JEB
Journal of Environmental Biology

Journal Home page : www.jeb.co.in
E-mail : editor@jeb.co.in

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Phenotypic and biochemical characterization of root nodule bacteria naturally associated with woody tree legumes in Saudi Arabia

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Publication Info

Paper received:
30 October 2013

Revised received:
21 January 2014

Accepted:
28 April 2014

Abstract

Thirty root-nodulating bacteria isolates were obtained from the roots of *Acacia ampliceps* (Maslin), *A. ehrenbergiana* (Hayne.), *A. saligna* (Labill.), *A. seyal* (Del.), *A. tortilis* (Forssk.), *A. tortilis* subsp. *raddiana* (Savi.), *Leucaena leucocephala* (Lam.) and *Vicia faba* (L.) trees growing in the Riyadh region. The isolates' phenotypic and biochemical properties were characterized by assessing colony appearance, growth rate, resistance to antibiotics and heavy metals, and tolerance to salinity, elevated temperature and pH. All isolates had same colony morphology and grew on yeast extract mannitol and tryptone yeast agar, but not MGS media. The results also revealed considerable diversity among the isolates, which exhibited different patterns of resistance to abiotic stresses. Most isolates tolerated temperatures up to 37°C and could grow from pH 5.5–8.5 and at a high NaCl concentration (2% w/v). The majority of isolates could utilize a variety of carbohydrates. Most of the isolates displayed resistance to antibiotics in the 75 µg ml^{-1} range, with ~100 µg ml^{-1} the maximum concentration at which growth was observed. All isolates were sensitive to aluminum and resistant to other heavy metals tested, and they were able to reduce nitrate and hydrolyze urea.

Key words

Legume trees, Phenotypes, *Rhizobium isolates*

Introduction

Rhizobia are bacteria that convert atmospheric nitrogen into a form that plants can use (Mylona *et al.,* 1995). Due to their ecological and economic importance, rhizobia have been investigated extensively over the last three decades. In particular, wild legumes and their symbionts are drawing attention because of their ability to tolerate extreme environmental conditions, such as elevated temperature, drought and salinity (Zahran, 2001). Rhizobia are capable of establishing symbioses with a wide range of legumes, such as trees (Khbaya *et al.,* 1998), shrubs (Donate-Correaet *et al.,* 2007), herbs (Giongo *et al*., 2008 and Odee *et al*., 1997) and aquatic plants. The taxonomy of bacterial endosymbionts of legume plants has experienced a profound series of extensions in the resent past (Young, 2009). Recently described nitrogen-fixing symbionts of legumes that belong to Alphaproteobacteria class include *Ochrobactrum* (Ngom *et al.,* 2004; Trujillo *et al.,* 2005), *Devosia* (Rivas *et al.,* 2003), *Blastobacter* (Van Brekum and Eardly, 2002), and *Methylobacterium* (Jaftha *et al.,* 2002; Jourand *et al.,* 2004); novel Betaproteobacteria (Benhizia *et al.,* 2004) include *Burkholderia* (Moulin *et al.,* 2001) and *Cupriavidus* (Vandamme and Coenye, 2004). Nevertheless, considerable work on rhizobium-legume symbiosis has been carried out, but little attention has been given to the symbionts of woody legumes, such as the *Acacia* species.

Saudi Arabia is located in a sub-tropical desert and its flora comprises of several introduced woody legume species, such as *Acacia karroo, A. saligna,* and *A. ampliceps*. Most of these species have adapted successfully to Saudi environmental conditions, due to the fact that they are fast-growing plants that thrive in areas with alkaline or saline soil (Fox, 1995) and as little rainfall as 250 mm per year. The aim of the present study was to investigate, isolate and characterize rhizobial strains naturally for

associated with woody tree legumes in the Riyadh region, located in central Saudi Arabia.

