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NODULATION PATTERN AND BIODIVERSITY OF RHIZOBIA OF SOME IMPORTANT LEGUMINOUS TREES AND SHRUBS OF ETHIOPIA

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ABSTRACT: A collection of 20 root nodule bacteria were isolated from the hitherto unexplored indigenous woody legumes from Ethiopia. Their diversity was evaluated using numerical analyses on different morphological and physiological characteristics. Most of the isolates were found to be slow-growing, sensitive to high concentration of NaCl, sterptomycin sulphate, but able to utilize a wide range of carbon sources, to grow at different temperatures and pH ranges implying that they can be good candidates for inoculation under varied conditions. They were grouped into one major cluster with a 20% average similarity level representing all but 2 isolates, and into four clusters representing a 60% average similarity level, except six isolates. In order to show the existence of cross-inoculation pattern between woody isolates and grain legume hosts, four selected isolates from *Erythrina brucei*, *Crotalaria*, *Indigofera*, and *Tephrosia spp* were inoculated into common bean (*Phaseolus vulgaris*) and cowpea (*Vigna unguiculata*) All, but one isolate from *Indigofera* (AURI30) elicited nodulation on both grain hosts with symbiotic effectiveness ranging from 41% to 100% against the Nitrogen-inoculated control. This suggests that the grain legumes can be cultivated in close association of the woody legumes in agroforestry systems without requiring their specific endosymbionts.

Key words/phrases: Cross-inoculation, Endosymbionts, Indigenous trees, Nodule bacteria.

INTRODUCTION

Leguminous plants are classified into one of the largest families of flowering plants, Leguminosae (Fabaceae). This family consists of three subfamilies (Mimosoideae, Caesalpinoideae and Papilionoideae) that collectively have 670 –750 genera and about 20,000 species of plants (Gepts *et al*., 2005). In the Ethiopian flora, 621 species of leguminous plants have been recorded, of which 553 species are indigenous and 48 are endemic (Thulin, 1989).

It is estimated that 30 % of the species of the Family Leguminosae are trees and shrubs of tropical and subtropical regions (Allen and Allen, 1981).

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These trees and shrubs are multipurpose plants that provide high heat-value fuel wood and charcoal, wood for poles, fence, pulp and construction, food, medicine, tannin, gum Arabic, and windbreak rows. They constitute browse species for domestic and wild animals and they are also used as a canopy cover and light shade for animals and understorey vegetation (for example, coffee, tea, and cocoa). Leguminous trees and shrubs are often fast growers, and play a key role in the rehabilitation of degraded and marginalized soils (Fassil Assefa, 1993; Herrera *et al*., 1993), since they are widely distributed in all tropical soils displaying a wide range of tolerance to acidity, alkalinity, water logging, mineral deficiency and drought (Rengel, 2002). They are also important in low-input agroforestry and plantation forestry due to their ability to fix atmospheric nitrogen in an endosymbiotic association with root-nodule rhizobia. The fixed nitrogen subsequently transfers to non-fixing associated plants as root exudate or as plant and animal residues after microbial transformations.

Although the Ethiopian highlands and lowlands are relatively rich in legumes (Thulin, 1989), studies on their nodulation status and nitrogen fixing potential is scarce (Fassil Assefa, 1993). Moreover, studies have concentrated on food and forage legumes – such as peas, beans, chickpeas, lentils, medics, and alfalfa – rather than woody legumes that offer several advantages with regard to rehabilitation of degraded and marginalized soils. Recently, nodulation analyses were carried out on some Ethiopian leguminous species of trees and shrubs of agroforestry importance (Fassil Assefa, 1993; Fassil Assefa and Kleiner, 1997; Endalkachew Wolde-meskel *et al*., 2004; Endalkachew Wolde-meskel *et al*., 2005). The results showed high rhizobial biodiversity associated with trees and shrubs. Moreover, rhizobial isolates from these legumes were also found to promiscuously nodulate tropical pulse crops such as common bean (*Phaseolus vulgaris*), ground nut (*Arachis hypogea*), soybean (*Glycine max*), and African yam bean (*Sphenostylis stenocarpa*) (Allen and Allen, 1981; Wang, 1989; Fassil Assefa and Kleiner, 1997). Some of the strains were found to be better nitrogen fixers under acidic conditions and high temperature regimes (Wang, 1989).

More work on the nodulation status and biodiversity of the endosymbionts of leguminous trees and shrubs of Ethiopia should be carried out. Further screening for the nodulation of wild legumes from their well-adapted indigenous environments serves as an additional reservoir to select high N-fixing strains for biological nitrogen fixing technology in sustainable agriculture and environmental management, and as a source of more strains

for taxonomic studies. Moreover, these legumes possess the ability to colonize marginalized and impoverished soils, and thus are recommended for agroforestry and reforestation programs (Dommergues, 1988).

Both the woody and grain legumes that were included in the current study possess great significance. Beside the particular use(s) of each type of woody legume, like the use of *Indigofera* species to produce indigo (a dye), the agronomic and ecological importance of woody species of Crotalaria (Graham and Vance,2003), *Dalbergia* and *Tephrosia* (Graham and Vance,2003), *Aeschynomene* (Giller, 2001), *Chamaecytisus*(Vinuesa, *et al*.,1998) and *Erythrina*(Legesse Negash, 2002; Graham and Vance,2003) was emphasized. The grain legumes: *V. unguiculata* and *P. vulgaris* are sources of proteins and calories for poor people of developing countries and they are also legume commodities (Gepts *et al*., 2005). Thus, the objectives of the present study were to isolate and characterize root-nodule bacteria from the hitherto unexplored, but the most diversified group of Ethiopia's indigenous woody legumes, and to evaluate the cross-inoculation patterns and symbiotic effectiveness of some of the isolates on cow pea (*Vigna unguiculata*) and common bean (*Phaseolus vulgaris*).

