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# GROUNDNUT NITROGEN FIXATION BY THREE SEROLOGICALLY AND MORPHOLOGICALLY DISTINCT RHIZOBIA

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**Summary**—Three cowpea miscellany rhizobia, a commercial strain (TAL 309) and two field isolates from Sudan (Wad Medani and Kadugli strains), were chosen for detailed studies because they could be separated by serological or morphological characteristics. The strains were screened in the greenhouse on three groundnut (*Arachis hypogaea* L.) cultivars, "Ashford" and "MH383" (both Virginia types), and "Barberton" (a Spanish type). Whenever the TAL 309 strain was included in inoculum mixtures, it consistently occupied the majority of nodules with all three groundnut cultivars. Although significant differences were found in top dry-matter weights, color ratings, nitrogenase activities, and total nodule number among the three cultivars tested, there were no host genotype-by-strain interactions either in growth measurements or in nodule occupancy. Correlation matrices indicated that several of the measures made could serve as reliable indicators of rhizobial efficiency. Both top dry-matter weight and plant color, and top dry-matter weight and $C_2H_2$ reduction were related at the 0.001 level of probability.

## INTRODUCTION

The groundnut plant relies on its symbiotic association with root-nodule bacteria for $N_2$ fixation. Differences in $N_2$-fixation efficiencies of groundnut rhizobia (Graham and Donawa, 1981; Weaver, 1974; Wynne *et al.*, 1980) and the role of the host plant in the symbiosis are well documented (Vincent and Waters, 1953; Caldwell and Vest, 1968). Before rhizobia can symbiotically fix $N_2$, they must colonize root surfaces and initiate nodule formation. Competition among *Rhizobium* strains to occupy nodules may be influenced by host genotype (May and Bohlool, 1983), *Rhizobium* strains (Trinick, 1982) and pH (Graham and Donawa, 1981; Weaver *et al.*, 1972).

Use of agglutination reactions is a common technique for strain identification (Vincent, 1970). Agglutination of *Bradyrhizobium japonicum* with specific antisera can be conveniently done by direct reaction with crushed nodules (Means *et al.*, 1964). Unfortunately, with groundnut nodules, we find the isolates must first be streaked on yeast extract mannitol agar (YEMA) before serological identity can be determined. If colony morphology can be used for identification of groundnut rhizobia, considerable time may be saved in determining nodule occupancy. Also, we found (Hadad and Loynachan, 1985), there is considerable cross-reactivity among isolates of groundnut rhizobia, making identification via agglutination reactions tedious. Ahmad *et al.* (1981) and Sinclair and Eaglesham (1984) reported a "wet" and "dry" type of colony morphology of West African cowpea rhizobia that varied in serological reactions and intrinsic antibiotic resistance.

During field studies in Sudan, we found that soils contained relatively high numbers of indigenous cowpea-type rhizobia (Hadad and Loynachan, 1985). Therefore, introduced rhizobia must be able to compete against a large native rhizobial population in order to occupy groundnut nodules. Greenhouse studies have been made to obtain data on factors that may influence nodulation and $N_2$ fixation of groundnut in Sudan. Three native Sudanese groundnut cultivars, two native and one commercial *Rhizobium* strains, and two pH levels that are characteristic of Sudanese soils were considered. Determination of nodule occupancy was based on colony morphology of rhizobial isolates, verified by serological reactions.

## MATERIALS AND METHODS

### *Rhizobial strains and groundnut cultivars*

Three serologically and morphologically distinct strains of groundnut-nodulating *Rhizobium* were identified in preliminary screenings. Two strains were from Sudan: The Wad Medani strain was isolated from a clay soil (pH 8.0) in central Sudan and the Kadugli strain from a clay soil (pH 6.5) in western Sudan. The commercial strain, TAL 309 (CB756), was supplied by Dr T. Wacek, North American Plant Breeders, Princeton, Illinois.

Seeds of the three Sudanese groundnut cultivars, "Ashford" and "MH383" (both Virginia types) and "Barberton" (a Spanish type), were obtained from the Agronomy Section, Agricultural Research Corporation, Wad Medani, Sudan. Seeds were surface-sterilized by immersion in 30% $H_2O_2$ for 15 min followed by five rinses in sterile water (Vincent, 1970) and were then germinated in sterile vermiculite. Seedlings with 1-cm radicles were transplanted into 4-l containers lined with autoclavable plastic bags, which contained sterile sand:vermiculite (1:1 by volume) and adjusted to pH 6.5. The mixture initially received 100 ml of a complete nutrient solution lacking nitrogen (Speidel and Wollum, 1980) and was watered as needed via a glass tube running to the bottom of the containers.

