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HOST SPECIFICITY AND CHARACTERIZATION OF FAST-GROWING RHIZOBIA FROM *MACROPTILIUM ATROPURPUREUM* cv. SIRATRO IN PAKISTAN

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Summary—The growth characteristics, intrinsic antibiotic resistance pattern, utilization of different C sources and symbiotic characteristics of 18 fast-growing rhizobial strains isolated from root nodules of *Macroptilium atropurpureum* (Siratro) were studied. Colonies on agar at 2–3 days were large, gummy, translucent and spreading (2–4 mm dia) and were acid producing. These strains did not utilize sodium citrate as a sole source of C and were sensitive to low concentrations of chloramphenicol (25 μg ml^{-1}), tetracycline (8 μg ml^{-1}) and streptomycin (12 μg ml^{-1}). The host specificity and effectiveness of these strains on different legume hosts revealed that they are different from other fast-growing strains. All strains failed to nodulate *Glycine max* (soybean cv. William, Lee and Peking). Sixteen of the isolates nodulated *Leucaena leucocephala* (ipil–ipil) and all 18 nodulated *Phaseolus vulgaris* (common bean). The fast-growing strains showed varying degrees of effectiveness in N_2 fixation on *M. atropurpureum*, *Vigna mungo* (blackgram) and *V. unguiculata* (cowpea). The isolates formed ineffective symbioses on two genotypes of *V. radiata* (mungbean).

INTRODUCTION

The identification of species within the genus *Rhizobium* was established initially on the basis of the legumes that they infect (Vincent, 1970). More recently, the genus was divided into two broad groups: *Rhizobium*, the fast-growers; and *Bradyrhizobium*, the slow-growers (Jordan, 1984). The cowpea-type strains are a less well defined group and consist mostly of slow-growing bradyrhizobia (Vincent, 1970; Gibson *et al.*, 1982). The concept that cowpea-type rhizobia are slow-growers may be inaccurate in the light of reports that fast-growing rhizobia also infect soybean, cowpea and mungbean (Sadowsky *et al.*, 1988; Chamber and Iruthayathas, 1988). We studied the isolation and characterization of fast-growing rhizobial strains from *Macroptilium atropurpureum*, cv. Siratro.

MATERIALS AND METHODS

Cowpea rhizobial/bradyrhizobial strains

Nodules were collected from plants of *Macroptilium atropurpureum* cv. Siratro that had been used for MPN counting of rhizobia in soil by a plant-infection technique using growth pouches (Asad *et al.*, 1991). The nodules were collected randomly from dilutions 4^{-1} to 4^{-8}. The soil was from a field experiment at the Nuclear Institute for Agriculture and Biology, Faisalabad in the semiarid region of Punjab, Pakistan. No leguminous crop had been grown in the soil previously. Rhizobia were isolated by streaking the content of surface-sterilized nodules on yeast extract mannitol (YEM) agar containing bromothymol blue and Congo red and incubating at 28 ± 2°C for 3–5 days. Single colonies from the plates were picked and further purified by streaking on the same medium. The purified cultures were authenticated by their infectivity on Siratro plants grown in growth pouches containing N-free nutrient solution. Rhizobia were reisolated from the nodules and finally pure cultures were maintained on YEM agar slants using standard techniques (Vincent, 1970). The reisolated strains were numbered Ma3 to Ma20. Strain Vr16, from *Vigna radiata* (Hafeez *et al.*, 1991), was included as a reference strain.

Growth studies

Cultures were grown in yeast extract mannitol broth (YEM) in 50 ml Erlenmeyer flasks. At late exponential growth (ca. 10^8–10^9 cells ml^{-1}), 0.5 ml was transfered into 100 ml Erlenmeyer flask containing 50 ml of YEM broth. Each strain was grown in 3 replicate flasks on a rotary shaker at 28 ± 2°C. Samples were drawn every 1 h for fast-growers and 4 h for slow-growers. Viable counts were made and the generation time of each strain was determined. The final pH was measured at an optical density of 0.9 at wavelength 660 nm.

Utilization of C sources

Hepes–Mes basal salts (AG) agar plates (Cole and Elkan, 1973), supplemented with different C sources, were used to assess the growth of the new isolates and

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reference strain. Individual C sources were filter sterilized (0.22 μm). The C sources (5 g l^{-1}) were L-arabinose, sucrose, mannitol, glucose, glycerol, sodium lactate, lactose, raffinose, dulcitol, sodium succinate and sodium citrate. The concentration of yeast extract was 1 g l^{-1}. Plates with no C source were used as controls. All strains were streaked in triplicate and incubated at 28°C. The presence and absence of growth was observed after 3–5 and 7–10 days for fast- and slow-growing strains, respectively.

