



Growth rate and Carbon source Utilization Patterns of Rhizobia isolated from *Cicer arietinum* L. and *Pisum sativum* L.

S. T. QURESHI⁺⁺, A. R. ABASSI*, A. YASMEEN**, A. J. PIRZADA, H. BUX, F. A. BUGHIO

Institute of Plant Sciences, Sindh University Jamshoro

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Abstract: Growth rate and carbon source utilization pattern are useful parameters for the estimation of genetic diversity among rhizobia. Nineteen chickpea and eleven green pea nodulating rhizobial strains were studied for growth rate and carbon source utilization. Isolates nodulating both crops were divided in to three groups on the basis of growth rate, although chickpea nodulating rhizobia were comparatively slow growers than green pea nodulating rhizobia. Most utilized carbon sources (100%) by chickpea isolates were L-arginine and urea where as green pea mainly depend on L-arginine and malic acid for carbon requirement. No chickpea strain consumed L-tryptophane as carbon source. Cluster analysis based on carbon source consumption divide 19 chickpea isolates with check strain Bio Power in to for major groups, where as green pea isolates were grouped in to three major clusters, and chickpea rhizobia are more selective and less diverse for their carbon source then green pea rhizobia.

Keywords: Growth Rate, Carbon Source, Rhizobium

1. INTRODUCTION

By the start of 1960's Green revolution technologies were introduced in Pakistan in by cultivation of high yielding varieties (HYV's). This technology soon become ineffective as most of HYV's need more irrigation, chemical fertilizers, pesticides and insecticides that result in cost of farmer. Recurrent irrigation severely damage soil micro and macro environments of which salinity was the major factor that badly affect cultivated agricultural land that results significant genetic erosion (Yasin, 2007). Fungicides applied to fields on one hand imposed toxic effects on symbiotic bacteria and their activities and caused serious allergies to human by consuming those food plants (Ayansina and Oso, 2006; Mubeen *et al.*, 2006). The constant use of pesticides usually causes resistance among pests and vectors and adversely affects non-target organisms. On the basis of so many direct and indirect hazardous effects of chemical fertilizers the scientific community was forced to try environmental friendly bio-fertilizer as safe alternatives. Now most of European countries used these alternate fertilizers and now even many research institutes in Pakistan like National Agriculture research center and Nuclear Institute of biology and genetic engineering produce biofertilizer at commercial level. There are different types of Biofertilizer such as Sulphar and nitrogen fertilizers.

Before rhizobia are made available commercially for field application it is necessary to properly characterize and identify them to get maximum biological nitrogen fixation (BNF) out of any legume-

rhizobium association (Sahgal and Johri, 2003). Many scientist all over world focused on genetic diversity within rhizobial natural populations more attentively (Satyaprakash and Annapurna, 2006; Küçük and Kivanc, 2008). Some other researchers have used carbon source utilization patterns to recognize efficient symbiotic strains among the *Rhizobiaceae* family (Küçük and Kivanc, 2008). Therefore present work is under taken to reveal growth rates and carbon source utilization patterns of Pakistani rhizobia before they are released commercially as bio-fertilizers.

2. MATERIAL AND METHODS

In order to study growth rate and patterns of carbon consumption 8–22 cm deep soil samples were collected initially from 27 localities from all over Pakistan and stones were removed manually. Metallic pistil and mortar was used to ground soils than samples were sieved and stored in plastic jars till used. Commercial strain of rhizobia for chickpea and green pea was isolated from commercial) biofertilizer (Bio-power) through root nodules. Seeds of chickpea (*Cicer arietinum* L.) and green pea (*Pisum sativum* L.) were surface sterilized with 70% alcohol for 2–3 minutes. Six seeds per pot (20 × 30 cm) of *Cicer arietinum* L. and *Pisum sativum* L. were sown in the soil. In case of commercial strain seeds were moisten first with distilled water and bio-power was sprinkled over them with a pinch of sugar. The seeds were then carefully transferred to pots containing sterile soil.

