ABUNDANCE AND CHARACTERIZATION OF COWPEA MISCELLANY *RHIZOBIUM* FROM SUDANESE SOILS

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Summary—Sudan is the fourth largest exporter of groundnuts in the world, yet little is known concerning the plant-rhizobial symbiosis. A study was made on the abundance of groundnut-nodulating rhizobia in the soils of Sudan as related to soil properties and the duration since groundnuts were last planted. Also, physiological, serological and nitrogen-fixing characteristics of Sudanese rhizobia are reported. All but one of 32 sites contained more than 300 rhizobia g^{-1} soil capable of forming nodules on siratro (*Macropitium atropurpureum*). Several of these soils had never been planted to groundnut. A correlation matrix indicated no relationship was present between soil rhizobial populations and any of the measured soil properties, or between soil rhizobial populations and the time since groundnuts were last planted in the rotation. Individual isolates of *Rhizobium* from six legumes: groundnut (*Arachis hypogaea*), mung bean (*Vigna radiata*), lubia (*Dolichos lablab*), cowpea (*Vigna unguiculata*), pigeonpea (*Cajanus cajan*) and bambara groundnut (*Voandzeia subterranea*) were obtained from four locations in Sudan. All isolates were able to nodulate each of the six legumes when grown in sterile vermiculite. The isolates grew in 0.1% NaCl-amended media, but growth was variable in 2.0% amended media. Most isolates grew after exposure to moist heat for 15 min at 50°C. Optimum pH for growth was, in general, between pH 6 and 8. Agglutination reactions indicated isolates from groundnuts, as well as isolates from other legumes, belonged to several serological groupings. Some isolates formed a large number of nodules on a Sudanese groundnut cultivar, whereas other isolates formed only few nodules.

INTRODUCTION

Rhizobium strains with little symbiotic specificity are more common in the tropics than in temperate regions of the world. Groundnut (*Arachis hypogaea* L.), which is an important food legume in several developing countries, is nodulated by members of the cowpea miscellany (Fred *et al.*, 1932; Buchanan and Gibson, 1974). This large heterogeneous group of rhizobia is characterized by a high degree of symbiotic promiscuity, and members are thought to be slow growing and alkaline producing. Some inconsistencies, however, were reported in Sudan by Habish and Kheiri (1968), who claimed that their groundnut *Rhizobium* isolates were fast growing and acid producing. Furthermore, their isolates did not nodulate cowpea, which is not characteristic of the cowpea group. Elsewhere, several strains of fast-growing rhizobia capable of nodulating cowpea and soybean have been reported (Zablotowicz and Focht, 1981). These findings emphasize that further work is needed in classifying rhizobia of the tropics.

The distribution of *Rhizobium* strains has been shown to vary with soil pH (Damirgi *et al.*, 1967), temperature (Ahmed *et al.*, 1981), available and organic nitrogen (Bezdicsek, 1972) and with the frequency of occurrence of soybeans in crop rotations (Weaver *et al.*, 1972). Only limited research, however, has been conducted on the effect of such soil factors on groundnut-nodulating rhizobia, especially in the tropics.

Rhizobia vary in effectiveness in fixing atmospheric nitrogen. Tropical rhizobia may nodulate crops readily but may not supply all the required nitrogen for maximum yields (Nambiar and Dart, 1982). Gathering basic information concerning cowpea-type

rhizobia may help in identifying the factors that limit the legume-*Rhizobium* symbiosis. Accordingly, this work was conducted to:

(1) assess abundance of cowpea-type rhizobia in different production regions of Sudan and correlate the data to soil properties and the place of groundnuts in crop rotations;

(2) obtain *Rhizobium* isolates from fresh nodules of the commonly grown legumes in Sudan and study their growth and serological characteristics. Test under greenhouse conditions the infectivity and effectiveness in nitrogen fixation of the isolates with a cultivar of groundnut from Sudan.