Intrinsic antibiotic resistance

Strains were streaked to the surface of AG agar plates containing individual antibiotics (μg ml^{-1}): chloramphenicol (12.5 and 25); tetracycline (4 and 8) neomycin (2.5); kanamycin (10); nalidixic acid (10); rifampicin (5 and 10); and streptomycin (2.5 and 10). Plates were incubated at 28°C and observed for growth after 3–5 and 7–10 days for fast- and slow-growing strains, respectively.

Host specificity and effectiveness

Host specificity and effectiveness of reisolated strains were studied on *M. atropurpureum* cv. Siratro, *V. radiata* cv. NHM 51 and cv. NHM 54, *V. mungo* cv. Mash 54-13, *V. unguiculata* cv. AC 58, *P. vulgaris* cv. Bush Bountiful, *G. max* cv. Lee, William and Peking, *L. leucocephala* cv. K8, *M. sativa* cv Florida 77, *T. subterraneum* cv. Mt. Baker, *C. arietinum* cv. CM 72 and *V. sativa*. Seeds were obtained from the Mutation Breeding Division of NIAB, Faisalabad, Pakistan and NifTAL, Paia, HI, U.S.A. Effective strains (TAL 169 for cowpea group, TAL 1797 for common bean and TAL 1145 for ipil–ipil) received from NifTAL were included as reference strains for each homologous host.

Surface sterilization of seed, seed scarification and plant growth conditions were as described by Asad *et al.* (1991). One ml inoculum (ca. 10^9 cells ml^{-1}) of each strain was applied to the roots of each test host growing in Leonard jars. There were 3 replicates of each strain and the control treatments. Plants without inoculation and with N (12 mM N as NH_4NO_3) were kept as controls. Plants were harvested after 30 days from sowing and N_2 fixation of each sample was determined by acetylene reduction assay (ARA) (Hafeez *et al.*, 1991). The H_2 evolution and the relative efficiency of N_2 fixation was calculated as defined by Schubert *et al.* (1977). Data on the number and dry weight of nodules and shoot dry weight were also recorded.

RESULTS AND DISCUSSION

The colonies of all 18 isolates from root nodules of Siratro were large (2–4 mm dia), gummy, translucent and spreading. They appeared within 2–3 days and turned the YEM–bromothymol blue agar plates, yellow and were designated as fast-growers and acid-producers. It was noted that all fast-growing strains had a lag phase of about 3–4 h and they reached stationary phase after 48–60 h. For the slow-growing strain Vr16, the lag phase was 12 h and stationary phase was reached after 5 days. Mean generation times ranged from 2.5–3.5 h for fast-growing strains and 8.7 h for the slow-grower. The pH of the medium for the fast-growing strains at 0.9 was 6.1–6.5.

The mean generation time, gum production and acid reaction in yeast mannitol indicated that these strains resembled other fast-growing species of rhizobia (Keyser *et al.*, 1982; Anand and Dogra, 1991).

Utilization of different C sources

All the fast-growing strains utilized glucose, arabinose, sucrose, lactose, raffinose, mannitol, sodium succinate, glycerol and yeast extract. Sodium lactate was not readily utilized and no strain utilized dulcitol or sodium citrate as a sole source of C for growth. Bradyrhizobial strain Vr16 utilized all the C sources including sodium citrate except raffinose, dulcitol and sodium lactate. Sadowsky *et al.* (1983) showed that fast-growing soybean rhizobial strains tended to use a wider variety of carbohydrates than the slow-growers. Similar findings have been reported by Anand and Dogra (1991) with fast- and slow-growing isolates from pigeon pea. In contrast, we did not observe any difference in the utilization of different C sources (except sodium citrate) between 18 fast- and 1 slow-growing strains. The utilization of di- and tri-saccharide by few *Bradyrhizobium* spp (*Vigna*) has been reported also by Eaglesham *et al.* (1987).

Intrinsic antibiotic resistance pattern

All the strains were resistant to neomycin, kanamycin, nalidixic acid and rifampicin but sensitive to chloramphenicol, tetracycline and streptomycin. Only 3 strains showed resistance to a low concentration of streptomycin. Cowpea bradyrhizobial strain Vr16 was resistant to all the tested antibiotics. Our results agree with the results of Anand and Dogra (1991), who reported that fast- and slow-growers differ in their intrinsic antibiotic resistance pattern.