Materials and Methods

Study areas : This study included twenty-one sites selected from seven areas in the Riyadh region, which is located in central Saudi Arabia. All *Acacia* and woody legume species studied grew wild in a natural forest setting. The coordinates of each site were determined by GPS (Fig.1).

Isolation of root nodule bacteria : Nodule collection was conducted from 2012–2013. Rhizobia strains were isolated from the root nodules of *A. ampliceps* (Maslin), *A. ehrenbergiana* (Hayne.), *A. saligna* (Labill.), *A. seyal* (Del.), *A. tortilis* (Forssk.), *A. tortilis* subsp. *raddiana* (Savi.), *Leucaena leucocephala* (Lam.), and *Viciafaba* (L.). Bacteria were isolated from healthy, unbroken, pink root nodules from samples collected in the field, or from seedlings grown in pots in the green houseThe method used to isolate the bacteria have been described by Vincent (1970).

Phenotypic characterization of rhizobia isolates : All isolates were stored on Yeast Extract Mannitol (YEM) agar slants (Vincent, 1970) at 4°C and in YEM broth containing 20% (v / v) glycerol at -20°C. Isolates were also stored in the laboratory at -20°C. Other test media used were Tryptone Yeast (TY) (Beringer, 1974) and MGS media.

Isolates' colony morphology was examined on YEM agar plates for 3–7 days of incubation at 28°C. Individual colonies were characterized based on size, color, shape, mucosity, transparency, borders and gram stain reaction (Vincent, 1970).

Each isolate was streaked on a YEM agar plate, which was incubated at 28°C and inspected daily. Generation time in culture was determined by inoculating each isolate into 100 ml sterile YEM broth, incubated in 250 ml conical flasks in a shaker at 200 rpm at 28°C. Growth was assessed by measuring optical density at 600 nm every 3 hrs. Three replicates were performed for each isolate. At the end of growth phase, growth curves were plotted and generation time at exponential phase was calculated

