CHAPTER 4

RESULTS

4. Results

4.1. Isolation of rhizobia

Assam a Northeastern state of India harbors diverse flora and fauna. For characterizing the diversity of rhizobia associated with legume plants of Assam two cultivated and two wild legume plants were selected. The cultivated legumes were *Cajanus cajan* and *Lablab purpureus*. Both the selected plants were agriculturally very significant and cultivated by the peoples of Assam since time immemorial. The wild legumes were *Mimosa pudica* and *Crotalaria pallida*. Root nodules of the selected plants were collected from 8 different geographical sites of Assam (Figure 9).

Figure 9: Sample collection. A) Nodule collection from Cachar; B) Nodule collection from Sivasagar; C-D) Collected nodule

The collected nodule samples were washed, sterilized, crushed, streaked on YEMA plate and incubated for 3 - 5 days. Pure cultures of all the isolates and reference strains were maintained in YEMA slants at 4°C for temporary storage and in 50% glycerol at -80°C for long-term storage (Figure 10). A total of 120 bacterial

isolates were obtained from the root nodule of selected leguminous plants. This includes, 32 isolates from root nodules of *L.purpureus,* 32 isolates from root nodules of *C. cajan*, 29 isolates from root nodule of *C. pallida* and 27 isolates from root nodule of *M. pudica*. Nomenclature of the isolates was carried out representing their location of sample collection, parent plant and isolate number (numeric Figure).

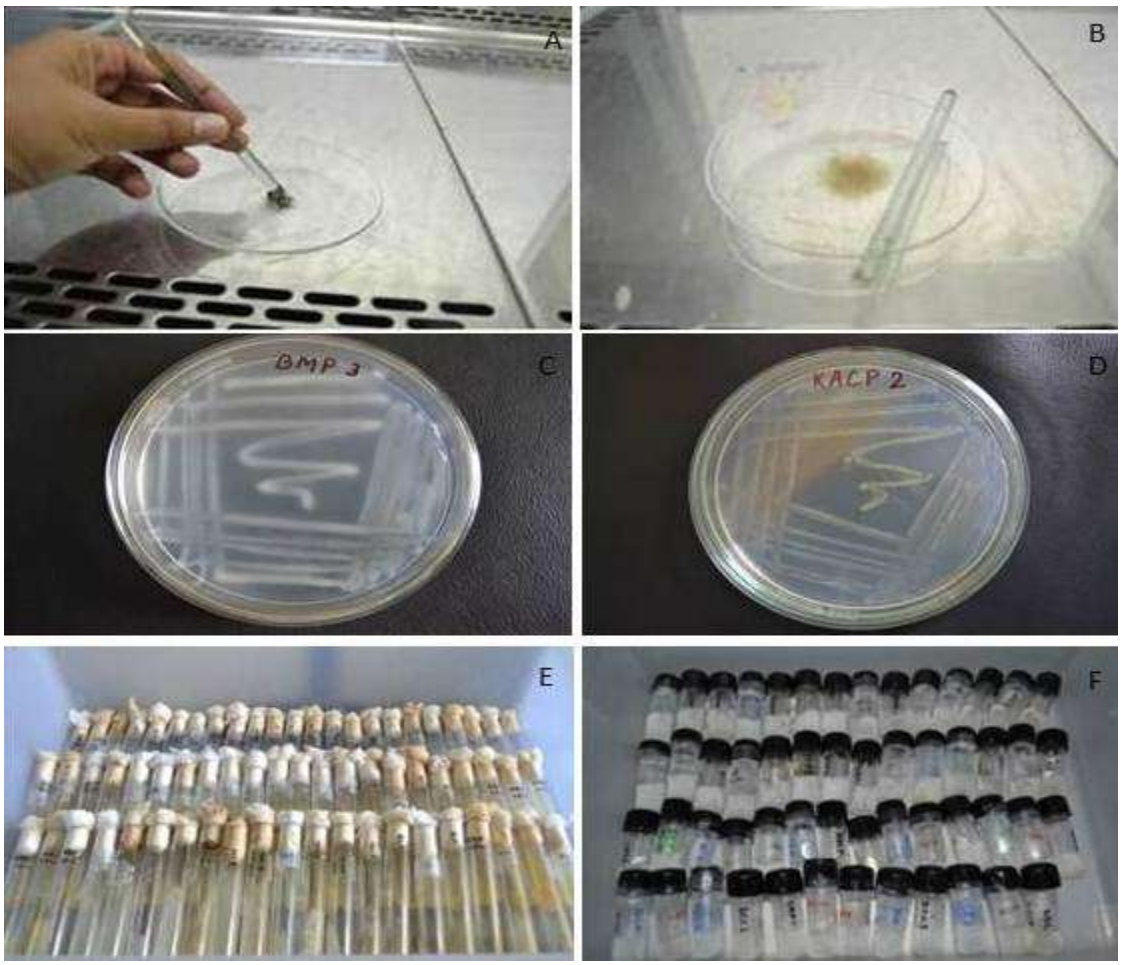

Figure 10:Isolation of bacteria. A) Crushing of nodules;B)Crushed nodule; C) Isolate on YEMA plate (*M. pudica*); D) Isolate on YEMA plate (*C. pallida*); E) Isolate on YEMA slants; F) Isolates on 50% glycerol

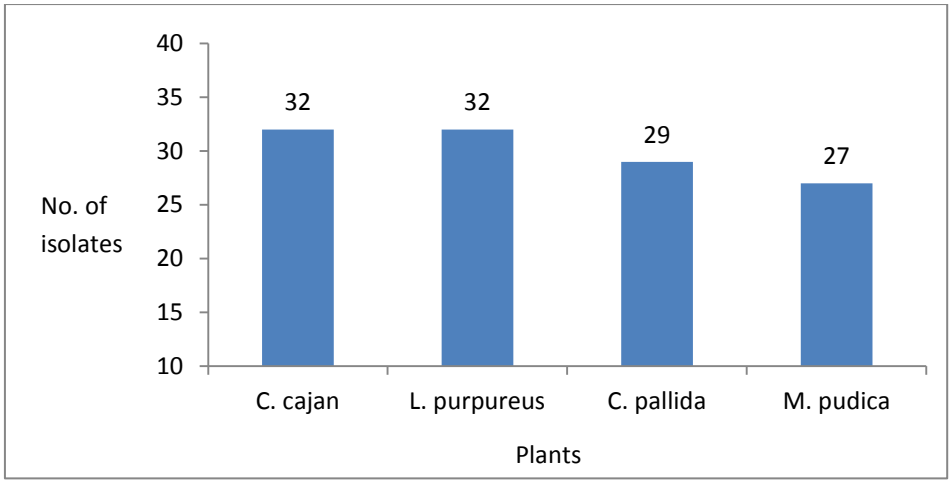

Figure 11: No of isolates obtained from the root nodules

4.2. Phenotypic characteristics and confirmatory tests

For preliminary characterization, the bacterial isolates were observed for their morphological and microscopic features and compared with the features of reference strains. In addition, 2 confirmatory tests were carried out for confirming the identity of the isolates as rhizobia.

4.2.1. Phenotypic characteristics and confirmatory tests of reference strains:

Results revealed that the reference strains formed round shaped, white color, slimy colonies on YEMA plate. The colony size of the reference strains ranges 0.5 - 3 mm in diameter (Figure 12). Microscopic observation revealed that all the reference strains showed gram negative staining and rod shaped cells (Table 9). On YEMA-Cr plate the reference strains showed white color colonies. All the reference strains showed negative results for ketolactose test.

Table 9: Phenotypic characteristics and confirmatory tests of reference strains

Reference strains	Morphological features		Microscopic features		Confirmatory tests	
	Colony color	Colony size (mm)	Gram staining	Cell shape	Color on YEMA-Cr	Ketolactose test
Rhizobium leguminosarum	White	1-3	-	Rod	White	-
Bradyrhizobium japonicum	White	1-2	-	Rod	White	-
Mesorhizobium thiogangeticum	White	1-3	-	Rod	White	-
Burkholderia cepacia	White	0.5-1	-	Rod	White	-

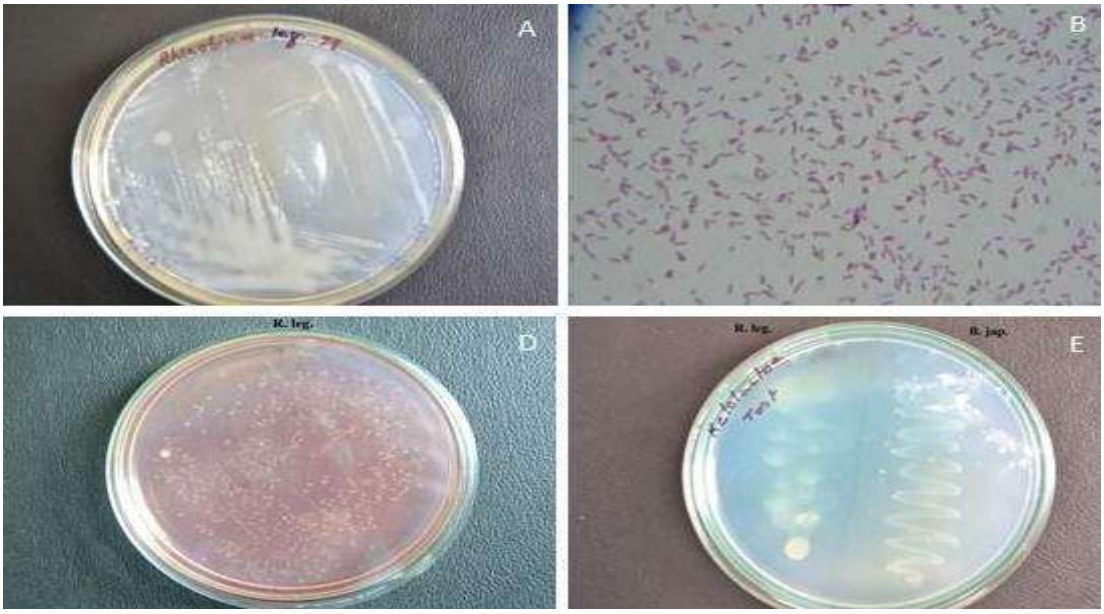

Figure12: Phenotypic characteristics and confirmatory tests of reference strains. A) Pure culture of *R. leguminosarum*; B) Gram staining image of *R. leguminosarum*; C) *R. leguminosarum*on YEMA-Cr plate; D) Ketolactose test of reference strains

4.2.2. Phenotypic characteristics and confirmatory tests of isolates obtained from *L. purpureus*:

The isolates obtained from *L. purpureus* formed white color colonies which range from 1 to 3 mm in diameter after 3-5 days of incubation on YEMA plate. Similar to reference strains, colonies of the isolates were round in shape, raised in elevation and produces mucus (Figure 13). Among 32 isolates studied, 27 isolates showed gram negative staining and 5 isolates showed gram positive staining. Most of the isolates showed rod shaped cells except 3 isolates which showed coccus shaped cells. On YEMA-Cr plate the isolates showed white color colonies except 6 isolates which absorbed the congo red dye and showed red color colonies. Ketolactose test revealed that 4 isolates were capable of producing ketolactose enzyme. The details of phenotypic characteristics and confirmatory tests are given in Table 10.

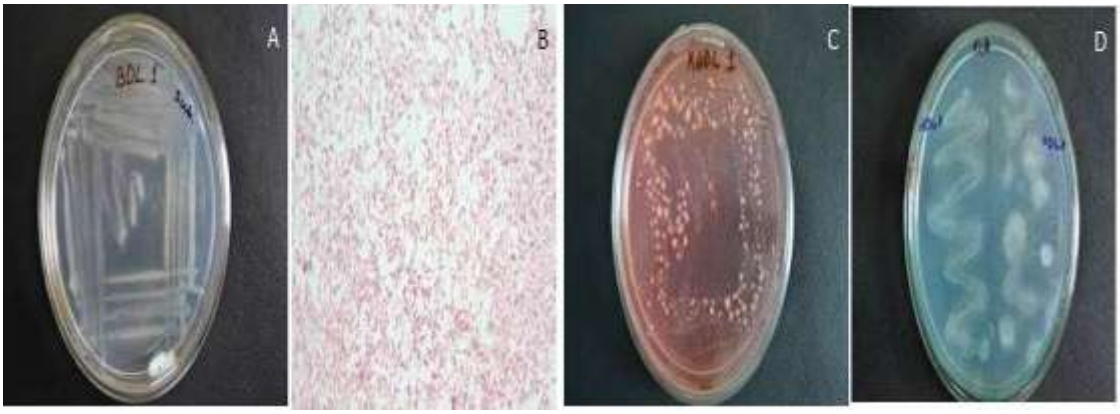

Figure 13: Phenotypic characteristics and confirmatory tests ofisolates (*L. purpureus*). A) Pure culture; B) Gram staining image (BDL1); C) Isolates on YEMA-Cr plate; D) Ketolactose test

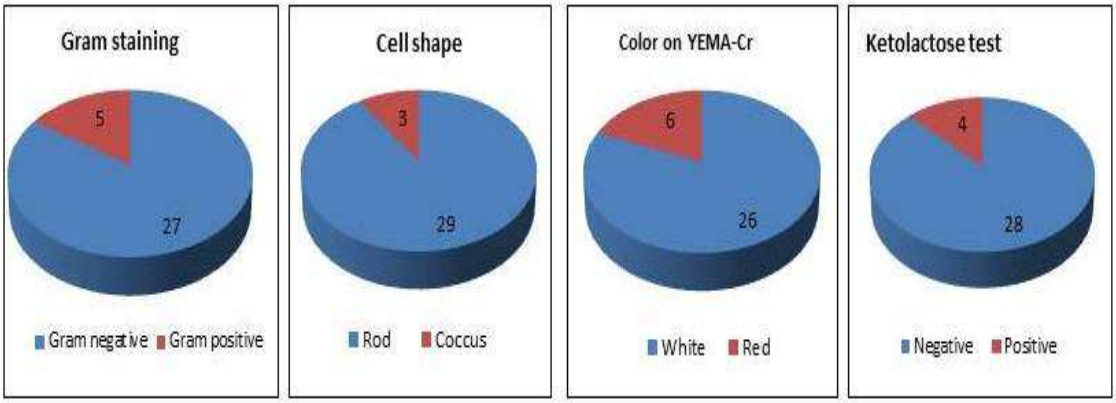

Figure 14: Pie chart of phenotypic characteristics and confirmatory tests of isolates (*L. purpureus*)

The features showed by the reference strains viz. Gram negative staining, rod shaped cells, white color colonies on YEMA media and negative results of ketolactose test were considered as standard criteria for screening the isolates. The results indicated that among the 32 isolates obtained from root nodules of *L. purpureus*, 25 isolate showed similarities in morphological, microscopic and confirmatory results with the reference strains (Figure 14). However, 7 isolates (KDL3, HIDL2, DDL3, TEDL1, TEDL2, GDL1 and UDL1) showed contrasting results of confirmatory tests. So, based on the results of primary screening 25 probable rhizobial isolates were selected for further study.

Table 10: Phenotypic characteristics and confirmatory test of isolates (*L. purpureus*)

Isolates	Sample collection site	Morphological features		Microscopic features		Confirmatory tests	
		Colony color	Colony size (mm)	Gram staining	Cell shape	Color on YEMA-Cr	Ketolactose test
RDL1	Cachar	White	1-2	-	Rod	White	-
RDL2	Cachar	White	1-3	-	Rod	White	-
IDL1	Cachar	White	1-2	-	Rod	White	-
IDL2	Cachar	White	1-3	-	Rod	White	-
KDL1	Karimganj	White	1-2	-	Rod	White	-
KDL2	Karimganj	White	1-3	-	Rod	White	-
KDL3	Karimganj	White	1-2	+	Rod	Red	+
HIDL 1	Hailakandi	White	1-2	-	Rod	White	-
HIDL2	Hailakandi	White	1-3	-	Coccus	Red	-
SIDL1	Hailakandi	White	1-2	-	Rod	White	-
SIDL 2	Hailakandi	White	1-2	-	Rod	White	-
HDL 1	Hailakandi	White	1-2	-	Rod	White	-
DDL1	N.C.Hills	White	1-2	-	Rod	White	-
DDL2	N.C.Hills	White	1-3	-	Rod	White	-
DDL3	N.C.Hills	White	1-2	-	Coccus	Red	-
NCDL 1	N.C.Hills	White	1-2	-	Rod	White	-
NCDL 2	N.C.Hills	White	1-3	-	Rod	White	-
TEDL1	Tezpur	White	1-2	+	Rod	Red	+
TEDL2	Tezpur	White	1-2	+	Rod	Red	+
TEDL3	Tezpur	White	1-2	-	Rod	White	-
SDL1	Tezpur	White	1-3	-	Rod	White	-
SDL2	Tezpur	White	1-2	-	Rod	White	-
GDL 1	Kamrup	White	1-3	+	Rod	Red	-
GDL2	Kamrup	White	1-2	-	Rod	White	-
UDL1	Kokrajhar	White	1-3	+	Coccus	White	+
UDL2	Kokrajhar	White	2-3	-	Rod	White	-
UDL3	Kokrajhar	White	1-2	-	Rod	White	-
KODL1	Kokrajhar	White	1-3	-	Rod	White	-
KODL2	Kokrajhar	White	1-2	-	Rod	White	-
BDL 1	Sivasagar	White	1-2	-	Rod	White	-
BDL 2	Sivasagar	White	1-2	-	Rod	White	-
BDL 3	Sivasagar	White	1-2	-	Rod	White	-

4.2.3. Phenotypic characteristics and confirmatory tests of isolates obtained from *C. cajan*:

The isolates obtained from *C. cajan* root nodules formed white color colonies and size of the bacterial colonies ranges 1-3 mm in diameter after 3-5 days of incubation on YEMA plate (Figure 15). The colonies of the isolates were round in shape, raised in elevation and produces high amounts of mucus. Microscopic observation revealed that among the 32 isolates investigated, 27 isolates were gram negative and 5 isolates were gram positive. 28 isolates showed rod shaped cells. The

confirmatory test revealed that, 27 isolates formed white colonies on YEMA-Cr media and in ketolactose test 26 isolates showed negative results. (Table 11).

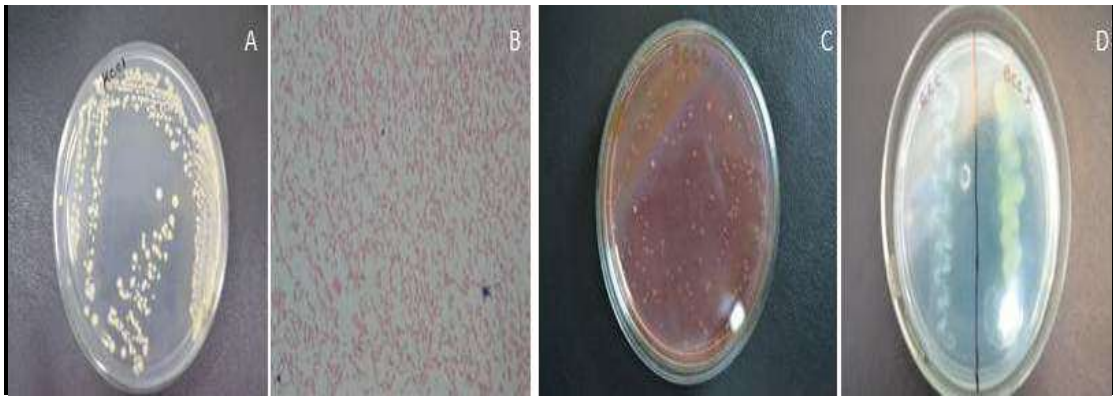

Figure 15: Phenotypic characteristics and confirmatory tests ofisolates (*C. cajan*). A) Pure culture; B) Gram staining image (KCC1); C) Isolate on YEMA-Cr plate; D) Ketolactose test

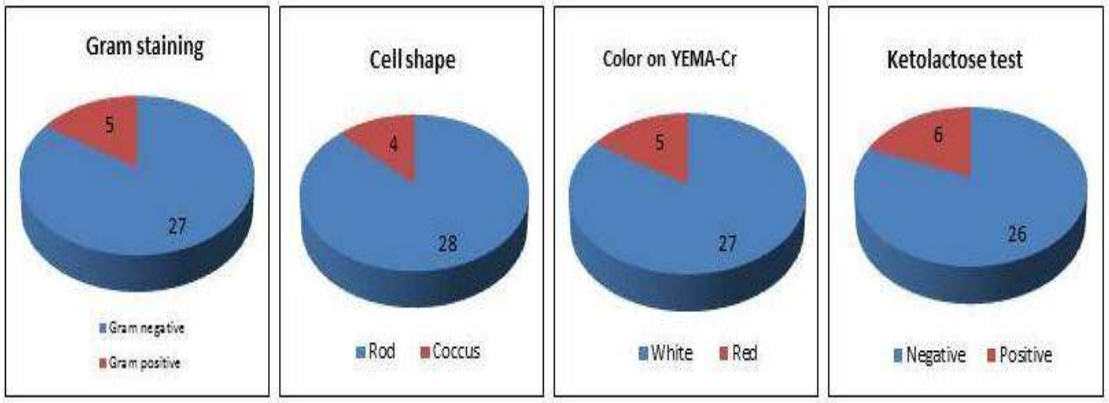

Figure 16: Pie chart of phenotypic characteristics and confirmatory tests of isolates (*C. cajan*)

The results clearly indicated that among the 32 isolates investigated, 23 isolate showed similarities in morphological, microscopic and confirmatory results with the reference strains (Figure 16). However, 9 isolates (KCC2, MKCC1, MKCC2, SICC2, HCC1, NCCC1, TECC1, JCC2 and BCC1) showed contrasting results of confirmatory tests when compared with the reference strains. So based on the results of primary screening 23 probable rhizobial isolates were selected.

