATERIALS AND METHODOLOGY

Sample collection

A total number of 22 leguminous plants of Fabaceae family were collected from different areas of Colombo (10 plants) and Anuradhapura (10 plants) districts of Sri Lanka.

Table 1. Sample collection details of 22 leguminous plants

Sample name	Scientific name	Sample code	Collected area
Beans	*Phaseolus vulgaris*	D01	Anuradhapura
Hummingbird	*Sesbania grandiflora*	D02	Colombo
Bush bean	*Phaseolus vulgaris*	D03	Colombo
Winged bean	*Psophocarpus tetragonolobus*	D04	Colombo
Soy bean	*Glycine max*	D05	Anuradhapura
Winged bean	*Psophocarpus tetragonolobus*	D06	Colombo
Cowpea	*Vigna unguiculata*	D07	Anuradhapura
Green gram	*Vigna radiate*	D08	Anuradhapura
Sunn hemp	*Crotalaria juncea*	D09	Anuradhapura
Sleeping grass	*Mimosa pudica*	D10	Colombo
Peanut	*Arachis hypogaea*	D11	Anuradhapura
Peanut	*Arachis hypogaea*	D12	Anuradhapura
Butterfly pea	*Clitoria ternatea*	D13	Colombo
Winged bean	*Psophocarpus tetragonolobus*	D14	Anuradhapura
Butterfly pea	*Clitoria ternatea,*	D15	Colombo

Long Beans	*Vigna unguiculata subsp. sesquipedalis*	D16	Colombo
Sleeping grass	*Mimosa pudica*	D17	Anuradhapura
Hummingbird	*Sesbania grandiflora*	D18	Colombo
Sleeping grass	*Mimosa pudica*	D19	Colombo
Soy bean	*Glycine max*	D20	Anuradhapura
Peanut	*Arachis hypogaea*	D21	Anuradhapura
Hummingbird	*Sesbania grandiflora*	D22	Colombo

Isolation of root nodulating bacteria

Initially, the roots of the samples were washed with running water to remove adjacent soil particles. Sample preparation and plate streaking were carried out under the laminar flow cabinet. The prepared YEAM plates were labelled properly with the sample code and date on the bottom of the lid, respectively each plate for each sample. Then the nodules of each samples were separated from the roots using a surgical blade and were transferred for immediate testing. First, the nodules were sterilized using 70% ethanol for few seconds followed by immersing the nodules in distilled water for 3-4 minutes. Sterile forceps were used to transfer the nodules. After the sterilizing process, the nodules were crushed using a glass rod to obtain a milky bacterial suspension. Then the inoculation loop was sterilised until it becomes red hot using the Bunsen burner and was left to cool. A loop full of the suspension was obtained from the sterile loop and was streaked on prepared YEMA media plates from each sample respectively using the four-streak quadrant method. The inoculation loop was sterilized after streaking each quadrant and before moving to the next sample. All streaked agar plates were parafilmed and incubated at 37 ˚C for 24 hours. After 24 hours of incubation, colony morphology (shape, size, colour, elevation, margin) of isolates were determined observing the colonies on YEMA plates.

Gram's Staining Procedure

A loop full of distilled water was positioned on the centre of a labelled slide. A colony from the sample was taken by the sterilized loop and positioned on the slide in a circular motion while spreading it about 1cm and a smear was obtained. The smear was air dried for few minutes and was heat fixed by passing the slide through the flame about two to three times, in order to adhere the smear to the glass slide properly. The smear was gently flooded by crystal violet drops and was left for 1 minute. The stain was washed off with distilled water and was air dried. Next gram's iodine was added to the smear and was left for 1 minute. It was washed off with distilled water and was air dried. Then few drops of gram's decolourizer were added to the smear and was left for 30 seconds. The solution was washed with distilled water and was air dried. Later, few drops of safranin was added to the smear and was left for 1 minute. It was rinsed with distilled water and the slide was permitted to air dry. Next the air dried, stained smear was observed under x40 and x100 (oil immersion) magnification of the light microscope.

Plate streaking on MacConkey agar media

The prepared MacConkey agar plates were labelled based on the sample number. The inoculation loop was sterilized by flaming until it became red hot using the Bunsen burner and was left to cool. Then a colony of each sample from the cultured YEMA agar plates were obtained and was

streaked on prepared MacConkey agar media plates accordingly on each sections of the plates in a pattern of 'zigzag' formation. After streaking, the plates were parafilmed and incubated at 37 ˚C for 24 hours. After incubation for 24 hours, colony characteristics were recorded on MacConkey agar plates.

Biochemical tests

Tryptophan test

The sterilized test tubes were labelled accordingly with sample codes and date. After autoclaving the broth media, 5ml of the broth was transferred into each test tube. Then the inoculation loop was sterilized and each test tube with 5ml of tryptophan broth was inoculated with the cultured bacterial samples which were obtained from the cultured YEMA media plates respectively after inoculation, the tryptophan inoculated broth tubes were incubated at 37 ˚C for 24 hours. After the incubation, 0.5ml of Kovac's indole reagent was added drop by drop gently to each broth cultured test tubes and results were observed within few seconds.

Starch hydrolysis test

The prepared starch agar plates were streaked at the centre of the agar media in a pattern of 'zigzag' formation using the cultured YEMA colonies of each sample. All streaked plates were parafilmed, inverted and incubated at 37 ˚C for 24 hours. After 24-hour incubation, the prepared cultured starch agar plates were transferred to the working area. Few drops of indole reagent were added to each cultured agar plate. Results were observed within 3-5 seconds.

Oxidase test

Wooden sticks for each sample were sterilized using 70% ethanol. A colony from each sample (cultured YEMA plates) was obtained by sterilized wooden sticks and was placed on the paper zone of the oxidase strips respectively. A development of deep purple-blue colour was observed in the paper zone of the strips within 5-10 seconds.