MATERIALS AND METHODS

Study sites, legume variety and nodule collection

Root nodules were obtained from seven genera of woody legumes collected from various regions of Ethiopia (Table 1) in October and November 2005, and brought to the Applied Microbiology Laboratory in sealed vials containing a desiccant (silica gel) covered with a 1 cm layer of cotton wool, as described by Lupwayi and Haque (1994). Voucher legume specimens were collected and brought to the Natural Herbarium, Biology Department, Addis Ababa University, for identification.

Isolation of rhizobia from root nodules

Dried, undamaged root nodules were imbibed with sterile distilled water and surface-sterilized using sodium hypochlorite by immersing for 4 minutes after exposure to 95 % ethanol for 10 seconds and then washed with sterile distilled water at least six times as described by Vincent (1974). The surface-sterilized nodules were aseptically crushed and their suspensions were streaked into sterilized plates of yeast extract mannitol agar (YEMA), the streaked plates were incubated at 28°C $\pm$ 2 for 4–10 days. Following this, all the plates were carefully examined and a single colony of each isolate was repeatedly streaked for purity. Purified colonies were preserved

at 4°C for further characterization. All isolates were Gram stained, and checked for ketolactose production, growth on peptone glucose agar medium, and congo-red absorption on YEMA as presumptive tests for rhizobia (Lupwayi and Haque, 1994).

Table 1**.** Woody legumes and the study sites.

Woody legume	Site	Geographic location
Dalbergia lactea	Belete forest	$7^0$8'22" N and $36^0$7'3" E
Indigofera species	Belete forest	$7^0$8'22" N and $36^0$7'3" E
	Fenchir	$8^0$30.2' N and $37^0$58.5' E
	Harawe	Not determined
	Ganda Chero	Not determined
	Konso	$5^0$20'5' N and $37^0$25"39' E
	Hammer	$5^0$11' 19" N and $36^0$32' 24" E
Crotalaria species	Gojeb	$7^0$24' N and $36^0$22'29" E
	Gelogalana	Not determined
	Errer Valley	$9^0$14' N and $42^0$15' E
	Grawa	Not determined
	Haramaya University campus	$9^0$24' N and $42^0$1' E
Crotalaria spinosa	Konso	$5^0$20'5" N and $37^0$25'39" E
	Afer town	$5^0$31'20" N and $36^0$43'51" E
Chamaecytisus palmensis	Dangla	Not determined
Tephrosia species	Addis Kidam	Not determined
Aeschynomen schimperi	Fogera field	Not determined
Aeschynomen elaphroxylon	Konso	$6.5^0$12'63" N and$33^0$41'93" E
Erythrina brucei	AAU, Science Faculty	$8^0$59'57" N and 38^0 4'18.5"

Characterization of the isolated rhizobial strains

All characteristics were assessed by inoculating them with 10^6 cells ml^{-1}, pH 6.8, and then incubating them for 4–10 days at 28°C$\pm$ 2, unless stated otherwise. All tests were made in triplicate, including the control plates. Colony morphology was determined on the basis of colony size (diameter), shape, texture, and gum production, according to Ahmed *et al*. (1984). The production of acid or alkali by a given strain was detected by incorporating 0.5 % bromthymol blue (BTB) into a litre of YEMA, as described by Lupwayi and Haque (1994).

One millilitre of isolate (10^6 cells), cultured for 48 hours, was inoculated into 100 ml sterilized YEMA broth in a 250 ml Erlenmeyer flask and placed on an orbital shaker at 150 rpm at room temperature. Optical density (OD_{540}) was measured every 6 hours using a spectrophotometer (Jenway, 6405 Uv/vis spectrophotometer). Samples were simultaneously diluted (10^{-1} to 10^{-10} with sterile distilled water), and a 0.1 ml sample from each dilution was spread onto sterilized YEMA plates to determine the colony forming units (CFUs) (Somasegaran and Hoben, 1994). Finally, the generation time (g) was calculated from the logarithmic phase according to White (1995).

The different physiological activities of isolates were assessed based on carbohydrate utilization, ecological response to temperature, pH and salt concentration, and antibiotic resistance. The ability of isolates to utilize different carbon sources was evaluated on monosaccharides (D-glucose, D-fructose, D-galactose, D-arabinose, D-mannose, and xylose), disaccharides (maltose, lactose, trehalose, D-sucrose, and cellobiose), sugar alcohols (mannitol, glycerol, and sorbital), and organic salts (sodium citrate and sodium glutamate), according to Somasegaran and Hoben (1994). D-glucose, D-fructose, lactose, D-sucrose and mannitol were sterilized by autoclaving at 121^0C for 15 minutes while all the rest were sterilized using a millipore filter (0.22 μm) since they are heat labile.

The evaluation of the growth of the rhizobial strains at different pH values was made by using Keyser-defined media, according to Lupwayi and Haque (1994). The pH of the medium was adjusted to the desired levels (pH 4–10.5) by using 1 N HCl or NaOH. Temperature tolerance was evaluated by inoculating the isolates on YEMA medium and incubating them at temperatures of 5, 10, 15, 20, 35, 40 and 45^0C. The ability of the rhizobial isolates to tolerate NaCl was also tested by streaking isolates into YEMA plates containing 1, 2, 3, 4, 5 and 6 % NaCl (w/v). Bacterial growth was evaluated qualitatively as (–) for no growth and (+) for positive growth.