Table 11. Phenotypic characteristics and confirmatory test of isolates (*C. cajan*)

Isolates	Sample collection Site	Morphological features		Microscopic features		Confirmatory tests	
		Colony color	Colony size (mm)	Gram staining	Cell shape	Color on YEMA-Cr	Ketolactose test
RCC1	Cachar	White	1-2	-	Rod	White	-
RCC2	Cachar	White	1-2	-	Rod	White	-
ICC1	Cachar	White	2-3	-	Rod	White	-
ICC2	Cachar	White	1-2	-	Rod	White	-
ICC3	Cachar	White	1-3	-	Rod	White	-
KCC1	Karimganj	White	1-2	-	Rod	White	-
KCC2	Karimganj	White	1-2	+	Coccus	Red	-
KCC3	Karimganj	White	1-3	-	Rod	White	-
MKCC1	Karimganj	White	2-3	+	Rod	Red	+
MKCC2	Karimganj	White	1-2	-	Coccus	Red	+
MKCC3	Karimganj	White	1-3	-	Rod	White	-
SICC1	Hailakandi	White	1-2	-	Rod	White	-
SICC2	Hailakandi	White	1-3	-	Rod	White	+
SICC3	Hailakandi	White	2-3	-	Rod	White	-
HCC1	Hailakandi	White	1-2	-	Rod	Red	-
HCC2	Hailakandi	White	1-3	-	Rod	White	-
HCC3	Hailakandi	White	1-2	+	Rod	White	-
DCC1	N.C.Hills	White	1-3	-	Rod	White	-
DCC2	N.C.Hills	White	1-2	-	Rod	White	-
NCCC1	N.C.Hills	White	1-3	-	Rod	Red	+
NCCC2	N.C.Hills	White	1-2	-	Rod	White	-
NCCC3	N.C.Hills	White	1-2	-	Rod	White	-
TECC1	Tezpur	White	1-2	+	Rod	White	-
TECC2	Tezpur	White	1-3	-	Rod	White	-
TECC3	Tezpur	White	1-2	-	Rod	White	-
SCC1	Tezpur	White	1-2	-	Rod	White	-
JCC1	Kamrup	White	1-2	-	Rod	White	-
JCC2	Kamrup	White	1-2	-	Coccus	White	+
KOCC1	Kokrajhar	White	1-2	-	Rod	White	-
KOCC2	Kokrajhar	White	1-2	-	Rod	White	-
BCC1	Sivasagar	White	1-2	+	Coccus	White	+
BCC2	Sivasagar	White	1-2	-	Rod	White	-

4.2.4. Phenotypic characteristics and confirmatory tests of isolates obtained from *C. pallida*:

Isolates obtained from *C. pallida* formed white colored, round shaped, slimy colonies on YEMA plate (Figure 17). The colony size of the isolates ranges in size from 1to3 mm in diameter after 4-5 days of incubation. Among the 29 isolates, 23 isolates showed gram negative staining and 6 isolates showed gram positive staining.

27 isolates showed rod shaped cells and 2 isolates showed coccus shaped cells. 26 isolates showed white colonies on YEMA-Cr plates, however 3 isolates absorbed the dye and showed red color colonies. In ketolactose test 5 isolate were capable of producing ketolactose enzyme (Table 12).

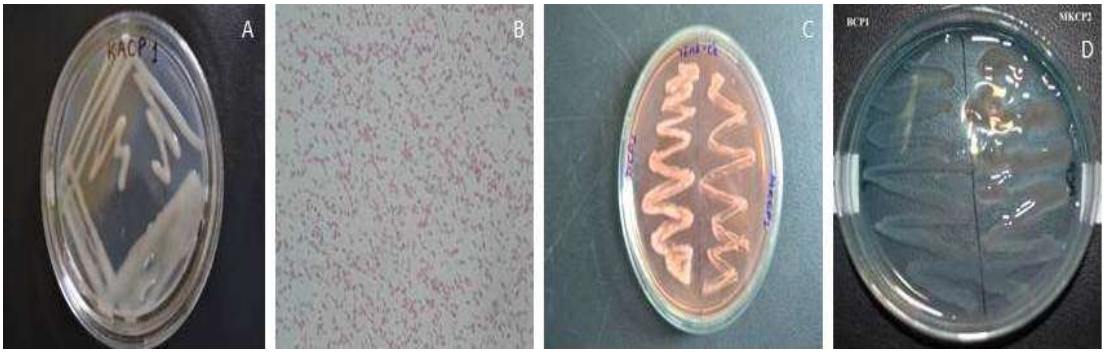

Figure 17: Phenotypic characteristics and confirmatory tests ofisolates (*C. pallida*). A) Pure culture; B) Gram staining image (KACP1); C) Isolate on YEMA-Cr plate; D) Ketolactose test

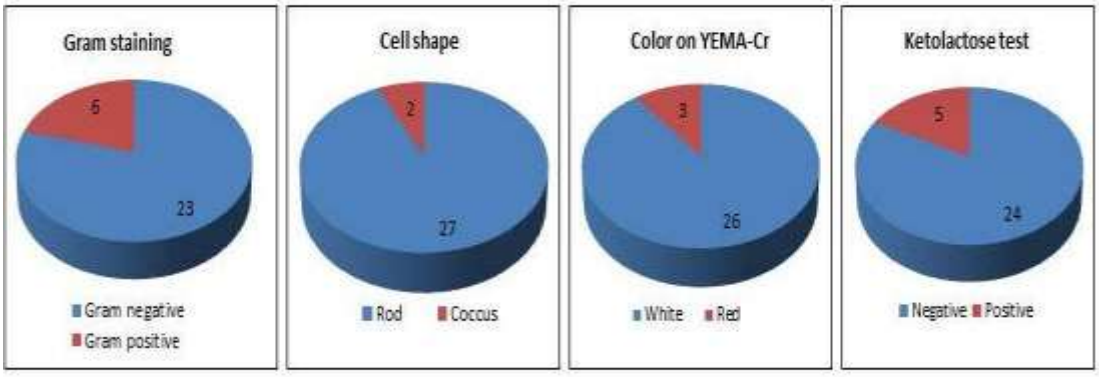

Figure 18: Pie chart of phenotypic characteristics and confirmatory tests of isolates (*C. pallida*)

The results indicated that among the 29 isolates obtained from root nodules of *C. pallida*, 22 isolate showed similarities in morphological, microscopic and confirmatory results with the reference strains (Figure 18). However, 7 isolates (BCP1, SICP1, TECP1, TECP2, HCP1, KCP2 and KOCP3) showed contrasting results of confirmatory tests when compared with the reference strains. So based on the results of primary screening 22 probable rhizobial isolates were selected.

Table 12. Phenotypic characteristics and confirmatory test of isolates (*C. pallida*)

Isolates	Sample collection Site	Morphological features		Microscopic features		Confirmatory tests	
		Colony color	Colony size (mm)	Gram staining	Cell shape	Color on YEMA-Cr	Ketolactose test
RCP1	Cachar	White	1-2	-	Rod	White	-
RCP2	Cachar	White	1-2	-	Rod	White	-
ICP1	Cachar	White	1-3	-	Rod	White	-
KACP1	Cachar	White	1-2	-	Rod	White	-
KACP2	Cachar	White	1-3	-	Rod	White	-
BCP1	Karimganj	White	1-2	-	Coccus	White	+
BCP2	Karimganj	White	1-2	-	Rod	White	-
MKCP1	Karimganj	White	1-3	-	Rod	White	-
MKCP2	Karimganj	White	2-3	-	Rod	White	-
GCP1	Hailakandi	White	1-2	-	Rod	White	-
SICP1	Hailakandi	White	1-2	-	Rod	Red	+
SICP2	Hailakandi	White	1-3	-	Rod	White	-
DCP1	N.C.Hills	White	1-2	+	Rod	White	-
DCP3	N.C.Hills	White	1-2	-	Rod	White	-
NCP1	N.C.Hills	White	1-2	+	Rod	White	-
NCP2	N.C.Hills	White	1-3	-	Rod	White	-
SCP1	Tezpur	White	1-2	-	Rod	White	-
SCP2	Tezpur	White	1-2	-	Rod	White	-
TECP1	Tezpur	White	1-3	-	Rod	White	+
TECP2	Tezpur	White	1-2	+	Rod	White	-
TECP3	Tezpur	White	1-2	-	Rod	Red	-
TECP4	Tezpur	White	1-2	-	Rod	White	-
HCP1	Sivasagar	White	1-3	+	Coccus	White	+
HCP2	Sivasagar	White	1-2	-	Rod	White	-
KCP1	Kamrup	White	1-3	-	Rod	White	-
KCP2	Kamrup	White	1-2	-	Rod	Red	-
KOCP1	Kokrajhar	White	1-2	-	Rod	White	-
KOCP2	Kokrajhar	White	1-2	-	Rod	White	-
KOCP3	Kokrajhar	White	1-3	+	Rod	White	+

4.2.5. Phenotypic characteristics and confirmatory tests of isolates obtained from *M. pudica*:

Theisolates showed white color, slimy colonies on YEMA plate. The colony size of the isolates ranges 0.5-2.0 mm in diameter after 4-5 days of incubation (Figure 19). Among the 27 isolates investigated, 23 isolates showed gram negative staining. Most of the isolates showed rod shaped cells except 3 isolates which showed coccus shaped cells. On YEMA-Cr plate 25 isolates showed white colonies and 24 isolate showed negative result of the ketolactose test (Table 13).

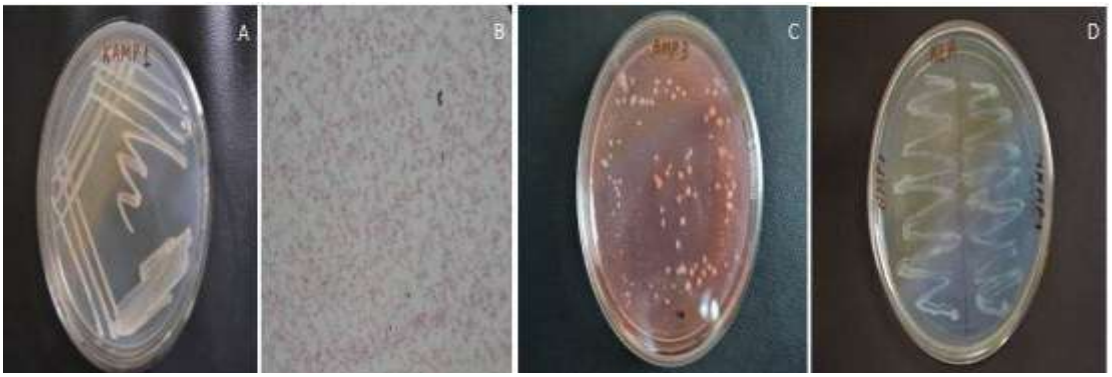

Figure 19: Phenotypic characteristics and confirmatory tests ofisolates (*M. pudica*). A) Pure culture; B) Gram staining image (KAMP1); C) Isolate on YEMA-Cr plate; D) Ketolactose test

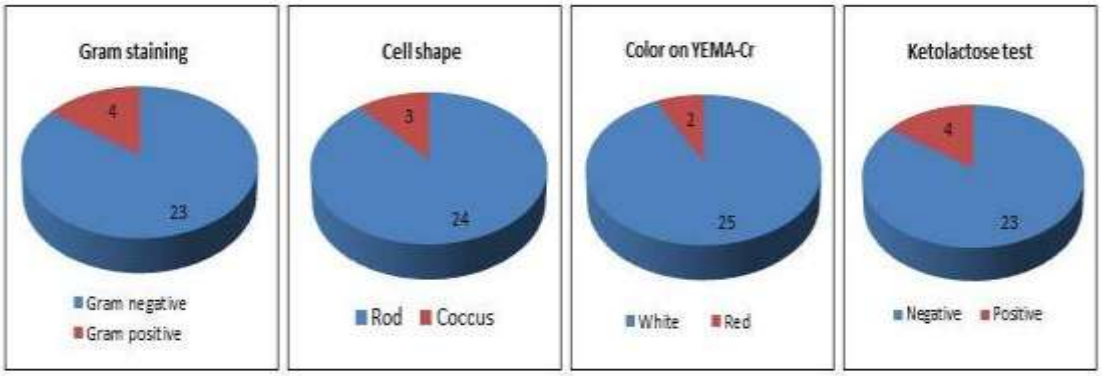

Figure 20: Pie chart of phenotypic characteristics and confirmatory tests of isolates (*M. pudica*)

The results indicated that among 27 isolates investigated, 22 isolate showed similarities in morphological, microscopic and confirmatory results with reference strains (Figure 20). However, 5 isolates (RMP1, HMP2, KMP2, SIMP2 and KOMP1) showed contrasting results of confirmatory tests when compared with the reference strains. So based on the results of primary screening 22 probable rhizobial isolates were selected.

Table 13. Phenotypic characteristics and confirmatory test of isolates (*M. pudica*)

Isolates	Sample collection Site	Morphological features		Microscopic features		Confirmatory tests	
		Colony color	Colony size (mm)	Gram staining	Cell Shape	Color on YEMA-Cr	Ketolactose test
KAMP1	Cachar	White	1-2	-	Rod	White	-
KAMP2	Cachar	White	1-2	-	Rod	White	-
RMP1	Cachar	White	1-2	+	Coccus	White	+
RMP2	Cachar	White	0.5-1	-	Rod	White	-
IMP1	Cachar	White	0.5-1	-	Rod	White	-
IMP2	Cachar	White	0.5-1	-	Rod	White	-
HMP 1	Cachar	White	1-2	-	Rod	White	-
HMP 2	Cachar	White	0.5-1	+	Coccus	White	+
KMP 1	Karimganj	White	0.5-1	-	Rod	White	-
KMP 2	Karimganj	White	0.5-1	-	Rod	Red	-
KMP 3	Karimganj	White	1-2	-	Rod	White	-
BMP1	Karimganj	White	0.5-1	-	Rod	White	-
SIMP 1	Hailakandi	White	0.5-1	-	Rod	White	-
SIMP 2	Hailakandi	White	0.5-1	+	Rod	Red	+
SIMP 3	Hailakandi	White	1-2	-	Rod	White	-
GMP 1	Hailakandi	White	1-2	-	Rod	White	-
NCMP 1	N.C.Hills	White	1-2	-	Rod	White	-
NCMP 2	N.C.Hills	White	1-2	-	Rod	White	-
DMP 1	N.C.Hills	White	1-2	-	Rod	White	-
DMP 2	N.C.Hills	White	0.5-1	-	Rod	White	-
TEMP1	Tezpur	White	0.5-1	+	Rod	White	-
SMP 1	Tezpur	White	0.5-1	-	Rod	White	-
SMP 2	Tezpur	White	1-2	-	Rod	White	-
KOMP1	Kamrup	White	1-2	-	Coccus	White	+
KOMP2	Kamrup	White	1-2	-	Rod	White	-
MKMP1	Sivasagar	White	1-2	-	Rod	White	-
MKMP2	Sivasagar	White	0.5-1	-	Rod	White	-

4.2. 6. Selection of isolates based on phenotypic and confirmatory test results

The results of phenotypic characterization and confirmatory tests of all the bacterial isolates obtained from the 4 legume plants revealed that the isolated bacterial samples include some contaminating strains which showed no resemblance with the features of reference strains. The contaminating strains were excluded from the study. So, a total of 92 probable rhizobial isolates were selected for further study. These include, 25 isolates obtained from *L. purpureus*, 23 isolates obtained from *C. cajan*, 22 isolate obtained from *C. pallida* and 22 isolates obtained from *M. pudica*.

4.4. Biochemical characteristics

4.3.1. Biochemical characteristics of isolates obtained from *L. purpureus*:

All the selected 25 bacterial isolates obtained from *L. purpureus* root nodules showed positive results of catalase test, oxidase test and negative results of starch hydrolysis test. The details of phenotypic characteristics and confirmatory tests are given in Table 14. Variation was observed in the results of nitrate, urease and citrate tests. Among 25 isolates investigated, 14 isolates showed positive results of nitrate reduction test, 22 isolates were capable of urease production and 7 isolates showed positive results for citrate utilization. In gelatin hydrolysis test 2 isolates, NCDL1 and NCDL2 were capable of hydrolyzing gelatin (Figure 21).