Catalase test

Cleansed glass slides were labelled accordingly with sample codes to be tested. A drop of hydrogen peroxide was added to the centre of each slide. Then the inoculation loop was sterilized to red hot and left to cool down. A colony from each sample culture YEMA plate was obtained and placed on the hydrogen peroxide drop on the respective slide. Results were observed within 3-5 seconds.

Voges Proskauer test

Sterilized test tubes were labelled with the sample code and date. After autoclaving, 3ml of Vogues Proskauer broth was added to each test tube. Then the inoculation loop was sterilized and each test tube was inoculated with the cultured bacterial samples which were obtained from the cultured YEMA media plates respectively. The inoculated broth tubes were incubated at 37 ˚C for 24 hours. After the 24-hour incubation of the cultured Vogues Proskauer broth tubes, was transferred to the working area. Then 600µl from the prepared 5%alpha naphthol was added to each of the cultured broth tubes followed by the addition of 200µl of the prepared 40% KOH using the 100-1000µl micro pipette and the solution was mixed well. Results were observed within 15-30 minutes after addition of reagents

Antibody sensitivity testing

The isolates were tested for antibiotic sensitivity by Kirby Bauer disc diffusion on prepared YEMA media plates. The four antibiotics Chloramphenicol (30µl), Kanamycin (30µl), Erythromycin (10µl) and Ampicillin (10µl) per disk were stored in the refrigerator at -2˚C.

First the 0.85% saline solution and 0.5 MacFarland standard was prepared. Afterwards, 5ml of the autoclaved saline solution was added to each labelled test tubes. Then a colony from the cultured YEMA sample plates were obtained and was inoculated in the 0.85% saline solution using a sterile inoculation loop. McFarland Turbidity Standard was compared for the accuracy to adjust bacterial suspensions by comparing the clarity of the lines on the Wickerham card. Afterwards, 200µl of inoculated saline solution of each sample was inoculated over the entire surface of prepared each YEMA media plate. Antibiotic disks were placed equidistantly at the centre of the 60 mm plates and were incubated at 37˚C for 24 hours. Data for antibiotic sensitivity was recorded by measuring the diameter of growth inhibition zone around the antibiotic disks after 24 hours of incubation. The data were subjected to one-way ANOVA using SPSS (2012) analysis program (version 21). The significant differences among mean diameters obtained for different antibiotics were analysed by conducting a post hoc turkey test. The statistical significance was set at the $P<0.05$ confidence level.

DATA ANALYSIS

Isolation of root nodulating bacteria and cultural characteristics

Bacterial colonies were observed after the sub culturing procedure in all of the 20 streaked plate samples after incubation. The observed colonies were characterized as per method described by Aneja, 2003 which includes colour, shape, margin, elevation, opacity and consistency. The observed white colonies were determined as nitrogen fixing bacterial colonies since YEMA media is used for the cultivation of symbiotic nitrogen fixing organisms such as Rhizobium species. Morphological characteristics of the colonies of all samples were identical to Rhizobium species presenting similar characteristics and were chosen for further characterization. The colonies were Circular in shape, entire margin, milky white, elevation with raised and convex, translucent opacity and with mucoid consistency.

Gram's staining results

The gram staining technique was considered as the initial identification test after the observation of bacterial cultures on YEAM media. The observation of stained bacteria under oil immersion microscopy (x100 magnification) differentiated the bacterial colonies into gram positive and gram-negative bacteria. The bacterial colonies of 20 samples on YEMA media were observed as gram negative rod-shaped bacteria (pink in colour) as shown in Figure 1 and two sample colonies (D04 and D14) revealed rod shape gram positive bacteria (purple in colour). Therefore, gram staining method of this research revealed 20 samples as the positive colonies for nitrogen fixing bacteria Rhizobium spp. with gram negative results and the two samples with gram positive results were not considered as Rhizobium spp. therefore were eliminated. All results of gram staining process are shown in table 2.

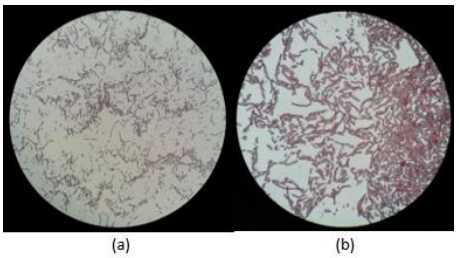

Figure 1. The observed Gram-negative bacteria of sample DO6. (a) x40 magnification, (b) x100 magnification

MacConkey agar media results

MacConkey agar media streaking process was considered to confirm the results of gram staining process for the 20 samples which revealed as gram negative bacteria on YEMA. Pink colour bacterial colonies were observed in all samples streaked on MacConkey agar media which were observed after incubation (Table 2). It confirmed the gram-negative result of the staining process of all sample isolates.

Biochemical Characteristics

Tryptophan test

The tryptophan test was performed to determine the ability of the bacteria to split amino acid tryptophan to form the compound indole. In this research study formation of indole was distinguish by the addition of Kovac's reagent which contains 4-dimethylaminobezaldehyde which reacts with indole to produce a red colour compound. According to the results of this study, a yellow/ colourless ring formation was observed in 18 samples indicating a negative result for the test which determined a positive result for Rhizobium spp. 2 samples were observed with red colour ring formations indicating a negative result for the identification of Rhizobium spp. The results of tryptophan test are shown in table 2 for all 20 gram negative samples.

Starch hydrolysis test

This test was performed to identify Rhizobium spp. by their ability to hydrolyse starch from the production of exoenzymes. All 20 samples subjected indicated a positive result for starch hydrolysis test with the presence of a clear halo formation (clear zone) around the bacterial colonies within 3-5 seconds of the addition of indole reagent.