The inherent antibiotic resistance of the strains was tested on YEMA medium by incorporating seven different filter-sterilized antibiotics (chloramphenicol, streptomycin sulfate, rifampcin, erythromycin, kanamycin monosulfate, neomycin sulfate, and ampicillin sodium sulfate, at 10, 20 and 30 μg/ml, following the method of Lupwayi and Haque (1994). The phosphate-solubilizing ability of the isolates was tested on Pikovskaya's medium (Tewari *et al*. 2004) and examined for the formation of clear zones around the colonies when sufficient growth was observed.

Numerical analysis was performed based on the phenotypic variabilities of the isolates using a similarity coefficient. A phenotypic dendrogram tree was constructed by using unweighed pair group method with the average, UPGMA (NT-SYS version 2.1) software.

Cross-inoculation and symbiotic effectiveness of isolates on tropical pulses

Bacterial isolates and host legumes

Four woody legume isolates : AURE2 (from *Erythrina brucei*), AURI30 (from *Indigofera*), AURC36 (from *Crotalaria*), and AURT2 (from

Tephrosia) were selected, since their host plants are more important agronomically and ecologically as well as more diversified and widely distributed, and tested for their ability to form root nodules and effective symbiosis with common bean (*P. vulgaris*) *and* cowpea (*V. unguiculata*). The seeds of *P. vulgaris* (Awash I Variety) and *V. unguiculata* (White Wonder Trealing Variety) were obtained from The Ethiopian Institute of Agricultural Research, Melkasa Research Center.

Four surface-sterilized seeds (exposed to 95% ethanol for 10 seconds and then to sodium hypochlorite for 4 minutes) were sown into acid-washed sand in a 1.5 kg capacity pot. The seedlings were thinned to three plants after 1 week. Each isolate was grown in YEMA broth in a 250 ml Erlenmeyer flask for 2–4 days, and 1 ml of a culture (10^9 cells) was pipetted to the base of a seedling 7 days after emergence (Vincent, 1970). Three pots, each fertilized with 120 ml of 0.05% KNO_3 solution once-weekly (Maatallah *et al*., 2002), were added as a positive control. Three unfertilized and uninoculated pots were also included as negative controls. The pots were arranged in a randomized complete block design in the greenhouse at the Science Faculty, with a 12-hour photoperiod, and average day and night temperatures of 31^o and 12^oC, respectively. Each pot was provided with quarter-strength N-free nutrient solution (Broughton and Dilworth, 1970) once a week and sterile distilled water every two days. The plants were harvested 8 weeks after planting, nodules were counted, and nodule and shoot dry weight was determined after drying them in paper bags at 70^oC for 48 hours (Somasegaran and Hoben, 1994). The total nitrogen content of the shoots was determined using the modified 'Wet' Kjeldahl method, as described by Sahlemedhin Sertsu and Taye Bekele (2000).

Data analysis

One-way analysis of variance (ANOVA) of the data was performed using version 10 of the SPSS statistical program. Mean separation was calculated using the Turkey's value when the F-test was significant at p=0.05. Symbiotic effectiveness of the tested isolates was calculated according to Lupwayi and Haque (1994), as percentage of shoot dry weight of plants inoculated with the test strain to shoot dry weight of plants supplied with nitrogen fertilizer.

RESULTS AND DISCUSSION

All the isolates were found to be Gram-negative rods; they failed to absorb Congo Red except AURI34, to produce 3-ketolactose, and to grow on peptone glucose agar, that are typical characteristics of root nodule bacteria

(Lupwayi and Haque, 1994). Most of the isolates formed dome-shaped colonies with a shiny appearance and a buttery texture. All of the isolates except AURC42 produced gelatinous colonies due to the production of various exopolysaccharides,.

The mean generation time of the isolates ranged from a minimum of 1.3 hours to a maximum of 11.4 hours (Table 2). Isolates of *Indigofera* (AURI30), *Tephrosia* (AURT2), *Aeschynomene schimperi* (AURA13), and *Aeschynomene elaphroxylon* (AURA28) achieved a mean generation time of less than 4 hours so that they were grouped as fast-growing rhizobia, whereas the rest of the isolates were grouped as slow growers according to Zerhari *et al*. (2000). The nodulation pattern of the isolates showed that *Indigofera* is nodulated by both slow-growing and fast-growing root-nodule bacteria; *Crotalaria*, *Dalbergia*, *Chamaecytisus palmensis*, and *Erythrina brucei* are nodulated by slow-growing rhizobia, whereas *Tephrosia* and *Aeschynomene* are nodulated by fast-growing rhizobia. Furthermore, the nodulation pattern clearly indicated that tree legumes are nodulated by both fast-growing and slow-growing rhizobia, in agreement with several investigators (Turk and Keyser, 1992; Fassil Assefa, 1993; Moreira *et al*., 1998; Endalkachew Wolde-Meskel *et al*., 2004; Endalkachew Wolde-Meskel *et al*., 2005). This is contrary to the early report of (Allen and Allen, 1936) that tree legumes are exclusively nodulated by slow-growing rhizobia.

Table 2. Cultural characterization of the rhizobial isolates.

