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Identification and Characterization of *Rhizobium* Associated with Woody Legume Trees Grown under Saudi Arabia Condition

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Abstract: Six woody legume tree species, *Acacia ehrenbergiana* (Hayne.), *Acacia nilotica* (Willd.), *Acacia saligna* (Labill.), *Acacia tortilis* (Forssk.), *Acacia tortilis* var. *reddiana* (Savi.) and *Leucaena leucocephala* (Lam.) were performed the growth characteristics and to identify the resistance of native isolated Rhizobium strains from root zone surrounding trees to environmental stresses- antibiotic, high temperature, salinity and acidity. Physiological properties of all isolated strains were fast growing and had the same colony morphology and produced high, slimy/mucous transparent to creamy colored colonies on YEMA plates after 3 days of incubation at 28°C. In addition to the strains failed to absorb Congo Red stain in the medium except for those isolated from *L. leucocephala* (LLR). Acid production was observed among the isolates after 72 hours. The generation span or longevity for YEMB cultures at 28°C ranged between 2.07 and 3.85 hours. The strains showed ability to the resistance of antibiotics, temperature, salinity and pH fluctuation. Based on the utilization of carbon and nitrogen as sole source of carbon and nitrogen, the results showed that those strains probably belonging to one of two groups, *Rhizobium* or *Sinorhizobium*. The isolates from present study may be useful to increase the symbiotic nitrogen fixation in legume trees. Therefore the study provides basis for further research on the phylogeny of Rhizobial strains nodulating the legume trees, as well as their use as inoculants to improve growth and nitrogen fixation in arid lands of the central region of Saudi Arabia.

Key words: Woody legume trees · *Rhizobium* characteristics · Identification · Environmental stress · KSA

INTRODUCTION

Nutrient enrichment of soils by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries. Scientific demonstration of this symbiosis was started in 19th century and it established the facts that bacteria present in nodules on legume roots are responsible for fixing atmospheric nitrogen [1]. *Rhizobium* spp. are well known group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops [2]. Legumes are regarded as the third largest family of angiosperm plants, including 17 000–19 000 species distributed world-wide with nearly 3000 species identified as potential N_2 fixers [3]. Nowadays, introduced woody species from their tropical origin to arid and semi arid temperate zones are major sources of N in semi-arid, arid and desert ecosystems: sequential cropping systems, agroforestry and silvopastoral systems (pastoralism), providing timber, fuel, pulp, fodder and even human food. Various published studied have been discussing the establishment and functioning of NFTs symbiosis [4]. The classical phenotypic characterization of *Rhizobia* has often been the first method employed when classifying unknown strains of *Rhizobia* [1, 5]. Though this method provides valuable information, it is laborious and time consuming. The Biology system, which is based on metabolic fingerprinting by determination of carbon source utilization, offered a less tedious alternative for large scale screening of isolates. It has been used to characterize and classify bacteria and bacterial communities in a wide range of environments, including marine, agricultural and nonagricultural soils and sludge [6, 7] The biology method has, for example,

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been reported to be the best among five typing ones for differentiating fluorescent *pseudomonas* [8]. It has also been applied to characterize members of Rhizobiaceae [4]. Many papers have been written pertaining *Rhizobia* taxonomy and phenotypic characteristics [9]; evolution [10] and the effects of environmental factors such as salinity [11] and acidity [12]. Previous work of Frioni [13, 14] showed the nodulation and nitrogen fixing capability of rhizobial isolates from of 17 species of legume trees in Uruguay. Milnitsky *et al*. [15] characterized some of these isolates according to their physiological properties, protein and plasmid profiles. Native herbaceous and legume trees from Saudi Arabia were studied in order to employ them in agronomic and silvopastoral practices. The characterization of indigenous populations of *Rhizobia* for their resistance to adverse or harsh environmental factors will be important before their selection for nursery inoculation. In temperate regions, like Saudi Arabia, there are scarce studies in this subject. Considering the above mention works, the present study was carried out to investigate the growth characteristics and resistance to environmental stresses-antibiotic, high temperature, salinity and acidity of *Rhizobium* isolates from some woody legume trees grown in Riyadh region.

