Proc. Indian natn. Sci. Acad. B **44**, pp. 71-77 (1978)

Microbiology

BIOCHEMICAL STUDIES ON *RHIZOBIUM TRIFOLII*

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The biochemical aspects like utilization of asparagine, reduction of potassium nitrite and tetrazolium triphenyl chloride were studied in order to know the efficiency of six strains of *R. trifolii*. Effect of different antibiotics on growth of different rhizobia revealed that chlorotetracycline was most inhibitory to *Rhizobium* while penicillin was least active. In pot culture studies, under both sterilized and unsterilized conditions, Aust T_6 strain showed an increase of 419·4% and 341·34% in fresh weight, 54·76% and 88·57% in dry weight and 137·41% and 152·4% in protein content respectively. These differences were, however, non-significant. The increase in chlorophyll-*a* and chlorophyll-*b* under sterilized and unsterilized conditions was, however, significant. The field experiment showed non-significant increase of 27·52% in fresh weight and 18·5% in dry weight of berseem. In protein content there was a significant increase of 36%.

INTRODUCTION

The strains of *Rhizobium* differ in nitrogen fixing capacity, nodule formation, chlorophyll content biochemical and serological characteristics. Efforts are being made by scientists to correlate the efficiency of *Rhizobium* with biochemical tests like utilization of asparagine (Domery & Alexander, 1969), reduction of tetrazobium triphenyl chloride and reduction of potassium nitrite. Various inhibitory substances or antagonistic micro-organisms have been reported to effect the efficiency of *Rhizobium* (Nilson, 1957; Keckes & Manninger, 1962). Keeping this in view, the present studies were undertaken to compare biochemical test with efficiency of *Rhizobium* and also to compare the performance of six strains of *Rhizobium trifolii* procured both from within and outside the country.

MATERIALS AND METHODS

Six strains of *Rhizobium trifolii* procured from various research stations were used in the present studies :

T_1 = *Rhizobium trifolii* 7001, Rothman's Experimental Station, U.K.
T_2 = *R. trifolii*, Department of Microbiology, PAU, Ludhiana
T_3 = *R. trifolii* 7003, T. A. I. Brochwell, Australia
T_4 = *R. trifolii*, Department of Microbiology, PAU, Ludhiana
T_5 = *R. trifolii* 7005, Institute of Soil Fertility, Netherland
T_6 = *R. trifolii* 7015, Dr Quick, Australia

All the cultures were maintained on yeast extract mannitol agar (YEMA) slants by fortnightly transfers.

Biochemical Studies

a) Utilization of nitrogenous compound (asparagine) — The strains were grown in 100 ml of the medium (mannitol, 10 g, K_2HPO_4, 0·5 g, $MgSO_47H_2O$, 0·2 g,

$CaCO_3$, 1·0 g, asparagine, 0·5 g and distilled water, 1000 ml) at 32·5°C. After a month the culture liquid was centrifuged and the nitrogen content of aliquot from the clear liquid was determined by the microkjeldhal method. The amount of nitrogen utilized was calculated from the difference in the original and final contents of the medium.

b) Reduction of 2, 3, 5 triphenyll tetrazolium chloride (TTC) — Test tubes containing 10 ml of yeast extract mannitol agar medium with 0·1 ml of 1% TTC were inoculated with 3 drops of freshly prepared culture of each strain and incubated at room temperature for 14 days.

c) Nitrite reduction — The nitrite booth (containing peptone 5·0 g, yeast extract, 3·0 g and KNO_2, 1·0 g in 1000 ml of distilled water) was inoculated with strains of *Rhizobium trifolii* and incubated for 2-7 days at 30°C. Reduction of nitrite was tested with sulphonite and L-nepthalamine.

d) Antibiotic sensitivity test — The antibiotic discs of chlorotetracycline (25 mcg) sulfadiazine (30 mcg), polymycin-B (300 mcg), penicillin-G (10 mg), streptomycin (10 mcg) and neomycin (30 mcg) per disc were tested at standard concentration for their effectiveness on different strains of *R. trifolii* using seeded medium technique. Discs were placed on the plates aseptically, then they were incubated under aerobic conditions upside down at 28°C for 48 hr. All results were recorded as zone of inhibition of various antibiotics, in mm.