MATERIALS AND METHODS

Soils and Rhizobium abundance

In June 1980, 32 sites in Sudan of varying soil types and management practices were sampled at the beginning of the rainy season (Table 1). The sites were chosen in geographical regions with existing groundnut production (central and south central Sudan) and with potential groundnut expansion (western Sudan, considered west of the Nile River; see Fig. 1). Three subsamples from each site were randomly taken from a 0- to 15-cm depth. Moisture and rhizobial numbers were determined within 24 h after sampling, and the rest of the soil samples were dried, crushed, sieved (<3 mm), and thoroughly mixed before chemical analyses were made.

Rhizobium numbers were determined in growth pouches (Weaver and Frederick, 1972) by using the MPN-technique (Vincent, 1970) with siratro (*Macropitium atropurpureum*) as the test plant. Ten g of each soil was added to 90 ml of sterile tapwater in

Table 1. Soil characteristics^a of the survey locations in Sudan

Soil	Location	Moisture (%)	pH		Organic		<i>Rhizobium</i> ^b No. (No. g ⁻¹)	Years ^c
			Suspension	Paste	C (%)	N (μg g ⁻¹)		
1	Abu Naama	9.89	8.2	7.0	0.431	599	1.1 × 10 ⁴	1
2	Abu Naama	10.13	8.9	7.9	0.463	588	2.4 × 10 ⁶	∞ ^d
3	Abu Naama	10.13	8.6	7.7	0.414	613	2.9 × 10 ⁴	1
4	Abu Naama	9.65	8.8	7.8	0.406	588	4.4 × 10 ⁴	1
5	Abu Naama	10.13	8.6	7.7	0.414	641	1.1 × 10 ⁵	1
6	Abu Naama	10.01	8.6	7.7	0.406	616	5.3 × 10 ⁴	2
7	Abu Naama	10.13	8.8	8.0	0.431	613	1.5 × 10 ⁴	1
8	Abu Naama	10.13	8.7	7.7	0.378	578	4.2 × 10 ⁴	2
9	Abu Naama	10.13	8.8	7.7	0.395	588	7.5 × 10 ⁴	4
10	Abu Naama	10.01	8.8	7.7	0.378	574	4.4 × 10 ⁴	3
11	Abu Naama	9.76	8.8	7.7	0.446	574	1.6 × 10 ⁵	1
12	Abu Naama	ND	ND	ND	ND	ND	1.1 × 10 ⁴	∞
13	Wad Medani	9.65	8.8	7.8	0.492	616	1.1 × 10 ⁴	∞
14	Wad Medani	15.20	8.6	7.9	0.481	602	1.5 × 10 ⁴	2
15	Wad Medani	10.25	8.9	8.0	0.492	630	2.0 × 10 ⁴	1
16	Wad Medani	6.26	9.0	8.0	0.424	574	3.5 × 10 ⁴	3
17	Wad Medani	6.38	9.0	8.5	0.460	564	3.5 × 10 ⁴	2
18	Wad Medani	7.52	8.7	7.9	0.427	588	2.1 × 10 ⁴	4
19	Wad Medani	6.04	8.6	8.0	0.416	602	7.3 × 10 ⁴	1
20	Sennar	6.49	8.4	7.8	0.378	560	1.5 × 10 ⁴	3
21	Sennar	9.52	8.5	7.9	0.359	613	1.1 × 10 ⁴	1
22	Sennar	5.93	8.9	8.2	0.424	620	2.4 × 10 ⁴	2
23	Sennar	6.38	8.5	8.0	0.323	560	1.5 × 10 ⁴	4
24	Sennar	6.83	8.7	7.9	0.402	557	2.0 × 10 ⁴	3
25	Sennar	6.26	8.7	7.9	0.323	585	2.0 × 10 ⁴	1
26	Rahad	6.95	8.8	7.8	0.543	700	1.5 × 10 ⁴	1
27	Rahad	10.37	8.5	7.7	0.477	641	1.5 × 10 ³	∞
28	Rahad	10.37	8.7	8.0	0.158	406	2.7 × 10 ³	∞
29	Rahad	8.93	8.0	7.4	0.101	333	ND	1
30	El Obeid	8.93	6.6	5.9	0.082	322	3.0 × 10 ²	1
31	El Obeid	8.93	6.9	6.5	0.101	333	1.1 × 10 ³	1
32	El Obeid	10.13	6.8	6.1	0.946	1246	1.5 × 10 ⁴	∞

