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Studies on fast and slow growing *Rhizobium* spp. nodulating *Cajanus cajan* and *Cicer arietinum*

BY E. S. P. BROMFIELD*

Soil Microbiology Department, Rothamsted Experimental Station, Harpenden, Herts AL5 2JQ, England

AND J. V. D. K. KUMAR RAO

International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India

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SUMMARY

Fast and slow growing *Rhizobium* spp. isolated from *Cajanus cajan* and *Cicer arietinum* were compared in terms of colony characteristics, utilisation of carbon sources, acid production, symbiotic effectiveness and nodulating competitiveness.

Fast growing isolates from *C. cajan* and *C. arietinum* formed 3–6 mm diameter colonies on yeast-extract mannitol agar after 4 days and were unlike the slow growers which produced colonies of *c.* 1 mm diameter after 7–10 days at 28 °C. The fast growing *Rhizobium* spp. from *C. cajan* utilised a wider range of carbon sources than the slow growing isolates from this host. Fast and slow growing strains from *C. arietinum* were able to utilise most of the carbon sources tested suggesting that the slow growers possessed glycolytic pathways similar to those in other fast growing species of *Rhizobium*. In culture, slow growing isolates from *C. cajan* produced a near-neutral to alkaline reaction (pH 6·6–7·5) whereas the fast growers from this host and both fast and slow growing isolates from *C. arietinum* produced an acidic reaction (pH 4·4–5·6). These data are discussed in the context of Norris' (1965) evolutionary concept of the Leguminosae. Under glassshouse conditions, fast and slow growing strains isolated from *C. cajan* and *C. arietinum* were equally effective on their respective hosts.

In competition with slow growing rhizobia, half of the fast growers formed more than 70% of the nodules on *C. cajan* grown in sand. In all but one instance similar results were obtained when plants were grown in soil. With *C. arietinum* grown in sand or soil, all fast growing isolates from this host formed more than 85% of the nodules in competition with slow growing strains.

INTRODUCTION

The genus *Rhizobium* is divided into species mainly on the basis of preferred legume hosts which they nodulate (Vincent, 1974). However, in the classification of the rhizobia, Bergey (1974) uses growth rate as a taxonomic characteristic to further divide the genus into two sub-groups. According to Bergey (1974) the first of these groups (*R. phaseoli*, *R. leguminosarum*, *R. trifolii* and *R. meliloti*) characteristically form 2-4 mm diameter colonies in 3–5 days at *c.* 25 °C on yeast-extract mannitol agar whereas the second group (*R. lupini* and *R. japonicum*) form small colonies (*c.* 1 mm diameter) after 7–10 days. However, in this classification Bergey (1974) does not consider a vast array of poorly defined rhizobia

* Present address: Chemistry and Biology Research Institute, Agriculture Canada, Ottawa, Ontario K1A OC6.

(*Rhizobium* spp.) which constitute the so-called 'cowpea miscellany' and nodulate legumes (cowpea group) mostly of tropical or sub-tropical origin. Although legume hosts for the fast and slow growing rhizobia are usually distinct, several legumes mostly of the cowpea group are known to be nodulated by both fast and slow growing strains of the same species of *Rhizobium*. *Lotus corniculatus* (Vincent, Nutman & Skinner, 1979) and *L. pedunculatus* (Pankhurst, 1977) are nodulated by both fast and slow growing *Lotus* rhizobia. Fast growing rhizobia have been isolated from *Lablab purpureus* (Trinick, 1980) and several species of *Acacia* (Dreyfus & Dommergues, 1981) which are usually nodulated by slow growing strains. *Glycine max* usually nodulates with slow growing *R. japonicum* (Bergey, 1976) or *Rhizobium* spp. (Bromfield & Roughley, 1980). However, several fast growing strains isolated from *G. soja* grown in China have been shown to nodulate *G. max* (Keyser, Bohlool, Hu & Weber, 1982). Other fast growing rhizobia have been isolated from *Cajanus cajan* (Raju, 1938; Anon., 1980) and *Cicer arietinum* (Raju, 1936; Okon, Eshel & Henis, 1972; Gaur & Sen, 1981) which are frequently nodulated by slow growing *Rhizobium* spp. However, there have been no comparative studies on the properties of the fast growing rhizobia which nodulate *C. cajan* and *C. arietinum* with the more usual slow growing microsymbiont. This paper reports on the colony characteristics, acid or alkali production, utilisation of carbon sources, symbiotic effectiveness and nodulating competitiveness of fast and slow growing *Rhizobium* spp. isolated from *C. cajan* and *C. arietinum*.