MATERIALS AND METHODS

Site and Woody Legume Trees Used in the Study: The present study was conducted during 2010 at Experimental Station of Collage of Food and Agriculture Sciences, King Saud University, included six woody legume trees viz., *Acacia ehrenbergiana* (Hayne.), *Acacia nilotica* (Willd.), *Acacia saligna* (Labill.), *Acacia tortilis* (Forssk.), *Acacia tortilis* var. *reddiana* (Savi.) and *Leucaena leucocephala* (Lam.) grown Dirab Valley South of Riyadh. Dirab valley (N. 24° 24' 33", E. 46° 39' 40") is a part of the Riyadh region, with many ecotypes of N_2-fixing trees, which could be used for the improved production of several agrosystems. The average temperature is 37°C in summer and about 5°C in winter with 50mm rainfall. Physical and chemical characteristics of experimental soil sites are given in Table 1.

Rhizobium Strains: Bacteria were isolated from the root zones of the six wood legume trees and listed using an abbreviation of the host tree followed by the name of *Rhizobium* strains i.e. *AER, ANR, ASR, ATR, ARR and LLR.* Strains were abbreviated as for isolates from *Acacia ehrenbergiana* (Hayne.), *Acacia nilotica* (Willd.), *Acacia saligna* (Labill.), *Acacia tortilis* (Forssk.), *Acacia tortilis* subsp *raddiana* (Savi.) and *Leucaena leucocephala* (Lam.) respectively. Nodules were isolated and sampled from roots of young trees and seedlings by using methods describing by Vincent [16]. After incubation for 3 days at 28°C single colonies were selected and transferred several times on to YEMA plates to ascertain purity of *Rhizobium* strains then conserved in YEMB (yeast extract-mannitol broth) liquid with 20% glycerol at -20°C until use.

Growth Characteristics of Isolated *Rhizobium*: Isolated strains were evaluated by measuring the size and time of appearance (days) of colonies in YEMA (medium with agar). The mean generation time (MGT) was estimated from the growth curve in the 3or 5 isolates from each host by measuring the optical density (650 nm) in 50 ml of YEM inoculated with 1 ml culture with 1×10^9 ufcml^{-1} and incubated at 28°C and 200 min^{-1}. The production of acid or alkali was determined in YEM medium with 25 µg bromothymol blue (BTB) ml^{-1}.

Identification of the *Rhizobium* Strains: API 20 NE test was used to identify the *Rhizobium* strains and for using the carbohydrate. This system is a standardized micro method combining 8 conventional tests and 12 assimilation tests for identification of non-fastidious gram-negative rod not belonging to the *Enterobacteriaceae*. The identification test method was described by Shetta [17].

Acid Resistance: Each isolated strain inoculated in solid medium acidified under sterile condition after autoclaving and sowed with rhizobial cultures from TY (tryptone-yeast extract medium) with less than 200 cfu in the inoculum (20 µl) [5]. The pH values test was estimated using pH values of 9, 8.5, 7.0, 6.5, 5.5 and 5.0 compared to pH of soil sample were adapting legumes trees grown.

Table 1: Physical and chemical analyses of soil sites

Particle size distribution (%)						Soluble cations (meq/L)				Soluble anions (meq/L)			
Sand	silt	clay	Soil texture	pH	EC (dS/m)	Na^+	K^+	Ca^{++}	Mg^{++}	HCO_3^-	CO_3-	SO_4^-	Cl^-
62.92	22.32	14.76	Sandy loam	7.86	6.53	30.43	2.47	25.0	7.40	2.16	-	7.20	30.60

Tolerant strains calculated according to the method described by Graham *et al*. [18].