Host woody legume	Rhizobial isolates	Mean generation time (hr)	Colony size (mm)	*BTB reaction
Dalbergia lactea	AURD2	11.4	1.4	blue
Indigofera species	AURI6	6.2	1.0	blue
Indigofera species	AURI14	5.9	1.0	blue
Crotalaria species	AURC22	9.6	2.4	yellow
Erythrina brucei	AURE2	6.2	2.4	blue
Crotalaria species	AURC23	5.1	2.0	blue
Indigofera species	AURI30	3.7	4.0	yellow
Indigofera species	AURI32	4.1	1.0	blue
Crotalaria species	AURC36	9.6	3.0	yellow
Crotalaria species	AURC42	10.3	1.0	blue
Crotalaria species	AURC48	5.0	2.0	blue
Chamaecytisus palmensis	AURCp2	4.2	1.5	blue
Tephrosia species	AURT2	3.5	3.0	yellow
Aeschynomene schimperi	AURA13	1.3	5.0	yellow
Indigofera spinosa	AURI21	4.8	1.5	blue
Crotalaria species	AURC24	11.0	1.4	blue
Aeschynomene elaphroxylon	AURA28	3.9	2.0	yellow
Indigofera spinosa	AURI34	10.5	2.4	blue
Indigofera spinosa	AURI36	6.1	3.2	blue
Crotalaria species	AURC37	5.1	1.8	blue

*- bromthymol blue.

The BTB reaction revealed that alkali production occurred in all of the isolates except the four fast growers and two slow growers (AURC22 and AURC36), which produced acid. Although acid production and alkali production are typically the characteristics of fast-growing and slow-growing rhizobia, respectively, Odee *et al*. (1997) and Endalkachew Wolde-Meskel *et al*. (2004) isolated some fast-growing alkali-producing and some slow-growing acid-producing rhizobia from woody legumes. However, fast-growing alkali-producing rhizobia were not encountered in this study.

Most of the isolates (86 %) catabolized and grew on all the tested carbon sources except sodium-citrate, which was utilized by only two of the isolates (Table 3). Both fast- and slow-growing isolates showed a similar pattern of carbon source utilization. The ability of isolates to utilize different carbon sources can give a competitive advantage when they live as saprophytes in the soil.

Table 3. Carbon source utilization of the isolates.

Rhizobial isolates	Carbon source															
	D–glucose	D–fructose	D–galactose	D–arabinose	D–mannose	Xylose	Maltose	Lactose	Trehalose	D–sucrose	Cellobiose	Mannitol	Glycerol	Sorbitol	Na–citrate	Na–glutamate
AURD2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURI6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURI14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURC22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURE2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURC23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURI30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURI32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURC36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURC42	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURC48	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURCp2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURT2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURA13	+	+	+	–	+	+	+	+	+	+	–	+	–	+	+	+
AURI21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	
AURA28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURI34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURI36	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
AURC37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+

(+) = growth and (–) = no growth

Only the *Aeschynomene schimperi* (AURA13) isolate was able to grow at pH values of 4 and 4.5, whereas all the isolates were able to grow at pH levels ranging from 5.5 to 9.5 (Table 4). The slow-growing alkali-producing

isolates grew better at alkaline pH levels (7.5–9.5) rather than at lower pH levels (4–5). Similarly, Grossman *et al*. (2005) reported the high acid susceptibility of slow-growing strains of rhizobia isolated from a leguminous tree of the genus *Inga*. However, the slow-growing bradyrhizobial isolates of *Acacia saligna* were found to be alkali-sensitive and acid-tolerant (Marsudi *et al*., 1999). This growth over a wider pH range implies that the isolates can be good candidates as inoculants over the indicated pH range.

Table 4. Growth of the isolates at different pH levels.

Rhizobial isolates	pH level											
	4	4.5	5	5.5	6	7.5	8	8.5	9	9.5	10	10.5
AURD2	–	–	+	+	+	+	+	+	+	+	+	–
AURI6	–	–	+	+	+	+	+	+	+	+	+	+
AURI14	–	–	+	+	+	+	+	+	+	+	+	+
AURC22	–	–	+	+	+	+	+	+	+	+	+	+
AURE2	–	–	+	+	+	+	+	+	+	+	+	+
AURC23	–	–	–	+	+	+	+	+	+	+	+	+
AURI30	–	–	–	+	+	+	+	+	+	+	–	–
AURI32	–	–	+	+	+	+	+	+	+	+	+	+
AURC36	–	–	–	+	+	+	+	+	+	+	+	+
AURC42	–	–	–	+	+	+	+	+	+	+	+	+
AURC48	–	–	–	+	+	+	+	+	+	+	+	+
AURCp2	–	–	+	+	+	+	+	+	+	+	–	–
AURT2	–	–	–	+	+	+	+	+	+	+	+	+
AURA13	+	+	+	+	+	+	+	+	+	+	+	+
AURI21	–	–	+	+	+	+	+	+	+	+	+	+
AURC24	–	–	+	+	+	+	+	+	+	+	+	–
AURA28	–	–	+	+	+	+	+	+	+	+	+	+
AURI34	–	–	–	+	+	+	+	+	+	+	+	+
AURI36	–	–	+	+	+	+	+	+	+	+	–	–
AURC37	–	–	+	+	+	+	+	+	+	+	+	+

(+) = growth and (–) = no growth

Only three isolates were able to grow at 5°C (Table 5), but more than 90 % and 47 % of the isolates tolerated and grew at 35°C and 40°C, respectively. Thus, most of the isolates can overcome high soil temperature, which is one of the major problems for biological nitrogen fixing in tropical and subtropical areas (Michiel *et al*., 1994). Eighty-one percent of the isolates failed to tolerate 1.0 % NaCl (w/v). Three isolates (AURA13, AURI21 and AURC24) tolerated up to 6 % NaCl (Table 6), similar to root-nodule bacteria from woody legumes such as *Acacia*, *Prospis*, and *Leucaena*, most of which were found to be tolerant up to 5 % NaCl (Lal and Khanna, 1995).