MATERIALS AND METHODS

Rhizobium strains

The fast and slow growing *Rhizobium* spp. were isolated from *Cajanus cajan* or *Cicer arietinum* and obtained from the International Crops Research Institute for the Semi-arid Tropics, Hyderabad, India.

Growth characteristics

Colony sizes and morphological characteristics of *Rhizobium* spp. were recorded after 4, 7 or 10 days at 28 °C on tryptone yeast-extract (TY) medium (Beringer, 1974) and yeast-extract mannitol agar (YEM) which was modified from Fred, Baldwin & McCoy (1932) by using 1 g l^{-1} dehydrated yeast-extract (Difco) and omission of $CaCo_3$.

Utilisation of carbon sources

Thirty *Rhizobium* spp. consisting of 12 fast and 18 slow growing isolates from *C. cajan* and *C. arietinum* were tested for utilisation of 16 sources of carbon. Tests were carried out in a defined liquid medium modified from Ronson & Primrose (1978) by using KNO_3 as the nitrogen source at 0·6 g l^{-1}. The medium was dispensed aseptically in 5 ml volumes to cotton-wool plugged glass tubes (100 × 12 mm) and the appropriate carbon source (filter sterile) added to a final concentration of 0·4% (w/v). Three replicate tubes for each strain and carbon source were inoculated with 50 μl cell suspension (*c.* 10^8 cells ml^{-1} in sterile water) washed from the surface of a YEM slope. Controls consisted of liquid medium inoculated with each strain but lacking carbon source. The tubes were placed in an orbital shaker at 28 °C for 7 days (fast growing isolates) or 14 days (slow growing isolates) and scored for growth (0–4 scale) by comparison with appropriate controls.

Acid production

Thirty-two fast and slow growing *Rhizobium* spp. from *C. cajan* and *C. arietinum* were tested for acid production by the method of Norris (1965). The acid production medium was modified from Norris (1965) by using 1 g l^{-1} dehydrated yeast-extract (Difco). Inocula were prepared by

washing bacteria from the surface of YEM slopes in sterile water to give *c.* 10^8 cells ml^{-1}. Four replicate tubes were inoculated with 100 μl cell suspension of each strain and randomised in racks.

Symbiotic effectiveness tests

Fast and slow growing *Rhizobium* spp. were tested for symbiotic effectiveness on *C. cajan cv* ICP-1 or *C. arietinum cv* G130. All seeds were surface sterilised (Vincent, 1970) and three of either host planted in 18 cm pots containing sterile sand and grit, 2 : 1 by volume. Immediately after planting, seeds of each host were inoculated with 1 ml of a YEM broth suspension of the appropriate *Rhizobium* strain; uninoculated controls were included. After 2 wk growth in a glasshouse at 26 °C (day) 19 °C (night) the seedlings were thinned to two per pot. The pots were supplied with minus nitrogen solution (Summerfield, Huxley & Minchin, 1977). To prevent cross-contamination by splashing, each pot was enclosed in a Polythene sleeve extending 10 cm from the surface of the sand and grit. The experimental design for each host inoculated with appropriate *Rhizobium* strains was a randomised complete block with four replications. Shoot dry weight, shoot N% and nodule dry weight were determined 8 wk after planting.