Intrinsic Antibiotic Resistance (IAR) and Salt Tolerance Level (STL): Determination of intrinsic antibiotic resistance (IAR) was evaluated according to Eaglesham's technique as described by Hashem *et al*. [19] in plates of YEM with different concentrations of antibiotics (25, 50, 75, 100, 150 and 200 µl/ml) of Spectinomycine ($C_{14}H_{24}N_2O_7.2HCl$) (Spe), Chloramphenicol ($C_{11}H_{12}C_{l2}N_2O_5$) (Chl), Rifampicin ($C_{43}H_{58}N_4O_{12.}$) (rif) and Kanamycin ($C_{18}H_{36}N_4O_{11}.H_2SO_4$) (Kan) (Sigma). Filter-sterilized aliquots of each antibiotic were added aseptically to sterile YEM medium at 50°C to give the final concentrations. Control plates contained no antibiotic. Each isolate was grown in YEM to late exponential phase and diluted to an inoculants size of 10^3 ufcml^{-1} at the point of inoculation on the agar surface, transferred by a multipoint inoculator, in three replications. Plates were incubated at 28°C for 7 days and the highest concentration where colony's diameter was similar to control assay was recorded as the resistance level. The salt tolerance level (STL) was determined with the same procedure on medium as antibiotic resistance, with 0.5, 1, 2, 3 and 4% of NaCl [20].

Statistical Analysis: The data obtained from five replicates were subjected to statistical analysis by using SAS computer program [21]. Morphological characterization data was converted into an absence/presence binary matrix (0, 1) using the method described by Rohlf [22].

RESULTS

Twenty six strains of *Rhizobia* were isolated from nodulating legume trees; *Acacia ehrenbergiana* (Hayne.), *Acacia nilotica* (Willd.), *Acacia saligna* (Labill.), *Acacia tortilis* (Forssk.), *Acacia tortilis* var. *reddiana*(Savi.) and *Leucaena leucocephala* (Lam.) grown in South Riyadh. Phenotypically all isolated strains had the same colony morphology and produced high, slimy/mucous transparent to creamy colored colonies on YEMA plates after 3 days of incubation at 28°C. The isolates were fast-growing and failed to absorb Congo Red in the medium except for those isolated from *Leucaena leucocephala* (LLR). Acid production was observed among the isolates after 72 hour at 28°C by changing the color to yellow (Table 2). Generation times for YEMB cultures at 28°C ranged between 2.07 and 3.85 hours (Table 2).

Table 2: Characterization of nodule-forming isolates form the studied woody legume trees

Host tree	*Rhizobia* strains	Number of isolates/ tree	Growth period (hours)	Generation time (hours)	Acid production	
					BTB	CR
Acacia ehrenbergiana (Hayne.)	AER	5	5 -7	2.51	+	-
Acacia nilotica (Willd.)	ANR	5	5 -7	2.44	+	-
Acacia saligna (Labill.)	ASR	5	3 -7	2.07	+	-
Acacia tortilis (Forssk.)	ATR	3	5 -7	3.79	+	-
Acacia tortilis var. *reddiana*(Savi.)	ARR	3	5 -7	3.85	+	-
Leucaena leucocephala	LLR	5	3 -7	2.65	-	+

(BTB) Bromothylmol Blue; (CR) Congo Red; (+) means positive growth/ present, (-) means no growth/ absent, strain isolated from *Acacia ehrenbergiana*(AER), strain isolated from *Acacia nilotica* (ANR), strain isolated from *Acacia saligna* (ASR), strain isolated from *Acacia tortilis* (ATR), strain isolated from *Acacia raddiana* (ARR), strain isolated from *Leucaena leucocephala* (LLR).

Table 3: Identification of *Rhizobia* isolates by using API 20 NE test

Test	Substrates	Reaction	Rhizobia strains					
			AER	ANR	ASR	ATR	ARR	LLR
NO_3	Potassium	Nitrites	-	-	-	-	-	-
	Nitrate	Nitrogen	-	-	+	+	+	-
TRP	Treptophane	Indole production	-	-	-	-	-	-
GLU	Glucose	Acidification	-	-	-	-	-	+
ADH	Arginine	Arginine dihydrolase	-	-	-	-	-	-
URE	Urea	Urease	+	-	+	-	-	-
ESC	Esculin	Hydrolysis	+	+	+	+	+	+
GEL	Gelatine	Protease	-	+	-	-	+	-
PNPG	P-nitrophenyl-BD glactopyranoside	B-galactosidase	+	+	+	-	-	+