Host specificity and effectiveness

The 18 new isolates showed considerable variation with respect to nodule number (data not shown), nodule mass (Table 1), plant dry weight (Table 2), nitrogenase activity (Table 3) and H_2 evolution (Table 4) with 5 cowpea-type host plants. A significant difference was noted in their ability to produce an effective symbioses with hosts that are normally field nodulated with slow-growing cowpea-type rhizobia (Tables 2 and 3) and is in accord with the findings of Trinick (1980). On Siratro, cowpea and blackgram most of these fast-growing isolates produced significantly more nodule mass and ARA than did the slow-growing strains. The Siratro isolates exhibited significantly higher shoot dry weight in cowpea than the uninoculated controls, while a significantly higher

Table 1. Effect of inoculation with fast-growing Siratro isolates on the nodule dry weight (mg plant^{-1}) of various host legumes

Strains	*M. atropurpureum*	*V. unguiculata*	*V. mungo*	*V. radiata*-1*	*V. radiata*-2*	*P. vulgaris*	*L. leucocephala*
Ma 3	13.4	24.4	11.9	0.0	0.7	53.8	11.8
Ma 4	11.0	32.6	13.1	0.0	2.1	61.2	19.2
Ma 5	15.9	17.4	9.5	5.9	5.1	92.3	4.0
Ma 6	9.8	32.5	14.3	1.0	0.0	77.7	5.1
Ma 7	15.9	27.9	16.7	1.4	4.1	12.1	4.3
Ma 8	11.9	23.8	18.5	5.2	4.7	70.4	12.7
Ma 9	22.0	18.6	13.1	0.3	1.3	61.5	4.6
Ma 10	12.2	19.8	14.3	1.3	0.0	36.9	3.9
Ma 11	11.0	17.4	19.0	1.5	2.2	52.4	5.3
Ma 12	8.5	22.1	10.7	4.1	0.0	57.5	2.4
Ma 13	8.5	15.1	19.0	1.5	8.1	46.2	15.1
Ma 14	11.0	25.6	9.5	0.3	6.9	44.2	8.0
Ma 15	7.4	34.9	14.3	0.0	7.8	60.2	2.6
Ma 16	13.4	33.7	13.1	4.3	5.7	10.8	5.5
Ma 17	15.9	19.8	19.0	0.0	5.3	35.0	4.7
Ma 18	11.0	34.1	19.0	2.0	5.9	0.4	15.1
Ma 19	6.1	12.8	10.7	0.0	0.0	7.5	0.0
Ma 20	11.0	32.6	19.0	6.3	7.4	0.4	0.0
Vr 16	4.9	18.6	13.1	16.7	16.2	49.6	1.5
TAL strain†	11.0	38.6	10.8	8.8	16.7	47.4	26.9
Uninoculated	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N control‡	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LSD(0.05)	3.2	4.4	3.4	4.9	3.6	3.7	2.4

**V. radiata*-1 = cv. NHM 51; *V. radiata*-2 = cv. NHM 54.
†TAL strain = TAL 1797 for *P. vulgaris*, TAL 1145 for *L. leucocephala* and TAL 169 for other hosts.
‡N control = 12 mM N as ammonium nitrate.

dry biomass was observed in the N control (Table 2). Most isolates formed only ineffective or partially effective symbioses with both mungbean genotypes NHM51 and 54, respectively (Table 2). Five and 3 isolates, respectively failed to nodulate with these 2 hosts (Table 1). Siratro is effectively nodulated by a wide range of root-nodule bacteria. It has been shown that Siratro, cowpea and mungbean were nodulated by fast-growing rhizobial strains (Chamber and Iruthayathas, 1988; Bromfield and Barran, 1990; Stanley and Cervantes, 1991). Anand and Dogra (1991) reported the presence of fast-growing rhizobial strains in pigeon pea nodules but did not indicate their effectiveness on other hosts. Specific activity of the nitrogenase (data not shown) indicated a high (98%) N_2-fixation efficiency. No H_2 was evolved when these hosts were nodulated by bradyrhizobial strains Vr16 and TAL 169. We observed low or no H_2 evolution from the nodules of cowpea-type hosts in symbioses with fast-growing isolates, indicating the importance of hydrogenase that can recycle H_2 which is formed by nitrogenase activity (Evans *et al.*, 1980). The new fast-growing Siratro isolates showed greater specificity with mungbean for invasiveness and effectiveness. Cowpea, blackgram and Siratro had the least similar profiles in terms of invasiveness and