Oxidase test

Oxidase test was performed to determine the presence of oxidase enzyme in bacterial isolates. All 20 samples conducted indicated a positive result by the observation of the development of deep purple-blue colour in the oxidase strip which was positive for identification of Rhizobium species.

Catalase test

All 20 samples conducted observed positive results for catalase test by the formation of bubbles within 3-5 seconds after sample addition to the respective hydrogen peroxide drop. If catalase is present, the H2O2 will be broken down into water and oxygen which result in the immediate formation of gas bubbles. Positive result confirmed that the isolated bacteria were aerobic which indicated a positive result for Rhizobium species.

Voges Proskauer test

The Voges Proskauer test resulted positive for 19 sample which represented by the development of a red colour on the surface of the medium after 15-30 minutes addition of the reagents. The positive result indicated the presence of diacetyl, the oxidation product of acetyl which is a characteristic of Rhizobium species. Only D20 isolate gave a negative result for the test.

Table 2. Comparison of YEMA, Gram's staining, MacConkey agar and biochemical tests results for the 20 samples

Sample code	YEMA results (presence of white colonies)	Gram staining	MacConkey agar results (presence of pink colonies)	Biochemical tests results				
				Tryptophan test	Starch hydrolysis	Oxidase test	Catalase test	VP test
D01	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D02	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D03	Positive	Gram negative	Positive	Positive	Positive	Positive	Positive	Positive
D05	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D06	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D07	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D08	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D09	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D10	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D11	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D12	Positive	Gram negative	Positive	Positive	Positive	Positive	Positive	Positive
D13	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D15	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D16	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D17	Positive	Gram negative	Positive	Positive	Positive	Positive	Positive	Positive
D18	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D19	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D20	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Negative
D21	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive
D22	Positive	Gram negative	Positive	Negative	Positive	Positive	Positive	Positive

All sample isolates except D04 and D14 were suspected as Rhizobium species after evaluating and identification through biochemical tests. Sample isolates from D12, D17 and D20 needed further test for the identification of Rhizobium species.

Antibiotic sensitivity test results

Antibiotic sensitivity pattern of all isolates against four different antibiotics, Chloramphenicol, Kanamycin, Erythromycin and Ampicillin was evaluated by disk diffusion method. All sample suspensions were compared with MacFarland standard turbidity test accordingly.

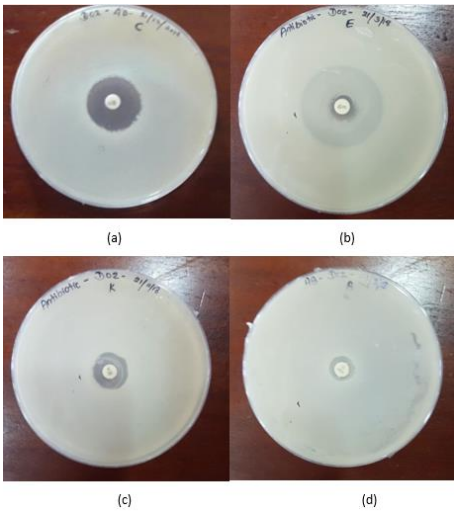

Figure 2. Inhibition zones of Rhizobium species of sample D02. (a) Chloramphenicol, (b) Erythromycin, (c) Kanamycin, (d) Ampicillin

Table 3. Results of antibiotic sensitivity testing zone of inhibition diameters (mm)

Sample code	Chloramphenicol (mm)	Kanamycin (mm)	Erythromycin (mm)	Ampicillin (mm)
D01	15	13	-	10
D02	23	13	07	-
D03	14	11	-	-
D05	11	-	15	-
D06	10	13	-	-
D07	10	11	07	-
D08	10	09	-	09
D09	10	11	09	12
D10	12	13	09	15
D11	12	13	07	07
D12	-	-	-	-
D13	07	16	19	-
D15	17	12	08	08
D16	08	-	-	15
D17	12	13	08	-
D18	10	10	-	-
D19	09	13	09	13
D20	16	13	-	-
D21	11	-	-	-
D22	-	-	-	-

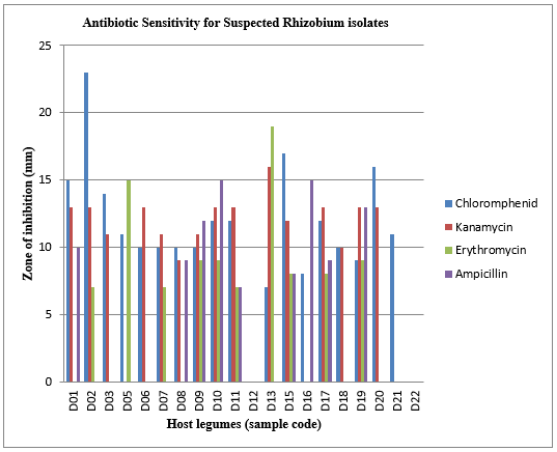

Figure 3. Effect of antibiotics on the growth of different suspected Rhizobium spp

Statistical analysis of antibiotic sensitivity test

Table 4. The statistical data for antibiotic sensitivity test

Descriptives

Zone of Inhibition

	N	Mean	Std. Deviation	Std. Error	Maximum
Chloramphenicol	20	10.8500	5.17357	1.15684	23.00
Kanamycin	20	9.2000	5.63448	1.25991	16.00
Erythromycin	20	4.9000	5.72988	1.28124	19.00
Ampicillin	20	4.4500	5.89804	1.31884	15.00
Total	80	7.3500	6.16051	.68877	23.00

ANOVA

Zone of Inhibition

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	601.700	3	200.567	6.361	.001
Within Groups	2396.500	76	31.533		
Total	2998.200	79			