Rhizobial cells were grown in yeast extract mannitol broth (YEMB) for 6 days at 28°C in a waterbath

Table 1. Treatments[a] used in evaluation of efficiency and competitive ability of groundnut *Rhizobium*

| Study I | | | Study II | | | |
|---|---|---|---|---|---|---|
| Trt No. | Strain | Inoculant size (Log no. $ml^{-1}$) | Trt No. | pH | Strain | Inoculant size (Log no. $ml^{-1}$) |
| 1 | Wad Medani (W.M.) | 8.3 | 1a | 6.5 | Wad Medani (W.M.) | 4.3 |
| | | | b | 8.0 | Wad Medani (W.M.) | 4.3 |
| 2 | Kadugli (K.) | 8.1 | 2a | 6.5 | Kadugli (K.) | 4.1 |
| | | | b | 8.0 | Kadugli (K.) | 4.1 |
| 3 | TAL 399 (T.) | 7.3 | 3a | 6.5 | Kadugli (K.) | 8.1 |
| | | | b | 8.0 | Kadugli (K.) | 8.1 |
| 4 | W.M./K. mixture | 8.0/7.8 | 4a | 6.5 | W.M./K. mixture | 4.0/7.8 |
| | | (1.6:1) | b | 8.0 | W.M./K. mixture | (1:6.3 × $10^3$) |
| 5 | W.M./T. mixture | 8.0/7.0 | 5a | 6.5 | Noninoculated control | None |
| | | (10:1) | b | 8.0 | Noninoculated control | None |
| 6 | K./T. mixture | 7.8/7.0 | 6a | 6.5 | Nitrogen control | None |
| | | (6.3:1) | b | 8.0 | Nitrogen control | None |
| 7 | Noninoculated control | None | | | | |
| 8 | Nitrogen control | None | | | | |

[a]Treatments applied to Sudanese cultivars Ashford, Barberton, and MH383 in Study I and to Ashford only in Study II. Values in parentheses under inoculant size are the arithmetical ratios of applied cells in mixed inoculants. Inoculant was added in 1 ml of concentrated, diluted, or mixed broth.

shaker and were counted using a Petroff–Hauser bacterial counter (Arthur H. Thomas Company, Philadelphia, Pennsylvania). Plate counts indicated good viability of all three strains at 6 days when grown under these conditions. Strains were tested singly and in mixtures (Table 1, Study I). Mixtures were prepared by adding an equal volume (0.5 ml) of the concentrated or diluted cultures. Because cell counts were not identical, the resulting ratios of added inocula varied from 1.6:1 to 10:1. Both non-inoculated and N controls (105mg N as $NH_4NO_3$ over 7 weeks) were established. Treatments were replicated four times and the containers positioned in the greenhouse is a completely randomized fashion.

At harvest, 7 weeks from sowing, plant color was rated by ranking 4 for dark green, 3 and 2 lighter shades of green, and 1 for yellow. The plant shoots were then dried at 72 C for 2 days and dry weights determined. Nitrogenase activity was measured by the acetylene reduction assay (Hardy *et al.*, 1968). Nodule weights and numbers were assessed and 20 nodules were sampled from each plant for determination of occupancy by the competing strains. Ten nodules from the taproot (main root) and ten nodules from lateral roots were chosen to represent small, medium and large nodules. Colony morphology and agglutination reactions were used for occupant identification. For antigen preparation, antiserum production, and agglutination reactions, the procedures described by Vincent (1970) were followed.

### *pH effect*

The soils of western Sudan have a neutral to slightly acid pH, whereas the soils in central Sudan characteristically contain free $CaCO_3$ and pHs are 8.0 and above (Hadad and Loynachan, 1985). In a separate study (Table 1, Study II), sand:vermiculite mixtures (1:1 by volume) were adjusted with $CaCO_3$ to pHs 6.5 and 8.0. The $CaCO_3$ was thoroughly mixed with the sand and vermiculite before placement in containers. The two Sudanese rhizobial isolates were evaluated on the Sudanese cultivar Ashford, the most popular cultivar planted in Sudan. The first two treatments represented levels of each strain frequently found in the field soil (Hadad and Loynachan, 1985), the third treatment tested a high level of the Kadugli inoculant strain, and the fourth treatment was a mixture of strains representing the more competitive strain (Wad Medani) at levels found $g^{-1}$ soil and a less competitive strain (Kadugli) at a higher inoculation level.