Pot Culture Experiment

Disease-free seeds of berseem (var. BL-1) were surface sterilized with 0·1% $MgCl_2$ following sterilized distilled water washings. These seeds were inoculated with 48 hr-old culture suspension and sown in earthen pots containing 8 kg of sandy loam soil of *p*H 8·0. The number of seedlings was thinned down to 7 per pot at the end of 6 days and again reduced to 5 at the end of 15 days so as to retain uniform number of plants in each pot. The plants were uprooted after 8 weeks and examined for nodulation, chlorophyll and dry weight.

Field Experiment

A field trial was conducted on sandy loam soils to find out most efficient strains of *Rhizobium trifolii.* Seeds after soaking in cold water were inoculated with two days old broth 10^9 cells/ml. These seeds were sown in randomized plots measuring 1/200 of an acre in quardruplicate by broadcast method. Uninoculated seeds served as control.

Observations on nodulation, chlorophyll and yield at different intervals of time (4, 8, 12 and 16 weeks) were taken.

RESULTS

Biochemical Characterization of Different Strains of R. trifolii

Different biochemical tests were carried out to know the efficiency of six strains of *R. trifolii.* Maximum utilization of asparagine was shown by Aust. T_6 (24 mg) and the minimum by LDH T_4 (0·8 mg). Strains utilized the asparagine from

1·4 to 2·4 mg/100 ml of the medium. Triphenyll tetrazolium chloride (TTC) was not reduced by any strain while the reduction of potassium nitrite was shown by all except Aust. T_6. It was also interesting to note that all the foreign strains showed the maximum utilization of asparagine when compared with local ones. The results are summarized in Table I.

TABLE I

Utilization of asparagine, reduction of TTC and nitrite by different strains of R. trifolii

Strains	Asparagine utilized mg/50 mg/ 100 ml medium	Reduction of Triphenyl tetrazolium chloride	Reduction of KNO_2
UK T_1	2·12	—	+
LDH T_2	1·40	—	+
AUST. T_3	2·20	—	—
LDH T_4	0·80	—	+
NETH T_5	2·30	—	+
AUST T_6	2·40	—	+

+ = Reduction
— = No Reduction

Effect of antibiotics on R. trifolii

The antibiotic resistance of short and broad spectrum by different strains of *R. trifolii* is shown in Table II. Among the six strains of Rhizobia, LDH T_2 was most sensitive with a sensitive zone of 2·7 and UK T_1 was least so having only 0·5 mm sensitive zone when tested against chlorotetracycline. Similarly sulfadiozine showed variable inhibition zone against various strains. The inhibition was 1·25 mm in LDH T_4; 1·75 mm in Aust. T_3; 1·20 mm in LDH T_4; 2·00 mm in Neth. T_5 and 0·75 mm in Aust. T_6 while there was no inhibition in Neth. T_5 and UK T_1 strains.

TABLE II

Effect of antibiotics on different strains of R. trifolii

Antibiotics	Standard concentration per disc in (mcg)	Diameter of inhibition zone in mm (Average of four replicates)					
		UK T_1	LDH T_2	AUST. T_3	LDH. T_4	NETH. T_5	AUTS. T_6
Chlorotetracycline	10	0·50	2·75	1·75	1·50	1·00	2·20
Sulfadiazine	30	—	1·25	1·75	1·20	2·00	0·75
Polymyxin-B	300	0·70	—	—	—	1·00	0·35
Penicillin-G	10	—	—	—	—	—	—
Streptomycin	10	—	1·50	0·45	0·75	1·20	1·50
Neomycin	30	1·75	1·75	1·50	1·28	1·50	0·50

— = No inhibition

Inhibition through polymyxin-B was 0·70 mm, 0·10 mm and 0·35 mm in UK T_1, Neth T_5 and Aust T_5 respectively. Other strains like LDH T_2, Aust T_5 and LDH T_4 were not affected. It was also observed that streptomycin produced inhibition zone of 0·45 to 1·50 mm and neomycin could inhibit from 1·28 mm to 1·75 mm while penicillin failed to inhibit any.