Table 5. Growth of the isolates at different temperature levels.

Rhizobial isolates	Température (°C)						
	5	10	15	20	35	40	45
AURD2	–	+	+	+	–	–	–
AURI6	–	+	+	+	+	–	–
AURI14	+	+	+	+	+	+	+
AURC22	–	+	+	+	+	–	–
AURE2	–	+	+	+	+	–	–
AURC23	–	–	+	+	+	+	+
AURI30	+	+	+	+	–	–	–
AURI32	–	–	+	+	+	+	–
AURC36	–	–	+	+	+	+	–
AURC42	–	–	+	+	+	+	+
AURC48	–	+	+	+	+	–	–
AURCp2	–	+	+	+	+	–	–
AURT2	–	–	+	+	+	–	–
AURA13	+	+	+	+	+	+	–
AURI21	–	–	+	+	+	+	–
AURC24	–	–	–	+	+	+	+
AURA28	–	+	+	+	+	+	–
AURI34	–	–	+	+	+	+	+
AURI36	–	+	+	+	+	–	–
AURC37	–	–	+	+	+	–	–

(+) = growth and (–) = no growth

Table 6. NaCl tolerance of the isolates.

Rhizobial isolates	Percentage NaCl (w/v)					
AURD2	1	2	3	4	5	6
AURI6	–	–	–	–	–	–
AURI14	–	–	–	–	–	–
AURC22	–	–	–	–	–	–
AURE2	–	–	–	–	–	–
AURC23	–	–	–	–	–	–
AURI30	–	–	–	–	–	–
AURI32	–	–	–	–	–	–
AURC36	–	–	–	–	–	–
AURC42	–	–	–	–	–	–
AURC48	–	–	–	–	–	–
AURCp2	–	–	–	–	–	–
AURT2	–	–	–	–	–	–
AURA13	+	+	+	+	+	+
AURI21	+	+	+	+	+	+
AURC24	+	+	+	+	+	+
AURA28	–	–	–	–	–	–
AURI34	+	+	+	–	–	–
AURI36	–	–	–	–	–	–
AURC37	–	–	–	–	–	–

(+) = growth and (–) = no growth

The majority of the isolates were found to be more sensitive to streptomycin sulfate, followed by kanamycin monosulfate, than the other antibiotics – all

tested at concentrations of 10, 20 and 30 µg ml^{-1} (Table 7). In contrast, all the isolates were tolerant to ampicillin sodium sulfate except AURI34, which failed to tolerate all the tested antibiotics at the indicated concentrations. Similarly, Odee *et al*. (1997) found that most rhizobia that were isolated from woody legumes in Kenya were more sensitive to streptomycin than kanamycin monosulfate and ampicillin.

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

Rhizobial isolates	Antibiotics and their concentrations (µg ml^{-1})																				
	Chloramphenicol			Streptomycin sulfate			Rifampicin			Erythromycin			Kanamycin monosulfate			Neomycin sulfate			Ampicillin sodium salt		
	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
AURD2	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+
AURI6	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURI14	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURE2	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+
AURC23	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURI30	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	+	+	+
AURI32	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC36	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC42	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURC48	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURCp2	+	+	+	–	–	–	+	+	+	+	–	–	–	–	–	+	–	–	+	+	+
AURT2	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	+	+	+	+	+	+
AURA13	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–	+	–	–	+	+	+
AURI21	+	+	+	+	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
AURC24	+	+	+	+	–	–	+	+	+	+	+	+	+	–	–	+	+	+	+	+	+
AURA28	+	+	+	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
AURI34	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
AURI36	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	+	+	+	+	+	+
AURC37	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

(+) = growth and (–) = no growth

On testing the phosphate-solubilizing ability of the isolates, three of the isolates (AURT2, AURI21, and AURCp2) formed narrow clear zones around their colonies, whereas AURI30 and AURA13 formed relatively wider clear zones, thus indicating better phosphate-solubilizing ability. Numerical analysis of the phenotypic data of the isolates also revealed their

diversity. At a boundary level of 20 % average similarity, all the isolates were grouped into one major cluster, except an isolate of the *Indigofera* species (AURI34) *and A. schemperi* (*AURA*13) (Fig. 1). The cluster contained 18 of the 20 isolates that were isolated from root nodules of *A. elaphroxylon, Chamaecytisus palmensis*, and *Erythrina brucei*, and species of *Dalbergia, Indigofera, Crotalaria*, and *Tephrosia*. At a boundary level of 60% similarity, all the isolates were grouped into four clusters, except isolates AURI34, AURI30, AURA13, AURT2, AURI36, and AURCp2, which were not clustered with any of the other isolates. In general, the isolates did not cluster on the basis of site or host of isolation, similar to the findings of Odee *et al*. (1997), and in contrast to the early groupings of rhizobia on the basis of cross inoculation group concept (Fred *et al*., 1932).