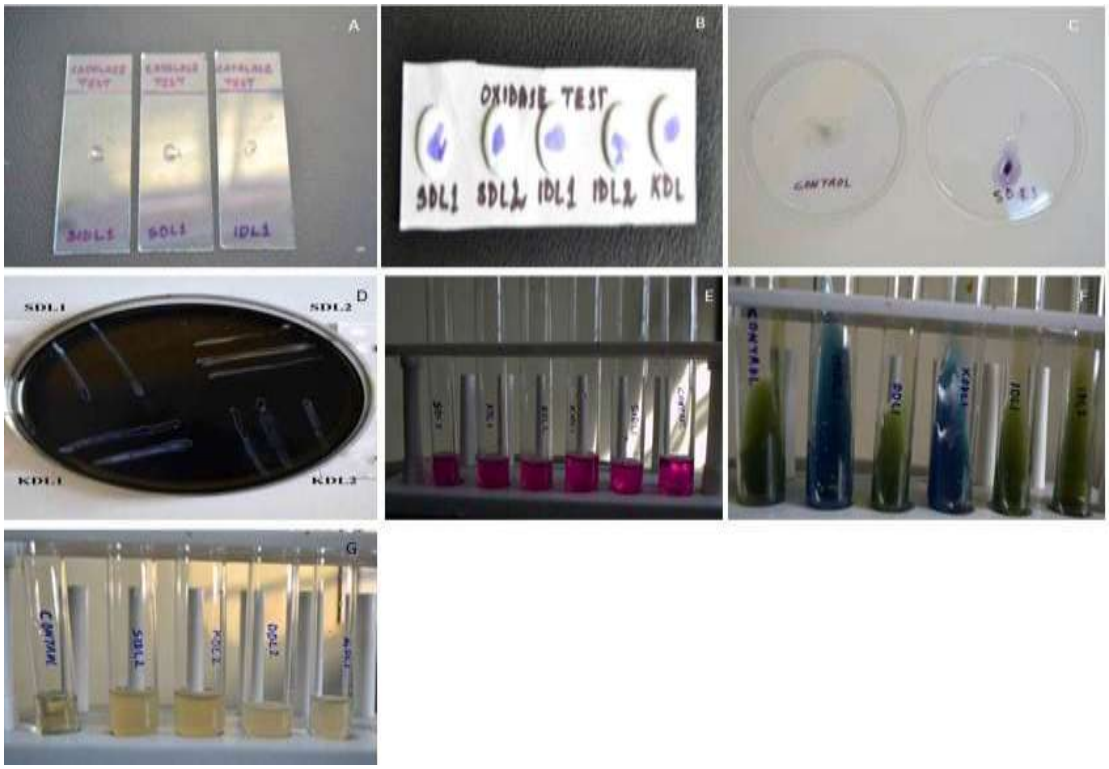

Figure 21: Biochemical characteristics of isolates (*L. purpureus*). A) Catalase test; B) Oxidase test; C) Nitrate test; D) Starch hydrolysis test; E) Urease test; F) Citrate test; G) Gelatin test

Table 14: Biochemical test results of isolates (*L. purpureus*)

Isolates	Catalase	Oxidase	Nitrate	Starch	Urease	Citrate	Gelatin
RDL1	+	+	-	-	+	-	-
RDL2	+	+	-	-	+	-	-
IDL1	+	+	-	-	+	-	-
IDL2	+	+	-	-	+	-	-
KDL1	+	+	+	-	+	-	-
KDL2	+	+	-	-	+	-	-
HIDL 1	+	+	+	-	+	+	-
SIDL1	+	+	+	-	+	+	-
SIDL 2	+	+	-	-	-	-	-
HDL 1	+	+	+	-	+	-	-
DDL1	+	+	-	-	+	-	-
DDL2	+	+	+	-	+	-	-
NCDL 1	+	+	+	-	+	+	+
NCDL 2	+	+	+	-	+	+	+
TEDL3	+	+	+	-	-	+	-
SDL1	+	+	+	-	+	-	-
SDL2	+	+	-	-	+	-	-
GDL1	+	+	-	-	+	-	-
UDL 2	+	+	+	-	+	-	-
UDL 3	+	+	+	-	+	-	-
KODL1	+	+	+	-	+	+	-
KODL2	+	+	+	-	+	-	-
BDL1	+	+	+	-	-	-	-
BDL2	+	+	-	-	+	+	-
BDL3	+	+	-	-	+	-	-

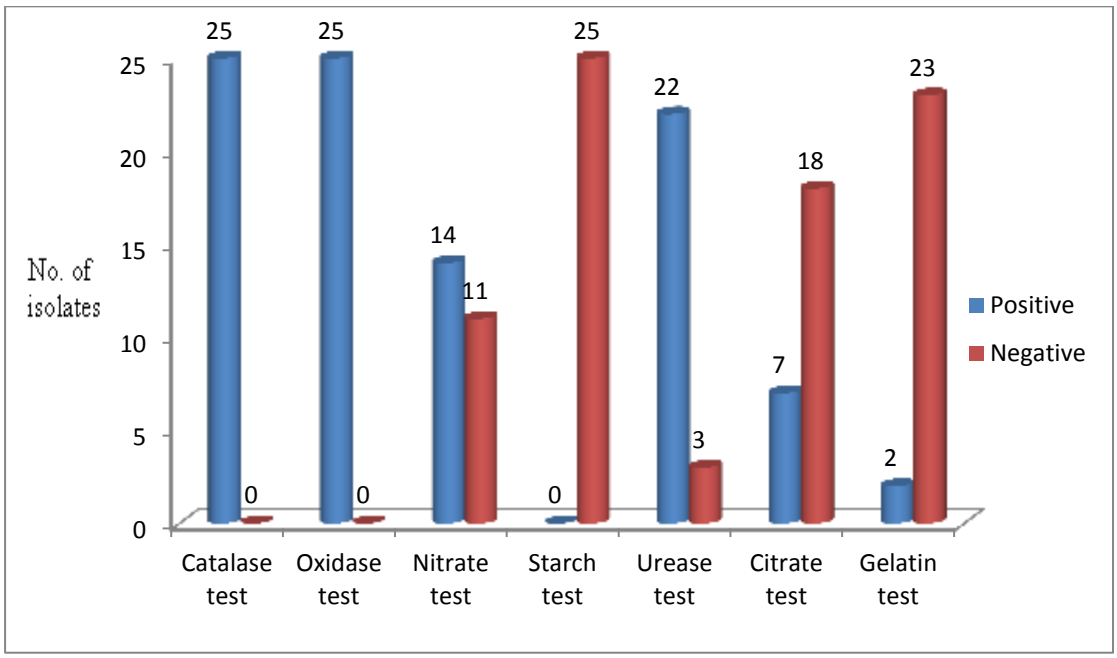

Figure 22: Bar diagram of biochemical test results of isolates (*L. purpureus*)

4.3.2. Biochemical characteristics of isolates obtained from *C. cajan*:

Similar to bacterial isolates obtained from *L. purpureus*, all the 23 isolates obtained from *C. cajan* showed positive results of catalase test, oxidase test and negative results of starch hydrolysis test and gelatin hydrolysis test (Table 15). The isolates showed variation in the results of urease, nitrate and citrate test. Among the 23 isolates investigated, 15 isolates showed positive results for nitrate reduction and urease production. Citrate test indicated that 5 isolates were capable of utilizing citrate (Figure 23).

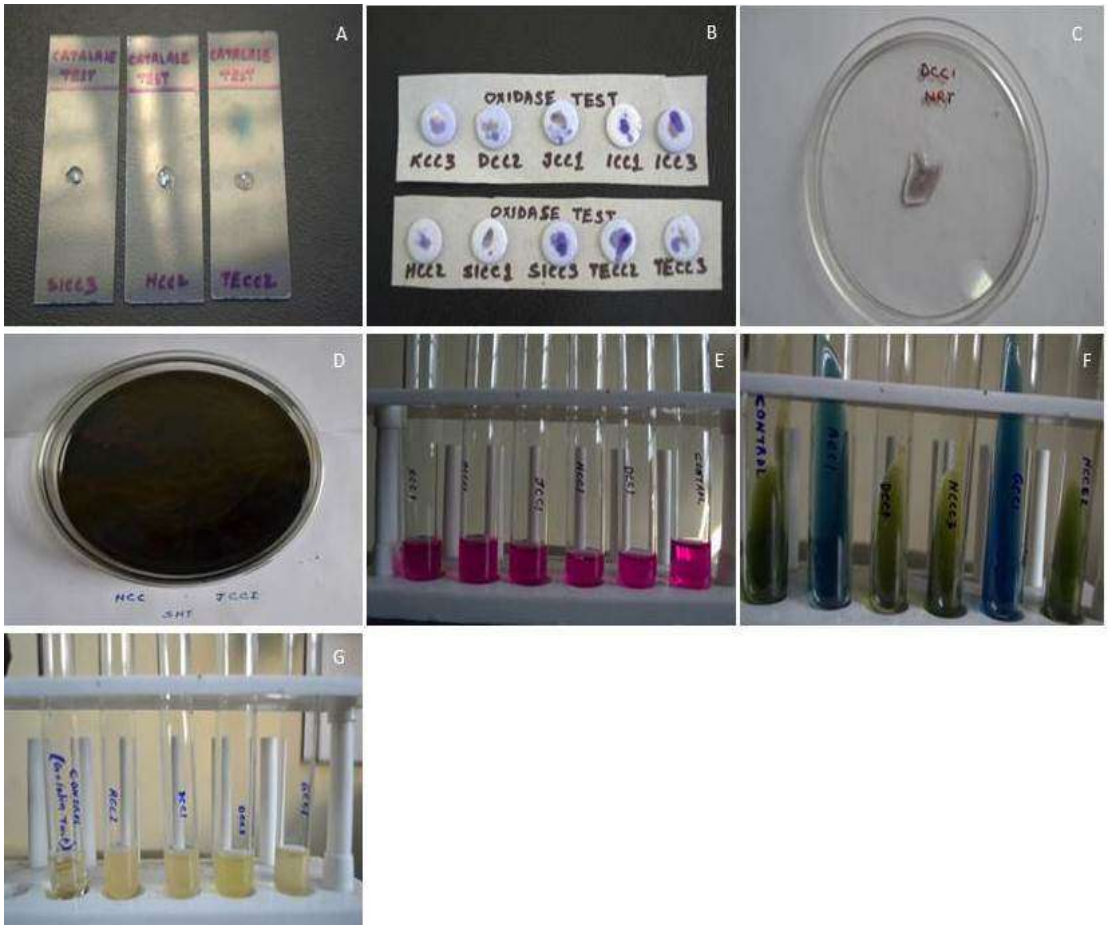

Figure23: Biochemical characteristics of isolates (*C.cajan*). A) Catalase tset; B) Oxidase test; C) Nitrate test; D) Starch hydrolysis test; E) Urease test; F) Citrate test; G) Gelatin test

Table 15: Biochemical test results of isolates (*C. cajan*)

Isolates	Catalase	Oxidase	Nitrate	Starch	Urease	Citrate	Gelatin
RCC1	+	+	+	-	+	+	-
RCC2	+	+	+	-	+	-	-
ICC 1	+	+	-	-	+	-	-
ICC 2	+	+	-	-	-	-	-
ICC3	+	+	+	-	+	-	-
KCC 1	+	+	-	-	+	-	-
KCC3	+	+	-	-	+	-	-
MKCC3	+	+	+	-	+	-	-
SICC1	+	+	+	-	+	+	-
SICC3	+	+	+	-	+	+	-
HCC 2	+	+	-	-	+	-	-
HCC3	+	+	+	-	+	-	-
DCC 1	+	+	+	-	-	-	-
DCC 2	+	+	+	-	+	-	-
NCCC2	+	+	+	-	+	-	-
NCCC3	+	+	-	-	+	-	-
TECC2	+	+	+	-	+	+	-
TECC3	+	+	-	-	+	-	-
SCC1	+	+	+	-	+	-	-
JCC 1	+	+	+	-	+	-	-
KOCC1	+	+	-	-	-	-	-
GCC1	+	+	+	-	+	+	-
JCC 2	+	+	+	-	+	-	-

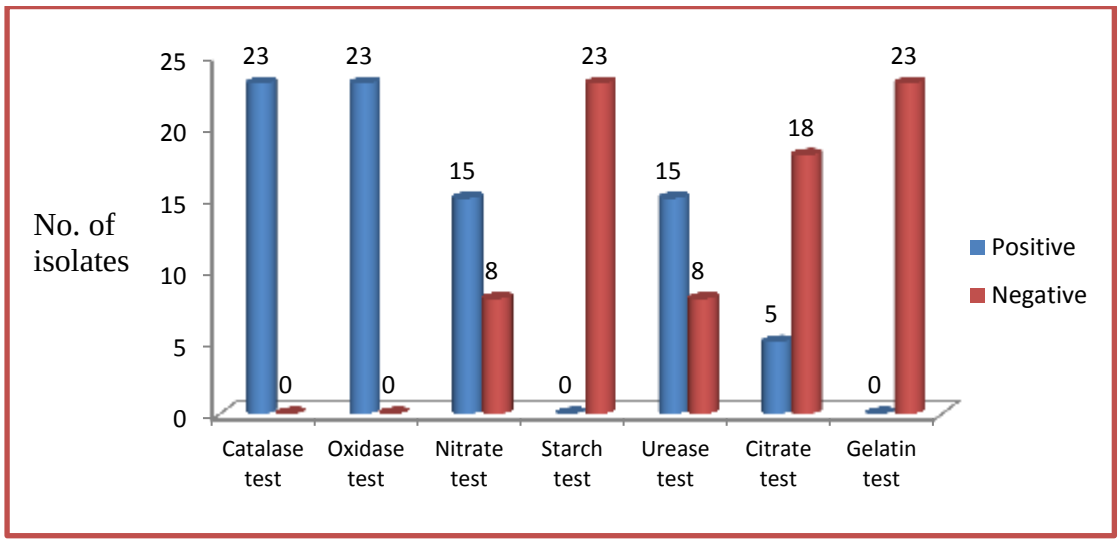

Figure 24: Bar diagram of biochemical test results of isolates (*C. cajan*)

4.3.4. Biochemical characteristics of isolates obtained from *C. pallida*:

All the selected 22 bacterial isolates showed positive results of catalase test, oxidase test and negative result of starch hydrolysis test. The details of phenotypic characteristics and confirmatory tests are given in Table 16. The remaining 4 tests showed variation in their results. Among the 22 isolates investigated, 11 isolates showed positive results of nitrate reduction test and 21 isolates showed positive results of urease test. In citrate test 5 isolates showed positive results. In gelatin hydrolysis test except ICP1 and DCP3, all the isolates showed negative results (Figure 25).

Figure25: Biochemical characteristics of isolates (*C. pallida*). A) Catalase test; B) Oxidase test; C) Nitrate test; D) Starch hydrolysis test; E) Urease test; F) Citrate test; G) Gelatin test

Table 16: Biochemical test results of isolates (*C. pallida*)

Isolates	Catalase	Oxidase	Nitrate	Starch	Urease	Citrate	Gelatin
RCP1	+	+	+	-	+	-	-
RCP2	+	+	-	-	+	-	-
ICP1	+	+	-	-	-	-	+
KACP1	+	+	+	-	+	-	-
KACP2	+	+	-	-	+	-	-
BCP2	+	+	+	-	+	-	-
MKCP1	+	+	-	-	+	+	-
MKCP2	+	+	+	-	+	+	-
GCP1	+	+	+	-	+	+	-
SICP2	+	+	-	-	+	-	-
DCP1	+	+	-	-	+	-	-
DCP3	+	+	+	-	+	+	+
NCP1	+	+	-	-	+	-	-
NCP2	+	+	-	-	+	-	-
SCP1	+	+	+	-	+	+	-
SCP2	+	+	+	-	+	-	-
TECP1	+	+	-	-	+	-	-
TECP4	+	+	+	-	+	-	-
HCP2	+	+	-	-	+	-	-
KCP1	+	+	-	-	+	-	-
KOCP1	+	+	+	-	+	-	-
KOCP2	+	+	+	-	+	-	-

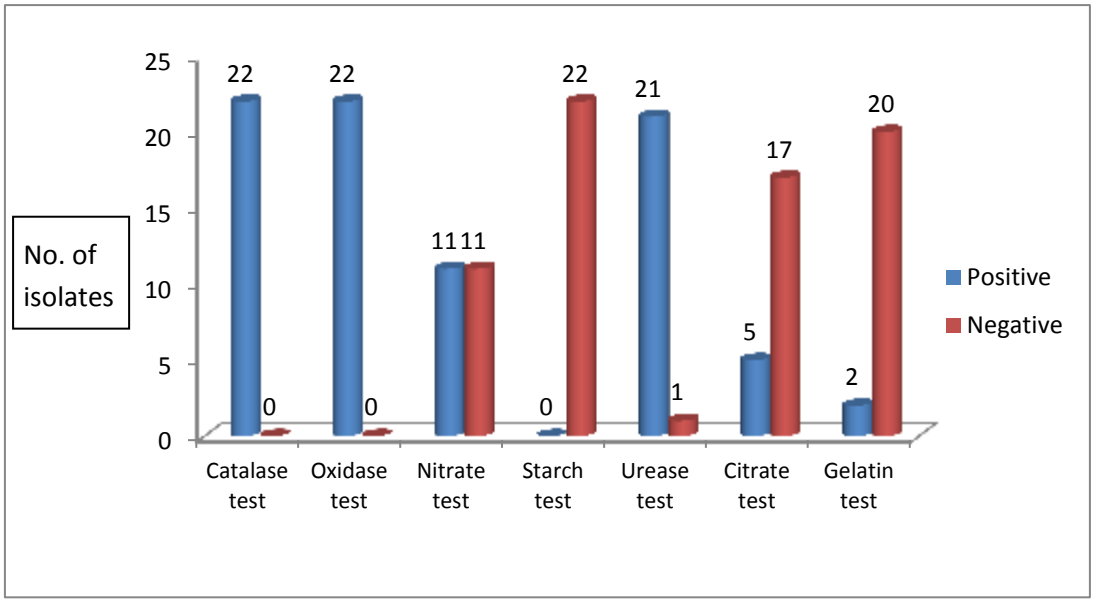

Figure 26: Bar diagram of biochemical test results of isolates (*C. pallida*)

4.3.4. Biochemical characteristics of isolates obtained from *M. pudica*:

All the selected 22 bacterial isolates obtained from *M. pudica* showed positive results of catalase, oxidase and negative results of starch hydrolysis test (Table 18). Variation was observed in the results of the remaining tests. Among the 22 isolates investigated, 20 isolates showed positive results of nitrate test, 4 isolates showed positive results of urease test, 18 isolates showed positive results of citrate test and 17 isolates showed positive results of gelatin hydrolysis test (Figure 27).

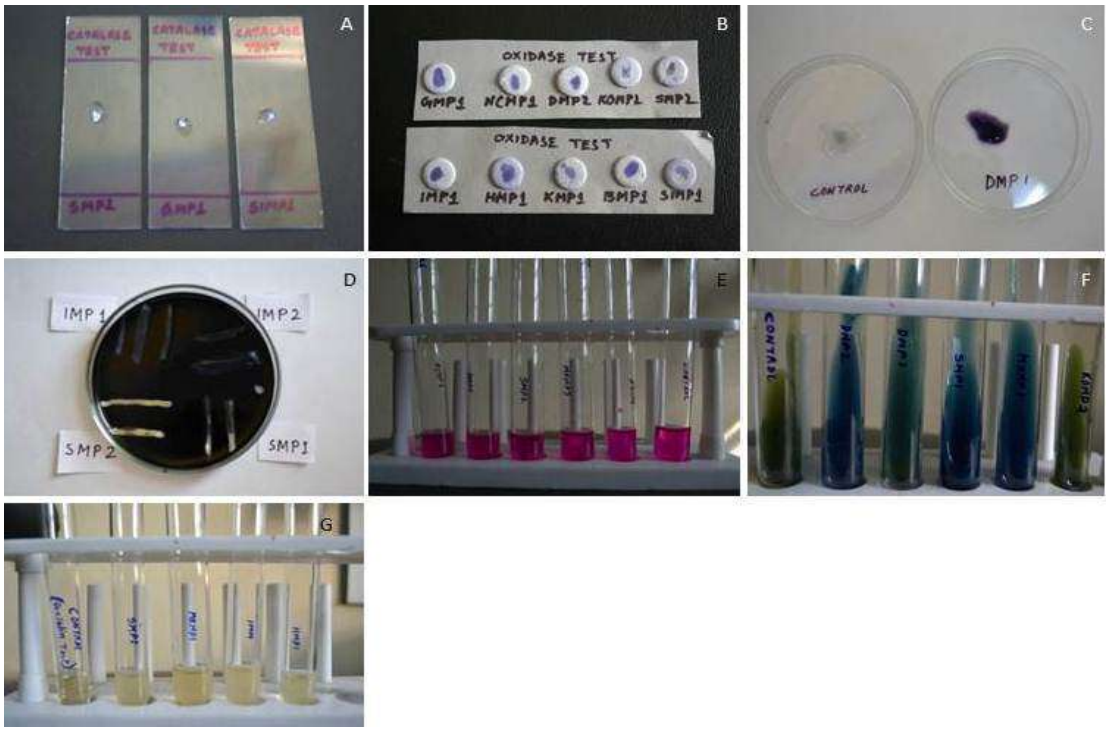

Figure27: Biochemical characteristics of isolates (*M. pudica*). A) Catalase test; B) Oxidase test; C) Starch test; D) Nitrate test; E) Urease test; F) Citrate test; G) Gelatin test

Table 17: Biochemical test results of reference strains

Reference strains	Catalase	Oxidase	Nitrate	Starch	Urease	Citrate	Gelatin
R. leguminoserum	+	+	+	-	+	-	-
B. japonicum	+	+	+	-	+	+	-
M. thiogangeticum	+	+	-	-	+	-	-
B. cepacia	+	+	+	-	-	+	+

Table 18: Biochemical test results of isolates (*M. pudica*)

Isolates	Catalase	Oxidase	Nitrate	Starch	Urease	Citrate	Gelatin
KAMP1	+	+	+	-	-	-	-
KAMP2	+	+	+	-	+	-	-
RMP2	+	+	-	-	-	+	+
IMP1	+	+	+	-	-	+	+
IMP2	+	+	+	-	-	+	+
HMP 1	+	+	+	-	-	+	+
KMP 1	+	+	+	-	-	+	+
KMP3	+	+	+	-	-	+	+
BMP1	+	+	+	-	-	+	+
SIMP 1	+	+	+	-	+	-	-
SIMP 3	+	+	+	-	-	+	+
GMP 1	+	+	+	-	-	+	+
NCMP 1	+	+	+	-	-	+	+
NCMP 2	+	+	-	-	+	+	+
DMP 1	+	+	+	-	-	+	+
DMP 2	+	+	+	-	-	+	+
TEMP1	+	+	+	-	-	+	+
SMP 1	+	+	+	-	-	+	-
SMP 2	+	+	+	-	-	+	+
KOMP2	+	+	+	-	+	-	-
MKMP1	+	+	+	-	-	+	+
MKMP2	+	+	+	-	-	+	+