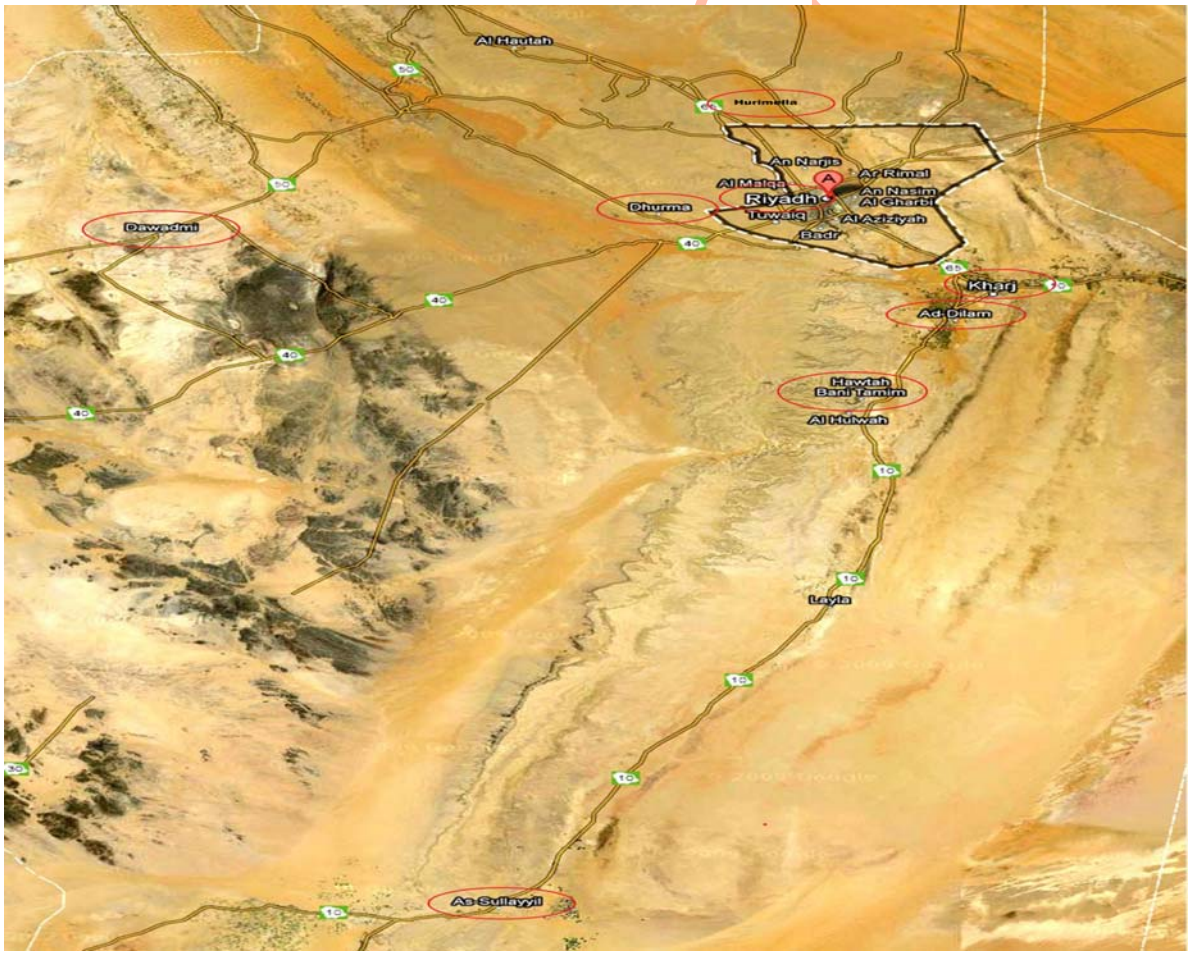

Fig. 1 : Map showing the collection sites

each isolate. The ability to grow on TY medium was also examined (Beringer, 1974).

All isolates were tested for an acid or alkaline reaction on YEM agar plates containing 0.25 mg bromothymol blue per litre after incubation at 28°C for 3–7 days (Vincent, 1970). A change in the color of bromothymol blue plates from green to yellow after 3 or 7 days of incubation indicates acid production. Each test strain was incubated for 7 days at 35, 37, 40, 42, 44 and 45°C (Hung *et al.,* 2005) on YEM agar plates to determine its optimal growth temperature. This test was carried out in three replicates. pH tolerance was determined by growing strains in acid or alkaline conditions. The pH of YEM agar was adjusted to 4.0, 5.0, 5.5 or 8.5 with either NaOH or HCl before being autoclaved (Küçük *et al*., 2006).

The ability of the bacteria to metabolize various carbon sources was tested using an API gallery (API 50 CH; BioMérieux, France); isolates were incubated for 48 hrs in YEM broth (Kersters *et al.,* 1984). Galleries were inoculated with isolates and incubated at 28°C for 24 hrs. The results were scored as positive when the color of the inoculated galleries changed. Tolerance to salt was assessed on YEM agar plates containing 1–5% NaCl (w/v). The isolates were grown in liquid medium incubated at 28°C for 7 days.

Resistance to antibiotics was tested on YEM agar plates. Sterile antibiotic solutions were added to the medium after sterilization: streptomycin (120 µg ml^{-1}), spectinomycin (120 µg/ml), kanamycin (100 µg ml^{-1}), chloramphenicol (200 µg ml^{-1}), rifampicin (100 µg ml^{-1}), ampicillin (100 µg ml^{-1}) or tetracycline (100 µg ml^{-1}). Isolates were inoculated and incubated at 28°C for 7 days (Kenenil *et al.,* 2010). Nitrate reduction was assessed in liquid TY medium as described by Lindstrom and Lehtomaki (1988). For isolates unable to grow in TY, YEM was utilized.