^aValues are the average of 3 determinations. The moisture percentage is the moisture content at time of sampling, suspension pH was measured with a glass electrode at a soil:H₂O ratio of 1:5, paste pH was measured in a saturated soil, organic carbon was measured by the Walkley-Black method, and organic nitrogen was measured by the Kjeldahl procedure.

^bPopulations are expressed on an oven-dry soil basis. The factor for determining 95% confidence limits is 4.68 (Cochran, 1950).

^cYears since last planted to groundnut.

^d∞ = Never planted to groundnut.

ND = Not determined.

dilution bottles at the Agricultural Research Corporation facilities at Wad Medani. One hundred, 3-mm dia glass beads were added, and each soil was shaken in a reciprocal shaker for 15 min. Serial

Fig. 1. Locations in Sudan where soil samples and rhizobial isolates were collected.

dilutions were made to give 10⁻³ to 10⁻⁶. The siratro seeds were surface sterilized with 95% ethanol and 0.2% HgCl₂ acidified with 5 ml HCl l⁻¹, followed by five washes in sterile water. Three seeds were then transferred to each growth pouch (Scientific Products, Division of American Hospital Supply Corporation, Moline, Illinois), which had been steamed for 15 min with flowing steam. One ml of the proper dilution for each soil was aseptically added to each pouch and replicated three times. Twenty ml of a sterile complete nutrient solution lacking nitrogen was added through alcohol-washed plastic straws to allow bottom watering. The growth pouches in rack holders were transferred to an air-conditioned room where the temperature was maintained at 30°C with a 14-h photoperiod provided by overhead fluorescent lighting. Sterile water was added as required through the straws. The pouches were inspected for nodulation after 2 months growth. The data were compiled and analyzed by constructing a correlation matrix.

Characterization of Sudanese rhizobia

In the second part of the study, *Rhizobium* isolations were made by using procedures described by Vincent (1970) from nodules collected in August during the rainy season from the commonly grown

Table 2. Legume of isolation and origin of the *Rhizobium* isolates used in the characterization study

Isolate no.	Legume of isolation		Origin*
	Common name	Scientific name	
1	Mung bean	<i>Vigna radiata</i>	Central Sudan (Wad Medani)
2	Pigeonpea	<i>Cajanus cajan</i>	Central Sudan (Wad Medani)
3	Cowpea	<i>Vigna unguiculata</i>	Central Sudan (Wad Medani)
4	Lubia	<i>Dolichos lablab</i>	Central Sudan (Wad Medani)
5	Bambara groundnut	<i>Voandzeia subterranea</i>	Western Sudan (El Obeid)
6	Groundnut	<i>Arachis hypogaea</i>	Central Sudan (Wad Medani)
7	Groundnut	<i>Arachis hypogaea</i>	South central Sudan (Sennar)
8	Groundnut	<i>Arachis hypogaea</i>	South central Sudan (Sennar)
9	Groundnut	<i>Arachis hypogaea</i>	Western Sudan (Kazgail)
10	Groundnut	<i>Arachis hypogaea</i>	Western Sudan (Kazgail)
11	Groundnut	<i>Arachis hypogaea</i>	Western Sudan (Kadugli)
12	8A11	—	Supplied by the Nitragin Company, Milwaukee, Wisconsin