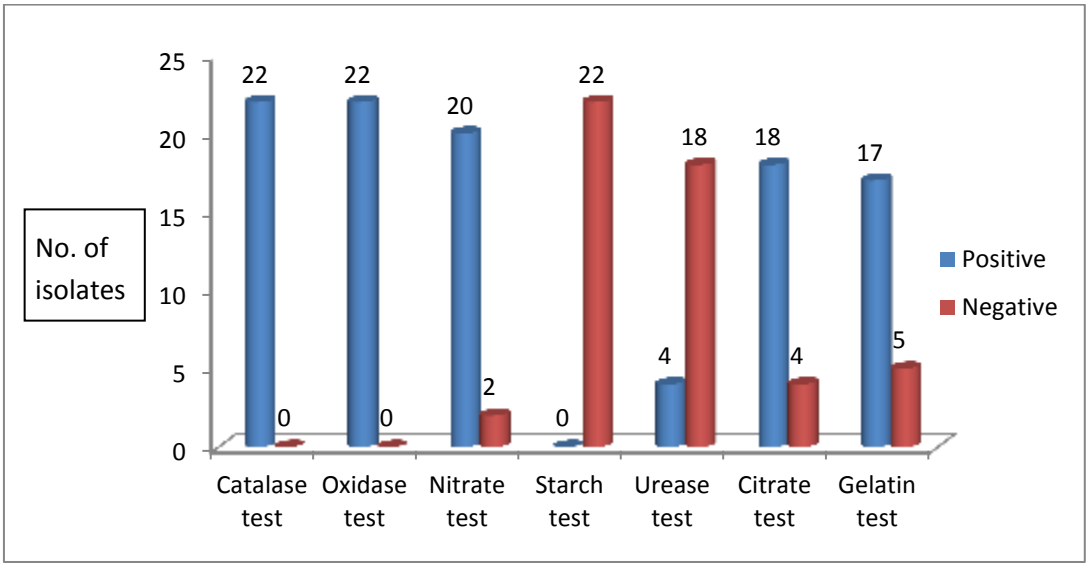

Figure 28: Bar diagram of biochemical test results of isolates (*M. pudica*)

4.5. Plant growth promoting (PGP) ability test

4.5.1. PGP ability of isolates obtained from *L. purpureus*:

Results revealed that 20 isolates obtained from *L. purpureus* produced IAA (Table 19) and SDL1 produces the highest amount of IAA(77 µg/ml). Similarly, 20 isolates were capable of phosphate solubilization. Clear halos of phosphate solubilization were seen around the bacterial colonies on Pikovskya's agar, which is due to dissolution of tricalcium phosphate (Figure 29). In addition 17 isolates showed positive results for ammonia production, which was confirmed by the development of brown color.

Table 19: PGP ability of isolates (*L. purpureus*)

Isolates	IAA (µg/ml)	Phosphate solubilization (E)	Ammonia production
RDL1	55	116	+
RDL2	-	-	+
IDL1	40	181	+
IDL2	38	114	+
KDL1	64	133	+
KDL2	37	80	+
HIDL 1	52	113	-
SIDL1	42	122	+
SIDL2	-	76	+
HDL 1	24	42	-
DDL1	26	-	+
DDL2	59	138	+
NCDL 1	41	-	+
NCDL 2	32	50	+
TEDL3	40	64	-
SDL1	77	127	-
SDL2	42	115	+
GDL1	48	122	+
UDL 2	-	-	-
UDL 3	-	72	-
KODL1	-	84	-
KODL2	30	72	+
BDL1	43	-	+
BDL2	39	66	+
BDL3	32	60	-

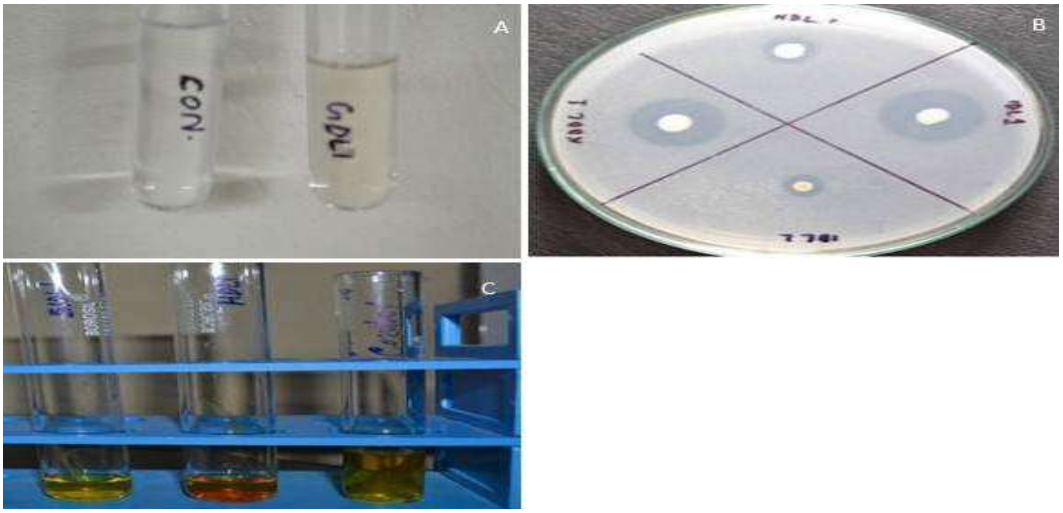

Figure 29: PGP ability of isolates (*L. purpureus*). A) IAA production; B) Phosphate solubilization; C) Ammonia production

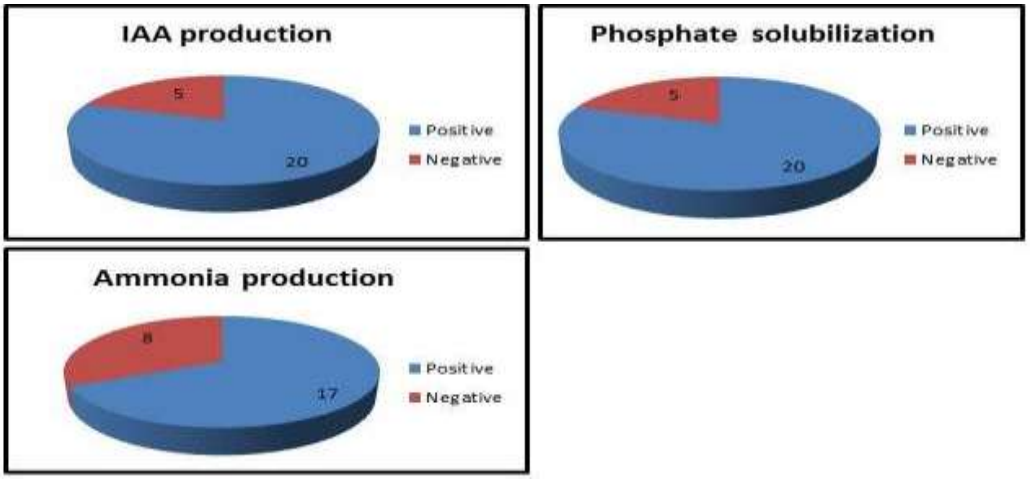

Figure 30: Pie chart ofPGP ability of isolates (*L. purpureus*)

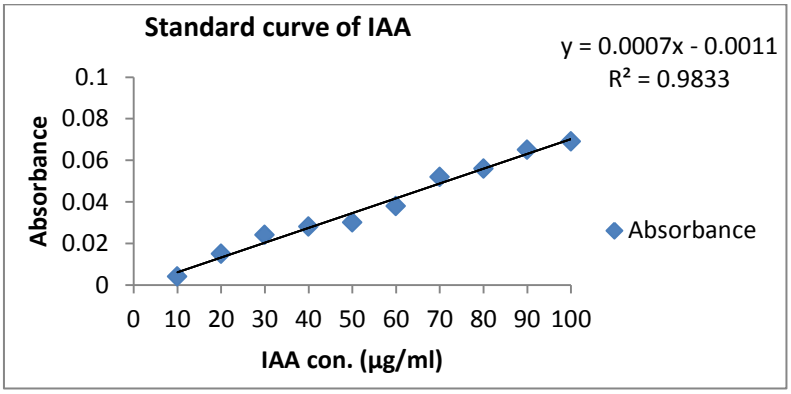

Figure 31: Standard curve of IAA production

4.5.2. PGP ability of isolates obtained from *C. cajan*:

Plant growth promoting ability test results of bacterial isolates obtained from *C. cajan* root nodules are documented in Table 20. The results revealed that 17 isolates were capable of producing IAA and TECC3 produced the highest amount of IAA (62µg/ml). Among the 23 isolates studied, 18 isolates were capable of phosphate solubilization which is confirmed by the formation of clear halo zones around the spot of inoculation. Isolate ICC1 showed highest phosphate solubilization efficiency. The PGP test results also confirmed that 18 isolates were capable of producing ammonia (Figure 32).

Table 20: PGP ability of isolates (*C. cajan*)

Isolates	IAA (µg/ml)	Phosphate solubilization (E)	Ammonia production
RCC1	24	75	-
RCC2	22	80	+
ICC 1	45	141	+
ICC 2	31	-	+
ICC3	41	114	+
KCC 1	25	-	-
KCC3	-	120	+
MKCC3	-	82	+
SICC1	55	133	+
SICC3	58	137	+
HCC 2	-	122	+
HCC3	-	-	-
DCC 1	13	70	+
DCC 2	40	116	+
NCCC2	-	-	+
NCCC3	-	66	-
TECC2	43	112	+
TECC3	62	113	+
SCC1	24	110	+
JCC 1	36	127	+
KOCC1	25	62	-
GCC1	31	-	+
GCC2	32	84	+

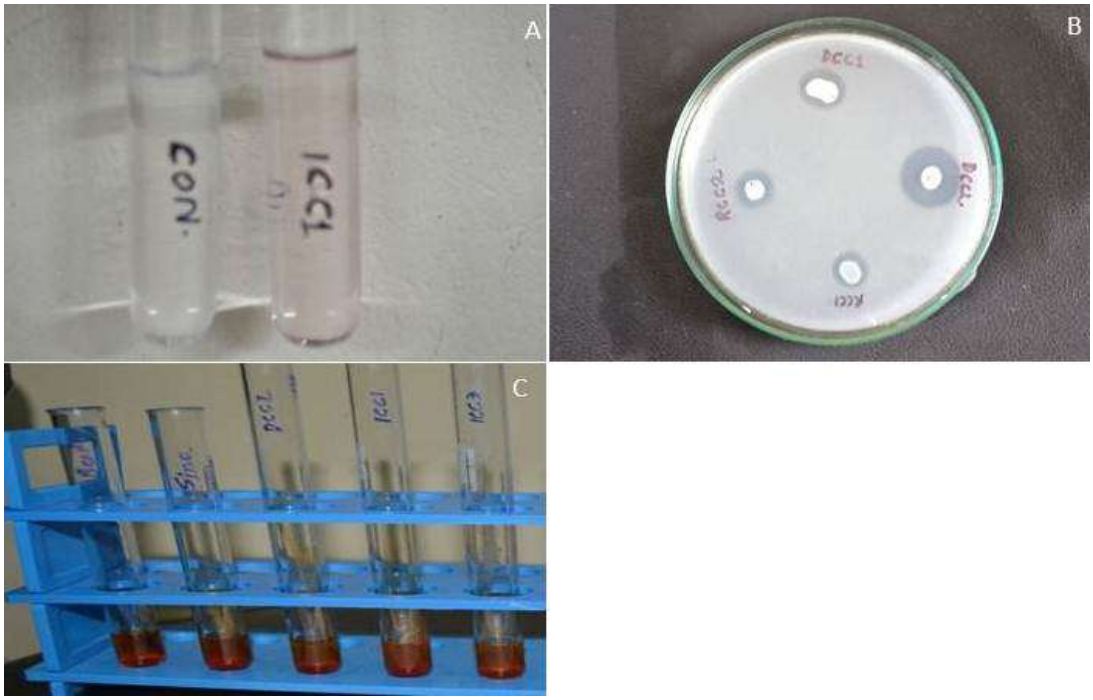

Figure 32: PGP ability of isolates (*C. cajan*). A) IAA production; B) Phosphate solubilization; C) Ammonia production

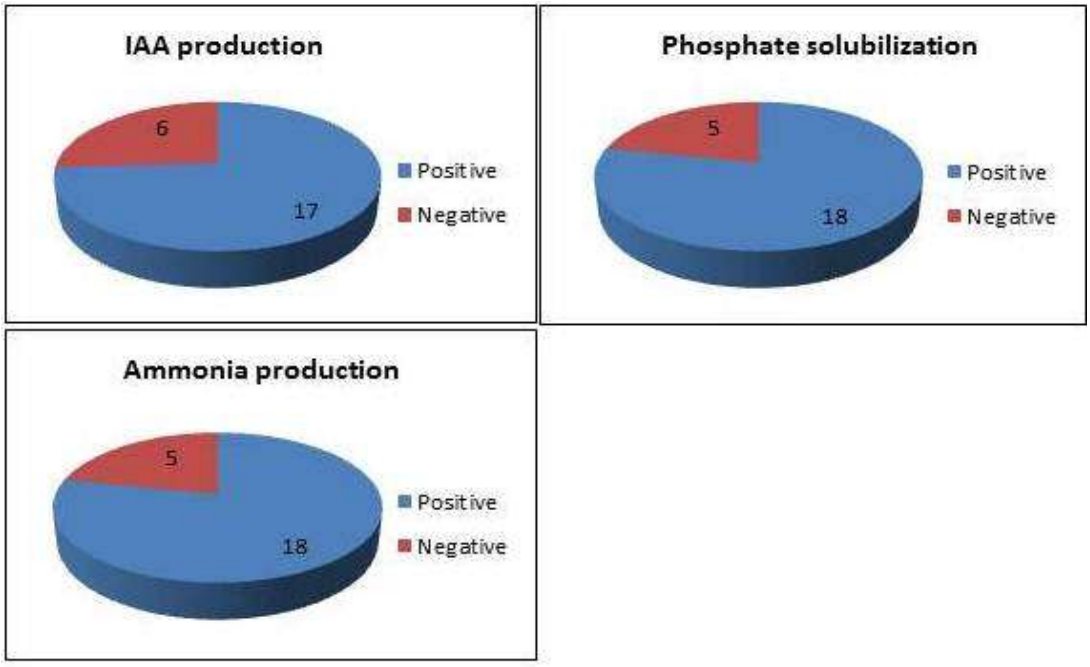

Figure 33: Pie chart of PGP ability of isolates (*C. cajan*)

4.4.3. PGP ability of isolates obtained from *C. pallida*:

The results of IAA production test of isolates obtained from *C. pallida* revealed that 16 isolates were capable of producing IAA which is confirmed by spectrophotometric analysis (Table 21). MKCP1 producedthe highest amount of IAA (66 µg/ml). In phosphate solubilization test 18 isolates showed positive results. The highest phosphate solubilization efficiency was shown by DCP1 (Figure 34). The results also confirmed that, 18 isolates were capable of ammonia production, which was confirmed by the development of brown color.

Table 21: PGP ability of isolates (*C. pallida*)

Isolates	IAA(µg/ml)	Phosphate solubilization (E)	Ammonia production
RCP1	32	62	+
RCP2	45	175	+
ICP1	30	80	-
KACP1	18	55	+
KACP2	0	143	+
BCP2	37	72	-
MKCP1	66	122	+
MKCP2	54	-	+
GCP1	0	125	+
DCP3	0	-	+
SICP2	55	140	+
DCP1	58	187	+
NCP1	40	155	+
NCP2	43	146	+
SCP1	62	163	+
SCP2	15	65	-
TECP1	36	127	+
TECP4	0	-	+
HCP2	49	70	-
KCP1	28	66	+
KOCP1	0	-	+
KOCP2	0	72	+

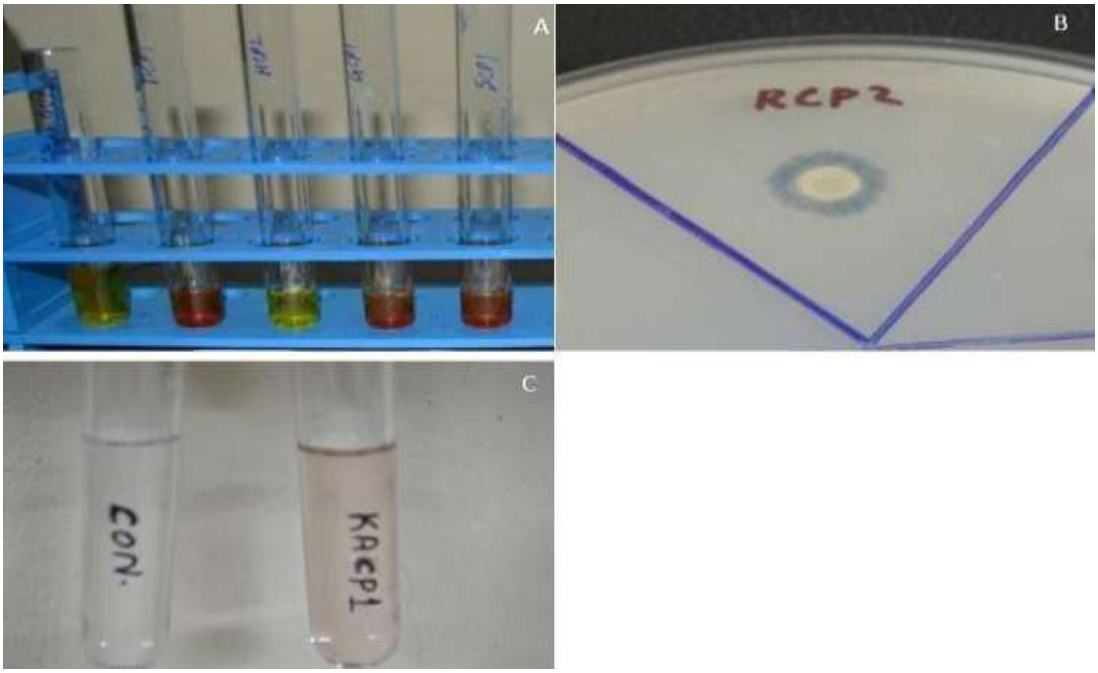

Figure 34: PGP ability of isolates (*C. pallida*). A) IAA production; B) Phosphate solubilization; C) Ammonia production

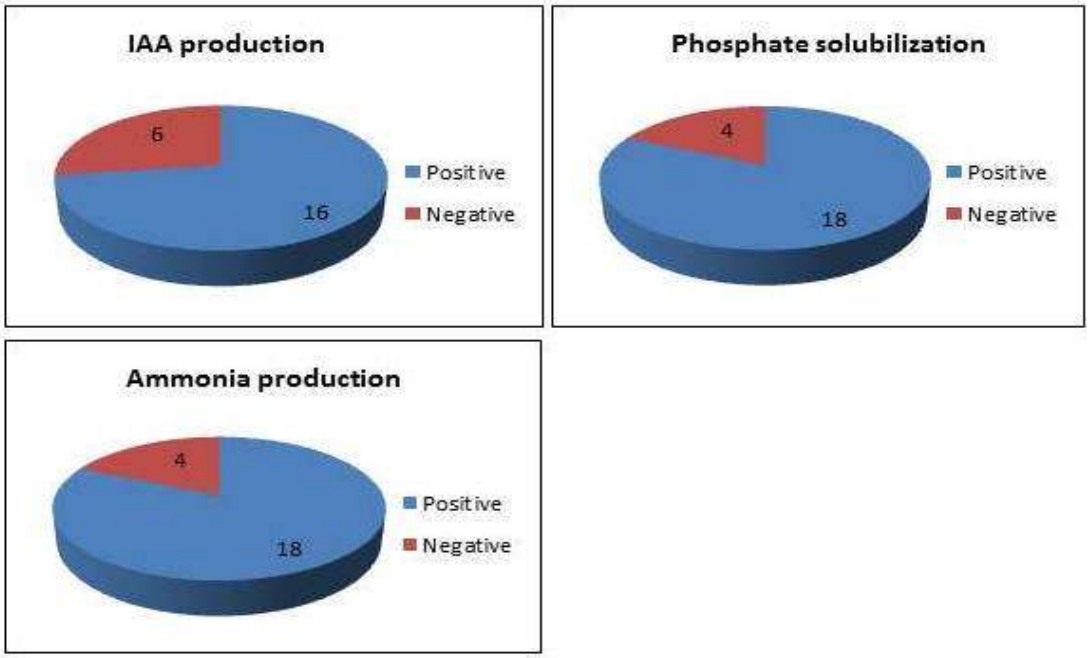

Figure 35: Pie chart ofPGP ability of isolates (*C. pallida*)

4.4.4. PGP ability of isolates obtained from *M. pudica*:

Plant growth promoting ability test of bacterial isolates obtained from *M. pudica* confirmed that 19 isolates were capable of producing IAA. The isolate HMP1 produced the highest amount of IAA (73 µg/ml). In phosphate solubilization test 19 isolates showed positive results and NCMP1 showed highest PSE. In ammonia production test 18 isolates showed positive results for ammonia production (Figure 36).