Resistance to heavy metals was assessed on solid YEM agar plates. Heavy metal solutions were filtered and added to the growth medium: 500 µg ml^{-1} $AlCl_3.6H_2O$; 100 µg ml^{-1} $CuCl_2.2H_2O$; 5 µg ml^{-1} $HgCl_2$ or 20 µg ml^{-1} $CdCl_2.2H_2O$. The isolates were inoculated with YEM agar plates and incubated at 28°C for 7 days (Küçük *et al*., 2006).

Statistical analysis : The morphological characterization data were converted into an absence/presence matrix following the method of Rohlf (1993).

Results and Discussion

The data obtained from the study indicated that only nine strains (KSA1, KSA2, KSA3, KSA4, KSA4R, KSA5, KSA6, KSA7, KSA8 and KSA9) were isolated from the studied sites (Table 1). All isolated strains had the same colony morphology and produced high, slimy/mucous, transparent to creamy colored colonies on YEM agar plates after 3 and 7 days of incubation at 28°C. The number of strains isolated from *Acacia* trees species was less than expected as compared with the large number of root nodules and the multi root nodule shapes. The small number of strains isolated from the legume plants indicate that the strains may be symbiotically competent with the studied species as they were with most other woody legumes tree species. These results were not compatible with the findings of Odee et al. (1997) and Jacobs et al. (2007) in *Acacia* species and Abril and González (1994) in *Prosopis* grown in Argentinean Chaco.

All isolates grew on YEM agar media, none grew on MGS, and most grew on TY (the exceptions being strains KSA5, KSA6, and KSA8 (Table 2). Acid production was indicated by a change in the colour of bromothymol blue plates from green to yellow after a 72 hrs of incubation at 28°C; all strains produced acid except for KSA4 and KSA8 (Table 2). The production of acid appears to be the characteristic of most rhizobia (Küçük *et al.,* 2006; Marsudi *et al.,* 1999).

In the present study, all isolates grew on YEM agar medium after incubation at 28°C for 3 days. All isolates had generation times ranging from 2.15–3.85 hrs except for KSA4 and KSA7, which grew slowly (Fig. 2). Thus, most isolates obtained in this study were fast-growers. The results are in agreement the study McCaig *et al.* (2001); they found that isolates from *A. ampliceps* grow either fast or very fast. Rhizobia associated with woody legumes have been described as slow-growing species,

Table 1 : Host plant and isolation sites of isolated root nodules bacteria

Isolate strains	Original host plant	Area of isolation	Sites of isolation
KSA1	*Acacia saligna*	Riyadh City	Wadi Derab
KSA2	*Acacia ampliceps*	Riyadh City	Wadi Derab
KSA3	*Acacia seyal*	Riyadh City	Wadi Derab
KSA4	*Acacia tortilis* subsp. *raddiana*	Al-Dawadmi	Jubullah
KSA4R	*Acacia tortilis* subsp. *raddiana*	Al-Dawadmi	Musloum
KSA5	*Vicia faba*	Riyadh City	Deraiaa,
KSA6	*Acacia tortilis* subsp. *tortilis*	Houtut bani Tamem	Aunther
KSA7	*Acacia tortilis* subsp. *raddiana*	Wadi Al-Dawaser	AS-Sulayyil
KSA8	*Leucaenia leucocephala*	Riyadh City	Wadi Derab
KSA9	*Acacia ehrenbergiana*	Riyadh City	Wadi Derab

Table 2 : Phenotypic characteristics of isolates: growth on various media and in response to abiotic stressors

Characteristic	Isolates								
	KSA1	KSA2	KSA3	KSA4	KSA5	KSA6	KSA7	KSA8	KSA9
Growth Media[a]									
YEM agar	+	+	+	+	+	+	+	+	+
MGS	-	-	-	-	-	-	-	-	-
TY agar	+	+	+	+	-	-	+	-	+
Acid production									
BTB[b]	+	+	+	-	+	+	-	+	+
Growth temperature (°C)[a]									
35	+	+	+	+	+	+	+	+	+
37	±	±	±	±	±	±	±	±	±
40	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-
45	-	-	-	-	-	-	-	-	-
pH[a]									
4.0	-	-	-	-	-	-	-	-	-
5.5	±	+	±	+	+	-	±	+	+
7.0	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+
8.5	±	+	±	+	+	+	+	+	±
NaCl (%)[a]									
1.0	+	+	+	+	+	+	+	+	+
2.0	+	+	+	+	+	+	+	+	+
3.0	±	±	±	±	±	±	±	±	±
4.0	±	-	-	-	-	±	-	±	-
5.0	-	-	-	-	-	-	-	-	-