Competition studies

Competition for nodulation between fast and slow growing *Rhizobium* spp. was examined on *C. cajan* cv. ICP-1 and *C. arietinum* cv. G130. The procedure and design of the experiments was similar to the above except that each host was grown in sterilised sand and grit and in soil/sterilised sand and grit mixture (1 : 1 by volume). The soil was a sandy loam (pH 6·9, water) collected from Woburn, Bedfordshire and was deficient in native *Rhizobium* spp. able to nodulate *C. cajan* and *C. arietinum*. Intended 1 : 1 mixtures of broth cultures of fast and slow growing *Rhizobium* spp. were each inoculated onto five replicate pots of the appropriate host. The actual proportion of strains in the inocula were determined from colony counts on YEM agar (Vincent, 1970). For nodule strain identity, isolates were made using a sterile needle (Franco & Vincent, 1970) and streaked on plates of YEM and TY medium. On these media fast and slow growing isolates of *Rhizobium* spp. could be readily differentiated by differences in morphology and growth rate; fast growing isolates appeared within 3–4 days whereas the slow growers took 7–10 days at 28 °C.

RESULTS

Colony characteristics

The slow-growing *Rhizobium* spp. formed colonies *c.* 1 mm diameter on YEM and TY medium after 7 days (isolates from *C. arietinum*) and 10 days (isolates from *C. cajan*). Fast growing *Rhizobium* spp. from both legumes formed colonies 3–6 mm in diameter on these media after 4 days. Colonies of the fast growing isolates from *C. cajan* were spreading, runny and generally misty in appearance. Fast growing *Rhizobium* spp. from *C. arietinum* and slow growing isolates from both legumes produced colonies which were circular, convex with entire edges and white in appearance.

Utilisation of carbon sources

Table 1 shows data for the growth responses of fast and slow growing isolates from *C. cajan* and *C. arietinum* tested on 16 carbon sources. The fast growing isolates from *C. cajan* utilised a wider range of the carbon sources tested than the slow growing isolates from this host. However, of the ten fast growing isolates tested, two failed to utilise maltose, eight dulcitol and one lactose, rhamnose, raffinose and sucrose. Several carbon sources also gave slight growth responses (scores of 1 or 2) with up to two isolates. In contrast, only arabinose, xylose, sodium pyruvate

Table 1. *Response of fast and slow growing* Rhizobium *spp. isolated from* Cajanus cajan *and* Cicer arietinum *to 16 carbon sources*

	Cajanus isolates										*Cicer* isolates									
	fast growers					slow growers					fast growers					slow growers				
No. of isolates tested:	10					10					2					8				
	No. of isolates showing growth response* to carbon source:																			
	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
Carbon source																				
L-Arabinose					10				5	5				1	1				1	7
D-Fructose					10	3	6	1							2				1	7
D-Glucose		1			9	3	5	2							2				2	6
D-Xylose					10			5	2	3					2				1	7
Maltose	2				8	7	3								2					8
Mannitol					10	3	5	2							2					8
Dulcitol	8		1		1	10					2					1	3	2	2	
Lactose	1	1	1	1	6	10						2				2		1	2	3
L-Rhamnose	1			1	8	3	7								2	1	1	2		4
D Galactose				1	9	1	2	6	1					2				1		7
Raffinose	1		2		7	10								2		7	1			
Trehalose					10	10									2					8
D-Mannose			1	4	5	1		8	1					2					2	6
Sucrose	1			1	8	9				1				1	1					8
Sodium pyruvate					10			1	6	3				1	1	2	2	1	3	
Sodium succinate			1	7	2				10						2	2	1		4	1
Control	10					10					2					8				

* 0, no growth (equal to control lacking C-source); 1, very slightly turbid; 2, slight-moderate turbidity; 3, turbid; 4, very turbid. Growth responses for each isolate are means of three replicates to the nearest whole number.

and sodium succinate were utilised by all slow growing isolates from *C. cajan*. Dulcitol, lactose, raffinose and trehalose did not support growth of any of these isolates and only one was able to utilise sucrose. Fast and slow growing isolates from *C. arietinum* were able to utilise most of the carbon sources tested although none of the fast growers utilised dulcitol and all showed only a slight growth response (scores of 1) with lactose.