Table 22: PGP ability of isolates (*M. pudica*)

Isolates	IAA (µg/ml)	Phosphate solubilization (E)	Ammonia production
KAMP1	22	56	+
KAMP2	43	64	-
RMP2	41	80	+
IMP1	42	145	+
IMP2	-	42	-
HMP 1	73	137	+
KMP 1	37	122	+
KMP 3	29	74	-
BMP1	68	140	+
SIMP 1	55	126	+
SIMP 3	33	62	+
GMP 1	-	148	+
NCMP 1	27	160	+
NCMP 2	-	54	+
DMP 1	40	72	+
DMP 2	33	157	+
TEMP1	24	-	+
SMP 1	31	-	-
SMP 2	65	138	+
KOMP2	40	152	+
MKMP1	26	-	+
MKMP2	24	110	+

Table 23: PGP ability of reference strains

Reference strains	IAA (µg/ml)	Phosphate solubilization (E)	Ammonia production
R. leguminosarum	45	125	+
B. japonicum	00	131	+
M. thiogangeticum.	00	121	+
B. cepacia	25	-	+

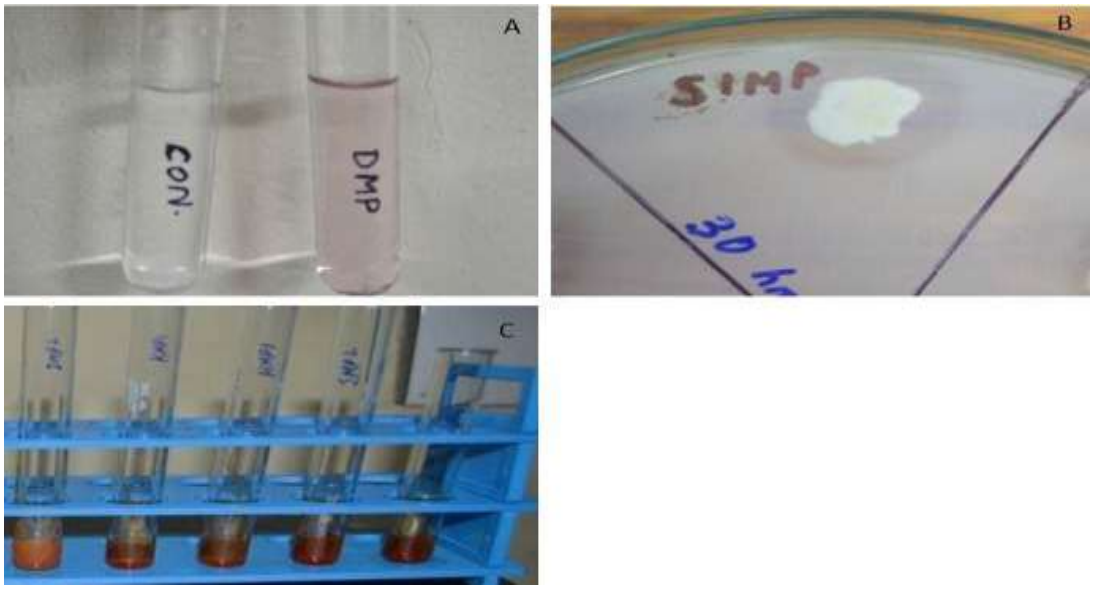

Figure 36: PGP ability of isolates (*M. pudica*). A) IAA production; B) Phosphate solubilization; C) Ammonia production

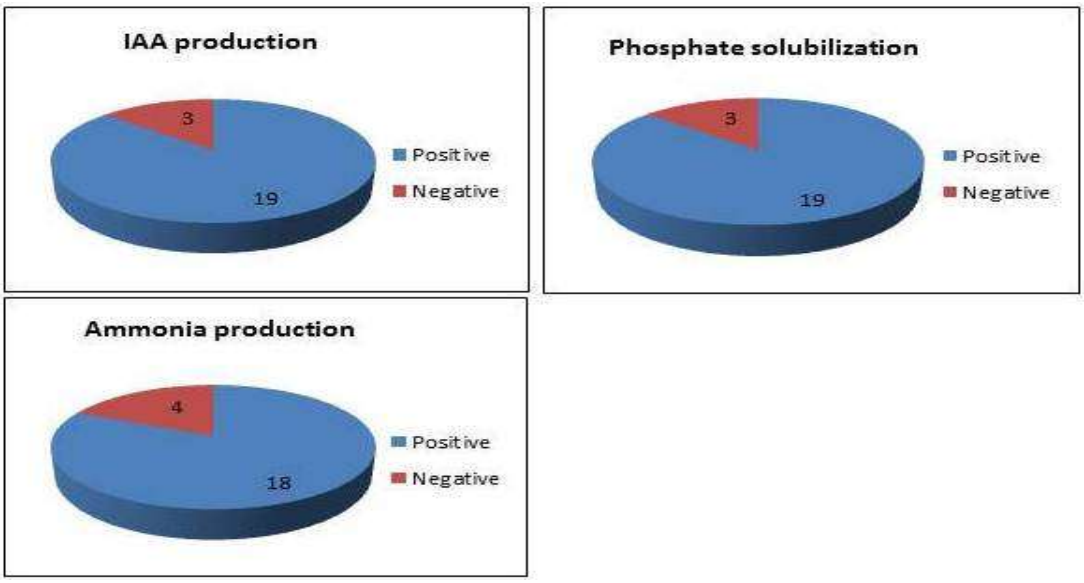

Figure 37: Pie chart ofPGP ability of isolates (*M. pudica*)

4.5. Acidic pH and salt tolerance test

4.4.2. Acidic pH and salt tolerance test of isolates obtained from *L. purpureus*:

The results revealed that most of the isolates survived and showed growth on YEMA medium adjusted at pH 5 and pH 6, but showed no visible growth on medium adjusted at pH 4 (Table 24). Among the 25 isolates investigated, all the isolates showed growth at pH 6 and 16 isolates showed growth at pH 5. The results also revealed that in salinity tolerance test isolates grew well on YEMA media adjusted to 1% and 2%NaCl. Among 25 isolates studied, all the isolates grew well at 1% NaCl, 17 isolates showed growth at 2% NaCl. However, no isolates survived on YEMA medium containing 3% NaCl (Figure 38).

Table 24: Acidic pH and salt tolerance ability of isolates (*L. purpureus*)

Isolate	pH tolerance			Salt tolerance (NaCl)		
	pH 4	pH 5	pH 6	1%	2%	3%
RDL1	-	+	+	+	+	-
RDL2	-	+	+	+	+	-
IDL1	-	-	+	+	+	-
IDL2	-	+	+	+	-	-
KDL1	-	+	+	+	+	-
KDL2	-	+	+	+	+	-
HIDL 1	-	-	+	+	+	-
SIDL1	-	-	+	+	-	-
SIDL2	-	+	+	+	+	-
HDL 1	-	+	+	+	+	-
DDL1	-	+	+	+	+	-
DDL2	-	+	+	+	+	-
NCDL 1	-	-	+	+	+	-
NCDL 2	-	-	+	+	+	-
TEDL3	-	+	+	+	+	-
SDL1	-	+	+	+	+	-
SDL2	-	-	+	+	+	-
GDL1	-	-	+	+	-	-
UDL 2	-	+	+	+	-	-
UDL 3	-	-	+	+	-	-
KODL1	-	+	+	+	-	-
KODL2	-	+	+	+	-	-
BDL1	-	+	+	+	+	-
BDL2	-	-	+	+	+	-
BDL3	-	+	+	+	-	-

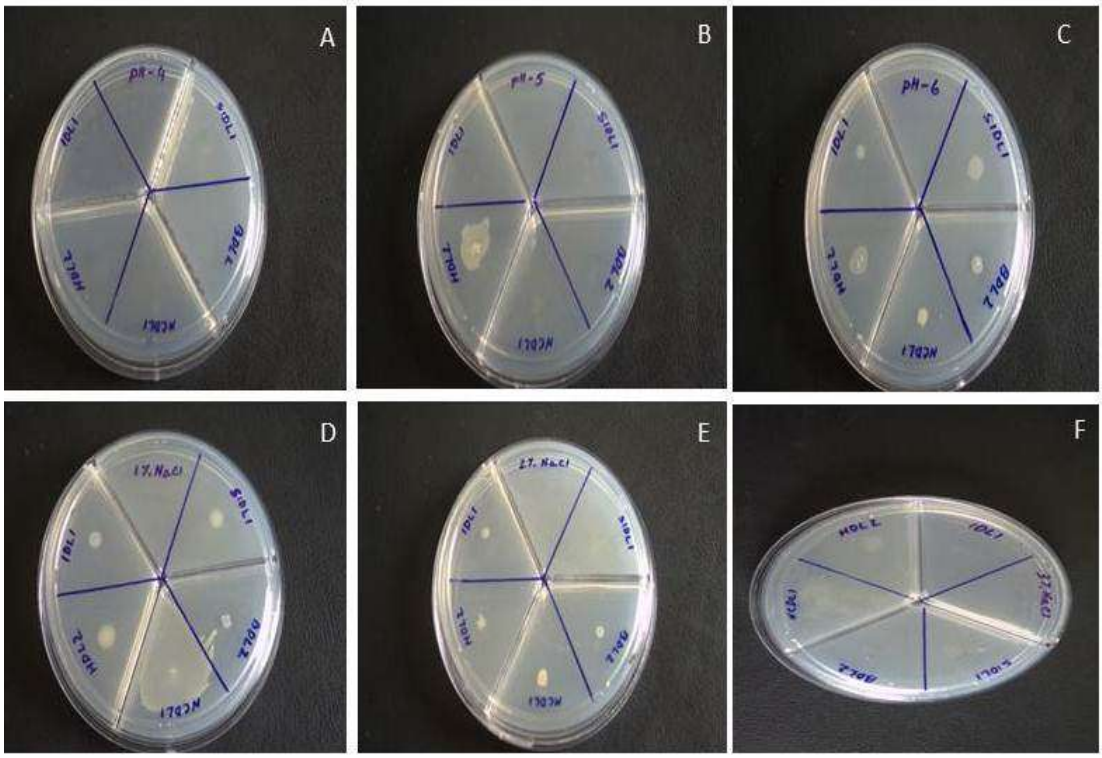

Figure 38: Growth of isolates (*L. purpureus*) at different pH and salt concentration. A) pH-4; B) pH-5; C) pH-6; D) 1% NaCl; E) 2% NaCl; F) 3% NaCl

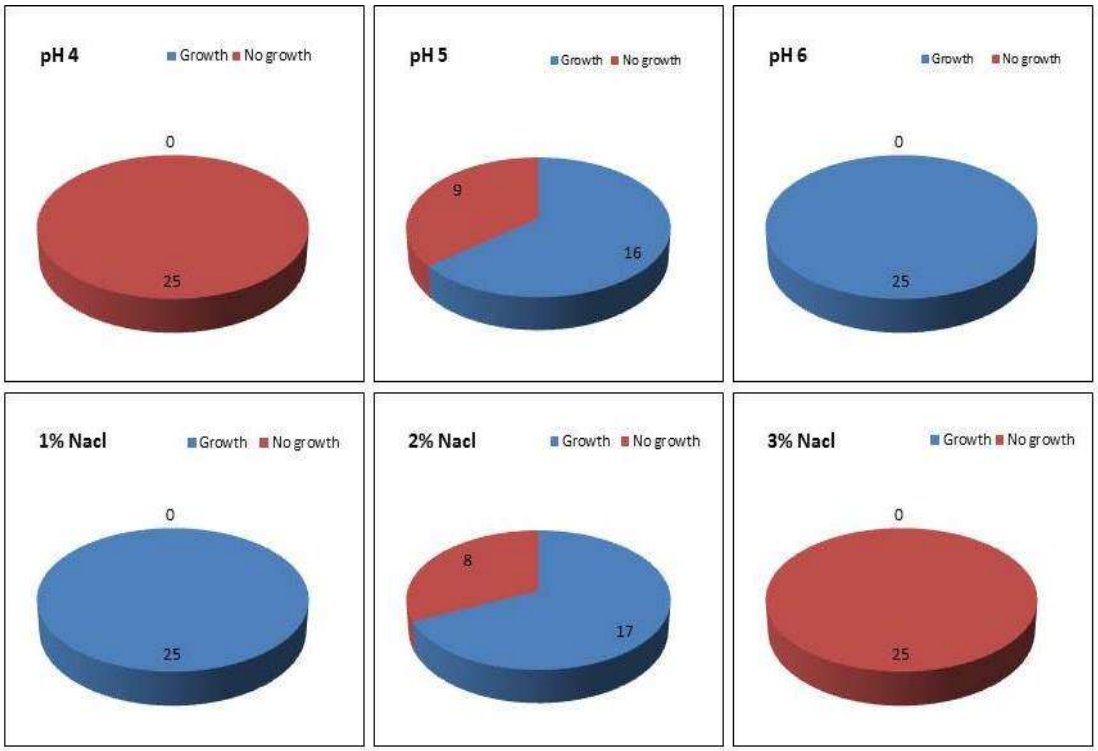

Figure 39: Pie chart of acidic pH and salt tolerance ability of isolates (*L. purpureus*)

4.5.2. Acidic pH and salt tolerance test of isolates obtained from *C. cajan*:

Similar to isolate obtained from *L. purpureus*, the isolates obtained from *C. cajan* showed growth on YEMA medium adjusted at pH 5 and pH 6, but unable to survive at pH 4 (Table 25). Among the 23 isolates investigated, all the isolates showed growth at pH 6 and 14 isolates showed growth at pH 5. In salinity tolerance test the isolates successfully tolerated and showed visible growth on YEMA medium containing 1% and 2% NaCl. Among the 23 isolates studied, all the isolates grew well on YEMA media containing 1% NaCl, 18 isolates showed growth at 2% NaCl, however at 3% NaCl no isolates survived (Figure 40).

Table 25: Acidic pH and salt tolerance ability of isolates (*C. cajan*)

Isolate	pH tolerance			Salt tolerance (NaCl)		
	pH 4	pH 5	pH 6	1%	2%	3%
RCC 1	-	+	+	+	+	-
RCC 2	-	-	+	+	+	-
ICC 1	-	-	+	+	+	-
ICC 2	-	+	+	+	-	-
ICC3	-	+	+	+	+	-
KCC 1	-	+	+	+	+	-
KCC3	-	+	+	+	+	-
MKCC3	-	+	+	+	+	-
SICC1	-	-	+	+	+	-
SICC3	-	+	+	+	-	-
HCC 2	-	+	+	+	+	-
HCC3	-	+	+	+	-	-
DCC 1	-	+	+	+	+	-
DCC 2	-	-	+	+	+	-
NCCC2	-	+	+	+	-	-
NCCC3	-	+	+	+	+	-
TECC2	-	+	+	+	+	-
TECC3	-	-	+	+	-	-
SCC1	-	+	+	+	+	-
JCC 1	-	-	+	+	+	-
KOCC1	-	-	+	+	+	-
GCC 1	-	-	+	+	+	-
GCC 2	-	-	+	+	+	-

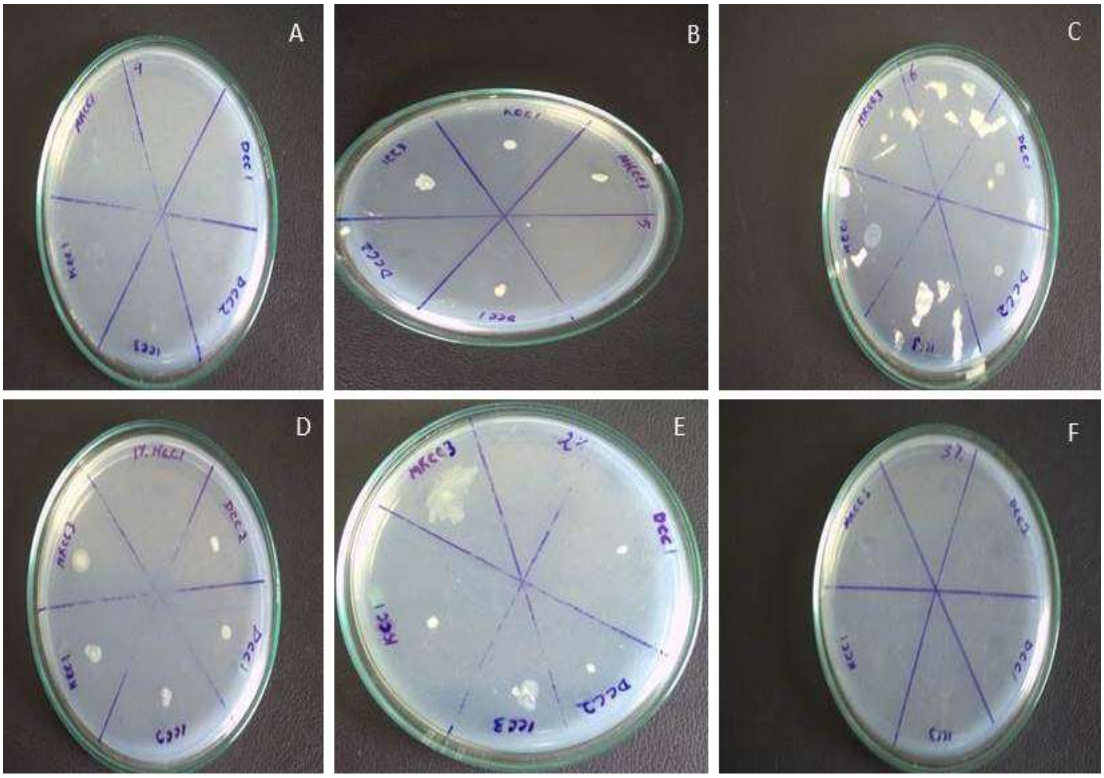

Figure 40: Growth of isolates (*C. cajan*) at different pH and salt concentration. A) pH-4; B) pH-5; C) pH-6; D) 1% NaCl; E) 2% NaCl; F) 3% NaCl

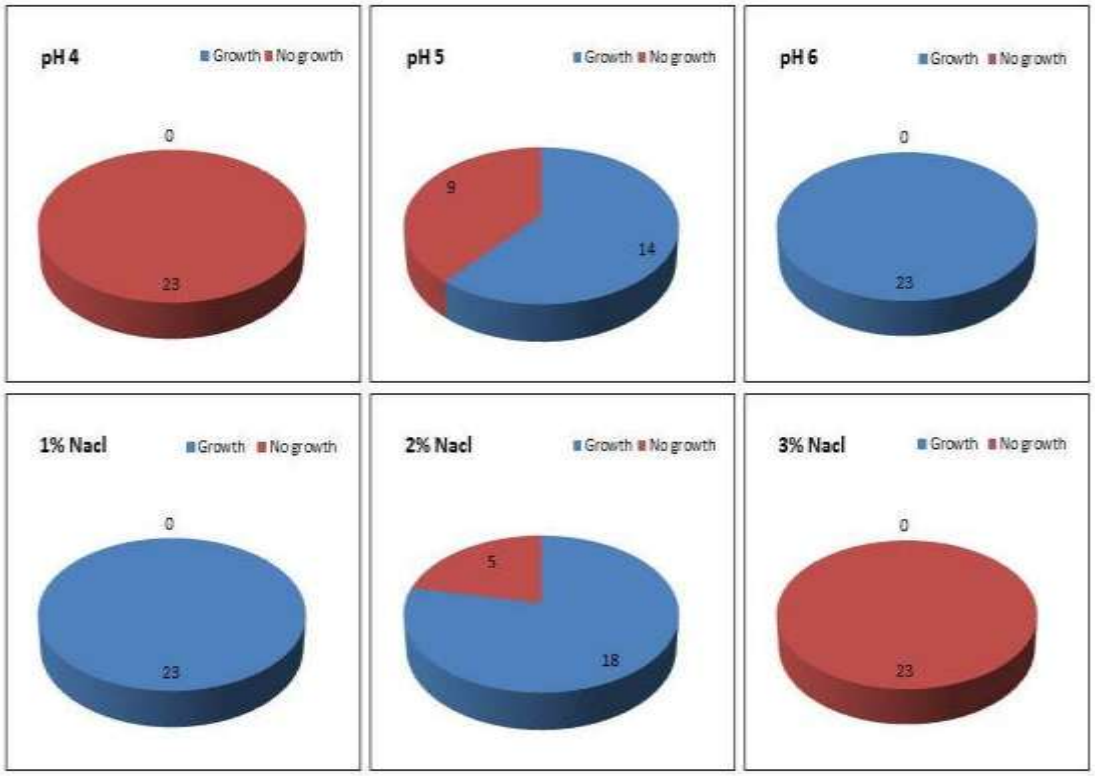

Figure 41: Pie chart ofpH and salt tolerance of isolates (*C. cajan*)

4.5.3. Acidic pH and salt tolerance test of isolates obtained from *C. pallida*:

Isolates obtained from *C. pallida* grew well on YEMA media adjusted at pH 6 and few isolates showed variable growth at pH 5 and pH 4 (Table 26). Among the 22 isolates investigated, all the isolates showed visible growth at pH 6, 8 isolates showed growth at pH 5 and 2 isolates (KACP2 and GCP1) showed growth at pH 4. In salinity tolerance test the isolates grew well on YEMA medium containing 1% and 2% NaCl. Among the 22 isolates studied, all the isolates showed growth at 1% NaCl and 13 isolates showed growth at 2% NaCl. Interestingly, 3 isolate (RCP2, GCP1 and SICP2) showed growth on YEMA medium containing 3% NaCl (Figure 42).