*Soils from central Sudan, south central Sudan, and Kadugli in western Sudan are classified as Vertisols; soils from El Obeid and Kazgail in western Sudan are classified as Alfisols.

legumes in Sudan (Table 2). The Kazgail and Kadugli sites (Fig. 1) were not sampled for *Rhizobium* counting in June, but nodules were collected in August. Colony characteristics were studied on yeast extract mannitol (YEM) agar medium containing bromthymol blue as a pH indicator. Growth in media with different pH's, different sodium chloride levels and after moist-heat exposure followed the methods of Graham and Parker (1964). Mannitol and glucose utilization was tested at 28°C in basal mineral medium (YEM minus carbohydrate) in Wheaton-Nephelo flasks placed on a waterbath shaker (140 rev min⁻¹). Daily changes in percentage transmittance were monitored with a spectrophotometer at 525 nm.

Agglutination reactions were used to study the serological identity of the isolates. For antigen preparation, antiserum production, and agglutination reactions, the procedures described by Vincent (1970) were followed. A total of eleven isolates representing five legumes and four geographical locations within Sudan were tested for antigenic identity. Antisera developed for two groundnut isolates (Wad Medani and Kazgail) and a cowpea isolate were used for these tests. Readings were reported as strong agglutination (4), partial agglutination (3, 2, 1), or no agglutinations (0).

Infectivity and the relative efficiency of the isolates in fixing atmospheric nitrogen were determined under greenhouse conditions at Iowa State University by using the Sudanese groundnut cultivar "Barborton". Four-liter cans were fitted with autoclavable plastic bags, and a 1:1 (volume basis) sand-to-vermiculite mixture was added. Each can contained a glass tube extending to the bottom of the can through which water and nutrient solution were aseptically added. The glass tube was covered with a plastic Kaput to avoid direct dust contamination. Two hundred ml of complete nutrient solution lacking nitrogen was added for initial wetting of the cans. The cans and contents were sterilized for 4 h at 121°C. Groundnut seeds were sterilized by immersing in 30% H₂O₂ for 15 min followed by five rinses in sterile water. One seedling was aseptically transferred to each can, and 1 ml of the proper *Rhizobium* suspension (>10⁶ rhizobia) was aseptically added. Sterile pea gravel was added to a depth of 0.5 cm to prevent surface contamination. A nitrogen control and an uninoculated control were included as treatment variables, and

each treatment (Table 2) was replicated 4 times. The cans were positioned 60 cm apart in the greenhouse in a completely randomized design; positions were changed weekly. The temperature was regulated to give 30 ± 2°C with a 14-h photoperiod. After 2 months of growth, nodulation and tissue dry weights were evaluated as indicators of nitrogen fixation.

RESULTS AND DISCUSSION

Rhizobium abundance as influenced by soil characteristics

The soils sampled in June varied considerably in the properties measured (Table 1). Soil pH (paste) ranged from 5.9 to 8.5. In general, the soils from central and south central Sudan were heavy clays (classified as Vertisols) and had neutral to alkaline pH, and the soils from western Sudan were sands and had acid pH. Also, the organic C content ranged from 0.08% for a sandy soil collected in western Sudan to a characteristic 0.3–0.4% C for soils from central and south central Sudan. One exception was a soil from western Sudan that was collected under Hashab (*Acacia senegal*) seedlings and had 0.9% organic C and 1246 µg organic N g⁻¹ soil.