Some slow growing isolates failed to utilise dulcitol, lactose, rhamnose, raffinose, sodium pyruvate and sodium succinate; several isolates showed only a slight growth response (scores of 1 or 2) with these carbon sources.

Acid production

Table 2 shows data for acid production on Norris' (1965) medium (pH 7·2 initially) by fast and slow growing isolates from *C. cajan* and *C. arietinum*. All fast growing isolates from *C. cajan* gave an acidic reaction (pH 5·2–5·6) whereas the slow growers produced a near-neutral to alkaline reaction (pH 6·6–7·5). There was variation in acid or alkali production within the fast and slowing growing isolates, e.g. IHP396 produced less acid ($P < 0·01$) than any of the fast growers and IHP2 and IHP20 produced more ($P < 0·05$) than all except IHP486; IHP309 produced more alkali ($P < 0·01$) than any of the slow growers and IHP41 and IHP53 less ($P < 0·01$) than all except IHP377, IHP156 and IHP456. The fast and slow growing isolates from *C. arietinum* all produced an acidic reaction (pH 4·4–5·6) on Norris' (1965) medium. Strains IC4, IC72 and IC94 (slow growers) produced a less acidic reaction ($P < 0·01$) than the remaining fast and slow growing isolates.

Table 2. *Acid or alkali production by fast and slow growing* Rhizobium *spp. isolated from* Cajanus cajan *and* Cicer arietinum

Host of isolation: *C. cajan*			*C. arietinum*		
Strain	Growth rate	final pH*	Strain	Growth rate	final pH*
IHP509	fast	5·64	IC2073	fast	4·64
IHP2	fast	5·16	IC122	fast	4·56
IHP100	fast	5·44	IC72	slow	5·58
IHP170	fast	5·32	IC74	slow	4·76
IHP20	fast	5·18	IC4	slow	4·38
IHP396	fast	5·84	IC55	slow	5·10
IHP502	fast	5·60	IC76	slow	4·56
IHP307	fast	5·32	IC70	slow	4·58
IHP486	fast	5·28	IC2060	slow	4·52
IHP38	slow	7·14	IC94	slow	5·22
IHP377	slow	6·70	IC2028	slow	4·78
IHP37	slow	7·06	CBH32	slow	4·66
IHP156	slow	6·74	IC35	slow	4·48
IHP95	slow	7·10	uninoculated	—	7·18
IHP309	slow	7·46	S.E.D. = 0·093 D.F. = 56		
IHP41	slow	6·64			
IHP456	slow	6·72			
IHP53	slow	6·66			
IHP202	slow	6·92			
uninoculated	—	7·20			
S.E.D. = 0·055 D.F. = 80					

* Values are means of four replicates.

Symbiotic effectiveness

Data for the symbiotic effectiveness of fast and slow growing *Rhizobium* spp. on *C. cajan* and *C. arietinum* are given in Table 3. The fast and slow growing isolates from *C. cajan* produced more shoot dry matter and shoot N% ($P < 0{\cdot}01$) than uninoculated plants and were equally effective on this host. The exceptions were IHP307 (fast growing isolate) which produced more ($P < 0{\cdot}05$) shoot dry matter than IHP38 (slow growing isolate) and IHP38 and IHP309 (slow growers) which produced significantly higher shoot N% ($P < 0{\cdot}05$) than IHP2 and IHP486 (fast growers). Strains IHP38 and IHP377 formed black pigmented nodules similar to those previously reported (Anon., 1978) for several isolates from *C. cajan*. The fast and slow growing *Rhizobium* spp. tested on *C. arietinum* also produced significantly more ($P < 0{\cdot}01$) shoot dry matter and shoot N% than uninoculated plants and were equally effective. The only exception was IC72 (slow grower) which produced more shoot dry matter ($P < 0{\cdot}05$) than IC2073 (fast grower).