Table 26: Acidic pH and salt tolerance ability of isolates (*C. pallida*)

Isolates	pH tolerance			Salt tolerance (NaCl)		
	pH 4	pH 5	pH 6	1%	2%	3%
RCP1	-	-	+	+	+	-
RCP2	-	+	+	+	+	+
ICP1	-	+	+	+	-	-
KACP1	-	-	+	+	-	-
KACP2	+	+	+	+	-	-
BCP2	-	+	+	+	+	-
MKCP1	-	-	+	+	+	-
MKCP2	-	-	+	+	-	-
GCP1	+	+	+	+	+	+
DCP3	-	+	+	+	+	-
SICP2	-	-	+	+	+	+
DCP1	-	-	+	+	+	-
NCP1	-	+	+	+	+	-
NCP2	-	-	+	+	+	-
SCP1	-	-	+	+	-	-
SCP2	-	-	+	+	+	-
TECP1	-	-	+	+	+	-
TECP4	-	+	+	+	+	-
HCP2	-	-	+	+	-	-
KCP1	-	-	+	+	-	-
KOCP1	-	-	+	+	-	-
KOCP2	-	-	+	+	-	-

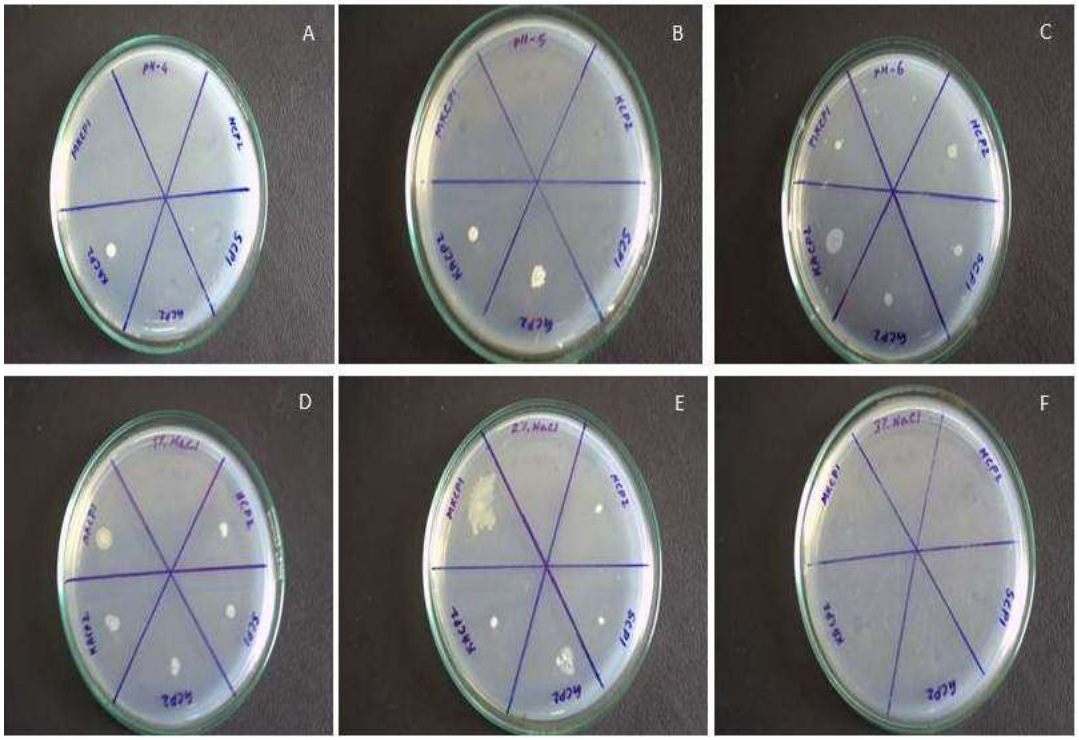

Figure 42: Growth of isolates (*C. pallida*) at different pH and salt concentration. A) pH-4; B) pH-5; C) pH-6; D)1% NaCl; E) 2% NaCl; F) 3% NaCl

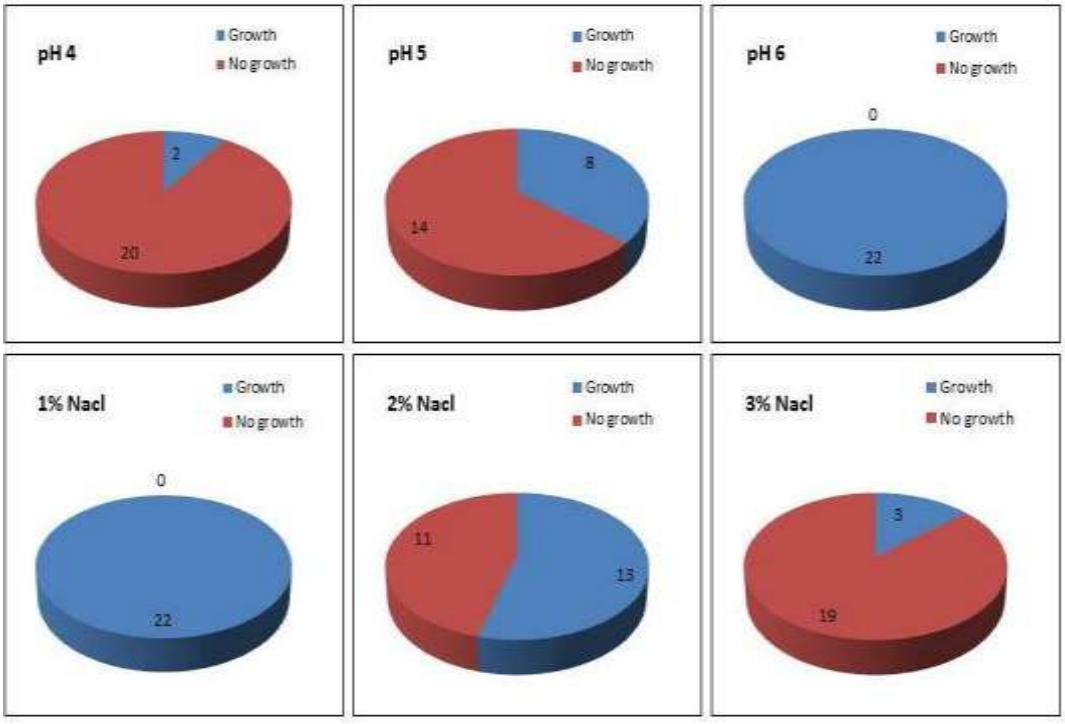

Figure 43: Pie chart of acidic pH and salt tolerance ability of isolates (*C. pallida*)

4.5.3. Acidic pH and salt tolerance test of isolates obtained from *M. pudica*:

All the isolates obtained from *M. pudica* grew well on YEMA media adjusted at pH 6, however the isolates showed variability in growth at pH 4 and 5 (Table 27). Among the 22 isolates, all the isolates showed growth at pH 6, 12 isolates showed growth at pH 5 and 3 isolates (RMP2, SIMP3 and KOMP2) showed growth at pH 4. In salinity test all the 22 isolates grew well on YEMA medium containing 1% NaCl, 11 isolates showed growth at 2% NaCl and 1 isolate (SMP2) showed growth at 3% NaCl (Figure 44).

Table 27: Acidic pH and salt tolerance ability of isolates (*M.pudica*)

Isolates	pH tolerance			Salt tolerance (NaCl)		
	pH 4	pH 5	pH 6	1%	2%	3%
KAMP1	-	-	+	+	+	-
KAMP2	-	+	+	+	+	-
RMP2	+	+	+	+	-	-
IMP1	-	-	+	+	+	-
IMP2	-	-	+	+	+	-
HMP 1	-	+	+	+	-	-
KMP 1	-	+	+	+	-	-
KMP 3	-	+	+	+	-	-
BMP1	-	+	+	+	-	-
SIMP 1	-	+	+	+	+	-
SIMP 3	+	-	+	+	-	-
GMP 1	-	-	+	+	-	-
NCMP 1	-	-	+	+	+	-
NCMP 2	-	+	+	+	+	-
DMP 1	-	+	+	+	+	-
DMP 2	-	-	+	+	+	-
TEMP1	-	-	+	+	-	-
SMP 1	-	+	+	+	-	-
SMP 2	-	+	+	+	+	+
KOMP2	+	+	+	+	-	-
MKMP1	-	-	+	+	-	-
MKMP2	-	-	+	+	+	-

Table 28: Acidic pH and salt tolerance ability of reference strains

Reference strain	pH tolerance			Salt tolerance (NaCl)		
	pH4	pH5	pH6	1%	2%	3%
R. leguminosarum	-	+	+	+	+	-
B. japonicum	-	-	+	+	-	-
M. thiogangeticum.	-	+	+	+	+	-
B. cepacia	-	-	+	+	-	-

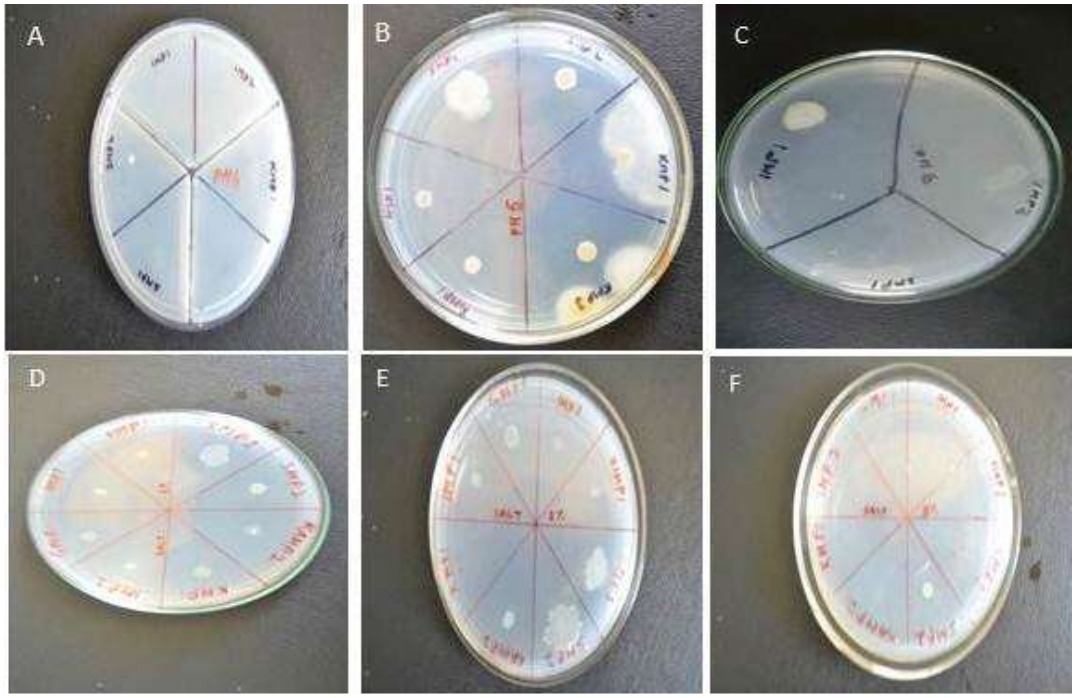

Figure 44: Growth of isolates (*M.pudica*) at different pH and salt concentration. A) pH-4; B) pH-5; C) pH-6; D) 1% NaCl; E) 2% NaCl; F) 3% NaCl

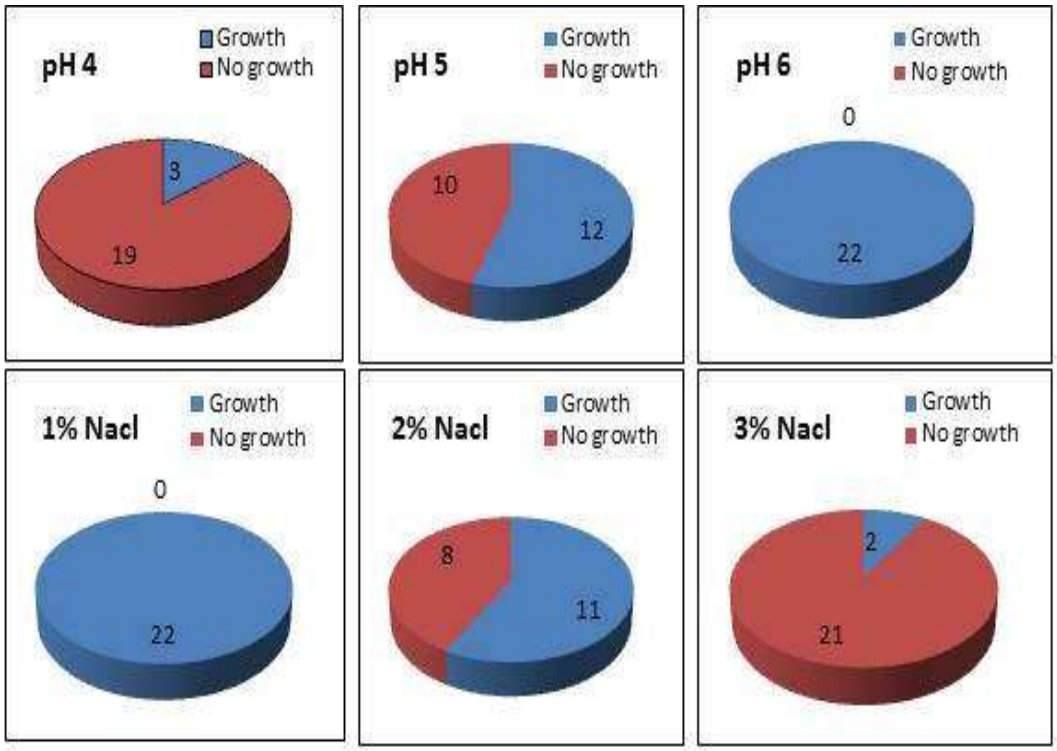

Figure 45: Pie chart ofacidic pH and salt tolerance ability of isolates (*M.pudica*)

4.6. Genomic DNA extraction

Based on the results of plant growth promoting ability, acidic pH tolerance ability and salt tolerance ability tests,40 best isolates (10 isolates from each plant) were selected for genotypic studies. For determining the presence of specific genes (*nifH*, *nodC* and 16S rDNA), genomic DNA was extracted from the selected 40 isolates and reference strains (Figure 46). The extracted DNA was visualized on 1% agarose gel after agarose gel electrophoresis. The photographic image of the gel was captured using gel documentation system. The selected isolates were investigated for the presence of *nifH* and *nodC* gene in their genome. Further PCR-RFLP analysis of 16S rDNA gene of the isolates was carried out.

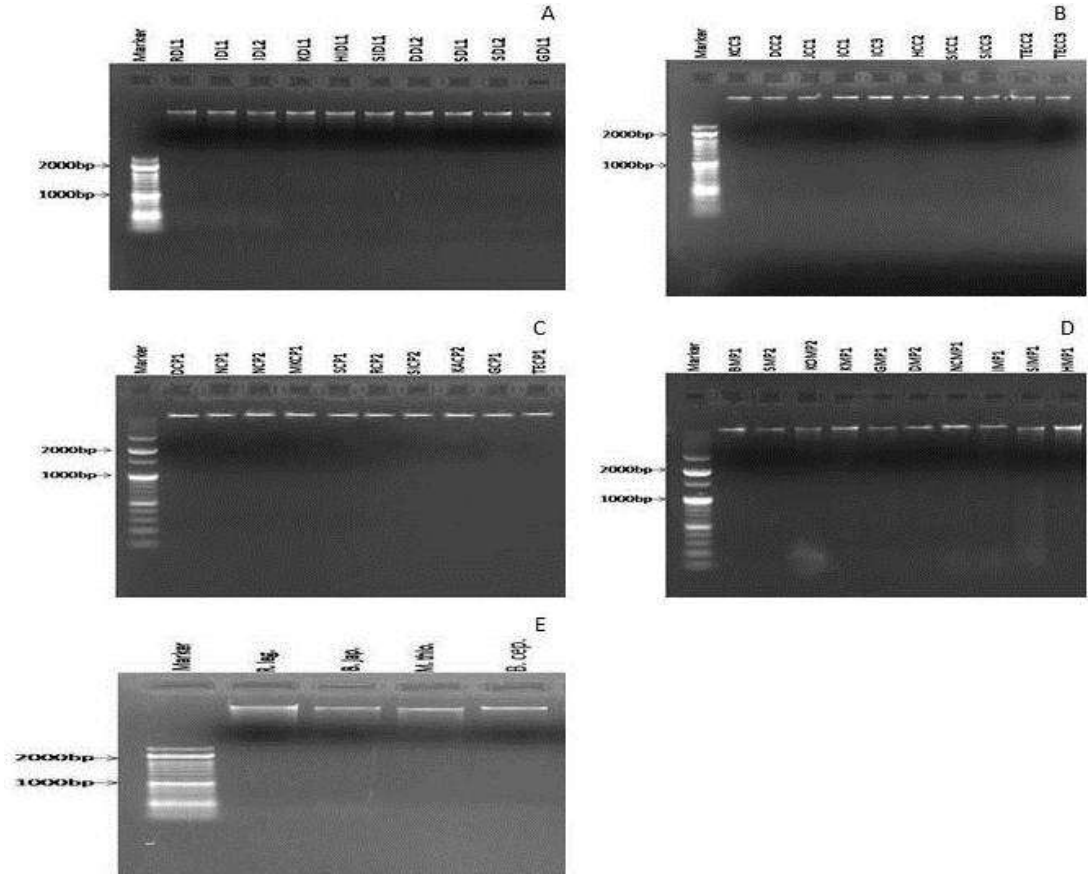

Figure 46: Extracted genomic DNA of bacterial isolates on 1% agarose gel. A) *L. purpureus*; B) *C. cajan*; C) *C. pallida*; D) *M. pudica*; E) Reference strains

4.7. *nifH* gene amplification

PCR amplification of *nifH* gene was carried out to determine the ability of isolates to produce nitrogenase enzyme. *nifH* gene amplification was carried out with the 40 selected isolates by using *nifH* gene specific primers. The amplified *nifH* gene was visualized on 1.5 % agarose gel after electrophoresis. The photographic image of the gel was captured using gel documentation system. Reference strain *R. leguminosarum* was used as a positive control in PCR amplification reaction. *nifH* gene positive isolates showed amplification of *nifH* gene at 360 bp as shown in the gel image below and it corresponds to the expected size of *nifH* gene (Figure 47). The study confirmed the presence of *nifH* gene in 7 isolates obtained from *L. purpureus*, 6 isolates obtained from *C. cajan*, 6 isolates obtained from *C. pallida* and10 isolates obtained from *M. pudica*.