Multiple Comparisons

Dependent Variable: Zone of Inhibition

Tukey HSD

(I) Antibiotic	(J) Antibiotic	Mean Difference (I-J)	Std. Error	Sig.
Chloramphenicol	Kanamycin	1.65000	1.77575	.789
	Erythromycin	5.95000*	1.77575	.007
	Ampicillin	6.40000*	1.77575	.003
Kanamycin	Chloramphenicol	-1.65000	1.77575	.789
	Erythromycin	4.30000	1.77575	.082
	Ampicillin	4.75000*	1.77575	.044
Erythromycin	Chloramphenicol	-5.95000*	1.77575	.007
	Kanamycin	-4.30000	1.77575	.082
	Ampicillin	.45000	1.77575	.994
Ampicillin	Chloramphenicol	-6.40000*	1.77575	.003
	Kanamycin	-4.75000*	1.77575	.044
	Erythromycin	-.45000	1.77575	.994

The mean difference is significant at the 0.05 level.

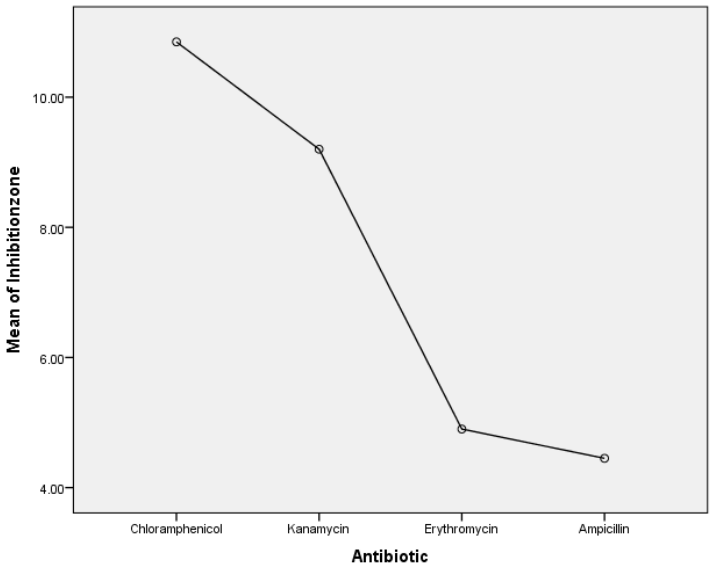

Figure 4. Mean plot for the mean diameter zone of inhibition against antibiotics

DISCUSSION

Isolation and identification of Nitrogen fixing Rhizobium species was the main concern of this study. Accordingly, strains of root nodulating Rhizobium bacteria were isolated from the root nodules of legume plants including beans, soybean, green gram, winged bean, cowpea, butterfly pea, peanut, hummingbird, long beans and sunn hemp growing in different areas of Colombo and Anuradhapura, Sri Lanka as presented in table 1. During this study primarily with YEMA media total number of 22 leguminous plant root nodule samples were examined where all samples which means 100% of total sample size were found to be nitrogen fixing root nodulating bacteria with similar colony morphological characteristics as Rhizobium species. Then Microscopic examination revealed that 20 of the sample isolates which means 91% were rod shaped and gram negative in nature indicating the presence of nitrogen fixing Rhizobium species (Anand and Dogra, 1991; Keyser, 1982; Singh, Kaur and Singh, 2008). Isolates of two samples (DO4 and D14) were observed as gram positive bacteria and therefore was ruled out and further biochemical tests were not carried out for Rhizobium identification in these respective samples. The 20 sample isolates which revealed a gram-negative result were further characterized by biochemical tests including tryptophan test, starch hydrolysis test, oxidase test, catalase test, voges proskauer test which revealed 17 sample isolates (85%) positive for Rhizobium species. However, 3 samples which means 15% of the 20 subjected samples for biochemical tests needed further investigations in order to confirm the presence and characteristics of Rhizobium species.

The legume plants were uprooted carefully during sample collection with root nodules 1-2 days prior to the sample preparation day and was stored in da refrigerator until the sterilization of nodules took place. Pinkish nodules were selected and were sterilized properly to remove adhesive solid particles and surface bound microorganisms. Pinkish nodules were selected since it indicates that an active nitrogen fixation had been established between the nodule bacteria and the legume plant (Pervin et al., 2017).

In this study, YEMA was used as the growth medium since this medium contains a source of nitrogen (in yeast extract) and carbohydrate (mannitol) it supports the growth of Rhizobium, thereby it serves as a selective media for root nodulating Rhizobium species which has also reported in early studies (Castro et al., 2003; Handley et al., 1998; Kucuk, Kivanc and Kinaci, 2006). Therefore, growth of bacteria on YEMA medium indicated their ability to fix atmospheric nitrogen to the plants. Colony morphological characterization was done to identify Rhizobium genera. This was detected due to the white colonies with mucilaginous, small (usually 2-4mm in diameter), circular, glistening convex, milky translucent colony morphological presentation in YEMA, which was as similar characteristics as Rhizobium species reported in Vincent, 1970 and Holt et al., 1994. The gummy appearance is because of extracellular polysaccharide production (Malisorn and Prasarn, 2014). This corelates with the Bergey's manual description of genus Rhizobium as forming "copious extracellular polysaccharide slime" (Kuykendall et al., 2015). It was observed that there was no variation between the morphological characteristics among the isolates. Therefore, all isolates were chosen for further characterization. It was also found that isolates were able to grow before 24-hour incubation. Therefore, most of the representative isolates were suggested to be fast growers.