(+) means that Rhizobia isolate has consumed the substrate (change the colour), (-) means that Rhizobia isolate had not consumed the substrate (no colour change). strain isolated from *Acacia ehrenbergiana*(AER), strain isolated from *Acacia nilotica* (ANR), strain isolated from *Acacia saligna* (ASR), strain isolated from *Acacia tortilis* (ATR), strain isolated from *Acacia raddiana* (ARR), strain isolated from *Leucaena leucocephala* (LLR).

Table 4: Used of carbohydrates by the *Rhizobia* isolates

		Rhizobia strains					
Test	Substrates	AER	ANR	ASR	ATR	ARR	LLR
GLU	Glucose	-	+	+	-	-	-
ARA	Arabinose	-	+	+	-	-	-
MNE	Mannose	-	+	+	-	-	-
MAN	Mantiol	-	+	+	+	-	-
NAG	N-acetyl- glucosamine	-	+	+	-	-	-
MAL	Maltose	-	+	+	-	-	-
GNT	Gluconate	+	+	+	-	-	-
CAP	Caprate	-	+	-	-	-	-
ADI	Adipate	-	+	+	-	-	-
MLT	Malate	-	+	+	-	-	-
CIT	Citrate	-	+	+	-	-	-
PAC	Phenyl-acetate	-	-	-	-	-	-

(+) means that Rhizobia isolate has consumed the substrate (change the colour), (-) means that Rhizobia isolate had not consumed the substrate (no colour change). strain isolated from *Acacia ehrenbergiana*(AER), strain isolated from *Acacia nilotica* (ANR), strain isolated from *Acacia saligna* (ASR), strain isolated from *Acacia tortilis* (ATR), strain isolated from *Acacia raddiana* (ARR), strain isolated from *Leucaena leucocephala* (LLR).

Table 5: Resistance of *Rhizobia* isolates to the temperature

	Temperature							
	28°C		30°C		37°C		45°C	
Rhizobia strains	3days	7 days	3days	7 days	3days	7 days	3days	7 days
AER	+	+	+	±	±	-	-	-
ANR	+	+	+	±	±	-	-	-
ASR	+	+	+	±	±	-	-	-
ATR	+	+	+	±	±	-	-	-
ARR	+	+	+	±	±	-	-	-
LLR	+	+	+	±	±	-	-	-

(+) means positive growth/ present, (±) means slight growth, (-) means no growth/ absent, strain isolated from *Acacia ehrenbergiana* (AER), strain isolated from *Acacia nilotica* (ANR), strain isolated from *Acacia saligna* (ASR), strain isolated from *Acacia tortilis* (ATR), strain isolated from *Acacia raddiana* (ARR), strain isolated from *Leucaena leucocephala*(LLR).

The responses of the strains varied among the reaction of the identification test (Table 3). The results indicated that, all *Rhizobia* strains did not utilize potassium nitrate (nitrites and nitrogen) except ASR, ATR and ARR strains. Regarding the TRP test (treptophane), all the strains did not produce indole, arginine and acidification except LLR strain. For the reaction of hydrolysis, all *Rhizobium* strains displayed hydrolysis reaction and B-glactosidase except for ATR and ARR strains. Most of the strains failed to produce protease and urease except ANR and LLR strains and AER and ANR, respectively.

Data presented in Table 4 indicated that *Rhizobium* strain ANR and ASR produced acid from glucose, arbinose, mannose, mantiol, N-acetyl-glucosamine, maltose, gluconate, adipate, malate and citrate, while AER, ATR, ARR and LLR did not produce acid, (which means that they do not use carbohydrates). For caprate and phenyl-acetate, the reaction of all *Rhizobium* strains was negative and they did not produce acid. AER strain was positive with gluconate, while ATR strain was positive with mantiol.