[a] +: Growth/presence, ±: Slight growth, -: No growth/absence; [b] BTB: bromothylmol blue, +: acid production (yellow colonies), -: alkaline production (green colonies)

but recent studies have shown that these populations also include fast and intermediate-growing strains (McCaig *et al.,* 2001).

The results indicate that the temperatures at which isolates could grow varied. All isolates grew well at 35°C but only slightly at 37°C (Table 2). No rhizobia isolates grew at 40°, 42°, 44° or 45°C. These results are in agreement with those of Hashem *et al.* (1998) and Marsudi *et al.* (1999) but differ from those reported by Hung *et al.* (2005) and Mohammed *et al.* (2000). Information on growth temperature range is useful in genetic engineering of nitrogen-fixing bacteria. As many other factors affect the competitiveness, establishment, and efficiency of strains, *in vitro* selection of temperature-tolerant root nodule bacteria is not considered (Hungria and Vargas, 2000).

Soil pH is an important parameter affecting rhizobial growth. Small variations in the pH of media can have significant effects upon bacterial growth (Singh *et al.,* 2008.). Most isolates grew at pH 5.5, 7.0, 8.0 and 8.5, but no we strains grew at pH 4.0 (Table 2). For most isolates maximal growth occurred at pH 8.0, although optimal pH ranged from 5.5–8.5. The data show that the studied isolates were globally tolerant to alkalinity and neutrality. These results are consistent with the findings of Berrada *et al.* (2012), Baoling *et al.* (2007) and Küçük *et al.* (2006), but differ from results of Graham *et al.* (1994) and Surange *et al.* (1997).

Salt stress in legumes results in reduction of nitrogen fixation and nodulation. As shown in Table 3, all isolates grew well at low NaCl concentrations (1–2% w/v), but at 3% salinity, only low levels of growth were observed. At 4% salinity, low growth levels were observed for KSA1, KSA6 and KSA8 after 10 days of

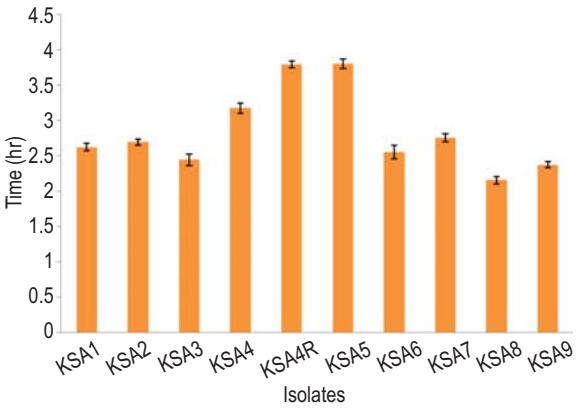

Fig. 2 : Generation period of the rhizobia isolates

Table 3 : Ability of rhizobia isolates to utilize various carbon sources[a]

Test substrates	Strains								
	KSA1	KSA2	KSA3	KSA4	KSA5	KSA6	KSA7	KSA8	KSA9
D-Glucose	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+	+
Manntiol	+	+	+	+	+	+	+	+	+
N-acetyl- glucosamine	+	-	+	+	+	+	+	+	+
Maltose	+	+	+	-	-	-	-	+	-
Gluconate	+	+	-	-	+	-	-	-	+
D- xylose	+	+	+	+	+	+	+	+	+
Starch	-	-	-	-	-	-	-	-	-
Glycérol	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	-	-	-	-	-	-
Melezitose	+	-	+	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-
Methyl-D-glucosde	-	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-	-
Lactose	+	+	+	-	+	-	-	-	-
Galactose	+	+	+	+	+	+	+	-	+