The correlation coefficients indicated no obvious relationship between MPN counts and soil properties, or between MPN counts and the length of time since groundnuts were last in the rotation (Table 3). Counts of the native rhizobial population capable of nodulating siratro showed that most of the soils in Sudan harbor a large population of rhizobia that potentially can nodulate groundnuts. Rhizobia were found in sites never planted to groundnuts (Table 1). With the exception of a sandy soil from western Sudan that contained fewer than 300 rhizobia g⁻¹ (lowest detection level of the test), 5 soils never before planted to groundnuts contained an average of 4.9 × 10⁵ rhizobia g⁻¹; one of the samples had 2.4 × 10⁶ rhizobia g⁻¹. This compares with averages of 2.3 × 10⁵, 3.6 × 10⁴, 2.9 × 10⁴ and 3.7 × 10⁴ rhizobia g⁻¹ obtained from soils last planted to groundnuts 1, 2, 3 and 4 years before sampling, respectively. Groundnut is the only legume commonly included in cultivated rotations in Sudan. Either rhizobia are naturally part of the indigenous soil population or native legumes (predominantly *Acacia* sp.) are serving as an inoculant source in

Table 3. Correlation coefficients (*r*) between soil characteristics and groundnut-nodulating rhizobia

Parameter	pH			Organic carbon	Total nitrogen	MPN	Years ^a
	Moisture	Suspension	Paste				
Moisture		-0.10	-0.20	-0.12	0.08	0.11	-0.02
pH (suspension)			0.94**	0.11	0.12	0.29	0.14
pH (paste)				0.04	0.05	0.16	0.13
Organic carbon					0.96**	0.11	0.18
Total nitrogen						0.08	0.14
MPN							-0.19

^aYears since last planted to groundnut.

**Significant at the 0.01 level of probability.

Sudan for rhizobia of the cowpea miscellany. The numbers of rhizobia in surface soil capable of nodulating siratro were higher in this study than those reported earlier from two soils in northern Nigeria, although Day *et al.* (1978) reported numbers to increase dramatically with increasing depth in the Nigerian soils.

Cultural and physiological characteristics

All the isolates tested were short, Gram-negative rods. The colonies were circular with entire margins and with raised to convex elevations. Though all the isolates tested were slow growers, some differences were evident. For example, isolates from lubia, bambara groundnut, groundnut (Kadugli) and pigeonpea could be placed in a group based on their first appearance on YEM-agar plates in 12 days and reaching a colony size of <1.0 mm in 14 days. The isolates from groundnut (Sennar) and the mung bean isolate appeared on plates in 10 days and reached a colony size of 1.0 mm in 14 days. The final group is represented by the Wad Medani strain, Sennar, and the Kazgail isolates (groundnut), which appeared on plates in 10 days and reached a colony size of >1.0 mm in 14 days of growth at 28°C.

Eight of 11 strains survived the moist-heat treatment for 15 min at 50°C. Graham and Parker (1964) reported no survival after this treatment, but Habish and Kheiri (1968) did report survival of some cowpea miscellany isolates.

Utilization of glucose and mannitol was similar except that the isolate from lubia utilized mannitol somewhat less efficiently than glucose. Was this related to less growth or less polysaccharide production from mannitol-grown cells? In a further experiment where cells were washed free of polysaccharide with phosphate buffered saline (5.6 g Na₂HPO₄, 2.7 g KH₂PO₄, and 4.1 g NaCl l⁻¹ adjusted to pH 7.2), and counted with a cell counter, results were similar to those without cell washing.

None of the strains grew at pH 4 and 10, but they did grow at pH 6 and 8. Isolates could not be differentiated by their relative rates of growth in media adjusted to the wide range of acidities tested.

Infectiveness of the isolates

All the isolates tested nodulated siratro and groundnut. This is at variance with the results of Habish and Kheiri (1968) who reported that groundnuts were not nodulated by isolates from cowpea, pigeonpea and lubia. The groundnut isolate from Medani gave a total of 161 nodules per plant, while the isolate from pigeonpea gave only five nodules per plant (Table 4). The two groundnut isolates from

Sennar (trts 7 and 8) and one isolate from Kazgail (trt 9) gave a relatively low number of nodules, suggesting that these strains were not as infective as other groundnut isolates.

