PHENOTYPIC AND PHYSIOLOGICAL CHARACTERIZATION OF RHOZOBIA STRAINS ISOLATED FROM DIFFERENT AREAS OF BIHAR INDIA

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ABSTRACT

The phenotypic and physiological diversity of ten rhizobia strains isolated from three different grain legumes was investigated. Colony morphology of different rhizobia and resistance to antibiotics, tolerance to salinity, to extreme temperatures and pH were studied under the present investigation. The dendrogram was obtained following unweighted pair group method using arithmetic averages. The clusters were identified at appropriate phenon level. The variation for optical appearance and consistency was less pronounced while forms, margin and elevation of colonies shows wide range of variation. Among all isolates of rhizobia, except isolates RA-1 and RA-2, rest were sensitive to high concentration of NaCl where as $CaCl_2$ shows positive effect on the growth of all isolates with maximum CFU by isolate RA-1. The best tolerance for high temperature was recorded in RA-1, RA-2 and RA-4, the isolates of *Cajanus cajan*. Rests of the isolates were sensitive to high temperature i.e. 42°C. 7.5 pH was noticed as optimum for excellent to good growth of all ten isolates of Rhizobia. These isolates were also suitable for wide range of pH. RA-6, the isolate nodulating *Vicia faba* showed best antibiotic i.e. ampicilin and Erythromycin tolerances along with isolates RA-1 and RA-10 which shows tolerance to ampicilin.

Keywords: Legumes, phenotypic diversity, physiological diversity, Rhizobia strains

INTRODUCTION

Soil micro-organism plays an important role in soil processes that determine plant productivity. Rhizobia, a symbiotic nitrogen fixing bacteria, had significant importance in agriculture. Rhizobia are classically defined as soil bacteria, which are non spore forming, gram negative, aerobic, chemo-organotrophic, free living and fixes atmospheric nitrogen symbiotically. The Rhizobia colonies are convex, circular, semi translucent, raised and mucilaginous (Jordan 1984). Rhizobia in root nodules are estimated to carry out 50 to 70 percent of the world's biological nitrogen fixation and the estimated annual biological fixation of atmospheric nitrogen varies between 100×10^6 and 180×10^6 metric tons per year (Phillips 1980; Burris and Roberts 1993). Unfavorable pH, high temperature, salinity had detrimental effect on legumes as well as microbes. Nearly 40 per cent of the world land surface can be categorized as having potential salinity problem (Cordovilla *et al.* 1994). Increasing salt concentration affect Rhizobia growth due to direct toxicity as well as osmotic stress (Tate 1995). In tropical and subtropical areas, high soil temperature was a major problem for biological nitrogen fixation of legume crops, as high temperature strongly affects bacterial infection. For most rhizobia, the optimum temperature range for growth in culture is 28 to 30°C and many are unable to grow at 30°C (Graham 1992). Rhizobia appear to be more tolerant to alkalinity in general but it was also found that some species of *Rhizobium* cannot do well at high pH. Slow-growing strains appear to be more tolerant to low pH than fast-growing strains (Jordon, 1984). However, some fast-growing strains such as *Rhizobium tropici* and *Mesorhizobium loti* can grow at a pH as low as 4 (Graham 1992; Gao *et al.* 1994). Rhizobia show difference in their response against antibiotics. In general the fast growing rhizobia are more sensitive than slow growing bradyrhizobia to many antibiotics (Jordan 1984). However, Chickpea rhizobial strains which are mixture of fast, slow and extra-slow grower, showed wide range of behaviors with regard to antibiotics and heavy metals and indicated the tolerance of strains to antibiotic and heavy metal is not correlated with their growth rate but it could be related to the bacterial species (Maatallah *et al.* 2002). The above information from different observation suggests that there are considerable amount of diversity found in Rhizobia, both at phenotypic as well as morphological level. Considering above fact, in the present study, we have investigated morphological and physiological diversity among ten different rhizobial strains isolated from three different crops root nodules of different region of Bihar.