[a] +: Isolate has consumed the substrate (Colour change), -: Isolate has not consumed the substrate (No colour change)

Table 4 : Antibiotic resistance of rhizobia isolates[a]

Antibiotic concentrations ($\mu g\ ml^{-1}$)		Isolates								
		KSA1	KSA2	KSA3	KSA4	KSA5	KSA6	KSA7	KSA8	KSA9
Ampicillin	50	+	+	+	+	+	+	+	+	+
	75	+	+	+	+	+	+	+	+	+
	100	+	+	+	+	+	+	+	+	+
	120	±	±	±	-	±	-	-	-	-
Chloramphenicol	50	±	+	±	+	+	+	+	+	+
	100	-	+	-	+	-	-	+	+	+
	150	-	-	-	-	-	-	-	-	-
	200	-	-	-	-	-	-	-	-	-
Kanamycin	50	+	+	+	+	-	+	+	±	±
	75	±	±	-	+	-	±	+	-	-
	100	-	-	-	-	-	-	-	-	-
	150	-	-	-	-	-	-	-	-	-
Rifampicin	50	+	+	+	+	+	+	+	+	+
	75	+	+	+	-	+	+	-	+	+
	100	+	-	±	-	±	±	-	-	-
	120	+	-	-	-	-	-	-	-	-
Spectinomycin	50	+	+	+	+	+	+	+	+	+
	75	+	+	+	±	+	+	±	+	+
	100	+	+	-	-	+	±	-	+	+
	120	+	±	-	-	-	-	-	+	+
Streptomycin	50	+	+	+	+	+	+	+	+	+
	75	+	+	+	±	+	-	±	+	+
	100	+	±	+	-	+	-	-	±	±
	120	+	-	±	-	-	-	-	-	-
Tetracycline	50	+	±	+	+	+	+	+	+	+
	75	+	-	±	+	±	+	+	+	+
	100	±	-	-	-	-	±	-	±	±
	120	-	-	-	-	-	-	-	-	-

[a] +: Growth/presence, ±: Slight growth, -: No growth/absence

Table 5 : Nitrate reduction test

Assay	Isolates								
	KSA1	KSA2	KSA3	KSA4	KSA5	KSA6	KSA7	KSA8	KSA9
Nitrate reduction									
Nitrate reduction	+	+	+	+	+	+	+	-	+
Hydrolsis of urea	+	+	+	+	+	+	+	+	+

[a]+: Growth/presence, ±: Slight growth, -: No growth/absence

Table 6 : Heavy metal resistance test[a]

Assay	Isolates								
	KSA1	KSA2	KSA3	KSA4	KSA5	KSA6	KSA7	KSA8	KSA9
Heavy metals									
$AlCl_3$	-	-	-	-	-	-	-	-	-
$CdCl_2$	+	-	+	+	+	-	+	-	+
$CuCl_2$	-	+	-	+	+	+	+	+	+
$HgCl_2$	+	+	+	+	-	+	+	-	-

[a]+: Growth/presence, ±: Slight growth, -: No growth/absence

incubation; no other strains were able to grow at such a high salt concentration. No rhizobia strains grew at NaCl concentrations greater than 4% (Table 2). The isolates capable of growing at 4% salinity may be good candidates for applications in high-saline soil. The results were consistent with the previous study of Hung *et al.* (2005), Küçük *et al.* (2006) and Singh *et al.* (2008).