Gram staining procedure was performed to ensure purity and eliminate any samples with gram positive bacteria. Gram staining technique revealed gram negative results indicating the positive results for rod shaped Rhizobium bacteria since the cell wall structure of Rhizobium is generally similar to that of the Gram-negative bacteria. The peptidoglycan of gram-negative bacteria consists of glutamic acid, alanine, diaminopimelic acid and amino sugars. Rhizobia also comprises an unusually complex composition of membrane phospholipids, among them phosphatidylcholine, and under conditions of phosphorus limitation, phospholipids can be replaced by membrane lipids that do not contain phosphorus (Lopez-Lara, Sohlenkamp and Geiger 2003). The two samples which revealed rod shaped gram-positive bacteria were confirmed as non-Rhizobium strains and were not subjected for further biochemical testing for the identification of Rhizobium species. MacConkey agar media was used as a selective media to confirm the gram-negative results obtained on YEMA in order to proceed with biochemical testing.

Tryptophan test was performed to determine the ability of Rhizobium bacteria to convert tryptophan to indole. 18 sample isolates were negative for the indole test indicating presence of Rhizobium according to previous studies Deka and Azad, 2006; Lalitha and Immanuel, 2013. However, two sample isolates of D03 and D17 interpreted positive for the indole test but these samples were not ruled out because rest of the biochemical tests were positive for Rhizobium in these respective samples, therefore further investigations should be carried out for the confirmation. All isolates obtained positive results from the starch hydrolysis assay. Iodine was used to determine capability of Rhizobium to use starch as a carbon source. On subjecting inoculated plates to iodine test, clear zones around the colonies were seen and the colonies turned yellow with a halo appearance, whereas blue colour appeared on no growth areas. This indicated that the isolates have the potential to hydrolyse starch present occurring in the medium. A study by De Oliveira et al., 2007 also observed that the Rhizobium strains utilize the starch obtained from the different sources supporting the present study. Kovac's reagent (1% N, N, N.N-tetramethyle-pphenylene diamine) saturated available oxidase strips were used in this study for the oxidase test to determine the presence of oxidase enzyme in Rhizobium isolates. Catalase test was performed to determine the presence of catalase enzyme in Rhizobium isolates. Both catalase and oxidase tests results were positive for all the 20 sample isolates which showed close confirmatory with findings of Datta, Singh and Tabassum, 2015; Naz, Bano and Hassan, 2009; Patil et al., 2014)

Also, 19 sample isolates out of the 20 samples were positive for the Voges-Proskauer (VP) Test indicating the presence of Rhizobium species. The positive result was due to the ability of Rhizobium to metabolize pyruvic acid to form acetyl-methyl carbinol (acetoin) neutral reacting end product which id converted into diacetyl in the presence of atmospheric oxygen and 40% potassium hydroxide. Diacetyl, under the catalytic action of alpha-naphthol and creatine, is converted into a red complex indicating a positive result. Positive VP test results for Rhizobium identification was supported by (Gopalakrishnan et al.,2015; Koneman et al., 2017). However, sample D20 of soybean legume plant isolates gave a negative result for the VP test which corelates with the findings of Patil et al., 2014 and Shahzad et al., 2012. Therefore, further tests were required for the confirmation of the respective Rhizobium strain. During the test alpha-naphthol was added prior to the addition of KOH to

make the test more sensitive and test results were not recorded beyond 30 minutes in order to eliminate false-positive VP results.

The physiological characterisation of the isolates under various biochemical tests (Table 2) categorically confirmed the purity of all the 20 sample isolates and 17 isolates indicated that they were closely related to Rhizobium species. Confirmation of purity of the Rhizobium isolates with the above tests was also made by Lalitha and Immanue, 2013; Gaur and Sen, 1981 which were helpful in presumptive identification of the Rhizobium genus of the isolates. Phenotypic characteristics of the 20 sample isolates were determined by antibiotic resistance pattern. McFarland standards were used as a reference to compare and adjust the turbidity of bacterial suspensions for standardize antimicrobial testing (Zapata and Ramirez-Arcos, 2015). The test tubes were closed tightly since the MacFarland standards are sensitive to air and light in order to avoid false positive results in comparison of the turbidity. All isolates were evaluated for their responses as resistant or sensitive against 4 different antibiotic disks including Chloramphenicol (30µl), Kanamycin (30µl), Erythromycin (10µl) and Ampicillin (10µl) per disk. Inhibition zone diameters indicated the sensitivity of the antibiotic against the respective Rhizobium species (Table 3). According to results, 18 isolates were sensitive to Chloramphenicol and the highest sensitivity with a 23mm inhibition zone (Figure 3) was observed against sample D02 isolate. Only two isolates (D12 and D22) were resistant towards Chloramphenicol. The highest sensitivity to Kanamycin (16mm) antibiotic was reported against the D13 isolate whereas sample isolates of D05, D12, D16, D21 and D22 were resistant against Kanamycin (Figure 3). Erythromycin was highly sensitive towards D13 (19mm) isolates and was resistant towards 10 sample isolates with no inhibition zone formation (Table 3). Ampicillin could not inhibit the growth of Rhizobium in most of the samples indicating resistance but D10 sample isolate was sensitive towards Ampicillin with a 15mm zone of inhibition (Figure 2). There are 3 known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge and amount of the antibiotic (Hungaria et al., 2000) and the Rhizobium that showed a high level of resistance did not take up the antibiotics. However detailed study is needed to evaluate this fact.