MATERIALS AND METHODS

1) Bacterial strains and culture conditions:

Strains of rhizobia were isolated from root nodules of different cultivars of arhar (*Cajanus cajan*), baqla (*Vicia faba* L.) and pea (*Pisum sativum*) growing in the crop fields of Saraiyan and Marvan blocks of Muzaffarpur district of Bihar. The details of different isolates taken in present investigation are given in Table 1. The Yeast Extract Mannitol (YEM) media was routinely used for isolation, purification and multiplication of these Rhizobia (Vincent 1970) as the basal medium during the experiment. YEMA basal medium was supplemented with different concentration of various adjuvants to generate an array of media. These isolates were identified by Gram staining and further by their oxidase (Steel 1961) and catalase activities (MacFaddin 1980). The purity of Rhizobia was ensured repeatedly by streaking and microscopic examination with gram staining. These pure isolates were maintained by repeatedly sub culturing on YEMA slant at 28±1°C and storing them at low temperature i.e. 5-7°C.

2) Morphological and Physiological studies:

Inoculation of pure isolates of Rhizobia on YEMA plate at 28±1°C results in colony formation after 36-40 hours. The colony morphology of isolates was studied after 2 days. Starter culture of the isolates was grown in YEM broth at 28±1°C for 48h and serial dilution upto 10^{-6} was done. From this, 1 ml of suspension was taken and transferred into YEMA reception plates with varying salt concentration, pH and temperature. Each of these experiments was performed in triplicate.

a) Salt tolerance:

Tolerance to Sodium Chloride (NaCl) and calcium chloride ($CaCl_2$) was tested through determining the growth on YEMA medium supplemented with NaCl and $CaCl_2$ at a concentrate of 1%, 2% and 3% (w/v) and 0.5 %, 1.0% and 1.5% (w/v) respectively.

b) pH tolerance:

Tolerance to extreme pH was tested on YEM agar medium set at different pH values i.e. 6.5, 7.0, 7.5 and 8.0 using NaOH.

c) Temperature tolerance:

Rhizobial growth pattern at different temperature was determined on YEM agar plates by incubating the bacterial cultures agar at 22±1°, 27±1°, 32±1°, 37±1° and 42±1°C.

d) Intrinsic Antibiotic Resistance profiles:

Resistance to antibiotics was tested on YEMA plates supplemented with the following filter sterilized antibiotics (μg/ml^{-1}) separately. Ampicillin (25, 50 and 60) and Erythromycin (30 and 60). The test for each antibiotic was carried out in triplicate.

3) Statistical Analysis

The data obtained from the laboratory analysis for each parameter were computed and then used for statistical analysis. The analysis was carried out with the help of RAUSTAT. The value of the standard error of mean (SEm±) of critical difference at 0.05 (5%) level of significance were computed among the various parameters which were obtained from the analysis for better interpretation of the results.

RESULTS AND DISCUSSION

a) Morphological analysis

The overall colony morphology has been summarized in Table.2. Most of the isolates were whitish and creamy; two were yellowish while a single isolate was colorless in nature. Colonies forms of five isolates were circular, three were irregular where as one muriform and one elongated form of colony was also observed. The elevations of colonies were convex, raised and flat types. Colony margin of the Rhizobia isolates showed variations. Four isolates had lobate, three had undulate, two had erose while one had entire margin. The optical appearance of the colony of the Rhizobia isolates were

Table 1: Rhizobia isolates collected from different crops of Muzaffarpur district

Sample No.	Crop	Soil type	Block
RA 1	Arhar	Upland Sandy Soil	Saraiya
RA 2	Arhar	Upland Sandy Soil	Saraiya
RA 3	Arhar	Upland Sandy Soil	Saraiya
RA 4	Arhar	Upland Sandy Soil	Saraiya
RA 5	Arhar	Upland Sandy Soil	Saraiya
RA 6	Bakla	Upland sandy/sandy loam soil	Saraiya
RA 7	Bakla	Upland sandy/sandy loam soil	Saraiya
RA 8	Arhar	Clay loam	Marvan
RA 9	Arhar	Clay loam	Marvan
RA 10	Pea	Clay loam	Marvan

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Table 2:Colony morphology of Rhizobia isolates grown on YEMA plate