The experiment was conducted in green house. Inoculated Plants were watered only while commercial

⁺⁺ Corresponding author Sadaf Tabasum Qureshi e-mail: qureshi_plantsciences@hotmail.com

*Department of Fresh Water Biology and Fisheries, Pakistan University of Sindh Jamshoro, Pakistan

**Department of Chemistry, Lahore College for Women University, Lahore, Pakistan

strain (Bio-power) were given nitrogen free nutrient media once a week. 6 weeks old plants were harvested to get nodules four to six healthy pink nodules. Initially nodules were washed thoroughly with distilled water followed by surface sterilization with 95% ethanol. At the end sodium hypochlorite 5.25% solution was used for sterilization suggested by Weaver and Graham (1994).

Crushing of nodules in sterilized water with glass was carried out aseptically in Laminar air flow chamber with help of glass rod until milky and resulted solution was streaked on yeast extract manitol agar (YMA) containing .0025% (w/v) Congo red plates (Vincent, 1970). The rhizobia that absorb little or no Congo red in the dark were considered rhizobia and finally subcultured after two days of growth to fresh plates of (YMA) without Congo red to obtain pure culture at 28°C. Fresh cultures were used for staining (Gram's staining) to confirm them as gram-negative rods. All the cultures were stored in 50% glycerol and YEM broth in 1:1 ratio solution at -70°C. Growth rate of chickpea and green pea rhizobia was monitored on (YEM) liquid medium incubated in shaker at 200- rev min⁻¹ after every 2 h by measuring the optical density at 600 nm. The estimate generation time logarithmic phase of growth was used (Maâtallaha *et al.*, 2002a).

API NE 20 kit was used to test the carbon source utilization patterns of selected rhizobia (Kücük and Kivanc 2008). The test was carried out according to the Manufacturer's protocol. Cultures were grown during 24 h in (YEM) liquid medium and uniform suspensions were prepared by suspending the cells in medium provided in kit. Suspensions for each isolate were standardized to 0.5% McFarland solution. Then 150 µl of suspension was inoculated into each well of the strip. The strips were incubated at 28°C for 48 h under static conditions in aseptic environment of growth chamber.

Statistical analysis

Carbon utilization patterns obtained were converted in a binary data matrix. Where 1 represent positive result and 0 for negative against each carbon source. Pair-wise comparisons of the isolates were used to generate similarity coefficients. The resulting similarity coefficients were further used to drive relationships among rhizobial strains of each crop in the form of cluster analysis using an un-weighted pair-group method with arithmetic averages. Finally a dendrogram was constructed using similarity metric. All the analysis was done using computer software Statistica (6.0).

3. RESULTS AND DISCUSSION

Nodulation test

Out of 27 soil samples, 19 chickpea and 11 green pea root nodule bacteria were isolated for the ability to

effectively nodulate in chickpea and green pea seedlings respectively when applied in sterile soil and sand mixture in pots.

Colony morphology

The gram staining revealed that all the isolated bacteria were gram negative rods and ultimately rhizobia (**Fig. 1-3**). All the chickpea (**Fig. 2**) and green pea (**Fig. 4**) rhizobial colonies were either shiny semi-transparent or milky-white with circular shape. Similar colony morphology are reported by many workers dealing with diversity of rhizobial strains (Kücük and Kivanc, 2008; Sajjad *et al.*, 2008).

Generation time

Time required by a strain to reach logarithmic growth phase is referred as generation time. From simple physiological studies to most complicated genomic techniques logarithmic phase broth cultures are used. As every strain has different growth rate ranging from slow, moderate to fast, therefore it is essential to carry out generation time studies before doing any type of study. According to present study both chickpea rhizobia and green pea fall under three groups. In case of chickpea 25% of the isolates were fast growers (50 min < GT < 3 h), 25% were slow growers (3 < GT ≤ 9) and 50% were very-slow growing bacteria with a generation time of more than 9 h (**Fig. 5**). While 33.3% of the green pea isolates were fast growers (50 min < GT < 3 h), 50% slow growers (3 < GT ≤ 9) and 16.66% were very-slow grower with a generation time of more than 9 h (**Fig. 6**). Different growth rate within chickpea as well as green pea rhizobia supported by the earlier work by Maâtallah *et al.* (2002a & b) who reported that 8.3% of the chickpea nodulating strains were fast-growing, 41.7% were slow growing and 50% were very slow growers.