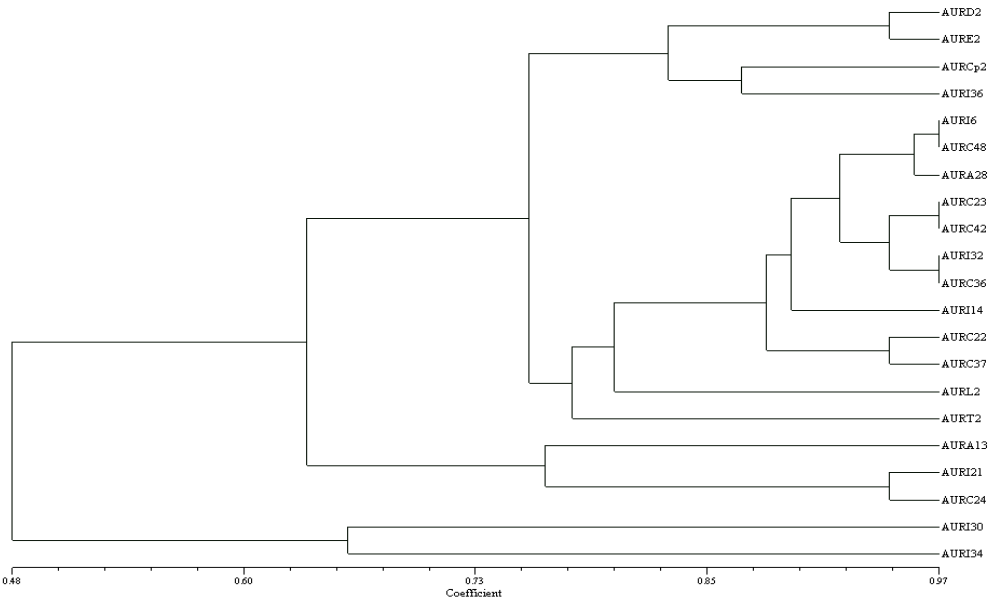

Fig .1. Dendrogram highlighting phenotypic similarity of the isolates.

All of the four inoculated isolates (AURE2, AURC36, AURT2, and AURI30) elicited root nodules on *P. vulgaris*, but AURI30 failed to do so on *V. unguiculuta* under the same greenhouse conditions. This failure is best explained in terms of genetic incompatibility of the isolate and the heterologous host legume. The nodulation of common bean (*P. vulgaris*) by all of the four tested isolates and cowpea (*V. unguiculata*) by three of the isolates (AURC36, AURE2, and AURT2) indicates the symbiotic

promiscuity of these grain legumes that has been reported by several researchers (Allen and Allen, 1981; Bala and Giller, 2001). It also indicates the ability of rhizobial isolates from tropical woody legumes to promiscuously nodulate tropical pulse crops (Allen and Allen, 1981; Wang, 1989; Fassil Assefa and Kleiner, 1997).

The nodulated cowpea and common bean plants showed better growth with greener leaves (Fig. 2 and Fig. 3), higher accumulated shoot dry weight, and total nitrogen than the uninoculated control plants (Table 8 and Table 9). Moreover, the isolates showed variations in nodule number, nodule dry weight, shoot dry weight, and total nitrogen, as well as symbiotic effectiveness (Table 8 and Table 9).

Table 8. Evaluation of symbiotic effectiveness of the nodulated isolates with *Vigna unguiculate.*

Treatment	NN/Plant	NDW (mg/plant)	SDW (mg/plant)	TN (%)	Symbiotic effectiveness (%)
AURC36	111[a]	54[ab]	473[ab]	1.57[b]	87
AURE2	132[a]	65[a]	356[bc]	1.91[a]	65
AURT2	25[b]	40[b]	225[cd]	1.20[c]	41
N+	–	–	543[a]	1.36[bc]	–
N–	–	–	89[d]	0.16[d]	–

Note:
NN = nodule number, NDW = nodule dry weight, SDW = shoot dry weight, TN = total nitrogen
Numbers are the means of variables of three replicates of three plants per pot.
Letters in the columns (a, b, c and d) are ranks of the means.
Numbers in the same column with different letters are significantly different at 0.05 level (Turkey HSD).

Table 9. Evaluation of symbiotic effectiveness of the nodulated isolates with *Phaseolus vulgaris.*

Treatment	NN/Plant	NDW (mg/plant)	SDW (mg/plant)	TN (%)	Symbiotic Effectiveness (%)
AURC36	120[a]	77[a]	409[a]	1.63[a]	112
AURE2	63[b]	28[b]	256[ab]	1.11[ab]	70
AURT2	79[b]	50[ab]	284[ab]	1.00[abc]	78
AURI30	73[b]	54[ab]	398[a]	0.65[bc]	109
N+	–	–	365[ab]	1.61[a]	–
N–	–	–	160[b]	0.20[c]	–

Note:
NN = nodule number, NDW = nodule dry weight, SDW = shoot dry weight, TN = total nitrogen.
Numbers are the means of variables of three replicates of three plants per pot.
Letters in the columns (a, b, c and d) are ranks of the means.
Numbers in the same column with different letters are significantly different at 0.05 level (Turkey HSD).

A.

B.

AURC36 AURE2 AURT2

Fig 2. A. *Vigna unguiculata* plants inoculated with isolate AURE2, AURC36, AURI30 and AURT2; and N+ and N- control plants.

B. Nodules induced by isolate AURC36, AURE2 and AURT2 on *Vigna unguiculate.*

A.

B.