### *Statistical analysis*

Correlation matrices were constructed to evaluate the relationships among the measurements made. Treatment differences within each study (Study I and II) were evaluated by using single degree-of-freedom contrasts (Chew, 1976; Nelson and Rawlings, 1983). Treatment means and a measure of data precision are indicated in tables.

## RESULTS AND DISCUSSION

Several of the measurements made in the assessment of $N_2$ fixation of groundnut were significantly related (Table 2). Top dry-matter weight and plant color, and top dry-matter weight and $C_2H_2$ reduction were related at the 0.001 level of probability in both studies, whereas total nodulation and $C_2H_2$ reduction were related at the 0.01 level. In the second study (values in parentheses, Table 2), nodule types were separated into lateral and main-root nodules to determine if pH affected the position of infection. Main-root nodule numbers ($P < 0.001$), lateral-root nodule numbers ($P < 0.05$), and main-root nodule weights ($P < 0.001$) were significantly related to nodule activity ($C_2H_2$ reduction). This confirms the results of Wynne *et al.* (1980) that one of several traits may be used to evaluate rates of $N_2$-fixation in the greenhouse.

### *Cultivar–strain interaction*

A combined analysis based on *F*-tests indicated there were differences in top dry-matter weights, color ratings, nitrogenase activities, and total nodulation among the three cultivars (each significant at the 0.01 level, data not shown); however, there was no host–genotype-by-strain interaction among the cultivars and strains tested. The lack of interaction between host and strain disagreed with the findings of Wynne *et al.* (1980); but in our study, all cultivars originated from one geographical region. Significant treatment differences were due mainly to the nitrogen

Table 2. Significant correlation coefficients ($r$) among measured parameters

| | Top dry weight | $C_2H_2$ reduction | Total nodule number | Main-root nodule number | Lateral-root nodule number | Main-root nodule weight |
|---|---|---|---|---|---|---|
| Color | 0.40*** (0.61)****[a] | NS[b] (0.24)* | NS (NS) | ND[c] (NS) | ND (NS) | ND (NS) |
| Top dry weight | | 0.63*** (0.53)*** | 0.35** (0.42)** | ND (NS) | ND (0.41)** | ND (0.48)** |
| $C_2H_2$ reduction | | | 0.29** (0.39)** | ND (0.58)*** | ND (0.28)* | ND (0.74)*** |
| Total nodule number | | | | ND (0.36)** | ND (0.98)*** | ND (0.39)** |
| Main-root nodule number | | | | | ND (NS) | ND (0.84)*** |
| Lateral-root nodule number | | | | | | ND (NS) |

[a]Bottom values in parentheses represent the correlation coefficients obtained in Study II. Top values are from Study I.
[b]NS = Not significant at the 0.05 level of probability.
[c]ND = Not determined; the subdivision of nodules into main- and lateral-root nodules was conducted only in Study II.
*, **, ***Significance at the 0.05, 0.01, and 0.001 levels of probability, respectively.

and noninoculation controls (Table 3). Single degree-of-freedom contrasts indicated that only total nodulation consistently varied with added rhizobial strain (mean squares not shown). The isolate from Wad Medani (W.M.) improved nodulation of these cultivars without obvious benefit to the plant (no improvement in top dry-matter weight or color rating), which implied ineffective nodulation. Strain TAL 309 (T) produced fewer nodules, but most of the nodules with TAL 309 were large and located on the main root.

Total nodulation was greater at pH 6.5 than at pH 8.0 (Table 4), with a much higher number of lateral than main-root nodules. Differences were significant for both total and lateral-root nodulation at the 0.01 level based on single degree-of-freedom contrasts (mean squares not shown). The average number of main-root nodules at pH 6.5 was 15.4, and the average number of lateral nodules was 87.1. At pH 8.0, the average number of main-root nodules was 14.5, whereas the average number of lateral-root nodules was 52.9. At 7 weeks when the study ended, the lateral-root nodules were still very small (for this reason, only main-root nodule weights were taken). The pH range used in our study, although reflective of pHs in Sudanese soils, may have been too narrow to cause major differences in most measurements made.