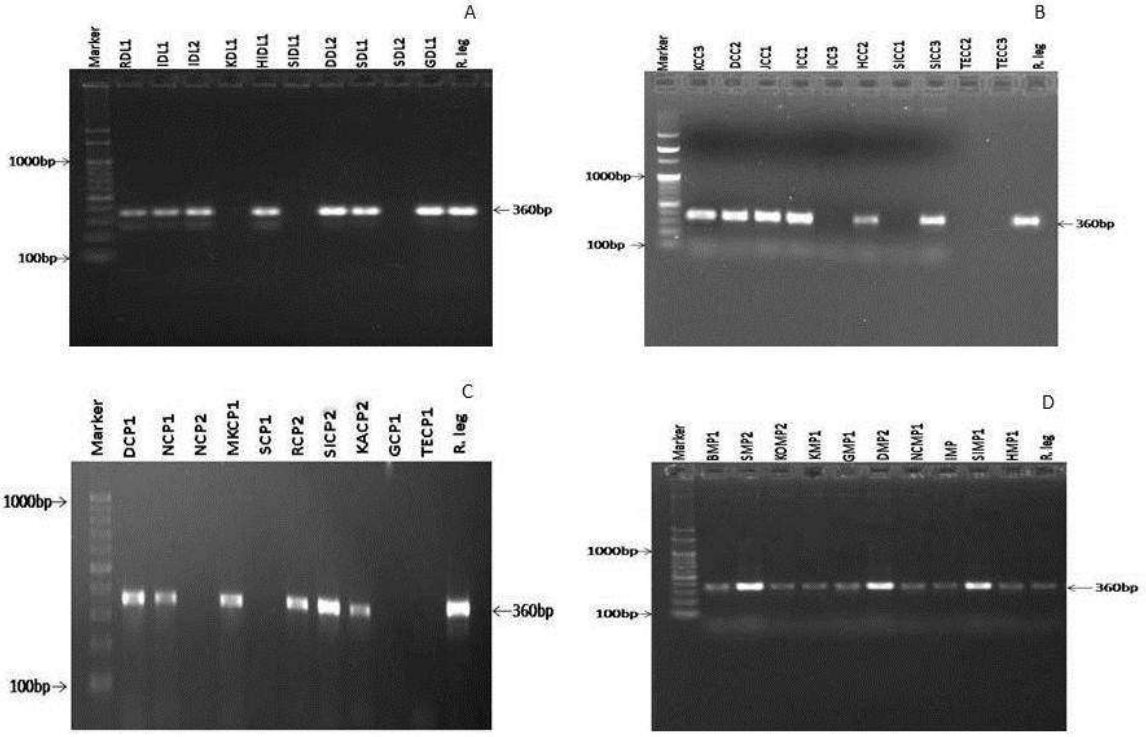

Figure 47: *nifH* gene amplification of bacterial isolates on 1.5 % agarose gel. A) *L. purpureus*; B) *C. cajan*; C) *C. pallida*; D) *M. pudica*

4.8. *nodC* gene amplification

PCR amplification of *nodC* gene was performed to screen the nodulation ability of isolates. *nodC* gene amplification was carried out with 40 selected isolates by using *nodC* gene specific primers. The amplified *nodC* gene was visualized on 1.5% agarose gel after electrophoresis. The photographic image of the gel was captured using gel documentation system. Reference strain, *R. leguminosarum* was used as a positive control in PCR amplification reaction. The results revealed the presence of *nodC* gene in 7 isolates obtained from *L. purpureous*, 6 isolates obtained from *C. cajan*, 4 isolates obtained from *C. pallida* and 10 isolates obtained from *M. pudica*. *nodC* gene positive isolates showed amplification of *nodC* gene at 500 bp as shown in the gel image below and it corresponds to the expected size of *nodC* gene (Figure 48).

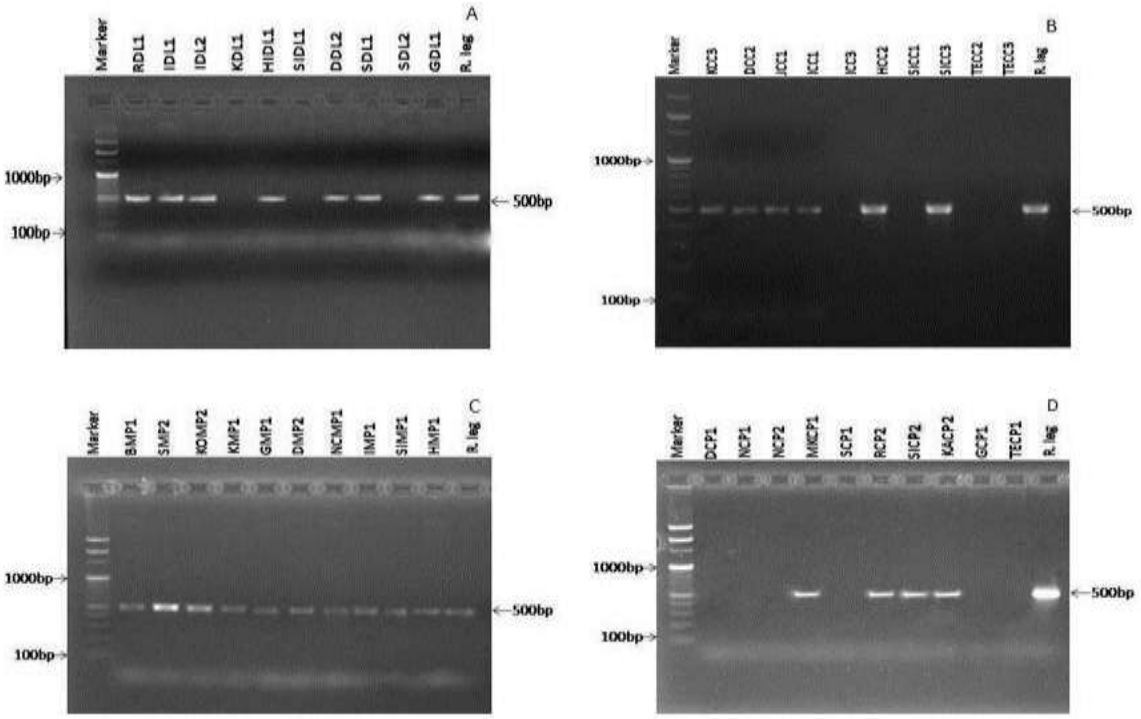

Figure 48: *nodC* gene amplification of bacterial isolates on 1.5 % agarose gel. A) *L. purpureus*; B) *C. cajan*; C) *C. pallida*; D) *M. pudica*

4.9. 16S rDNA gene amplification

For genotypic characterization of the bacterial isolates, 16S rDNA amplification was carried out by using universal primers 27F and 1492r. The genomic DNA extracted from the 40 rhizobial isolates and reference strains were used as a DNA template for 16S rDNA amplification. The amplified 16S rDNA was visualized on 1.5% agarose after electrophoresis. The photographic image of the gel was captured using gel documentation system.The results of PCR amplification generated single fragment of about 1, 500 bp as shown in gel image below (Figure 49) and it corresponds to the expected size of 16S rDNA.

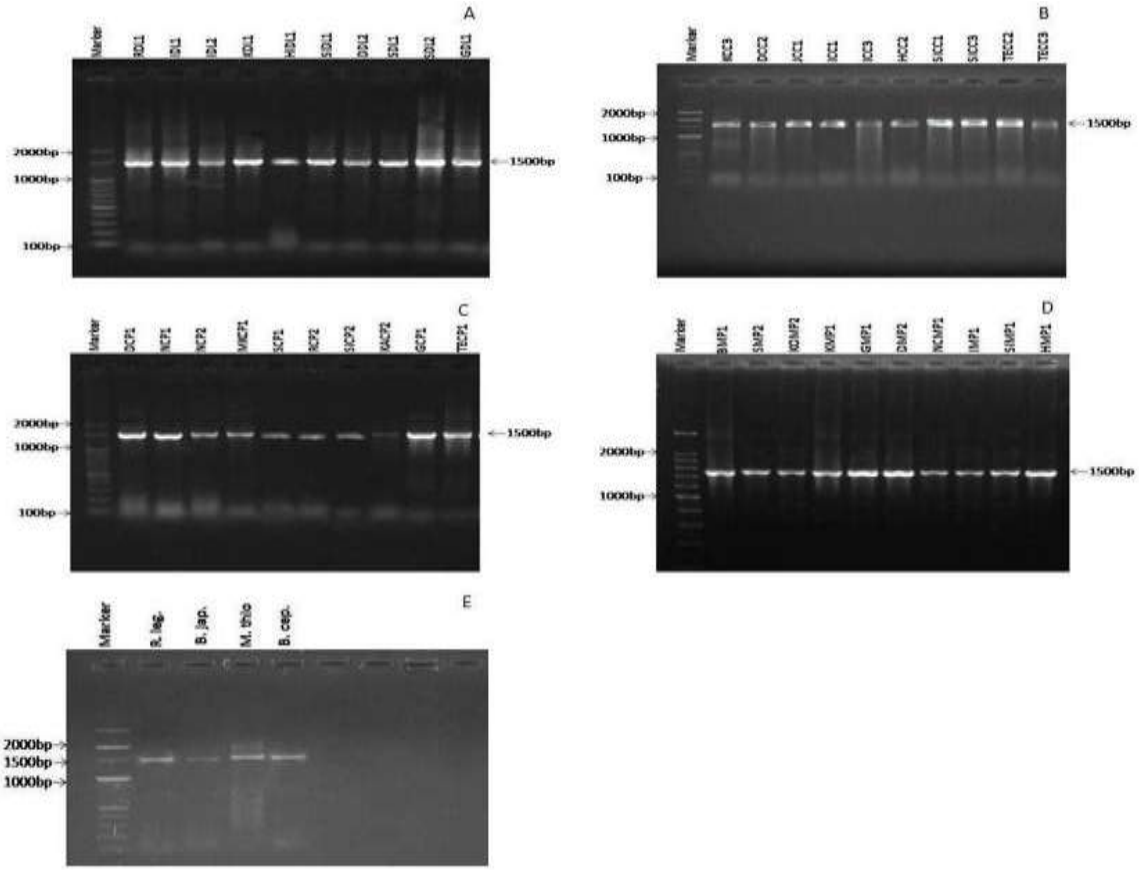

Figure 49: 16S rDNA gene amplification of bacterial isolates on 1.5 % agarose gel. A) *L. purpureus*; B) *C. cajan*; C) *C. pallida*; D) *M. pudica*; E) Reference strains

10. 16S rDNA PCR-RFLP analysis

4.10.1. 16S rDNA PCR-RFLP analysis of isolates obtained from *L. purpureus*:

Restriction digestion was carried out by *AluI* restriction enzyme. The restriction pattern revealed the presence of several DNA bands which ranges in size from 200 bp to 900 bp (Figure 50). Based on this restriction pattern an UPGMA tree was constructed by using NTSYS software. Dendrogram derived from the PCR-RFLP analysis grouped the isolates into 2 different clusters (Figure. 51). Cluster I comprised of 7 isolates (RDL1, IDL1, IDL2, HIDL1, SDL1, SDL2 and GDL1) and grouped with reference strains *R. leguminosarum* and *B. japonicum.* Cluster II comprised of 3 isolates (KDL1, SIDL1 and DDL2) and grouped with the reference strain *M. thiogangeticum*.

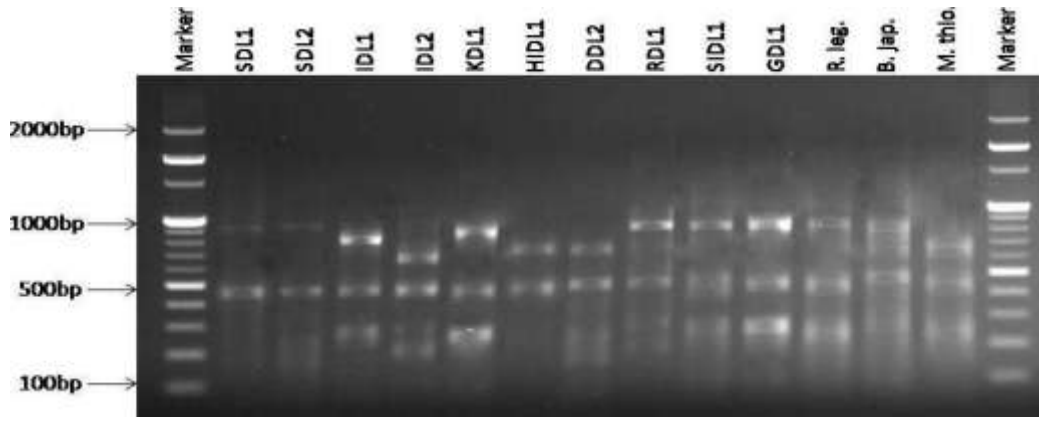

Figure 50: PCR-RFLP restriction profile on 2% agarose gel (*L. purpureus*)

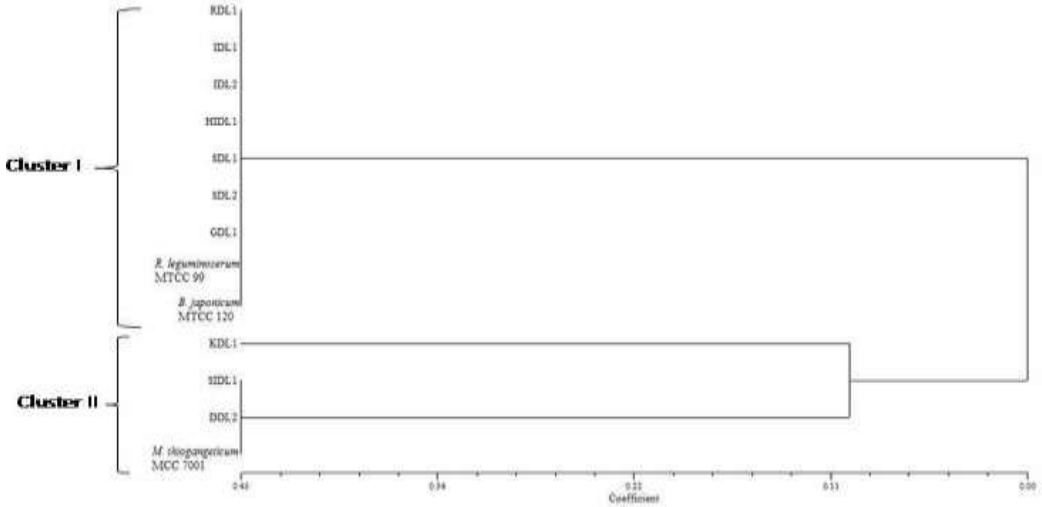

Figure 51: UPGMA dendrogram based on RFLP analysis of isolates (*L. purpureus*)

4.10.2. 16S rDNA PCR-RFLP analysis of isolates obtained from *C. cajan*:

Restriction digestion was carried out by *AluI* restriction enzyme. The restriction DNA bands range in size from 300 bp to 1000 bp (Figure 52). Dendrogram derived from the PCR-RFLP analysis grouped the isolates into 2 clusters (Figure 53). Cluster I comprised of 4 isolates (ICC1, TECC2, ICC3 and TECC3) and grouped with the reference strain *B. japonicum*. Cluster II comprised of 6 isolates (KCC3, JCC1, SICC1, DCC2, HCC2 and SICC3) and grouped with reference strains *R. leguminosarum* and *M. thiogangeticum*.

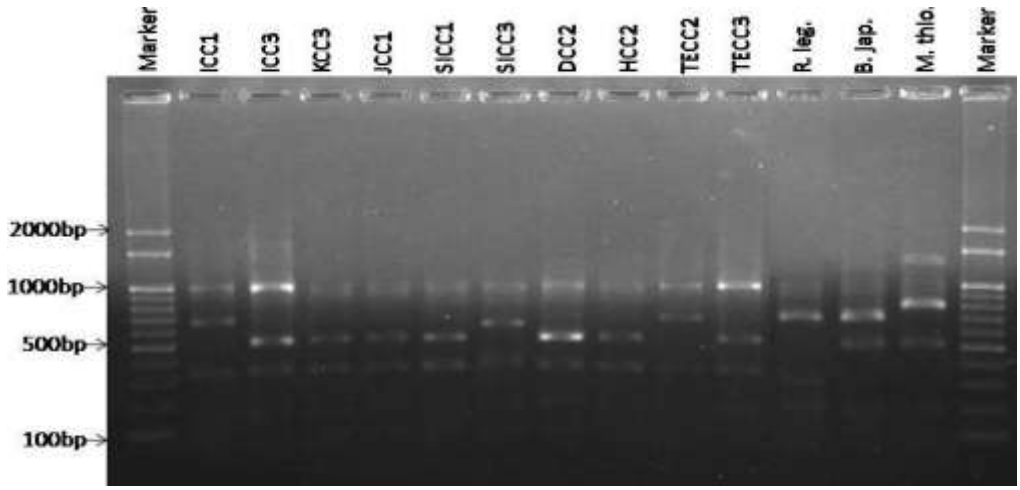

Figure 52: PCR-RFLP restriction profile on 2% agarose gel (*C. cajan*)

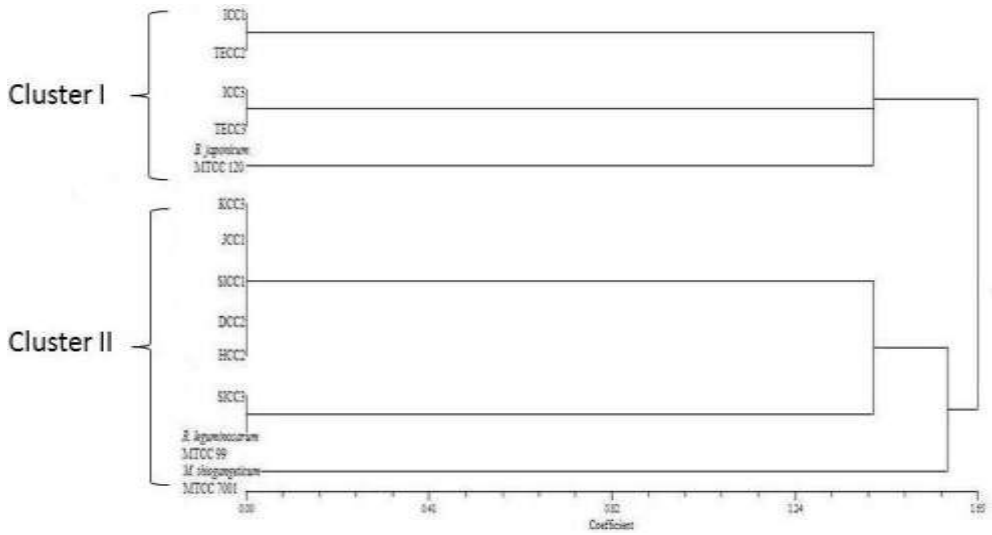

Figure 53: UPGMA dendrogram based on RFLP analysis of isolates (*C. cajan*)

4.10.3. 16S rDNA PCR-RFLP analysis of isolates obtained from *C. pallida*:

Restriction digestion was carried out with *HinfI* restriction enzyme. Digested DNA band ranges in size from 100 bp to 1000 bp (Figure 54).The patterns obtained from the PCR-RFLP analysis indicated a high genetic diversity among the isolates. UPGMA dendrogram analysis grouped the isolates into 3 different clusters (Figure 55). 2 isolates (MKCP1 and DCP1) clustered with *B. japonicum*, 6 isolates (RCP2, NCP1, NCP2, SICP1, TECP1 and SCP1) clustered with *R. leguminosarum* and 2 isolates (KACP2 and GCP1) clustered with *M. thiogangeticum*.