Competition studies

Table 4 shows data for the proportion of strains in the nodules of *C. cajan* cv. ICP-1 and *C. arietinum* cv. G130 grown in sand or soil and inoculated with intended 1 : 1 mixtures of fast and slow growing *Rhizobium* spp. For strain identity, 30 nodules selected at random were taken from each of five replicates for each host grown in sand or soil and inoculated with each mixture of strains. Fast growing *Rhizobium* spp. formed the majority (70–80%) of the nodules with half of the inoculated combinations of strains on *C. cajan* grown in sand, whereas slow growing isolates formed most of the nodules (80–90%) with the remaining two inoculum mixtures. Similar results were obtained with *C. cajan* grown in soil except with one inoculum mixture

Table 3. *The response of* Cajanus cajan, *cv. ICP-1 and* Cicer arietinum *cv. G130 to inoculation with fast or slow growing* Rhizobium *spp. (means of four replicates)*

Inoculum	shoot dry weight g plant^{-1}	Shoot N%	Nodule dry weight mg plant^{-1}
	Host: *C. cajan* cv. ICP-1		
IHP2F*	0·62	3·13	95
IHP307F	0·73	3·31	119
IHP486F	0·57	3·21	114
IHP509F	0·61	3·28	111
IHP53S	0·72	3·43	112
IHP38S	0·59	3·58	97
IHP309S	0·65	3·54	101
IHP377S	0·68	3·40	107
uninoculated	0·41	1·92	—
S.E.D.	0·08	0·15	14·65
	D.F. = 24	D.F. = 24	D.F. = 21
	Host: *C. arietinum* cv. G130		
IC2073F	0·98	3·55	110
IC122F	1·10	3·67	122
IC72S	1·25	3·71	154
IC74S	1·00	3·48	134
uninoculated	0·69	2·12	—
S.E.D.	0·11	0·13	18·47
	D.F. = 12	D.F. = 12	D.F. = 9

* F = fast growing strain; S = slow growing strain.

Table 4. *Proportion of nodules on* Cajanus cajan *cv. ICP-1 and* Cicer arietinum *cv. G130 grown in sand or soil and inoculated with mixtures of fast and slow growing* Rhizobium *spp.*

Inoculum (fast growing strain + slow growing strain)	Host	% fast growing strain in inoculum	% nodules due to fast growing strain on plants grown in: sand	soil
IHP486 + IHP309	*C. cajan*	58	72*	39**
IHP509 + IHP377	*C. cajan*	57	81**	77**
IHP307 + IHP38	*C. cajan*	43	23**	20**
IHP2 + IHP53	*C. cajan*	54	8**	19**
IC2073 + IC72	*C. arietinum*	72	89**	94**
IC122 + IC74	*C. arietinum*	71	86**	98**

Asterisks denote level of significance (*, $P < 0{\cdot}05$; **, $P < 0{\cdot}01$) in χ^2 tests (D.F. = 1) comparing ratios of strains in each inoculum with those in nodules of plants grown in sand or soil.

(IHP486 + IHP309) where the majority of the nodules were formed by the slow growing strain contrasting to a majority formed by the fast grower on this host grown in sand. Chi squared analyses showed that in every instance the ratios of strains in the nodules deviated significantly from the ratio in each inoculum due to either fast or slow growing strains producing the majority of the nodules.

The fast growing strain in each inoculum mixture formed the majority of the nodules on *C. arietinum* grown in sand or soil. Chi squared analyses showed that the ratios of strains in the nodules deviated ($P < 0{\cdot}01$) from the ratios in the inocula.

DISCUSSION

The fast growing *Rhizobium* spp. from *C. cajan* and *C. arietinum* used in this investigation produced 3–6 mm diameter colonies after 4 days and were quite unlike slow growing isolates which formed *c.* 1 mm diameter colonies after 7–10 days. Fast growing species of *Rhizobium* have been reported to utilise a wide range of carbon sources whereas slow growing species are more specialised in their requirements (Vincent, 1974). The fast growing *Rhizobium* spp. isolated from *C. cajan* and used in our experiments similarly demonstrated very different carbon source utilisation and were able to grow with a wider range of these compounds than the slow growing isolates from this host. However, most fast and slow growing isolates from *C. arietinum* were able to utilise the majority of carbon compounds tested. In this respect the slow growing isolates from *C. arietinum* differ from the other slow growing species of *Rhizobium* (Graham, 1964; Glenn & Dilworth, 1981) in their ability to utilise disaccharides. This suggests that the slow growing isolates from *C. arietinum* possess disaccharide hydrolytic enzymes and uptake systems similar to those reported for the fast growers (Glenn & Dilworth, 1981).