Carbon source utilization

One of the most initial tests to distinguish strains among the *Rhizobiaceae* family is carbon utilization patterns. A number of carbon substrates were metabolized by most of the tested chickpea rhizobia strains as carbon substrates (**Fig. 7**). L-arginine and urea were the most utilized carbon sources (100%) followed by L-arabinose (95%) and potassium gluconate, D-maltose (90%). Only L-tryptophane was not utilized by any of the chickpea nodulating rhizobia (**Table 1**).

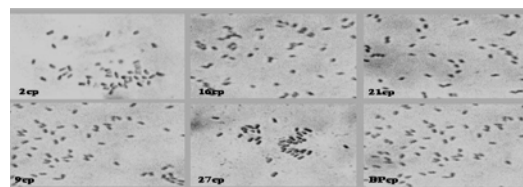


Fig. 1: Gram's staining of chickpea root nodule bacteria (cp) (40x)

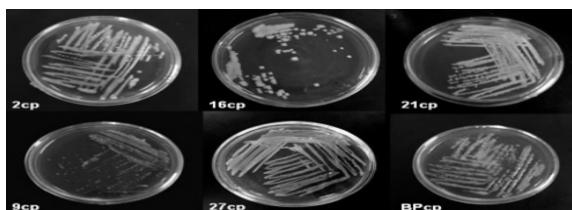


Fig. 2: Colony morphology of chickpea root nodule bacteria (cp) and commercial strain (BPcp)

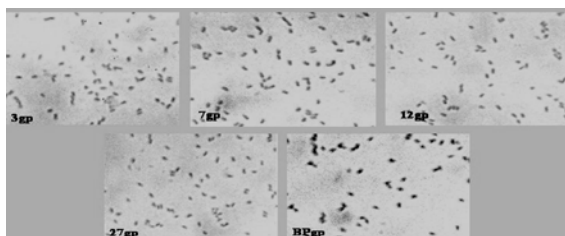


Fig. 3: Gram's staining of green pea root nodule bacteria (gp) (40x)

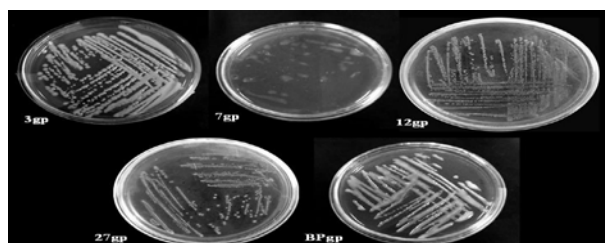


Fig. 4: Colony morphology of green pea root nodule bacteria (gp) and commercial strain (BPgp)