The statistical analysis was conducted by a one-way between subjects ANOVA to compare the effect of the four antibiotics on the growth of suspected Rhizobium isolates of different legume host samples (Zone of inhibition diameter). Table 4 (a) indicated that the mean diameter zone of inhibitions against chloramphenicol ($M = 10.85$, $SD = 5.17$) significantly differed among kanamycin ($M = 9.20$, $SD = 5.63$), erythromycin ($M = 4.90$, $SD = 5.73$) and ampicillin ($M = 4.45$, $SD = 5.90$). However, erythromycin did not significantly differ from ampicillin. Findings from the ANOVA Table (b) proved that there was a statistically significant difference at the $p<0.05$ level in the diameters of inhibitions zones for the four antibiotics [$F(3, 76) = 6.361$, $p = 0.001$] since p value obtained was less than 0.05 significance level as shown in Table 4 (b). Therefore, to evaluate the most effective antibiotic on the sensitivity (zone of inhibition) a multiple comparison post hoc Turkey HSD test was conducted to compare the mean diameters of the antibiotics. Table 4 (c) indicates that there is a statistically significant difference between chloramphenicol and erythromycin sensitivity, since its $p = 0.007$ which is less than 0.005 significance level. Also, the highest statistically

significant difference among the groups were found between chloramphenicol and ampicillin zone of inhibitions since its p = 0.003 which is less than 0.005 significance level. However, there were no statistically significant difference between Chloramphenicol and kanamycin sensitivity (p = 0.789) also between Kanamycin and erythromycin sensitivity (p = 0.082) and between erythromycin and ampicillin sensitivity (p = 0.994) since these p values were greater than 0.05 significance level. It was observed that there was a statistically significance difference between kanamycin and ampicillin sensitivity since its p = 0.044 which is less than 0.05 significance level.

Moreover, it was observed as figure 4 explains that most of the Rhizobium isolates were susceptible to chloramphenicol and kanamycin and most were resistant to erythromycin and ampicillin at the amount of antibiotics under observation. These results were also supported by the findings in Datta, Singh and Tabassum, 2015 and Singh, Kaur and Singh, 2008. Different antibiotics have target sites and function in legume symbionts. Susceptibility explains since chloramphenicol targets the 23S rRNA (peptidyl transferase) which prevent protein chain elongation whereas kanamycin targets 16S rDNA which misreads and inhibits the translocation of the tRNA – mRNA complex and prevent protein synthesis of Rhizobium bacteria. Ampicillin function includes the irreversible inhibition of enzyme trans-peptidase which id needed by Rhizobium for cell was synthesis and erythromycin targets 23S rDNA which leads to the inhibition of protein synthesis of Rhizobium bacteria which further explains the above findings regarding antibiotic sensitivity. (Naamala, Jaiswal and Dakora, 2016; Salian et al., 2012). Sample isolate of D12 and D22 were resistant towards all four antibiotics. Intrinsic antibiotic resistance may result from genes including ampC b-lactamase found in gram-negative bacteria which naturally occur in the Rhizobia bacterium's chromosome of these isolates. A study by Cole and Elkan, 1973 reported that R. japonicum now known as Bradyrhizobium japonicum carries extra chromosomal antibiotic resistance genes. Antibiotic resistant Rhizobium increases Rhizobium survivability in the soil to occupy high number of nodules in legumes. So that, these antibiotics resistant Rhizobium species can survive antibiotic stressed conditions and help to increase soil productivity (Nahar, Begum and Akhter, 2017).

Finally evaluating all the results, positive Rhizobium isolates were identified and according to literature it can be suspected that few sample isolates D01, D05, D08, D11, D12 and D21 are related with host specific Rhizobium species. D01 sample isolate from Phaseolus vulgaris (Bean) can be suspected with R. leguminosarum phaseoli host specific strain of Rhizobium species (Kucuk, Kivanc and Kinaci, 2006) and D08 sample isolate from Vigna radiata (Green gram) can be suspected with Rhizobium phaseoli host specific Rhizobium strain (Datta, Singh and Tabassum, 2015). D05 sample isolate from Glycine max (Soybean) can be suspected with Bradyrhizobium japonicum host specific strain of Rhizobium species (Delamuta et al., 2013; Javaid and Mahmood, 2010) depending on the positive test results. Also, D11, D12 and D21 sample isolates from Arachis hypogaea (Peanut) can be suspected with Bradyrhizobium arachidis host specific strain of Rhizobium species (Wang et al., 2013) However, further tests are needed for the identification and confirmation of specific Rhizobium strain of all suspected Rhizobium isolates.

CONCLUSION

According to the present study, the presence of nitrogen fixing bacteria were cultivated in YEMA media and determined by Gram's staining technique, MacConkey agar streaking followed by the biochemical tests to isolate and identify Rhizobium species from different 22 leguminous plant root nodules. 17 sample isolates were positive and identified as Rhizobium species. The study also facilitated to observe and identify the antibiotic sensitivity pattern of all isolates of Rhizobium species against four different antibiotics by disk diffusion method. However, further tests such as Glucose peptone agar (GPA) and lactose assay, Salt, temperature and pH tolerance assay, urea hydrolysis, citrate utilization are needed for the identification and confirmation of specific Rhizobium strain of all suspected Rhizobium isolates. Also molecular methods including polymerase chain reaction (PCR) and 16S rRNA sequencing are more reliable and specific for identification of Rhizobium isolates. Once the pure cultures of Rhizobium strains are confirmed and established with further tests preparation of biofertilizer can be conducted as Rhizobium biofertilizers are recommended for the growth and development of grain legumes to improve the productivity and to augment soil nitrogen status.

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REFERENCES

Al-Mujahidy, S.M.J., Hassan, M.M., Rahman, M.M. and Mamun-or-Rashid, A. (2013) Study on Measurement and Statistical Analysis of Adherent Soil Chemical Compositions of Leguminous Plants and Their Impact on Nitrogen Fixation. International Journal of Biosciences, 3(6), pp.112-119.

Anand, R.C. and Dogra, R.C. (1991). Physiological and biochemical characteristics of fast and slow growing Rhizobium sp., from pigeon pea (Cajanus cajan). Journal of Applied Bacteriology, 70(1), pp.197-202.