Effect of Temperature on Growth of *Rhizobium* Strains: The isolated *Rhizobium* strains AER, ANR, ASR, ATR, ANR and LLR were restricted to thrive on YMA and incubated for 3 and 7 days at different temperature degrees (28, 30, 37 and 45°C) to test their tolerance to higher temperature. The results indicated that, all *Rhizobium* strains grew at 28 and 30°C after 3 and 7 days of the incubation. At 37°C, all the strains were slightly grown after 3 days of incubation while after 7 days no growth was recorded. At 45°C, no strains were found after 3 and7 days of the incubation (Table 5). The optimum temperature for the growth of most isolates was 37°C.

Table 6: Effect of extreme pHs on Rhizobium growth

	pH							
Rhizobia strains	5.0	5.5	6.5	7	8.0	8.5	9.0	12
AER	-	±	+	+	+	+	±	-
ANR	-	±	+	+	+	+	±	-
ASR	-	+	+	+	+	±	±	-
ATR	-	-	+	+	+	+	±	-
ARR	-	-	+	+	+	+	±	-
LLR	+	+	+	+	±	±	-	-

(+) means positive growth/ present, (±) means slight growth, (-) means no growth/ absent, strain isolated from *Acacia ehrenbergiana* (AER), strain isolated from *Acacia nilotica* (ANR), strain isolated from *Acacia saligna* (ASR), strain isolated from *Acacia tortilis* (ATR), strain isolated from *Acacia raddiana* (ARR), strain isolated from *Leucaena leucocephala* (LLR).

Table 7: Intrinsic antibiotic resistance (IAR) of the isolates

		Rhizobia strains					
Antibiotic concentrations µl/ml		AER	ANR	ASR	ATR	ARR	LLR
Spectinomycine	25	+	+	+	+	+	+
	50	+	+	+	+	+	+
	75	+	+	+	+	+	+
	100	+	+	+	+	+	+
	150	+	+	+	+	+	+
	200	+	+	+	+	+	+
Chloramphenicol	25	-	+	+	-	-	+
	50	-	-	-	-	-	±
	75	-	-	-	-	-	-
	100	-	-	-	-	-	-
	150	-	-	-	-	-	-
	200	-	-	-	-	-	-
Rifampicin	25	+	+	+	+	+	+
	50	+	+	+	+	+	+
	75	+	+	+	-	-	+
	100	+	+	+	-	-	+
	150	-	-	-	-	-	-
	200	-	-	-	-	-	-
Kanamycin	25	-	+	+	+	+	+
	50	-	-	-	-	-	-
	75	-	-	-	-	-	-
	100	-	-	-	-	-	-
	150	-	-	-	-	-	-
	200	-	-	-	-	-	-

(+) means positive growth/ present, (±) means slight growth, (-) means no growth/ absent, strain isolated from *Acacia ehrenbergiana* (AER), strain isolated from *Acacia nilotica* (ANR), strain isolated from *Acacia saligna* (ASR), strain isolated from *Acacia tortilis* (ATR), strain isolated from *Acacia raddiana* (ARR), strain isolated from *Leucaena leucocephala*(LLR).

Table 8: Salt tolerance level (STL)

	Salt concentrations%					
Rhizobia strains	0.5	1.0	2.0	3.0	4.0	Control
AER	+	+	+	+	-	+
ANR	+	+	+	+	-	+
ASR	+	+	+	+	±	+
ATR	+	+	+	+	-	+
ARR	+	+	+	±	-	+
LLR	+	+	+	+	±	+

(+) means positive growth/ present, (±) means slight growth, (-) means no growth/ absent, strain isolated from *Acacia ehrenbergiana* (AER), strain isolated from *Acacia nilotica* (ANR), strain isolated from *Acacia saligna* (ASR), strain isolated from *Acacia tortilis* (ATR), strain isolated from *Acacia raddiana* (ARR), strain isolated from *Leucaena leucocephala*(LLR).

Effect of Extreme Phs on *Rhizobium* Growth: The sensitivity of the isolates to extreme pHs was examined. Most of the isolates grew well at pH of 6.5,7 and 8, but only a few isolates grew at pH 5.5 and 9. No growth was recorded at pH 5 and 12 (Table 6). The optimum pH for the growth of most isolates was 9.