Table 2. Effect of inoculation with fast-growing Siratro isolates on the shoot dry weight (g plant^{-1}) of various host legumes

Strains	*M. atropurpureum*	*V. unguiculata*	*V. mungo*	*V. radiata*-1*	*V. radiata*-2*	*P. vulgaris*	*L. leucocephala*
Ma 3	0.15	0.43	0.26	0.13	0.17	0.17	0.18
Ma 4	0.15	0.42	0.20	0.12	0.16	0.38	0.17
Ma 5	0.11	0.29	0.25	0.14	0.18	0.38	0.20
Ma 6	0.08	0.42	0.19	0.14	0.18	0.80	0.20
Ma 7	0.08	0.39	0.18	0.13	0.17	0.20	0.18
Ma 8	0.12	0.46	0.27	0.11	0.14	0.40	0.15
Ma 9	0.07	0.38	0.20	0.15	0.20	0.21	0.21
Ma 10	0.10	0.40	0.27	0.14	0.18	0.40	0.20
Ma 11	0.10	0.35	0.28	0.15	0.20	0.26	0.21
Ma 12	0.06	0.34	0.15	0.14	0.18	0.34	0.20
Ma 13	0.07	0.28	0.25	0.13	0.17	0.38	0.18
Ma 14	0.09	0.23	0.21	0.12	0.16	0.30	0.17
Ma 15	0.08	0.50	0.22	0.19	0.25	0.30	0.25
Ma 16	0.10	0.40	0.19	0.15	0.20	0.50	0.12
Ma 17	0.10	0.40	0.31	0.14	0.18	0.30	0.11
Ma 18	0.09	0.40	0.28	0.13	0.17	0.30	0.21
Ma 19	0.08	0.26	0.24	0.17	0.22	0.20	0.14
Ma 20	0.09	0.44	0.26	0.12	0.16	0.40	0.10
Vr 16	0.06	0.34	0.26	0.24	0.31	0.04	0.19
TAL strain†	0.12	0.38	0.21	0.13	0.17	0.33	0.23
Uninoculated	0.05	0.08	0.13	0.16	0.21	0.03	0.11
N control‡	0.23	0.74	0.49	0.27	0.36	1.10	0.34
LSD(0.05)	0.08	0.17	0.15	0.14	0.18	0.17	0.15

**V. radiata*-1 = cv. NHM 51; *V. radiata*-2 = cv. NHM 54.
†TAL strain = TAL 1797 for *P. vulgaris*, TAL 1145 for *L. leucocephala* and TAL 169 for other hosts.
‡N control = 12 mM N as ammonium nitrate.

Table 3. ARA (μmol ethylene plant^{-1} h^{-1}) by different legume hosts inoculated with fast-growing Siratro isolates

Strains	*M. atropurpureum*	*V. unguiculata*	*V. mungo*	*V. radiata*-1*	*V. radiata*-2*	*P. vulgaris*	*L. leucocephala*
Ma 3	12.5	5.9	9.8	0.0	0.3	0.3	3.6
Ma 4	5.1	16.3	4.8	0.0	0.3	0.1	2.0
Ma 5	6.7	6.3	3.6	1.2	1.4	0.0	1.3
Ma 6	5.0	12.4	4.9	1.6	0.0	2.2	3.1
Ma 7	16.4	9.8	9.0	1.2	0.1	1.1	0.1
Ma 8	6.4	14.0	13.8	0.1	1.7	8.4	4.9
Ma 9	8.1	8.9	10.0	0.2	0.1	31.9	0.4
Ma 10	7.9	2.7	16.9	0.4	0.0	2.9	0.0
Ma 11	10.0	8.0	19.1	6.8	1.4	3.7	0.0
Ma 12	5.7	8.6	8.4	1.7	2.0	3.6	0.0
Ma 13	1.8	2.2	17.0	0.2	2.5	0.0	0.1
Ma 14	5.6	10.3	1.2	0.2	1.2	1.7	0.3
Ma 15	3.1	10.9	9.0	0.1	1.2	10.2	0.1
Ma 16	6.7	10.2	5.3	0.7	0.8	1.7	0.1
Ma 17	11.1	21.5	9.4	0.3	1.4	9.6	0.0
Ma 18	9.3	8.7	1.7	4.4	1.8	0.0	0.2
Ma 19	5.3	6.6	6.5	0.1	0.0	0.0	0.0
Ma 20	6.4	19.9	17.0	3.0	3.0	0.0	0.0
Vr 16	2.8	3.1	4.8	2.6	3.6	0.0	0.0
TAL strain†	2.0	2.2	1.3	1.7	0.3	13.5	12.0
Uninoculated	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N control‡	0.0	0.0	0.0	0.0	0.0	0.0	0.0
LSD(0.05)	6.4	7.4	8.4	2.1	1.5	5.2	2.0