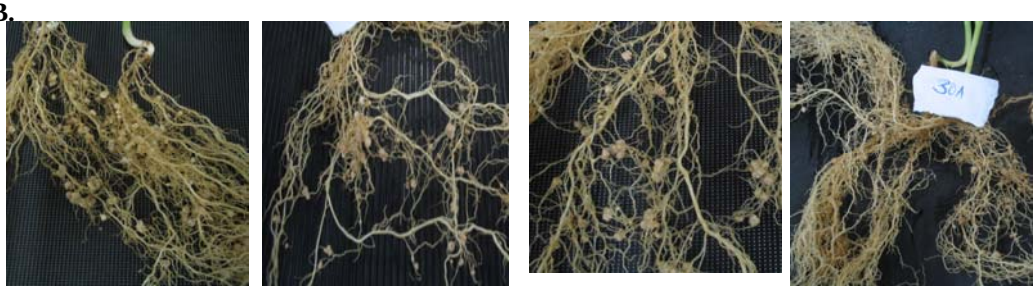

Fig. 3. A. *Phaseolus vulgaris* plants inoculated with isolate AURC36, AURI30, AURE2, and AURT2; and N+ and N- control plants
B. Nodules induced by isolate AURC36, AURE2 and AURT2 and AURI30 on *Phaseolus vulgaris*

Isolates of *Crotalaria* (AURC36) accumulated the maximum shoot dry weight of 473 mg plant^{-1} on *V. unguiculata* and 409 mg plant^{-1} on *P. vulgaris*. Symbiotic effectiveness varied from a minimum of 41 % in the *V. unguiculata*–AURT2 (from *Tephrosia*) association to a maximum of 112 % in the *P.vulgaris*–AURC36 (from *Crotalaria*) association (Table 8 and Table 9). AURC36 also achieved higher symbiotic effectiveness (87 %) than the other inoculated isolates on cowpea. Variation in the symbiotic effectiveness of the isolates suggests the presence of specific host-strain requirements. The maximum total nitrogen (1.91 %) was accumulated by isolates of *Erythrina brucei* (AURE2) on *V. unguiculata*, followed by AURC36 (from *Crotalaria*) on *P. vulgaris*

Shoot dry weight was found to be positively correlated with total nitrogen (Pearson's correlation, r=0.6) at a significance level of 0.01 (two-tailed) both in the case of *P. vulgaris* and *V. unguiculata (*Table 10). However, higher shoot dry weight was not necessarily associated with higher total nitrogen percent. For instance, *V. unguiculata* plants that were fertilized with N_2 and that acquired a mean shoot dry weight of 543 mg plant^{-1}, or those that had been inoculated with AURC36 and that had acquired a mean shoot dry weight of 473 mg plant^{-1}resulted in a significantly (α = 0.05) lower total N_2 percent than those inoculated with AURE2 and that acquired a mean shoot dry weight of 356 mg plant^{-1}. Such observations were explained by Bala and Giller (2001) in terms of the nitrogen use efficiency to produce dry matter per unit nitrogen accumulated.

Table 10. Correlation test of shoot dry weight and total nitrogen percent of common bean (A) and cowpea (B) plants.

A.

		Shoot dry weight	Total nitrogen percent
Shoot dry weight	pearson correlation	1	0.6**
	Sig.(2-tailed)	.	0.002
	N	24	24
Total N-percent	pearson correlation	0.6**	1
	Sig.(2-tailed)	0.002	.
	N	24	24

B.

		Shoot dry weight	Total nitrogen percent
Shoot dry weight	pearson correlation	1	0.6**
	Sig.(2-tailed)	.	0.0
	N	45	45
Total N-percent	pearson correlation	0.6**	1
	Sig.(2-tailed)	0.0	.
	N	45	45

** Correlation is significant at the 0.01 level (2-tailed).

CONCLUSION

Variations in the morphological and physiological characteristics of root nodule bacterial isolates of woody legumes included in the study showed their diversity. The growth response of the isolates to various carbon sources, antibiotics, pH and temperature values, and NaCl concentrations can assist one in their laboratory culturing and can give clue regarding the potential of the isolates to use as field inoculants, though laboratory observations may not necessarily be extrapolated to field conditions. The selected, cross-inoculated isolates also differed from failure to inducing root nodules and achieving symbiotic effectiveness higher than 100% on grain legumes. This observed difference is practically significant to decide which of the grain legumes should be better cultivated in close association to which of the woody legumes in agroforestry, in tree fallow or alley cropping system, to improve the yield of the grain.

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REFERENCES

Ahmed, M., Rafique, M. and Mc Langhlin, W. (1984). Characterization of indigenous rhizobia from wild legumes. *FEMS Microbiol. Lett*. **24**: 197-203

Allen, O.N. and Allen, E.K. (1936). Root nodule bacteria from some tropical leguminous plants. I. Cross nodulation study with *Vigna sinensis*. *Soil Sci.*. **42**:61-77.

Allen, O.N. and Allen, E.K. (1981). **The Leguminosae: A source book of characterstics, uses and nodulation.** University of Wisconsin Press; Madison, Wi and Mac Millan , London, 812 pp.

Bala, A. and Giller, K.E. (2001). Symbiotic specificity of tropical tree rhizobia for host legumes. *New Phytol.* **149**: 495–507.

Broughton, W.J. and Dilworth, M.J. (1970). Control of leghaemoglobin synthesis in snake beans. *Biochem. J*. **125**:1075–1080.

Dommergues, Y.R. (1988). Future direction for biological nitrogen fixation research. *Plant Soil*. **108**:191–199.

Endalkachew Wolde-meskel, Berg, T., Peters, N.K. and Frostegard, A. (2004). Nodulation status of *Acacia* spp. and other native woody legumes, and phenotypic characteristics of associated rhizobia in soils of southern Ethiopia. *Biol. Fert. Soils*. **40**:55–66.

Endalkachew Wolde-meskel, Zewdu Terefework, Frostegard, A. and Lindstrom, K. (2005). Genetic diversity and phylogeny of rhizobia isolated from woody legumes in southern Ethiopia. *Int. J. Syst. Evol. Micr.* **55**:1439–1452.

Fassil Assefa (1993). **Nodulation and nitrogen fixation by *Rhizobia* and *Bradyrhizobia* spp. of some indigenous tree legumes of Ethiopia.** PhD. dissertation. University of Bayreuth, Germany,108 pp.