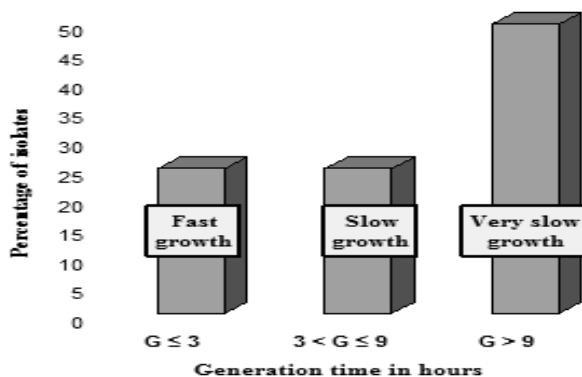


Fig. 5: Generation times of tested chickpea nodulating bacteria

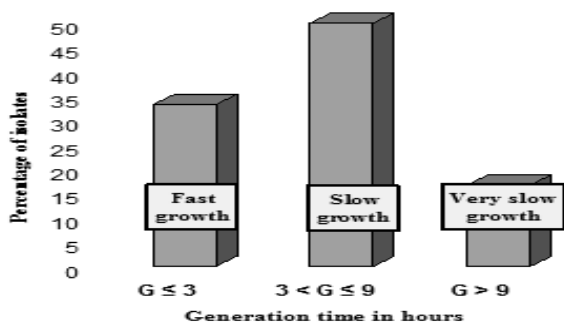


Fig. 6: Generation times of tested green pea nodulating bacteria

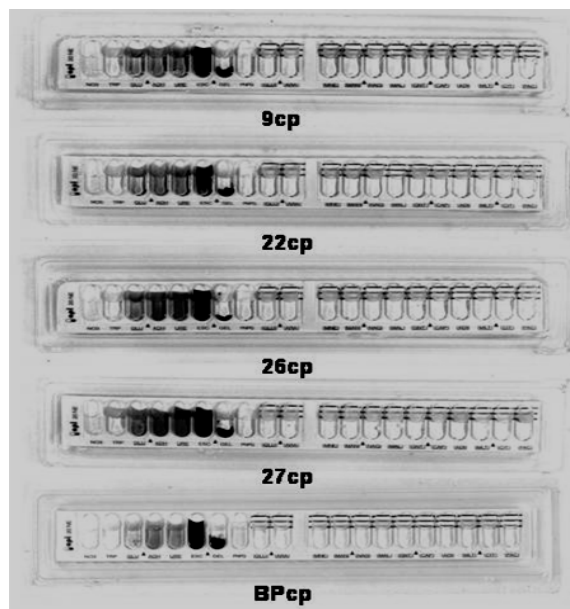


Fig. 7: API NE 20 kit strips showing differential response for carbon source utilized among chickpea rhizobia and commercial strain

Table 1: Carbon source utilized by chickpea rhizobia (percentage)

Carbon source	Isolates (%)
L-arginine	100
L-arabinose	95
D-maltose	90
Potassium gluconate	90
Malic acid	80
D-galactose	75
D-mannose	75
D-mannitol	75
N-acetyl-glucosamine	70
Tri sodium citrate	70
n-capric acid	60
Adipic acid	35
Phenylacetic acid	35
D-Glucose	20
L-tryptophane	0

Green pea rhizobial strains were also diverse for their carbon (Fig. 6). All the isolates (100%) utilized L-arginine and D-glucose and malic acid followed by L-arabinose, D-mannose, D-mannitol, D-maltose and potassium gluconate (92%). D-glucose was the least utilized carbon source (17%) (Table. 2). It is obvious from result that most of tested chickpea and green pea rhizobia were capable consuming multiple carbohydrates although they were either slow or very slow growers. Dealing with chickpea rhizobia Nour *et al.* (1994) and Maâtallah *et al.* (2002a) also come to the same conclusion that slow growers rhizobia have many alternate carbon sources metabolisms. Formerly, it was believed by Stowers (1985) and van Rossum *et al.* (1995) that only fast growing strains can utilize broad range of carbon sources whereas slow-growth was attributed to their selective carbon source utilization ability.

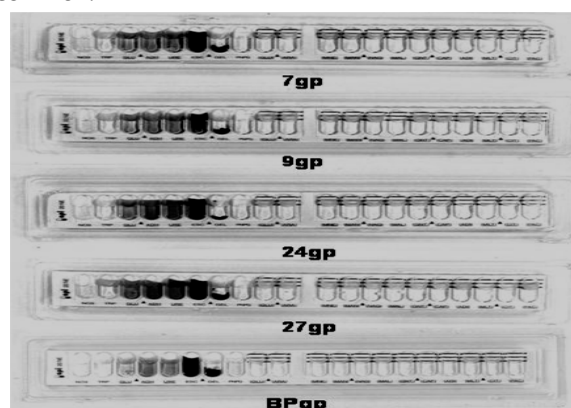
Table 2: Carbon source utilized by green pea rhizobia (percentage)

Carbon source	Isolates (%)
L-arginine	100
D-galactose	100
Malic acid	100
L-arabinose	92
D-mannose	92
D-mannitol	92
D-maltose	92
Potassium gluconate	92
N-acetyl-glucosamine	83
Tri sodium citrate	75
n-capric acid	67
Adipic acid	58
Phenylacetic acid	50
L-tryptophane	42
D-glucose	17

The distance matrix for carbon source utilization by chickpea rhizobia is organized in **(Table 3)**. Cluster analysis based on distance matrix (Fig. 7) divide 20 chickpea isolates in to for major groups at the distance of 1.3. Cluster 1 included five isolates (6cp, 7cp, 8cp, 9cp, 13cp) that were unable to metabolized sodium citrate and D-glucose. They shared six carbon sources in common. Cluster 2 consisted of five chickpea nodulating bacteria (11cp, 12cp, 22cp, 26cp, 27cp). This cluster did not utilize L-tryptophane and D-glucose. Cluster 3 comprised of six isolate (3cp, 5cp, 14cp, 24cp, 19cp, BPcp). All those that were unable to utilize L-

tryptophane, adipic acid and phenylacetic acid but all shared seven carbon sources. Cluster 4 included four isolates (2cp, 16cp, 21cp, 25cp). Only five carbon sources were utilized by all isolates.