Rhizobia isolates	Colour	Form	Elevation	Margin	Mucilaginous/ stickiness	Transparency	Crop source	Soil type
RA-1	Whitish	Circular	Convex	Entire	Less slimy	Semi translucent	Arhar	Upland sandy
RA-2	Whitish	Circular	Convex	Undulate	Slimy	Translucent	Arhar	Upland sandy
RA-3	Yellowish	Muriform	Raised	Undulate	Gummy	Translucent	Arhar	Upland sandy
RA-4	Creamy	Irregular	Raised	Undulate	Non-slimy/ lessgummy	Semi translucent	Arhar	Upland sandy
RA-5	Creamy	Circular	Flat	Lobate	Gummy	Translucent	Arhar	Upland sandy
RA-6	Creamy	Circular	Raised	Erose	Gummy	Translucent	Bakla	Sandy loam sandy
RA-7	Yellowish	Circular	Convex	Lobate	Less gummy	Translucent	Bakla	Sandy loam sandy
RA-8	Colourless	Irregular	Raised	Erose	Gummy	Semi translucent	Arhar	Clay loam
RA-9	Creamy	Irregular	Flat	Lobate	Less gummy	Translucent	Arhar	Clay loam
RA-10	Creamy	Elongated	Raised	Lobate	Gummy	Translucent	Pea	Clay loam

Table 3: Effect of different conc. of NaCl on growth (CFU) of Rhizobia isolates

Treatments	Rhizobia Isolates										Mean
	RA-1	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-8	RA-9	RA-10	
1.0%	84.66	78.00	72.66	77.33	75.00	80.00	72.33	66.66	67.66	66.33	74.06
2.0%	71.33	66.66	40.33	50.33	56.33	67.00	58.66	58.00	38.66	46.33	53.36
3.0%	41.33	46.33	12.66	0.00	11.00	2.33	4.33	16.33	9.33	0.00	14.36
Control	115.33	97.33	86.00	85.00	86.33	97.33	82.66	80.00	67.33	75.00	87.23
S.E.m (±)	2.7215										
CD at 5%	2.8977										
CV	15.0313										

Table 4 Effect of different conc. of $CaCl_2$ on growth (CFU) of Rhizobia isolates

Treatments	Rhizobia Isolates										Mean
	RA-1	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-8	RA-9	RA-10	
0.5%	114.33	68.33	88.33	125.00	69.66	98.66	104.33	80.33	80.66	96.33	92.596
1.0%	119.00	76.33	96.33	134.66	79.00	110.33	121.33	91.66	97.33	103.66	102.963
1.5%	130.66	92.33	101.00	139.66	90.66	129.00	126.00	109.33	112.33	115.33	114.630
Control	115.33	90.33	86.0	85.00	86.33	97.33	82.66	80.00	67.33	75.00	87.231
SEm (±)	3.31163										
CD at 5%	9.6102										
CV	10.5402										

Table 5: Effect of different pH on growth (CFU) Rhizobia isolates

Treatments	Rhizobia Isolates										Mean
	RA-1	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-8	RA-9	RA-10	
6.5	86.66	75.66	74.66	68.00	66.33	86.33	63.66	61.33	55.66	67.00	70.529
7.0	115.66	97.00	85.33	94.66	87.66	87.33	83.66	79.33	77.66	86.66	89.495
7.5	137.33	105.66	96.33	117.33	108.33	128.00	94.66	89.00	86.66	102.66	106.596
8.0	166.00	127.66	106.66	130.00	129.66	165.00	127.33	100.33	105.66	108.33	126.663
SEm (+)	2.7796										
CD at 5%	8.0663										
CV	8.9400										

Table 6: Growth (CFU) of Rhizobia isolates at different temperature

Treatments	Rhizobia Isolates										Mean
	RA-1	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-8	RA-9	RA-10	
22±1°C	86.66	83.33	45.66	67.33	61.33	67.66	45.66	44.33	35.66	50.33	58.795
27±1°C	124.00	98.66	89.00	87.33	88.00	97.33	85.33	79.66	67.33	75.66	89.230
32±1°C	84.66	85.33	68.66	81.66	79.66	76.66	50.66	47.33	45.66	54.33	17.795
37±1°C	27.66	32.66	0.00	22.33	19.33	16.66	18.66	16.33	5.66	16.33	17.562
42±1°C	9.33	8.66	0.00	5.33	0.00	0.00	0.00	0.00	0.00	0.00	2.332
SEm (±)	2.5788										
CD at 5%	7.1481										
CV	17.3229										

Table 7: Sensitivity of Erythromycin against growth (CFU) of Rhizobia isolates

Treatments	Rhizobia Isolates										Mean
	RA-1	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-8	RA-9	RA-10	
30 µg/ml	0.00	0.00	0.00	0.00	7.66	26.66	0.00	0.00	1.33	15.66	5.131
60 µg/ml	0.00	0.00	0.00	0.00	0.00	8.33	0.00	0.00	0.00	0.00	0.833
Control	115.33	97.33	86.00	85.00	86.33	97.33	82.66	80.00	67.33	75.00	87.231
SEm (±)	2.8070										
CD at 5%	8.3405										
CV	28.5746										