**V. radiata*-1 = cv. NHM 51; *V. radiata*-2 = cv. NHM 54.
†TAL strain = TAL 1797 for *P. vulgaris*, TAL 1145 for *L. leucocephala* and TAL 169 for other hosts.
‡N control = 12 mM N as ammonium nitrate.

effectiveness by these isolates and supports the findings of Thies *et al.* (1991).

Sixteen and 18 isolates of Siratro elicited nodules on ipil–ipil and common bean, respectively. These new isolates formed very large nodules (7–9 mm) and greater nodule mass on common bean than on other hosts (2–5 mm). They were significantly less effective on both hosts than the reference strains (TAL 1145 and 1797). Only one isolate, Ma 9, showed highly significant difference in the production of ethylene (31.9 μmol plant^{-1} h^{-1}, Table 3) and H_2 evolution (7.3 μmol g^{-1} h^{-1}, Table 4) in common bean. The relatively high N_2-fixing efficiency of these rhizobial strains on common bean ranged from 85 to 100%. The bradyrhizobial strain Vr16 formed ineffective nodules on both hosts. No correlation was observed in any of the variables studied in all the hosts only a poor correlation was found between nodule mass and ARA ($r = 0.66$) in common bean and nodule number and nodule mass ($r = 0.65$) in cowpea.

The new fast-growing isolates and bradyrhizobial strain Vr16 failed to nodulate gram, clover, alfalfa, vetch and soybean (cv. William, cv. Lee and cv. Peking). The absence of nodules on soybean cv. Peking suggests that they are different from *R. fredii* (Chamber and Iruthayathas, 1988). Each reference NifTAL strain of *Rhizobium*/*Bradyrhizobium* formed effective nodules on its respective homologous legume

Table 4. H_2 evolution (μmol H_2 g^{-1} module fresh wt h^{-1}) by different legume hosts inoculated with fast-growing Siratro isolates

Strains	*M. atropurpureum*	*V. unguiculata*	*V. mungo*	*P. vulgaris*
Ma 3	0.91	0.18	2.40	0.00
Ma 4	0.00	1.80	1.70	2.40
Ma 5	0.08	0.13	0.00	0.00
Ma 6	0.27	0.55	1.20	0.85
Ma 7	0.62	0.24	0.86	0.00
Ma 8	0.20	1.29	0.83	1.78
Ma 9	0.33	0.72	1.30	7.33
Ma 10	0.40	0.00	0.83	0.00
Ma 11	0.67	1.00	1.90	1.16
Ma 12	0.86	0.73	0.89	1.56
Ma 13	0.00	0.15	2.60	0.00
Ma 14	0.89	0.00	0.00	0.00
Ma 15	0.13	0.39	0.67	0.00
Ma 16	0.55	0.33	0.33	0.00
Ma 17	1.20	1.23	0.25	0.00
Ma 18	0.89	0.14	0.00	0.00
Ma 19	1.20	0.35	0.00	0.00
Ma 20	0.67	0.63	0.88	0.00
Vr 16	0.00	0.00	0.00	0.00
TAL strain*	0.00	0.00	0.00	0.00
Uninoculated	0.00	0.00	0.00	0.00
N control†	0.00	0.00	0.00	0.00
LSD(0.05)	0.86	0.94	1.68	1.39

*TAL strain = TAL 1797 for *P. vulgaris*, TAL 1145 for *L. leucocephala* and TAL 169 for other hosts.
†N control = 12 mM N as ammonium nitrate.

host and all uninoculated control plants were without nodules.

The host specificity and effectiveness results indicate that fast-growing rhizobial strains isolated from Siratro nodules are different from other *Rhizobium* spp, including *R. fredii*, *R. loti*, *R. leguminosarum* bv. *trifolii*, *viceae* and *phaseoli*, *R. meliloti* and *Rhizobium* strain NGR 234 (Jordan, 1984; Eardly *et al.*, 1985; Chamber and Iruthayathas, 1988; Bromfield and Barran, 1990; Somasegaran and Bohlool, 1990; Stanley and Cervantes, 1991).

We believe this is the first report of isolation of fast-growing rhizobial strains from a tropical forage legume grown in the semiarid region of Pakistan.

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