Fassil Assefa and Kleiner, D. (1997). Nodulation of African yam bean (*Sphenostylis stenocarpa*) by *Bradyrhizobium* spp. isolated from *Erythrina brucei*. *Biol. Fert. Soils* **25**:209–210.

Fred, E.B., Baldwia, I.L. and Mc Copy, E. (1932). **Root nodule bacteria and leguminous plants.** Madison, WI, USA. University of Wisconsin Press.

Gepts, P., Beavis, W.D., Brummer, E.C., Shoemaker, R.C., Stalker, H.T., Weeden, N.F. and Young, N.D. (2005). Legumes as a model plant family: Genomics for food and feed report of cross-legume advances through genomics conference. *Plant Physiol.* **137**:1228–123.

Giller, K.E. (2001). **Nitrogen fixation in tropical system.** CABI publishing, Wallingford, UK.

Graham, P.H. and Vance, C.P. (2003). Legumes: Importance and constraints to greater use. *Plant Physiol*. **131**:872-877.

Grossman, J.M., Sheaffer, C., Wyse, D., Brucciarelli, B., Vance, C. and Graham, P.H. (2005). An assessment of nodulation and nitrogen-fixation in inoculated *Inga oestediand*, a nitrogen-fixing tree shading organically grown coffee in Chiapas, Mexico. *Soil Biol. Biochem.* **20**:1–16.

Herrera, M.A., Swamanca, C.P and Barea, J.M. (1993). Inoculation of woody legumes with selected mycorrhizal fungi and rhizobia to recover desertified Mediterranean ecosystems. *Appl. Environ. Microbiol*. **59**:129–133.

Lal, B. and Khanna, S. (1995). Selection of salt tolerant Rhizobium isolates of *Acacia nilotica*. *World J. Microb. Biot.* **10**:637–639.

Legesse Negash (2002). Review of research advances in some selected African trees with special reference to Ethiopia. *Ethiop. J. Biol .Sci*. **1**(1):81-127.

Lupwayi, N.Z. and Haque, I. (1994). **Legume-rhizobium technology manual.** Working document. No. 29. Environmental Science Division. International Livestock Center for Africa, Addis Ababa, Ethiopia. pp. 1-93

Maatallah, J., Berroho, E.B., Sanjuan, J. and Lluch, C. (2002). Phenotypic characterization of rhizobia isolated from chickpea(*Cicer arietinum*) growing in Moroccan soil. *Agronomie* **22**: 321-329.

Marsudi, N.D.S., Glenn, A.R. and Dilworth, M.J. (1999). Identification and characterization of fast- and slow-growing root nodule bacteria from southwestern Australian soils able to nodulate *Acacia saligna*. *Soil Biol. Biochem.* **31**:1229–1238.

Michiel, J., Verrech, C. and Vanderleyden, J. (1994). Effect of temperature stress on bean nodulating rhizobium strains. *Appl. Microbiol.* **60**:1206–1212.

Moreira, F.M.S., Haukka, K. and Young, J.P.W. (1998). Biodiversity of rhizobia isolated from a wide range of forest legumes in Brazil. *Mol. Ecol.* **7**:889–895.

Odee, D.W., Sutherland, J.M., Makatiani, E.T., McInroy, S.G. and Sprent, J.I. (1997). Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. *Plant Soil* **188**:65–75.

Rengel, Z. (2002). Breeding for better symbiosis. *Plant Soil* **245**:147-162.

Sahlemedhin Sertsu and Taye Bekele (2000). **Procedures for Soil and Plant Analysis.** National Soil Research Center, Ethiopian Institute ofAgricultural Research, Addis Ababa.

Somasegaran, P. and Hoben, H.J. (1994). **Handbook for Rhizobia: Methods in legume-rhizobium technology**. Springer-Verlay, New York. 441 pp.

Tewari, R.P., Hoodal, G.S. and Tewari, R. (2004). **Laboratory techniques in microbiology and biotechnology.** Abhishek Publications, Chandigarh (India).

187 pp.

Thulin, M. (1989). Fabaceae. In: **Flora of Ethiopia**, Vol. 3. pp. 71–96 (Hedberg, M. and Edwards, S., eds.). The National Herbarium, Addis Ababa University.

Turk, D. and Keyser, H.H. (1992). Rhizobia that nodulate tree legumes: Specificity of the host for nodulation and effectiveness. *Can. J. Microbiol.* **38**:451–460.

Vincent, J. M. (1970). **A manual for practical study of root nodule bacteria.** Blackwell Science Publication, Oxford. 164 pp.

Vinuesa, P., Rademark,W., deBruijn, F.J. and Werner, D. (1998). Genotypic characteristics of Bradyrhizobium strains endemic to woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16s rRNA(16s rDNA) and 16s-23s rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic finger printing and partial 16s rDNA sequencing. *Appl. Environ. Microbiol*. **64**(6): 2096-2104.

Wang, S.S. (1989). Response of groundnut (*Arachis hypogea*) to inoculation with rhizobium strains isolated from wild arboreal legumes. *MIRCEN J. Appl. Microbiol. Biotechnol*. **5**:135–141.

White, D. (1995). **The physiology and biochemistry of prokaryotes.** Oxford, Oxford University Press. 378 pp.

Zerhari, K., Aurag, J., Khbaya, B., Kharchaf, D. and Filali-Matouf, A. (2000). Phenotypic characterstics of rhizobia isolates nodulating *Acacia* species in the arid and Saharan regions of Morocco. *Lett. Appl. Microbiol.* **30**:351–357.