The infectiveness of the isolates in forming nodules varied within as well as among locations. Whereas one isolate from Kazgail (western Sudan) gave 107 nodules per plant, the other isolate from the same field produced significantly (*P* < 0.05) fewer nodules (48 nodules per plant). Based on these nodulation results, it is justifiable to classify all the tested rhizobia as belonging to the cowpea miscellany.

Nitrogen fixation

Wynne *et al.* (1980) reported that nodulation, plant weight, nitrogenase activity and N content of greenhouse-grown groundnut were significantly correlated with each other. The isolated rhizobia in this study varied significantly in the number of nodules and top dry weights produced (Table 4). The groundnut isolate from Medani (trt 6) produced significantly more nodules than the other isolates, including the standard strain 8A11, but most of the nodules in this treatment had white interiors, and approximately 90% were small and located on lateral roots. The greater number of nodules did not result in greater top dry weights. Other groundnut isolates that produced many nodules were Kazgail (trt 10) and Kadugli (trt 11). The nodules of these isolates were mostly located on the main root, had red interiors, and were associated with large top dry weights. These results agree with those of Bromfield and Roughley (1980),

Table 4. Nitrogen-fixation traits of groundnut after inoculation with Sudanese *Rhizobium* isolates

Treatment	Isolate	Nodules ^a (no. plant ⁻¹)	Top dry weight ^a (g plant ⁻¹)
1	Mung bean	54.0def	0.62ab
2	Pigeonpea	5.0g	0.53ab
3	Cowpea	62.3cde	0.40b
4	Lubia	31.3efg	0.57ab
5	Bambara groundnut	93.3bcd	0.68ab
6	Groundnut (Wad Medani)	161.3a	0.61ab
7	Groundnut (Sennar)	36.0efg	0.42b
8	Groundnut (Sennar)	19.0fg	0.41b
9	Groundnut (Kazgail)	48.3ef	0.46ab
10	Groundnut (Kazgail)	107.0b	0.67ab
11	Groundnut (Kadugli)	109.7b	0.82a
12	8A11	95.0bc	0.60ab
13	Uninoculated control	0.0g	0.58ab
14	Nitrogen control	1.0g	0.81a

^aValues represent an average of 4 replications; means within the same column having a common letter are not significantly different at the 0.05 level of probability by the Duncan Multiple-Range Test.

Table 5. Serological cross reactivity of rhizobia isolated from different legumes and different locations in Sudan

Antigen	Antiserum		
	Groundnut (Wad Medani)	Groundnut (Kazgail)	Cowpea
Groundnut (Wad Medani)	4 ^a	4	4
Groundnut (Kazgail)	1	4	2
Groundnut (Kazgail)	0	0	0
Groundnut (Sennar)	4	2	2
Groundnut (Sennar)	2	0	0
Groundnut (Kadugli)	0	0	0
Cowpea	4	2	4
Green gram	0	4	4
Pigeonpea	1	0	4
Lubia	0	0	0
Bambara groundnut	0	0	0

^a Reactivity was evaluated by agglutination with 4 = strong agglutination, 3, 2, 1 = partial agglutination, and 0 = no agglutination.

who worked with cowpea miscellany isolates from Nigeria, in that the isolates varied in their nitrogen fixation efficiency but, in this study, differences among isolates in top dry weights generally were small. This perhaps was related to the lack of robustness of the Sudanese cultivar used in the tests.

Serological identity of the isolates

Several of the groundnut rhizobia reacted with antisera produced from isolates from Medani, Kazgail and the cowpea isolate (Table 5). Cross reactivity was found and several of the strains evidently had common antigenic sites leading to partial agglutination. Four isolates failed to react with any of the tested antisera.

This was an initial attempt, based on a limited sampling, to characterize Sudanese rhizobial isolates. Cowpea-type rhizobia obviously vary at different locations, and alternate hosts are capable of providing rhizobia that can nodulate groundnut. Rhizobial numbers in the soil were not related to measured soil properties or to the length of time since groundnut was last planted.

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