According to the evolutionary concept of the Leguminosae (Norris, 1965), the slow growing alkali producing *Rhizobium* represents the 'ancestral' form and is typically associated with symbiotically unspecialised tropical legumes inhabiting acid soils of low exchange capacity. The fast growing acid producing species of *Rhizobium* are considered to be associated with the taxonomically advanced temperate legumes which are specific in their nodulating requirements and have a special requirement for high soil pH, available calcium and nutrient status. The slow growing isolates from *C. cajan* used in our experiments produced an alkaline reaction in culture in accordance with this concept. However, all the fast growing isolates from *C. cajan* were acid producers and may represent intermediate forms between the 'ancestral' *Rhizobium* as defined by Norris (1965) and the fast growing species (*R. leguminosarum*, *R. phaseoli*, *R. trifolii* and *R. meliloti*) which form a symbiosis with the taxonomically advanced and symbiotically specialised temperate legumes. The fact that the fast growing *Cajanus* isolates were as effective in nitrogen fixation as the slow growers on this host indicates that this was not a 'relic association' which Norris (1965) describes as being occasionally formed by fast growing species of *Rhizobium* retaining their 'ancestral' ability to nodulate tropical legumes whilst normally losing their capacity to form an effective symbiosis. Norris (1965) also considers that symbiotic specialisation of the host is strongly correlated with acid production by its characteristic *Rhizobium*. Our finding that the fast and slow growing isolates from *C. arietinum* were acid producers and the fact that this host is highly specific in its nodulating requirements (Guar & Sen, 1979) lends support to this concept.

The method used in this paper to identify strains in nodules, based on differences in growth rate, did not permit the detection of double strain occupancy of nodules involving fast and slow growing isolates because the slow growing component tended to be overgrown on reisolation by the fast growing strain. However, Bromfield & Jones (1980) have demonstrated that dual strain occupancy of nodules is of rare occurrence in soil. The results of the competition studies with *C. cajan* and *C. arietinum* showed that in all but one instance the proportion of nodules formed by fast growing strains on plants grown in sand was similar to or less than the proportion on plants grown in soil indicating that double infections involving fast and slow growers occurred infrequently in sand. However, the single aberrant result with *C. cajan* where a fast growing strain (IHP486) formed a greater proportion of nodules in sand than in soil may have been partly due to undetected double infections on the plants grown in sand.

If the fast growing isolates from *C. cajan* are considered intermediate forms between the 'ancestral' and 'advanced' *Rhizobium* as defined by Norris (1965) then it might be expected that competition for nodulation between fast and slow growers would reveal almost exclusive selection by the host for the slow growing strains. However, our results showed that half of the fast growing isolates when inoculated in mixtures with slow growers, formed the majority of

nodules on *C. cajan* perhaps indicating an even closer evolutionary relationship with the slow growing, alkali producing rhizobia which nodulate tropical legumes.

With *C. arietinum*, all fast growing isolates inoculated in mixtures with slow growers, formed the majority of nodules, a result which when considered in conjunction with their symbiotic efficiency suggests a high degree of compatibility with this host.

Although only a limited number of strains were examined, it is probable on the basis of these results that indigenous fast growing rhizobia able to nodulate *C. arietinum* are more frequently encountered than normally anticipated and may have been unconsciously excluded from culture collections because their morphology deviates from the slow growing type usually expected to be associated with this host.

In this respect a field survey determining the proportion of fast and slow growing isolates able to nodulate *C. arietinum* might well repay investigation.

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