Euclidean distances for carbon source utilization in green pea rhizobia are cluster analysis in **(Fig. 8)**. At the distance of 3.8 isolates were divided in to three major clusters. Cluster 1 included two strains (18gp, 19gp). L-tryptophane and phenylacetic acid were not utilized by any isolate while seven carbon (L-aragnine, D-glucose, L-arabinose, D-mannitol, D-maltose, potassium gluconate and malic acid) sources were utilized in common.

**Fig. 8: API NE 20 kit strips showing differential response for carbon source utilized among green pea rhizobia and commercial strain****Table 3: Euclidean distances between 19 isolates and commercial strain of chickpea for carbon source utilization pattern**

Isolates	2cp	3cp	5cp	6cp	7cp	8cp	9cp	11cp	12cp	13cp	14cp	16cp	19cp	21cp	22cp	24cp	25cp	26cp	27cp	BPcp
2cp	0																			
3cp	0.43	0																		
5cp	0.43	0	0																	
6cp	0.77	0.77	0.77	0																
7cp	0.65	0.86	0.86	0.1	0															
8cp	0.77	0.77	0.77	0	0.1	0														
9cp	0.77	0.77	0.77	0.23	0.33	0.23	0													
11cp	0.43	0.66	0.66	0.81	0.89	0.81	0.58	0												
12cp	0.43	0.66	0.66	0.81	0.89	0.81	0.58	0	0											
13cp	1.26	1.04	1.04	0.49	0.62	0.49	0.49	1.04	1.04	0										
14cp	0.66	0.43	0.43	0.81	0.89	0.81	0.81	0.73	0.73	1.04	0									
16cp	0.55	0.55	0.55	0.9	0.99	0.9	0.68	0.35	0.35	1.15	0.58	0								
19cp	0.77	0.77	0.77	0.45	0.55	0.45	0.68	0.81	0.81	0.49	0.58	0.9	0							
21cp	0.3	0.54	0.54	0.71	0.78	0.71	0.71	0.38	0.38	1.15	0.63	0.22	0.71	0						
22cp	0.49	0.49	0.49	0.43	0.49	0.43	0.43	0.36	0.36	0.88	0.67	0.73	0.73	0.6	0					
24cp	0.43	0.43	0.43	0.77	0.86	0.77	0.77	0.43	0.43	1.26	0.2	0.33	0.77	0.3	0.49	0				
25cp	0.33	0.55	0.55	0.9	0.99	0.9	0.68	0.35	0.35	1.15	0.81	0.23	0.9	0.22	0.73	0.55	0			
26cp	0.49	0.49	0.49	0.43	0.49	0.43	0.43	0.36	0.36	0.88	0.67	0.73	0.73	0.6	0	0.49	0.73	0		
27cp	0.66	0.66	0.66	0.59	0.66	0.59	0.32	0.24	0.24	0.77	0.8	0.59	0.86	0.72	0.16	0.66	0.59	0.16	0	
BPcp	0.39	0.39	0.39	0.59	0.66	0.59	0.59	0.52	0.52	0.77	0.24	0.59	0.32	0.43	0.52	0.39	0.59	0.52	0.63	0

Cluster 2 is divided in two A and B groups. Group A contains only one strain (26gp) that utilized narrow range of carbon sources; Group B comprised of five isolates (7gp, 8gp, 12gp, 23gp, 24gp). This group utilized wide range of carbon sources except L-tryptophane, D-glucose and gelatin.

Cluster 3 included four strains (3gp, 9gp, 27gp, BPgp) that were unable to utilize D-glucose and phenylacetic acid, capric acid and adipic acid. It can be inferred from the results that narrow range and broad range carbon utilizing ability could be found within same species. The result proves that the evolution of rhizobial strain can be influenced by environmental conditions such as horizontal gene transfer, geographical isolation and soil type and available carbon sources.

4. CONCLUSION

It is concluded that chickpea nodulating rhizobia are comparatively slow growers than green pea nodulating rhizobia and chickpea rhizobia are more selective and less diverse for their carbon source than green pea rhizobia.

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