Andrews, M. and Andrews, M. (2017). Specificity in Legume-Rhizobia Symbioses. International Journal of Molecular Sciences, 18(4), pp.705-744.

Aneja, K. (2003). Experiments in microbiology plant pathology and biotechnology. 3rd ed. New Delhi: New age International (p) Ltd. Publishers, pp.178-185.

Castro, I. V., Ferreira, E. M. and McGrath, S. P. (2003). Survival and plasmid stability of rhizobia introduced into a contaminated soil. Soil Biology and Biochemistry, 35(2), pp. 49–54.

Cole, M. and Elkan, G. (1973). Transmissible Resistance to Penicillin G, Neomycin, and Chloramphenicol in Rhizobium japonicum. Antimicrobial Agents and Chemotherapy, 4(3), pp.248-253.

Datta, A., Singh, R. and Tabassum, S. (2015). Isolation, Characterization and Growth of Rhizobium Strains under Optimum Conditions for Effective Biofertilizer Production. International Journal of Pharmaceutical Sciences Review and Research, 32(1), pp.199-2018.

Deka, A. and Azad, P. (2006). Isolation of Rhizobium strains: Cultural and Biochemical characteristics. Legume Research, 29(3), pp.209-212.

Delamuta, J., Ribeiro, R., Ormeno-Orrillo, E., Melo, I., Martinez-Romero, E. and Hungria, M. (2013). Polyphasic

evidence supporting the reclassification of Bradyrhizobium japonicum group strains as Bradyrhizobium diazoefficiens sp. International Journal of Systemic and Evolutionary Microbiology, 63(9), pp.3342-3351.

Deora, G. and Singhal, K. (2010). Isolation, biochemical characterization and preparation of biofertilizers using Rhizobium strains for commercial use. Bioscience Biotechnology Research Communications, 3(2), pp.132-136.

Diagne, N., Swanson, E., Pesce, C., Fall, F., Diouf, F., Bakhoum, N., Fall, D., Ndigue Faye, M., Oshone, R., Simpson, S., Morris, K., Thomas, W., Moulin, L., Diouf, D. and Tisa, L. (2017). Permanent Draft Genome Sequence of Rhizobium sp. Strain LCM 4573, a Salt-Tolerant, Nitrogen-Fixing Bacterium Isolated from Senegalese Soils. Genome Announcements, 5(18), pp.285- 304.

Gaur, Y. and Sen, A. (1981). Cultural and Biochemical Characteristics of Root Nodule Bacteria of Chickpea (Cicer arietinum L.). Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Zweite Naturwissenschaftliche Abteilung: Mikrobiologie der Landwirtschaft, der Technologie und des Umweltschutzes, 136(4), pp.307-316.

Gopalakrishnan, S., Sathya, A., Vijayabharathi, R., Varshney, R., Gowda, C. and Krishnamurthy, L. (2015). Plant growth promoting rhizobia: challenges and opportunities. 3 Biotech, 5(4), pp.355-377.

Handley B A, Hedges A J and Beringer J E 1998 Importance of host plants for detecting the population diversity of Rhizobium leguminosarum biovar viciae in soil. Soil Biology and Biochemistry 30, pp. 241-249

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T and Williams, S.T. (1994). In Bergey´s Manual of Determinative Bacteriology. Williams and Wilkins Press, Baltimore. USA.

Huang, C., Liu, C., Chen, S. and Kao, W. (2016). Phylogenetic Identification, Phenotypic Variations, and Symbiotic Characteristics of the Peculiar Rhizobium, Strain CzR2, Isolated from Crotalaria zanzibarica in Taiwan. Microbes and Environments, 31(4), pp.410-417.

Hungria, M., Andrade, D., Chueire, L., Probanza, A., Guttierrez-Mañero, F. and Megías, M. (2000). Isolation and characterization of new efficient and competitive bean (Phaseolus vulgaris L.) rhizobia from Brazil. Soil Biology and Biochemistry, 32(11-12), pp.1515-1528.

Javaid, A. and Mahmood, N. (2010). Growth nodulation and yield response of Soybean to biofertilizers and organic manures. Pakistan Journal of Botany, 42(2), pp.863-871.

Jia, R., Zhang, R., Wei, Q., Chen, W., Cho, I., Chen, W. and Li, Q. (2015). Identification and Classification of Rhizobia by Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry. Journal of Proteomics & Bioinformatics, 08(06), pp.98-107.

Keyser, H.H., Bohlool, B.B. and Weber, D.F. (1982). Fast-Growing Rhizobia Isolated from Root Nodules of Soybean. Science, 215(4540), pp.1631-1632.

Koneman, E.W., Procop, G.W., Hall, D.G., Janda., W.M. (2017). 7th ed. Color Atlas and Textbook of Diagnostic Microbiology, J.B. Lippincott Company, Philadelphia, PA.

Kucuk, C., Kivanc, M. and Kinaci, E. (2006). Characterization of Rhizobium Sp. Isolated from Bean. Turkish Journal of Biology, 30, pp.127-132.

Kuykendall, L., Young, J., Martínez-Romero, E., Kerr, A. and Sawada, H. (2015). Rhizobium. Bergey's Manual of Systematics of Archaea and Bacteria, pp.1-36.

Lalitha, S. and Immanuel, S. (2013). Biochemical characterization of Rhizobium and its impact on black gram and green gram plants. 2013(9), pp.1-6.

Lopez-Lara, I., Sohlenkamp, C. and Geiger, O. (2003). Membrane Lipids in Plant-Associated Bacteria: Their Biosyntheses and Possible Functions. Molecular Plant-Microbe Interactions, 16(7), pp.567-579.