Weight of Nodules

In pot culture experiment the weight of nodules was observed under sterilized and unsterilized conditions. There was a significant difference in the weight of nodules of plants inoculated with the strains Aust. T_6 ; Neth. T_5 ; UK T_1 and Aust. T_3. But there was no significant difference in LDH T_2 and T_4 in sterilized and unsterilized conditions. The weight of nodules increased from 158% of control (in unsterilized conditions) to 464%. It was also interesting to see that the values were significantly higher in sterile conditions than in unsterilized ones except for Aust. T_6. The performance of imported cultures was better than the local ones—the increase ranged from 112 to 158% in the locals and 221 to 464% in the foreign ones — the order of efficiency being T_6, T_5, T_3, T_1, T_2 and T_4.

Chlorophyll Content

The chlorophyll content much varied in the sterilized plants of *Trifolium alexandrinum* inoculated with *R. trifolii*. Plants inoculated with six strains of *R. trifolii* had higher chlorophyll content than the control ones, both under sterilized and unsterilized conditions (Table III).

Fresh, Dry Weight and Protein Content of Plants

As could be seen from Table III, plants grown in soil inoculated with different strains of *R. trifolii* showed a marked and significant increase in fresh, dry weight and protein content. In these the plants showed significant symbiosis with strains Aust. T_6, Neth. T_5, Aust. T_3 and UK T_1 and to a lesser extent with LDH T_2 and T_4. The increase in fresh weight in Aust. T_6, Neth. T_5 and Aust. T_3 ranged from 114·90 to 419%. Protein contents of different strains also showed an increase over the control under sterilized and unsterilized conditions. The trend of performance with different strains in all the observations was similar and all strains showed a significant increase in protein and dry matter also. The efficiency of nitrogen fixation by local diazotrophs was poorer than the foreign diazotrophs and exhibited different ecological status under natural and green house conditions.

Field Experiments

When various strains showed favourable results under pot culture experiments, they were tried under field conditions also and the findings of the trial are summarized in Table IV. It was encouraging to note that the plants inoculated with *R. trifolii* give higher yield than the control ones. A maximum yield was shown by Aust. T_6. This was followed by Neth. T_5, and Aust. T_3, UK T_1, Ldh. T_2 and Ldh. T_4. The differences in the yield in Aust. T_6, Neth. T_5 and UK T_1 were significant while it was not so in Ldh. T_2 and Ldh. T_4. It confirmed

TABLE III

Effect of Rhizobium inoculation on various characteristics of berseem after 8 weeks of growth

Treatments	Weight of nodules mg/5 plants		Chlorophyll				Fresh weight g/5 plants		Dry weight g/5 plants		Protein mg/5 plants	
			Sterilized		Unsterilized							
	Sterilized	Unsterilized	a	b	a	b	Sterilized	Unsterilized	Sterilized	Unsterilized	Sterilized	Unsterilized
Control	—	140	17·81	23·54	7·5	12·87	7·625	8·225	0·525	0·428	50·4	46·3
UK T_1	465	400	42·15	68·50	14·63	26.43	15·796	13·942	0·675	0·634	95·4	79·4
Ldh. T_2	372	362	36·95	64·97	9·96	18·12	13·750	11·492	0·650	0·534	79·3	77·7
Aust. T_3	498	459	44·55	74·21	15·43	28·77	16·500	15·400	0·718	0·635	100·5	102·7
Ldh. T_4	282	291	33·90	63·25	9·83	14·80	14·014	10·450	0·638	0·475	69·6	65·8
Neth. T_5	720	563	48·27	85·25	17·93	30·96	17·050	15·400	0·750	0·700	110·5	102·9
Aust. T_6	755	790	53·40	100·40	18·25	31·56	17·875	18·050	0·813	0·825	127·2	109·8
F test		N.S.		N.S.				N.S.		N.S.		N.S.
C.D. at 5% level		42·46		14·29				—		—		—