Table 8: Sensitivity of Ampicillin against growth (CFU) of Rhizobia isolates

Treatments	Rhizobia Isolates										Mean
	RA-1	RA-2	RA-3	RA-4	RA-5	RA-6	RA-7	RA-8	RA-9	RA-10	
25 µg/ml	30.00	0.00	0.00	0.00	16.33	28.66	17.00	0.00	0.00	27.33	11.965
50 µg/ml	12.33	0.00	0.00	0.00	3.33	12.33	4.33	0.00	0.00	11.33	4.365
60 µg/ml	2.33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.66	0.599
Control	115.33	97.33	86.00	85.00	86.33	97.33	82.66	80.00	67.33	75.00	87.231
SEm (±)	2.5266										
CD at 5%	7.3322										
CV	30.6832										

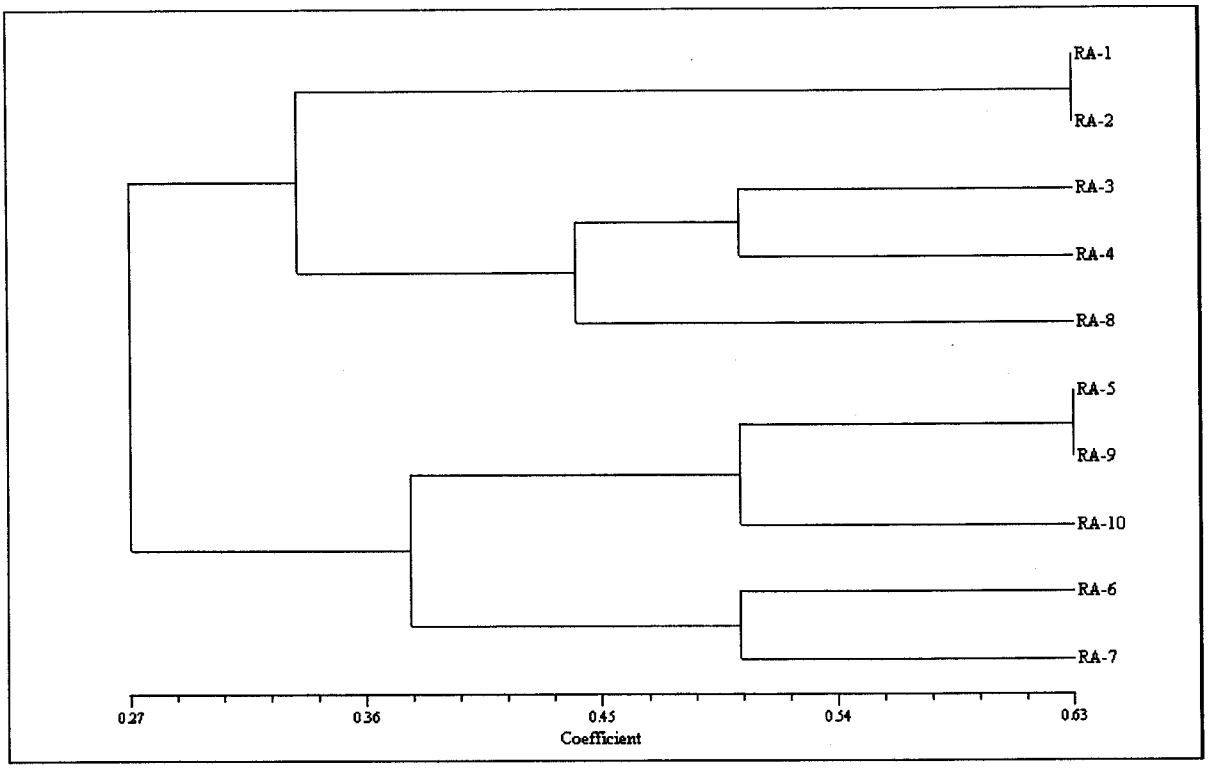

Fig. 1 Dendrogram based on eight morphological characters of ten Rhizobia isolates