Increasing the inoculant size of the Kadugli strain $10^4$ times (trt 2 vs 3) increased the number and weight of main-root nodules and nitrogenase activity (Table 4).

*Competition for nodule sites*

The percentage of nodules occupied by each strain in Study I and the ratios of rhizobia applied and recovered are detailed in Table 5. When the Wad Medani, TAL 309, or Kadugli strain was added singly, all nodules were occupied by the applied strain.

Table 3. Nitrogen-fixing traits as influenced by single-strain *Rhizobium* additions to three Sudanese groundnut cultivars (Study I)

| Trt[a] No. | Strain | Plant growth: Dry top wt (mg plant$^{-1}$) | Plant growth: Color rating | Nitrogenase activity ($\mu$mol plant$^{-1}$ h$^{-1}$) | Total nodulation (No. plant$^{-1}$) |
|---|---|---|---|---|---|
| | | | Ashford | | |
| 1 | W.M. | 630[b] | 2.5 | 352 | 103.0 |
| 2 | K. | 600 | 3.1 | 362 | 69.0 |
| 3 | T. | 650 | 2.6 | 424 | 56.0 |
| 7 | Noninoculated control | 570 | 2.0 | NN[c] | NN |
| 8 | N control | 730 | 3.8 | NN | NN |
| | SE | 30 | 0.1 | 27 | 8.2 |
| | | | Barberton | | |
| 1 | W.M. | 400 | 1.6 | 237 | 70.0 |
| 2 | K. | 450 | 2.0 | 254 | 35.0 |
| 3 | T. | 440 | 2.3 | 286 | 34.0 |
| 7 | Noninoculated control | 390 | 2.1 | NN | NN |
| 8 | N control | 660 | 3.4 | NN | NN |
| | SE | 20 | 0.1 | 23 | 6.1 |
| | | | MH 383 | | |
| 1 | W.M. | 530 | 3.1 | 286 | 71.0 |
| 2 | K. | 510 | 3.3 | 321 | 62.0 |
| 3 | T. | 550 | 2.7 | 331 | 28.0 |
| 7 | Noninoculated control | 440 | 2.8 | NN | NN |
| 8 | N control | 700 | 3.7 | NN | NN |
| | SE | 30 | 0.1 | 28 | 8.2 |

[a]Treatments are explained in Table 1 (Study I).
[b]Values are averages of four replications.
[c]NN indicates no nodules were present.



3

Table 4. Influence of pH and inoculum size on nitrogen-fixing parameters of the Sudanese cultivar Ashford (Study II)

| Trt[a] No. | Strain | pH | Plant growth: Tissue dry wt (mg plant$^{-1}$) | Plant growth: Root dry wt | Plant growth: Color rating | Nodulation: Total | Nodulation: Main root (No. plant$^{-1}$) | Nodulation: Lateral root | MR wt[b] (mg plant$^{-1}$) | $N_2$-ase activity ($\mu$mol plant$^{-1}$ h$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1a | W.M. | 6.5 | 440[c] | 200 | 3.2 | 98.0 | 9.2 | 88.7 | 21.8 | 28 |
| b | W.M. | 8.0 | 390 | 190 | 2.9 | 76.4 | 5.7 | 70.7 | 10.0 | 6 |
| 2a | K. | 6.5 | 450 | 190 | 3.0 | 111.0 | 8.5 | 102.5 | 19.0 | 121 |
| b | K. | 8.0 | 490 | 230 | 3.2 | 65.0 | 11.7 | 53.3 | 28.4 | 210 |
| 3a | K. | 6.5 | 530 | 190 | 3.9 | 111.0 | 19.7 | 91.3 | 64.0 | 276 |
| b | K. | 8.0 | 540 | 240 | 3.9 | 56.4 | 17.7 | 38.7 | 67.8 | 313 |
| 4a | W.M./K. | 6.5 | 350 | 200 | 2.7 | 90.0 | 24.0 | 66.0 | 95.5 | 207 |
| b | W.M./K. | 8.0 | 500 | 190 | 3.3 | 72.0 | 23.0 | 49.0 | 89.6 | 212 |
| | SE | | 20 | 10 | 0.2 | 7.1 | 1.5 | 6.6 | 6.0 | 23 |

[a]Treatments are explained in Table 1 (Study II).
[b]Main-root nodule dry weight.
[c]Values are averages of four replications.