TABLE IV

Effect of Rhizobium inoculation on fresh and dry weight yield of berseem at various periods

Treatments	Fresh weight Kg/plot (weeks)				Dry weight Kg/plot (weeks)			
	4	8	12	16	4	8	12	16
Control	11·8	24·3	26·7	32·8	1·32	3·26	4·14	8·03
UK T_1	13·8	27·12	29·8	39·2	1·45	3·60	4·50	8·06
Ldh. T_2	12·2	26·7	27·8	38·3	1·40	3·56	4·51	8·04
Aust. T_3	15·7	28·5	31·5	41·5	1·52	3·66	4·50	9·15
Ldh. T_4	11·0	24·5	27·8	38·2	1·39	3·36	4·21	8·80
Neth. T_5	12·6	29·6	32·5	42·8	1·62	3·51	4·87	9·15
Aust. T_6	18·1	33·1	35·5	42·0	1·72	3·90	4·96	9·21
F test			N.S.				S	
C.D. at 5% level			3·15				0·484	

the findings of pot culture experiment. In this respect four cultures received from foreign countries were better than the local ones. This will be clear from Table IV.

Discussion

The ability to reduce potassium nitrite and inability to metabolize TTC was shown by all the strains except Aust. T_3. Similar results were obtained by Habbal and Elkan (1967) while correlating the efficiency of nitrogen fixation by *R. japonicum* and its inability to metabolize nitrite and failure to reduce TTC. This was, however, contradictory to their earlier report of reduction of nitrite by *Rhizobium* sp. Gupta and Sen (1963) also reported several strains of *Rhizobium* sp. from gram, which reduced nitrite. The attempt of correlating biochemical property of *R. trifolii* with efficiency was partially successful. The hypotheticated positive correlation of the organic nitrogen utilization with nitrogen fixation is confirmed here.

In antibiotic inhibition test, maximum inhibition was shown by chlorotetracycline, and no inhibition by pencillin. It was, however, possible to correlate the efficient strains, Aust. T_6, Neth. T_5, Aust. T_3 and UK T_1 with the degree of resistance against the antibiotics. The local strains showed resistance against only two antibiotics. No correlation could, however, be drawn between effectiveness of Rhizobia and antibiotic action. This is in line with the findings of Abdel Ghaffar and Allen (1950). It was also interesting to note that the different strains of *R. trifolii* showed different degree of sensitivity when tested against 6 antibiotics, including short and broad spectrum. Similar observations were also made by Keckes and Manninger (1962); Sethi (1967, 1971), and Sethi and Subha Rao (1972).

Inoculation with six strains of *R. trifolii* including four foreign and two local ones increased the yield, weight of nodules, chlorophyll and protein content. All the foreign strains had better edge over the local ones. However, the values were significant in case of Aust. T_6 only. The correlation between the weight of nodules, chlorophyll and protein content was seen to be positive and significant in case of Aust. T_3, Neth. T_5 and Aust. T_6 whereas it was non-significant in case of Ldh. T_2 and Ldh. T_4. The values obtained under sterilized and unsterilized conditions were very close. However, the values obtained under sterilized conditions were numerically higher than the unsterilized ones. The results were contrary to the findings of Sethi (1971) and Sethi and Subba Rao (1972). It is now a well known fact that inoculation increases the weight of nodules, dry weight, yield and protein control. All these characters varied in magnitude depending upon the efficiency and competitiveness of isolates.

Yield was the ultimate analysis for the efficiency of various strains of *R. trifolii* inoculation under field conditions. Highest yield was obtained on the Aust. T_6 (32·12 kg/plot) followed by Neth. T_5 (29·38 kg/plot), Aust. T_3 (29·0kg/plot) UK T_1 (27·9 kg/plot); Ldh. T_2 (26·28 kg/plot) and Ldh. T_4 (25·64 kg/plot). The same parameter was taken into consideration by Gibson (1968) also, while considering the efficiency of different legume strains. It indicated that efficient

foreign diazotrophs could compete with native rhizobia and could tolerate the change in ecological conditions for their symbiotic performance, There was a similar trend in the dry weight and the protein content due to inoculation.

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