Determination of Intrinsic Antibiotic Resistance (IAR) and Salt Tolerance Level (STL): Fresh cultures of isolated *Rhizobium* strains were grown onto YMA containing spectinomycine, chloramphenicol, rifampicin and kamamycin to determine the intrinsic antibiotic resistance (IAR). Data in Table 7 indicated that all strains were resistant to 25, 50, 75,100 and 200 µg/ml spectinomycine. *Rhizobiun* strains ANR, ASR and LLR were resistant to 25 µg/ml chloramphenicol, while strain LLR was slightly resistant at 50 µg/ml. No activities were observed for the strains under higher concentrations. Strains AER, ANR, ASR and LLR were able to grow until 100 µg/ml of rifampicin, while strain ATR and ARR could grow at 50 µg/ml. At higher concentration of rifampicin (more than 100 µg/ml), no cells were observed. Strain AER was sensitive to all concentrations of kanamycin, while the maximum concentration of kanamycin at which the *Rhizobium* was able to grow was 25 µg/ml. All isolated strains were sensitive to kanakmycin at concentration higher than 25 µg/ml.

Salt tolerance level of *Rhizobium* strains (STL) was examined under different NaCl concentrations at 28°C. Table 8 showed that, all of the isolates grew in the presence of high concentrations of NaCl (2% w/v), while most of the isolates grew in high concentrations of NaCl (3% w/v). The strains ASR and LLR slightly grew at 4% salinity after 3 days of incubation.

DISCUSSION

Early reports of *Rhizobia* associated with woody legumes described them as species that belong to the slow-growing, but more recent reports have shown that this population includes a very diverse type of *Rhizobia* including fast, intermediate and slow-growing bacteria. In the present study, all the isolates were throve on YEM agar medium after incubation at 28°C for three days. The isolates were fast grow and having sticky appearance. The results indicated that all isolated *Rhizobium* were acid producers but bacteria isolated from *Leucaena leucocephala* (LLR) produced no acid. The pH of the medium and broth during growth of isolates changed from 7 to 6, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during the growth [23, 24]. General microscopic view of the *Rhizobium* isolates showed them to be rod cells and gram negative in nature [2]. The generation time for isolated strains isolated from the studied trees at 28°C ranged between 2.07 and 3.85 hours; this included two extra growth categories, very fast and, fast to accommodate isolates from Australian *Acacia* spp and African *Acacia* species, this did not fit into the traditional fast- and slow-growing types [25]. The scarce number of *Rhizobium* strains isolated from woody legumes in Riyadh region may suggest that they are not as symbiotically competent as *Rhizobium* in nodulating with the majority of our woody species, these results were in harmony with the finding of Odee[20] in *Acacia* and Abril and González[26] in *Prosopis* from Argentinean Chaco.

Carbohydrate utilization assays indicated that *Rhizobium* isolates obtained from legume tree roots were able to utilize different carbohydrate sources, thus it was assumed that they may produce important enzymes like amylase and cellulases. *Rhizobium* strains were able to utilize glucose and sucrose more efficiently than normal YEM medium [2, 27]. Utilization of L-glutamine was only one characteristic that can distinguish between strains of *Rhizobium* and *Sinorhizobium* next the glucose. *Sinorhizbium* and *Bradyehizobium* could be distinguished based on utilization of D-maltose, lithium lactate, dextrose, glycerol, trehalose, adonitol as sole carbon source, the utilization of DL-aspartic acid, glycine, DL- threonine, DL- alanine, sodium nitrate, calcium nitrate and L-arganine, ammonium nitrate and L-glutamine as sole nitrogen source, while none of these characteristic could distinguish between *Rhizobium* and *Bradyrhizobium* [28, 29]. The obtained results showed that those strains might belong to one of two groups, *Rhizobium* or *Sinorhizobium,* based on the utilization of carbon and nitrogen as sole source of carbon and nitrogen respectively. The results of our study suggests that bacteria of different genera may adapt to the environmental conditions influenced by root exudates from their hosts- root exudates are composed of both low and high components, including an array of primary and secondary metabolites, portions and peptiodes [30, 31], that vary in quantity and chemical structure depending on the plant selective environments for a specific group of bacteria.