In these tests, strain TAL 309 seemed quite competitive when compared with the Sudanese strains on all three cultivars. The ratio of applied cells with Wad Medani/TAL 309 was 10:1, but the ratios of nodule occupants were 1.3–1.6:1. The ratio of applied cells with Kadugli/TAL 309 was 6.3:1, but the ratios of nodule occupants were 0.1–1.2:1. In comparing the Sudanese strains, it appeared that the Wad Medani strain was more competitive. Relatively consistent occupancy data were obtained with all three cultivars. Under field conditions when strain TAL 309 was applied to groundnuts in a Sudanese soil populated with the Wad Medani strain, TAL 309 was competitive and occupied 30 and 40% of the main and lateral-root nodules, respectively.

In study II trt 4, regardless of pH, when a high inoculant level of the less competitive Kadugli strain was added together with a lower inoculant level of the more competitive Wad Medani strain, all nodules tested contained the Kadugli strain. The use of ultrahigh inoculum rates, as suggested by Kapusta and Rouwenhorst (1973), seems promising as an effective means of improving nodule occupancy of less-competitive strains of groundnut rhizobia. Work

Table 5. Influence of Sudanese groundnut cultivars on nitrogen fixation and nodule occupancy

| Trt[a] No. | Strain | Total nodulation (No. plant$^{-1}$) | Nitrogenase activity ($\mu$mol plant$^{-1}$ h$^{-1}$) | Percentage occupancy[b]: W.M. | T. (%) | K. | Applied ratio | Ratio recovered |
|---|---|---|---|---|---|---|---|---|
| | | | | Ashford | | | | |
| 4 | W.M./K | 63.0[c] | 404 | 86.7 ± 6.6[d] | 0.0 | 13.3 ± 6.6 | 1.6:1 | 6.5:1 |
| 5 | W.M./T. | 132.0 | 349 | 56.3 ± 6.3 | 43.8 ± 6.3 | 0.0 | 10.0:1 | 1.3:1 |
| 6 | K./T. | 58.0 | 383 | 0.0 | 73.3 ± 14.7 | 26.7 ± 14.7 | 6.3:1 | 0.4:1 |
| | SE | 8.2 | 27 | | | | | |
| | | | | Barberton | | | | |
| 4 | W.M./K. | 49.0 | 310 | 66.7 ± 9.3 | 0.0 | 26.7 ± 9.3 | 1.6:1 | 2.5:1 |
| 5 | W.M./T. | 98.0 | 245 | 61.5 ± 6.9 | 38.5 ± 6.9 | 0.0 | 10.0:1 | 1.6:1 |
| 6 | K./T. | 40.0 | 217 | 0.0 | 45.5 ± 6.3 | 54.5 ± 6.3 | 6.3:1 | 1.2:1 |
| | SE | 6.1 | 23 | | | | | |
| | | | | MH 383 | | | | |
| 4 | W.M./K. | 78.0 | 333 | 92.3 ± 6.6 | 0.0 | 7.7 ± 6.6 | 1.6:1 | 12.0:1 |
| 5 | W.M./T. | 114.0 | 306 | 55.6 ± 7.4 | 44.4 ± 7.4 | 0.0 | 10.0:1 | 1.3:1 |
| 6 | K./T. | 83.0 | 373 | 0.0 | 90.9 ± 22.5 | 9.5 ± 22.5 | 6.3:1 | 0.1:1 |
| | SE | 8.2 | 28 | | | | | |

[a]Treatments are explained in Table 1 (Study I).
[b]Mixed infections accounted for 6.6%.
[c]Values are averages of four replications.
[d]Means ± SE.

Table 6. Characteristics of the *Rhizobium* isolates

| *Rhizobium* strain | Time for appearance on YEMA (days) | Colony size at day[a] (mm): 8 | 12 | 16 | Colony margin | Colony elevation | BTB[b] absorption | Polysaccharide secretion[c] |
|---|---|---|---|---|---|---|---|---|
| Kadugli | 12 | 0 | <1.0 | 1.5 | Entire | Raised | +4 | Slight |
| TAL 309 | 8 | <1.0 | 1.0 | >3.0 | Irregular | Flat | +1 | Abundant |
| Wad Medani | 10 | 0 | <1.0 | 2.0 | Entire | Convex | +2 | Slight |

[a]Growth was at 28°C on YEMA amended with 25 $\mu$g bromthymol blue (BTB) ml$^{-1}$ as a pH indicator.
[b]All isolates were alkaline producers; BTB rating was +4 for maximum absorption, +2 for moderate absorption, and +1 for slight absorption.
[c]Determined by visual appearance of sliminess on YEMA.