The ability to utilize various carbon sources can be used to identify the taxon to which a rhizobial isolate belongs. Table 3 indicates that polysaccharides and monosaccharides (e.g. glycerol, L-arabinose, maltose, galactose, D-glucose, D-mannose, mannitol and D-xylose) were utilized by most isolates whereas disaccharides were not used. Starch, glycogen, D-glucoside, inulin and melezitose were poorly assimilated by most isolates. The results demonstrated that rhizobia isolates obtained from legume tree roots could utilize different carbohydrate sources, facilitating their ability to produce cellulose and important enzymes such as amylase. Küçük *et al.* (2006) and Singh *et al.* (2008) reported that rhizobia strains were able to metabolize glucose and sucrose more efficiently than the other nutrients present in YEM medium. Pongsilp and Leelahawong (2010) and Pongsilp and Boonkerd (2007) demonstrated that utilization of L-glutamine was the only characteristic that distinguished strains of *Rhizobium* and *Sinorhizobium*. *Sinorhizbium* and *Bradyehizobium* can also be distinguished the basis of carbon sources. *Sinorhizbium* has the ability to use D-maltose, lithium lactate, dextrose, glycerol, trehalose, or adonitol as the sole carbon source. Based on the study of Pongsilp and Leelahawong (2010) and Pongsilp and Boonkerd (2007) results, it appeared that the isolates were either *Rhizobium* or *Sinorhizobium* .The present study, which demonstrates that the isolates were capable of metabolizing a variety of carbon sources, is in agreement with the earlier report by Bias *et al.* (2006), who found that bacteria of different genera adapt to the environmental conditions created by their hosts' root exudates. Root exudates are composed of a variety of low and high molecular weight molecules, including an array of primary and secondary metabolites and peptides.

Intrinsic Antibiotic Resistance (IAR) profile can be useful in identification of strain. Table 4 shows that most rhizobia isolates were resistant to antibiotics at concentrations of 50 and 75 µg ml^{-1}, while at concentrations greater than 100 µg ml^{-1}, resistance of the isolates varied. No growth was observed when the concentration of chloramphenicol, kanamycin or tetracycline exceeded 120 µg/ml. In general, the maximum antibiotic concentration at which most strains grew was 75 µg ml^{-1}. All isolates were sensitive to antibiotics at higher concentrations. Some isolates were resistant to all concentrations of spectinomycin tested, and it appeared that the isolates studied were more susceptible to chloranphenicol and kanamycin than rifampicin. This result was in line with the study of Shetta *et al.* (2011) and Berrada *et al.* (2012) , who report that, the fast growing rhizobium strains are more sensitive to antibiotics. Depending on the difference in antibiotics resistance pattern, this technique could be successfully employed for the field of ecological studies, particularly in recovery and enumeration of rhizobia introduced into soil (Gauri *et al.,* 2011). However, more study is needed to establish this fact (Hungria and Vargas, 2000).

All isolated strains except KSA8 reduced nitrate, while urea was hydrolyzed by all strains (Table 5). This result was consistent with the findings of Mohamed *et al.* (2000), but disagreed with the finding of Jensen and Schrøder (1965).

No growth was detected on plates containing $AlCl_3$. Most strains were tolerant to $CdCl_2$, $CuCl_2$ and $HgCl_2$ (Table 6). This result is in accordance with previous reports, of respectively Mohamed *et al.* (2000); Carrasco *et al.* (2005) and Al- Kadeeb (2007).

In the present study, phenotypic and biochemical study showed large biodiversity between rhizobia isolates from legume trees in the Riyadh region of Saudi Arabia. The studied isolates belonged to fast-growing Rhizobia, with an exception of an isolate from *A. tortilis subsp. raddiana*. The isolates exhibited a variable resistance to antibiotics, high temperature, and salinity and could grow over a range of pH. This study provides a base for further research on the genetic structure and development of the rhizobia strains that nodulate legume trees; in addition, the isolates gathered may be used as inoculants to improve legume tree growth and nitrogen fixation in Saudi Arabia.

Acknowledgment

This work was supported by NSTIP strategic technologies program number (AGR-1270-02) in the Kingdom of Saudi Arabia.

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