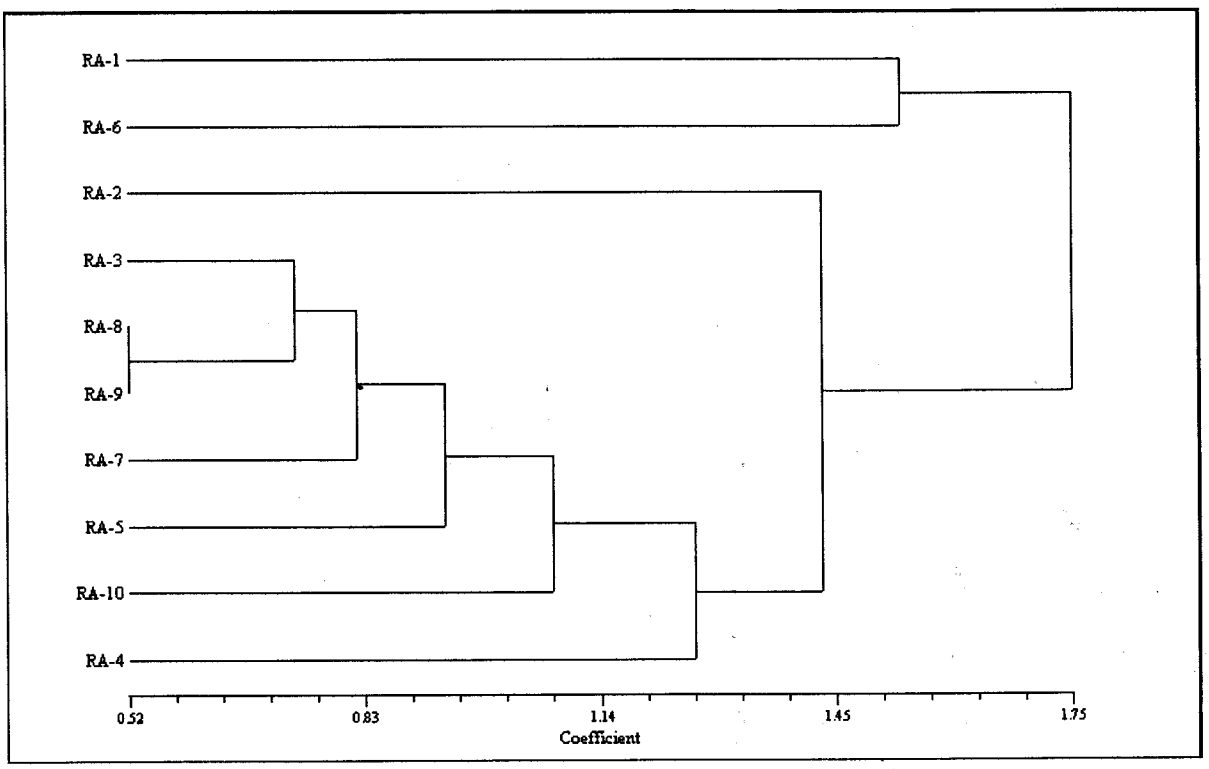

Fig. 2: Dendrogram based on taxonomic distance for physiological response parameters of Rhizobia isolates

either semi translucent or translucent. The consistency of the Rhizobia colony had variation. Five isolates were gummy, two were less gummy, two were slimy where as one was non-slimy in nature. Maatallah *et al.* (2002) observed both phenotypic and genetic variation in the chickpea rhizobia isolates collected from different areas of Morocco. Kucuk *et al.* (2006) also observed significant differences for physiological characteristics of *Rhizobium sp.* isolated from bean.