4

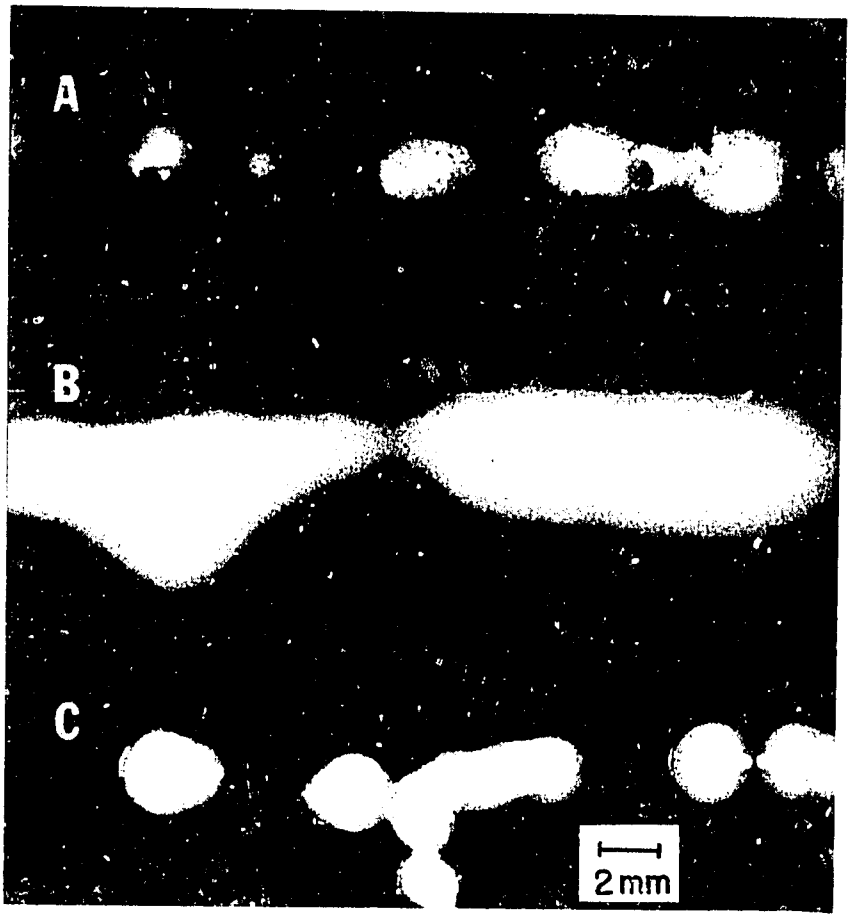


Fig. 1. Colonial morphology of Kadugli (A), TAL 309 (B) and Wad Medani (C) *Rhizobium* strains.

is needed to quantify the inoculum size needed with the Kadugli strain to compete successfully with the Wad Medani strain under field conditions.

*Strain identification*

The *Rhizobium* strains used varied considerably in their growth characteristics when isolated from nodules and grown on YEMA containing 25 $\mu$g bromthymol blue ml$^{-1}$ as a pH indicator. Time of appearance, colony shape, size, elevation and absorption of the bromthymol blue varied among the strains (Fig. 1 and Table 6). Absorption of the bromthymol blue, most evident by the Kadugli strain, resulted in a decided yellowishness of the colony. In fact, the Kadugli and Wad Medani strains could easily be separated based solely on color.

We observed that many of our isolates from groundnut share common surface somatic antigens. This complicates identification via agglutination because of partial agglutination reactions where results are intermediate between the nonantigen control and the homogeneous antigen control. In these studies, we intially chose three rhizobial strains that did not share common antigens and, later, discovered that the strains could quite easily be separated by cultural characteristics. Morphological identification greatly reduced the tedious task of serological identification. In all cases, complete positive agreement was obtained between colony morphology and agglutination tests.

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