Most of the isolates in this study possessed optimum growth at 30°C, but some of the isolated strains were slightly able to grow at 37°C. No bacterial growth was recorded at higher temperature than 37°C. Indeed, the

present results are in agreement with previous work of Hashem *et al*. [19] and Marsudi *et al* [32], while it disagree with those of Hung *et al*. [33]. However, since many other factors affect the competitiveness, establishment and efficiency of strains, *in vitro* selection of temperature-tolerant root nodule bacteria is not considered as a promising approach for field applications [34]. The survey of such characteristics can be useful for improvement of inoculants by genetic engineering of strains with symbiotic nitrogen fixation.

Extremes of pH can be a major factor limiting microorganism in soil. The pH is an important parameter for the growth of the organism. Slight variation in pH of the medium might have significant effects on the growth of bacteria or organism [2]. The results indicated that most of the isolates grew well at pH of 6.5, 7.0 and 8.0, but only a few isolates grew at pH 5.5 and 9.0. No growth was recorded at pH 5 and 12.0. The optimum pH for the growth of most isolates was 9.0. Thus medium with pH 5.0 and 9.0 was the optimum parameters for growth of the isolated *Rhizobium* strains. Our results were in agreement with previous studies of Kucuk *et al*. [27] and Baoling *et al*. [24], while disagree with the finding of Graham *et al*. [18]and Surange *et al*. [35].

The isolates were resistant to all level of spectinomycine at all used concentrations of tested antibiotics. However, it was susceptible to chloranphenicol and kanamycin than rifampicin. This might have resulted in the isolation of *Rhizobium* strains to a wide spectrum of antibiotics to be included in soil microbial analysis Döbereiner *et al*. [36] ascribed the increased resistance of rhizobial strains to the presence of antibiotics in the soil as a consequence of microbial activities such as *Streptomyces*, above all. There are three known determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge and amount of the antibiotic and *Rhizobium* that showed a high level of resistance did not take up the antibiotics. However detailed study is needed to evaluate this fact [34]. Furthermore, the pattern of IAR is useful in the strain identification [37].

Salinity is one of the major factors restricting the symbiotic nitrogen fixation [17]. It is known that salt stress significantly reduces nitrogen fixation and nodulation in legumes. In the present study, most of the isolates were persisted under salt concentrations of 3.0%. Hence, these isolates may be the candidates for applications in the saline influenced soil. The results were in harmony with results of Hung *et al*. [33], Kucuk *et al*.[27] and Singh *et al*.[2]. More biodiversity studies are warranted to screen nitrogen fixing organisms from different ecosystems, with emphasis on their biochemical characteristics and genetic diversity.

CONCLUSION

In conclusion, it can be said that phenotypically, the *Rhizobium* isolates from legume trees are belong to the fast growing groups. The strains showed resistance to antibiotics, temperature, salinity and pH. The isolates from present study may be useful to increase the symbiotic nitrogen fixation in legume trees. This study therefore provides the basis for further research on the phylogeny of *Rhizobium* strains nodulating the legume trees, as well as their use as inoculants to improve growth and nitrogen fixation in arid lands of central region of Saudi Arabia. The study suggesting adaptability of isolates to different ecological environments with many factors at stress levels. To confirm the identity, as well as the symbiotic and nitrogen fixation, additional experimental work would be necessary, for instance DNA-DNA hybridization, multilocus sequences analysis of housekeeping genes. Continued efforts should be made to understand the complex association between legumes and their symbiotic partners, with an emphasis on their ecological role, co-evolution, selection of symbiotic partners, acquisition, soil fertility, evolution and transfer of nitrogen fixation (nif) or nodulation (nod) genes and ultimately to employ efficient strains in the sustainable agricultural practices in Saudi Arabia.

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