b) Phenotypic traits analysis

The various phenotypic traits viz. salt, pH, temperature tolerance and intrinsic antibiotic resistance, of the ten rhizobia isolates are summarized in Table 3, 4, 5, 6, 7 and 8 respectively. However the response of different conc. of NaCl on growth (CFU) of Rhizobia isolates indicated that all conc. (1-3%) of NaCl were inhibitory to all isolates of the Rhizobia as compared to control. But at 1% conc. of NaCl, RA-1 had maximum CFU 84.66 followed by RA-6 (80.00), RA-2 (78.00), RA-4 (73.33), and RA-5 (75.00). However other isolates produced CFU in a range of 66-77. As regard 2% conc. the growth (CFU) ranged from 71.33 to 38.66. But of 3% conc. no growth (CFU) was observed in RA-4 and RA-10. The result supports the results of Abdel-Wahab and Zaharn (1979) where they have reported that the strains of *Rhizobium leguminosarum* found to be tolerant to NaCl upto 4.27% ,whereas, the bacterial colonization of *Vicia faba* were reduced in the presence of 0.61 to 1.02% NaCl as observed by Zahran and Sprent (1986). Simlarly, Chein *et al.* (1992) reported that the mutant strain of *Rhizobium leguminosarum bv. viciae* which grew at 2.04% NaCl formed ineffective nodules. The other isolates showed excellent sensitivity against 3% conc. NaCl whereas all isolates showed positive response to $CaCl_2$ at all concentration. However, maximum CFU was observed at 1.5% conc. in all isolates as compared to control. RA-1 gave maximum CFU at all three conc. i.e. 0.5 to 1.5% $CaCl_2$ ranged from 114.33 to 130.66 and significantly superior to all isolates in response to $CaCl_2$. Therefore, it was showed that upto 1.5% conc., $CaCl_2$ has no negative effect on Rhizobia isolates collected from different part of Bihar. The growth performances of all isolates were best at $27\pm1^{0}C$. Increasing temperature range showed decreasing trend of growth of isolates. However, for temperature ranging from $22\pm1^{0}C$ to $37\pm1^{0}C$, the growth in rhizobia was observed. Excellent growth of all isolates was observed at $27\pm1^{0}C$. Medium growth was observed at $22\pm1^{0}C$ and $32\pm1^{0}C$. But poor to very poor growth was recorded at $37\pm1^{0}C$ and $42\pm1^{0}C$. No CFU was noticed at $42\pm1^{0}C$ in RA-3, RA-5 to RA-10. Therefore all isolates can be grown at temperature range of $22\pm1^{0}C$ to $37\pm1^{0}C$. The effect of pH on growth (CFU) of ten rhizobia isolates indicated that pH 6.5, 7.0, 7.5 and 8.0 supported the growth of all rhizobia isolates but 7.5 pH was noticed as optimum pH for excellent to good growth. These isolates of rhizobia were suitable for wide range of pH. It was noticed that RA-6 showed maximum tolerance to 30 and 60 mg/ml of Erythromycin. Very little growth was observed in RA-5, RA-9 and RA-10 at 30 mg/ml. Rest showed sensitivity with Erythromycin at both concentrations as compared to control whereas isolates RA-2, RA-3, RA-4, RA-8 and RA-9 were found to be most sensitive against Ampicilin at 25, 50 and 60 µg/ml as compared to other isolates. RA-1 and RA-10 was found resistant at all concentration but RA-5, RA-6 and RA-7 were resistant upto to 50 mg/ml.

Classification based on Morphological Character and Physiological Response:

The dendrogram (Fig. 1) obtained from numerical taxonomic analysis based on morphological characters of ten Rhizobia isolates indicated that the isolates could be grouped into 8 clusters when phenon line was drawn taking into consideration 75 similarity units as cut off point. Out of the eight clusters resulted, clusters B, C, D, F, G and H were monoisolate clusters having RA-3, RA-4, RA-8, RA-10, RA-6 and RA-7 isolates, respectively. The isolates RA-1 and RA-2 were accommodated in cluster A, whereas the isolates RA-5 and RA-9 could be placed into cluster E. Classification of Rhizobia isolates was also done on the basis of their response to the environmental conditions, to which the strains were exposed. The classification was made using taxonomic distance as a measure of dissimilarity in numerical taxonomic approach for the classification of isolates.

The method for tree building in the analysis involved sequential agglomerative hierarchical non-overlapping clustering based on distance matrices. The dendrogram was obtained following unweighted pair group method using arithmetic averages. The clusters were identified at appropriate phenon level (Fig. 2). A close perusal of dendrogram obtained for ten Rhizobia isolates (Fig.4.9) revealed that isolates could be classified into seven clusters when phenon line was drawn taking into consideration 25 dissimilarity units as cutoff point. Out of the seven clusters resulted, six clusters namely; B, C, D, E, F and G were monoisolate clusters and comprised RA-5, RA-10, RA-4, RA-2, RA-6 and RA-1 isolates, respectively (Table 4.10). The isolates RA-3, RA-7, RA-8 and RA-9 could be included in the cluster A. The nature of response of three isolates, namely, RA-1, RA-2 and RA-6 seemed to be most distinct from the physiological response of remaining isolates while the isolates RA-8 and RA-9 were found to be most closely related isolates amongst the Rhizobia isolates included in the present study.

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