Malisorn, K. and Prasarn, C. (2014). Isolation and characterization of Rhizobium spp. from root of legume plants species. Khon Kaen Agriculture Journal, 4(2014), pp.157-160.

Martínez- Romero, E. (2003). Diversity of Rhizobium-Phaseolus vulgaris symbiosis: overview and perspectives. Plant Soil. 252, pp.11-23.

Menge, D., Lichstein, J. and Ángeles-Pérez, G. (2014). Nitrogen fixation strategies can explain the latitudinal shift in nitrogen-fixing tree abundance. Ecology, 95(8), pp.2236-2245.

Naamala, J., Jaiswal, S. and Dakora, F. (2016). Antibiotics Resistance in Rhizobium: Type, Process, Mechanism and Benefit for Agriculture. Current Microbiology, 72(6), pp.804-816.

Nahar, N., Band, A. and Akhter, H. (2017). Isolation, identification and molecular characterization of Rhizobium species from Sesbania bispinosa cultivated in Bangladesh. African Journal of Agricultural Research, 12(22), pp.1874-1880.

Naz, I., Bano, A. and Hassan, T. U. (2009). Morphological, biochemical and molecular characterization of Rhizobia from Halophytes of Khewea salt range attock. Pakistan Journal of Botany, 41(6), pp.3159-3168.

Niste, M., Vidican, R., Puia, C., Rotar, I. and Pop, R. (2015). Isolation and Biochemical Characterization of Rhizobium Leguminosarum bv. Trifolii and Sinorhizobium Meliloti using API 20 NE and API 20 E. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Agriculture, 72(1), pp.173-178.

Oliveira, A., Oliveira, L., Andrade, J. and Chagas Júnior, A. (2007). Rhizobia amylase production using various starchy substances as carbon substrates. Brazilian Journal of Microbiology, 38(2), pp.208-216.

Patil, S., Patil, D., Patil, M., Gaikwad, P., Patil, P. and Bhamburdekar, S. (2014). Isolation, characterization and salt tolerance activity of Rhizobium sp. from root nodules of some legumes. International Journal of Current Microbiology and Applied Sciences, 3(5), pp.1005-1008.

Pervin, S., Jannat, B., Sanjee, S. and Farzana, T. (2017). Characterization of Rhizobia from Root Nodule and Rhizosphere of Lablab purpureus and Vigna sinensis in Bangladesh. Turkish Journal of Agriculture - Food Science and Technology, 5(1), pp.14-19.

Salian, S., Matt, T., Akbergenov, R., Harish, S., Meyer, M., Duscha, S., Shcherbakov, D., Bernet, B., Vasella, A., Westhof, E. and Böttger, E. (2012). Structure-Activity Relationships among the Kanamycin Aminoglycosides: Role of Ring I Hydroxyl and Amino Groups. Antimicrobial Agents and Chemotherapy, 56(12), pp.6104-6108.

Shahzad, F., Shafee, M., Abbas, F., Babar, S., Tariq, M. and Ahmad, Z. (2012). Isolation and Biochemical characterization of Rhizobium meliloti from root nodules of Alfalfa (Medico sativa). The Journal of Animal & Plant Sciences, 22(2), pp.522-524.

Shetta, N., Al-Shaharani, T. and Abdel-Aal, M. (2011). Identification and Characterization of Rhizobium Associated with Woody Legume Trees Grown under Saudi Arabia Condition. American-Eurasian Journal of Agricultural & Environmental Sciences ..., 10(3), pp.410-418.

Singh, A., Bhatt, R., Pant, S., Bedi, M. and Naglot, s. (2011). Characterization of Rhizobium isolated from root nodules of Trifolium alexandrinum. Journal of Agricultural Technology, 7(6), pp.1705-1723.

Singh, B., Kaur, R. and Singh, K. (2008). Characterization of Rhizobium strain isolated from the roots of

Trigonella foenumgraecum (fenugreek). The African Journal of Biotechnology, 7(20), pp.3672-3676.

Somasegaran, P. and Hoben, H. (1994). Handbook for rhizobia. 1st ed. London: Springer.

Thrall, P., Laine, A., Broadhurst, L., Bagnall, D. and Brockwell, J. (2011). Symbiotic Effectiveness of Rhizobial Mutualists Varies in Interactions with Native Australian Legume Genera. PLoS ONE, 6(8), pp.1-11.

Tyagi, A., Kumar, V., shottam, P. and Tomar, A. (2017). Isolation, Identification, Biochemical and Antibiotic Sensitivity Characterization of Rhizobium Strains from Vigna mungo (L) Hepper, Cicer arietinum L and Vigna radiata (L) R Wilczek in Muzaffarnagar, Uttar Pradesh, India. International Journal of Current Microbiology and Applied Sciences, 6(12), pp.2024-2035.

Vincent, J. (1970). A manual for the practical study of the root-nodule bacteria. London: International Biological Programme.

Wang, R., Chang, Y., Zheng, W., Zhang, D., Zhang, X., Sui, X., Wang, E., Hu, J., Zhang, L. and Chen, W. (2013). Bradyrhizobium arachidis sp. nov., isolated from effective nodules of Arachis hypogaea grown in China. Systematic and Applied Microbiology, 36(2), pp.101-105.

Willems, A. (2006). The taxonomy of rhizobia: an overview. Plant and Soil, 287(1), pp.3-14.

Zahran, H. (1999). Rhizobium-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in an Arid Climate. Journal of Microbiology and Molecular Biological Reviews, 63(4), pp.968-989.

Zapata, A. and Ramirez-Arcos, S. (2015). A Comparative Study of McFarland Turbidity Standards and the Densimat Photometer to Determine Bacterial Cell Density. Current Microbiology, 70(6), pp.907-909.