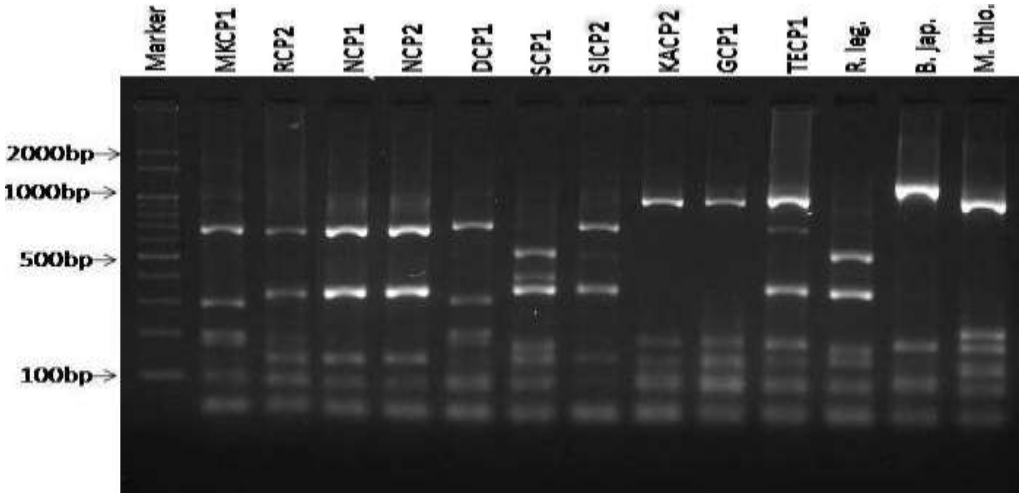

Figure 54: PCR-RFLP restriction profile on 2% agarose gel (*C. pallida*)

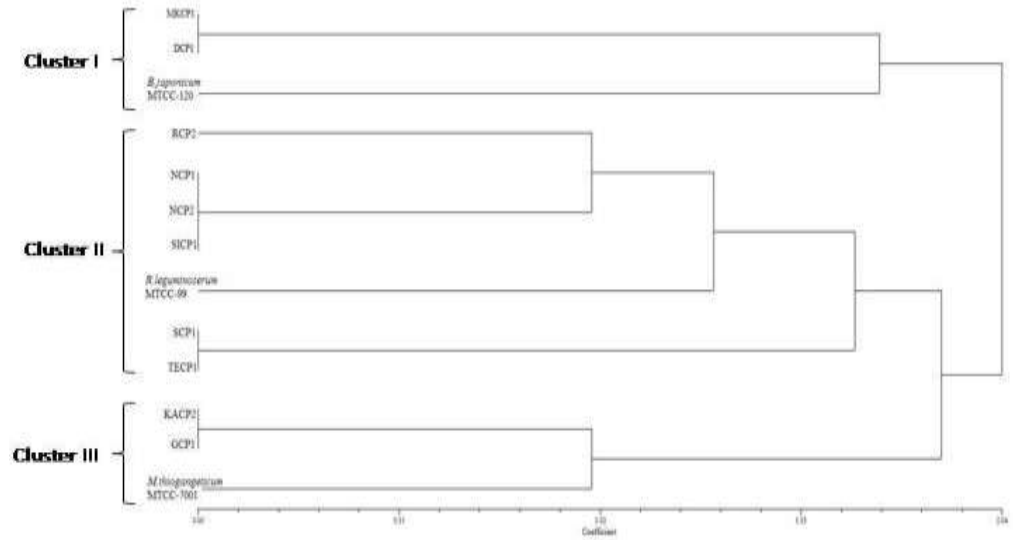

Figure 55: UPGMA dendrogram based on RFLP analysis of isolates (*C. pallida*)

4.10.4. 16S rDNA PCR-RFLP analysis of isolates obtained from *M. pudica*:

Restriction digestion was carried out by *AluI* restriction enzyme.The restriction pattern revealed the presence of several DNA bands which ranges in size from 200 bp to 700 bp (Figure 56). The UPGMA dendrogram analysis revealed that the isolates can be grouped into 3 different clusters (Figure 57). Cluster I comprised of 4 isolates (BMP1, NCMP1, SMP2 and HMP1) and reference strain *B. cepacia*. Cluster II comprised of 5 isolates (KOMP2, GMP1, IMP1, SIMP1 and DMP2). Cluster III comprised of 1 isolates (KMP1) and 3 reference strains *M. thiogangeticum*, *R. leguminosarum* and *B. japonicum*.

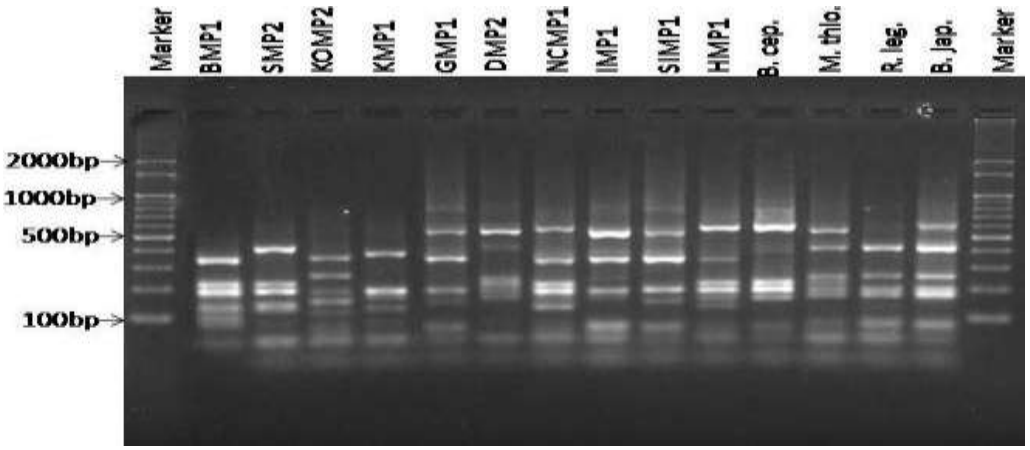

Figure 56: PCR-RFLP profile on 2% agarose gel (*M. pudica*)

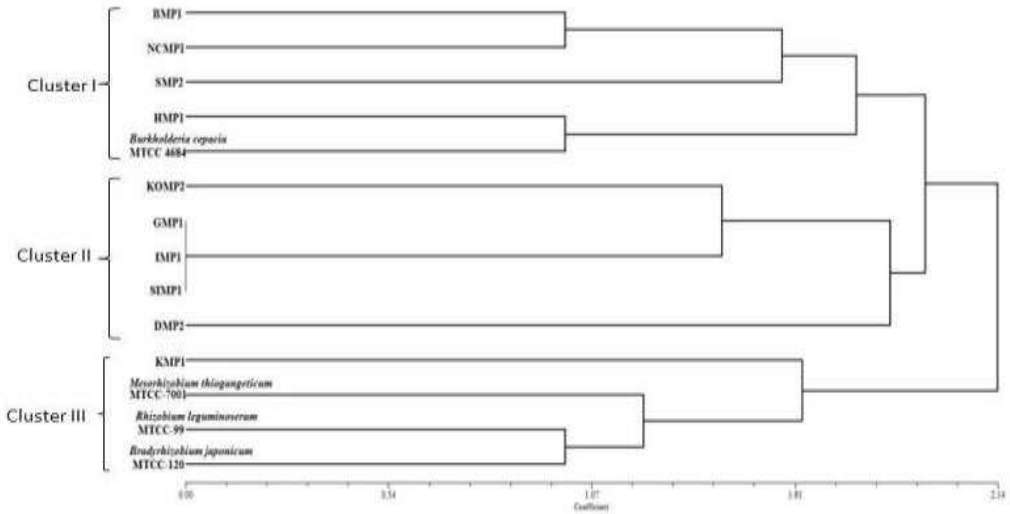

Figure 57: UPGMA dendrogram based on RFLP analysis of isolates (*M. pudica*)

4.11. 16S rDNA sequencing analysis

To elucidate the phylogenetic identity of the bacterial isolates sequencing of 16S rDNA gene was carried out. Based on the results of 16S rDNA PCR-RFLP analysis results, 4 isolate from each plant representing different PCR-RFLP cluster was selected for sequencing.16S rDNA gene sequence was checked and edited by BioEdit software. The NCBI-BLAST program was used to search for similar 16S rDNA sequences in the NCBI-GenBank databases.

4.11.1. 16S rDNA sequencing analysis of isolates obtained from *L. purpueus*:

For determining the phylogenetic identity of the isolates obtained from *L. purpureus* 16S rDNA gene sequencing of 4 isolate (HIDL1, SDL1, SIDL1, DDL2) which represents the 2 PCR-RFLP clusters were carried out. In NCBI-BLAST analysis the isolates HIDL1, SDL1, SIDL1 and DDL2 showed closest similarity with *Bradyrhizobium elkanii*, *Bradyrhizobium lablabi*, *Bradyrhizobium pachyrhizi* and *Bradyrhizobium elkanii* respectively (Table 29). Alignment of 16S rDNA sequences of the isolates with other most similar sequences available in GenBank (NCBI) generated a phylogenetic tree (Figure 58). The phylogenetic tree revealed the formation of 3 separate clusters. Cluster I comprised of 4 reference strains obtained from GenBank. Cluster II comprised of all the 4 sequenced isolates and 3 reference strains (*B. retamae*, *B. pachyrhizi* and *B. lablabi*). Reference strain *B. elkanii* form a separate cluster III. The phylogenetic tree was in agreement with the BLAST analysis results. 16S RDNA sequences of the 4 isolates were deposited in the GenBank databasethrough Sequin submission format. The GenBank accession number of the 4 isolates is provided in table 29.

Table 29: Isolates, GenBank accession number and their closest match in NCBI

Isolates	GenBank accession no.	PCR-RFLP cluster type	Closest match in NCBI	Similarity
HIDL1	**KT_900891**	Type I	*Bradyrhizobiumelkanii*NBRC 14791 (NR_112927)	99%
SDL1	**KT_900892**	Type I	*Bradyrhizobiumlablabi* CCBAU 23086(NR_117513)	99%
SIDL1	**KT_900893**	Type II	*Bradyrhizobiumpachyrhizi* PAC48 (NR_043037)	98%
DDL2	**KT_900890**	Type II	*Bradyrhizobiumelkanii* NBRC 14791 (NR_112927)	99%

Table 30: Reference 16S rDNA sequences retrieved from NCBI for phylogenetic study

Reference strain from NCBI	**GenBank accession no.**	**Source**
Bradyrhizobiumelkanii NBRC 14791	NR_112927	NCBI
Bradyrhizobiumlablabi CCBAU 23086	NR_117513	NCBI
Bradyrhizobiumpachyrhizi PAC48	NR_043037	NCBI
Bradyrhizobiumelkanii NBRC 14791	NR_112927	NCBI
Bradyrhizobiumliaonigese 2281	NR_041785	NCBI
Bradyrhizobiumlupine USDA3051	NR_134836	NCBI
Bradyrhizobiumjaponicum USDA6	NR_112552	NCBI
Bradyrhizobiumdenitrificans LMG8443	NR_118770	NCBI

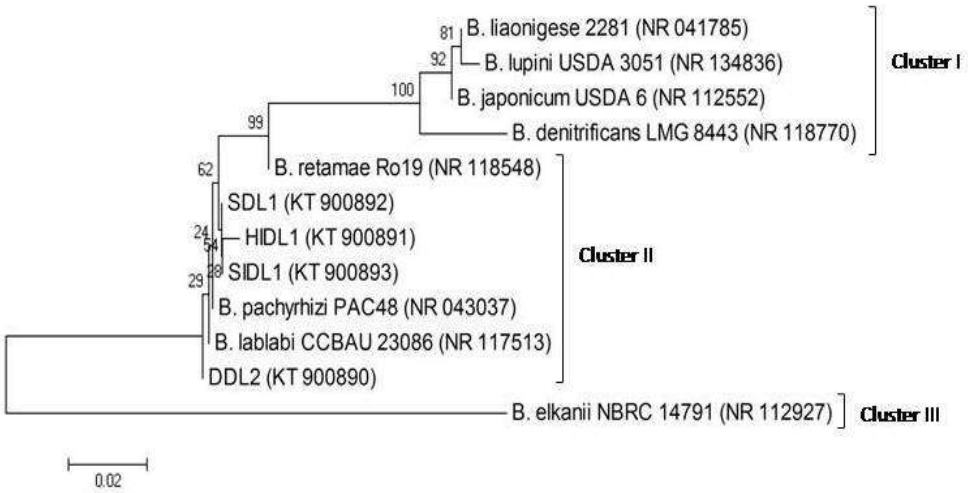

Figure 58: Phylogenetic (neighbor-joining) tree depicting the relationship ofisolates obtained from *L. purpureus* and closest reference strains retrieved from NCBI. The species nameis followed by their GenBank accession numbers. The numbers shown next to each bifurcation are bootstrap percent values based on 1000 replications

4.11.2. 16S rDNA sequencing analysis of isolates obtained from *C. cajan*:

Similarly, 4 isolates obtained from *C. cajan* (ICC1, ICC3, DCC2, SICC1) representing the 2 clusters of PCR-RFLP analysis were selected for 16S rDNA gene sequencing. In BLAST analysis the isolate DCC2 showed closest similarity with *Rhizobium mayense* strain CCGE526. Likewise, BLAST result of ICC1 showed its closest similarity with *Mesorhizobium loti* strain MAFF 303099 which is a common legume symbiont. ICC3 and SICC1 showed close similarity with *Burkholderia cenocepacia* strain AU 1054 and *Burkholderia anthina* strain W92B respectively, both of which belongs to a group called *Burkholderia cepacia* complex. The phylogenetic tree revealed the formation of 2 separate clusters (Figure 59). All the reference strains grouped together and form cluster I. In contrast to blast analysis result the 4 isolates form a separate cluster II. The acquired 16S RDNA sequences of the 4 isolates were deposited in the GenBank database through Sequin submission format. The GenBank accession number of the isolates is provided in table 31.

Table 31: Isolates,GenBank accession number and their closest match in NCBI

Isolates	GenBank accession no.	PCR-RFLP cluster type	Closest Match in NCBI	Similarity
ICC1	**KR_611429**	Type I	***Mesorhizobium loti* strain MAFF 303099(NR_074162)**	99%
ICC3	**KR_107938**	Type I	***Burkholderia cenocepacia* strainAU 1054(NR_074686)**	99%
DCC2	**KR_107936**	Type II	***Rhizobium mayense* strain CCGE526(NR_109703)**	98%
SICC1	**KR_107937**	Type II	***Burkholderia anthina* strain W92B (NR_104975)**	99%

Table 32: Reference 16S rDNA sequences retrieved from NCBI for phylogenetic study

Reference strain from NCBI	GenBank accession no.	Source
Mesorhizobium loti strain MAFF 303099	NR_074162	NCBI
Burkholderia cenocepacia strainAU 1054	NR_074686	NCBI
Rhizobium mayense strain CCGE526	NR_109703	NCBI
Burkholderia anthina strain W92B	NR_104975	NCBI
Rhizobium jaguris CCGE525	NR_109700	NCBI
Rhizobium grahamii CCGE502	NR_118140	NCBI
Mesorhizobium australicum WSM2073	NR_102452	NCBI
Mesorhizobium ciceri NBRC100389	NR_113894	NCBI
Burkholderia metallica R-16017	NR_042636	NCBI
Burkholderia lata	NR_102890	NCBI

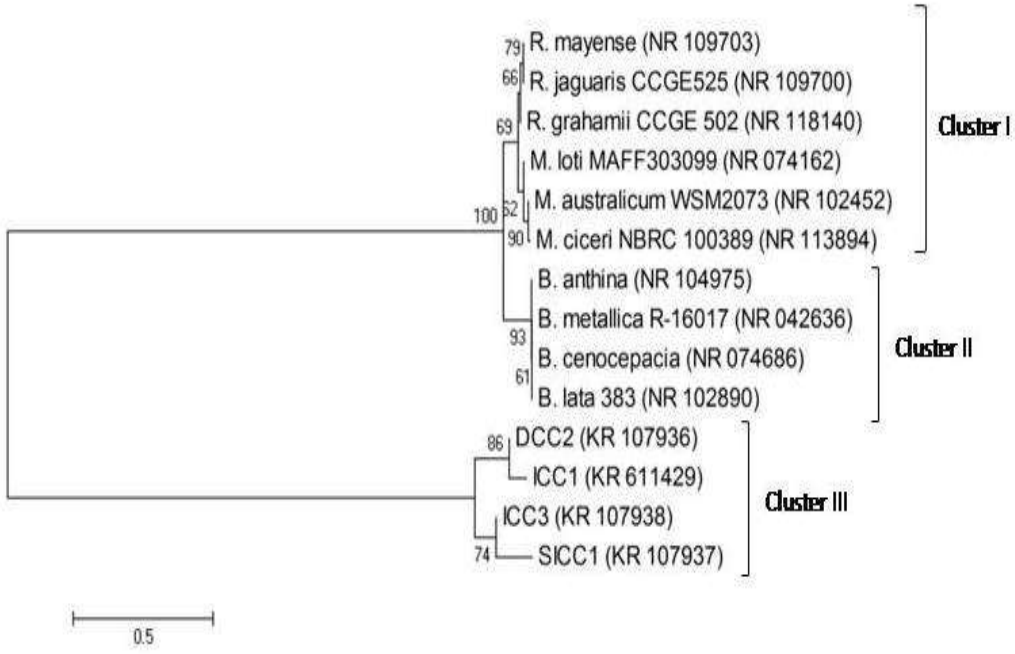

Figure 59: Phylogenetic (neighbor-joining) tree depicting the relationship ofisolates obtained from *C. cajan* and closest reference strains from NCBI. The species nameis followed by their GenBank accession numbers. The numbers shown next to each bifurcation are bootstrap percent values based on 1000 replications

4.11.3. 16S rDNA sequencing analysis of isolates obtained from *C. pallida*:

Likewise, 4 isolates obtained from *C. pallida* (NCP1, RCP2, KACP2, DCP1) representing the 3 clusters of PCR-RFLP analysis were selected for 16S rDNA gene sequencing. NCBI-BLAST analysis of the sequences revealed that DCP1 showed closest similarity with *Bacillus megaterium* NBRC 15308, RCP2 was closely related to *Mesorhizobium jarvisii*, NCP1 was closely related to *Bacillus koreensis* BR030 and KACP2 was closely related to *Mesorhizobium jarvisii*.. The GenBank accession number of the isolates is provided in Table 33. In the phylogenetic tree 2 isolates DCP1 and NCP1 that showed close similarity with *Bacillus megaterium* and *Bacillus koreensis* strains forms a cluster with *Bacillus* strains. Isolate KACP2 which showed close similarity with *M. jarvisii* forms a cluster with *Mesorhizobium* strains. Interestingly isolate RCP2 which also showed close similarity with *M. jarvisii* forms a separate cluster (Figure 60). The acquired 16S rDNA sequences of the 4 isolates were deposited in the GenBank database through Sequin submission format.

Table 33: Isolates,GenBank accession number and their closest match in NCBI

Isolate	PCR-RFLP cluster type	GenBank accession no.	Closest Match in NCBI	Similarity
DCP1	Type I	KX_774273	***Bacillus megaterium* NBRC 15308 (NR_112636)**	98%
RCP2	Type I	KX_964648	***Mesorhizobium jarvisii* (NR_135858)**	97%
NCP1	Type II	KX_774274	***Bacillus koreensis* BR030 (NR_116851)**	98%
KACP2	Type III	KX_774271	***Mesorhizobiumjarvisii* (NR_135858)**	97%

Table 34: Reference 16S rDNA sequences retrieved from NCBI for phylogenetic study

Reference strain from NCBI	GenBank accession no.	Source
Bacillus megaterium NBRC 15308	NR_112636	NCBI
Mesorhizobium jarvisii	NR_135858	NCBI
Bacillus koreensis BR030	NR_116851	NCBI
Bacillus firmus NBRC15306	NR_112635	NCBI
Mesorhizobium huakuii NBRC15243	NR_113738	NCBI
Mesorhizobium erdmanii	NR_135857	NCBI

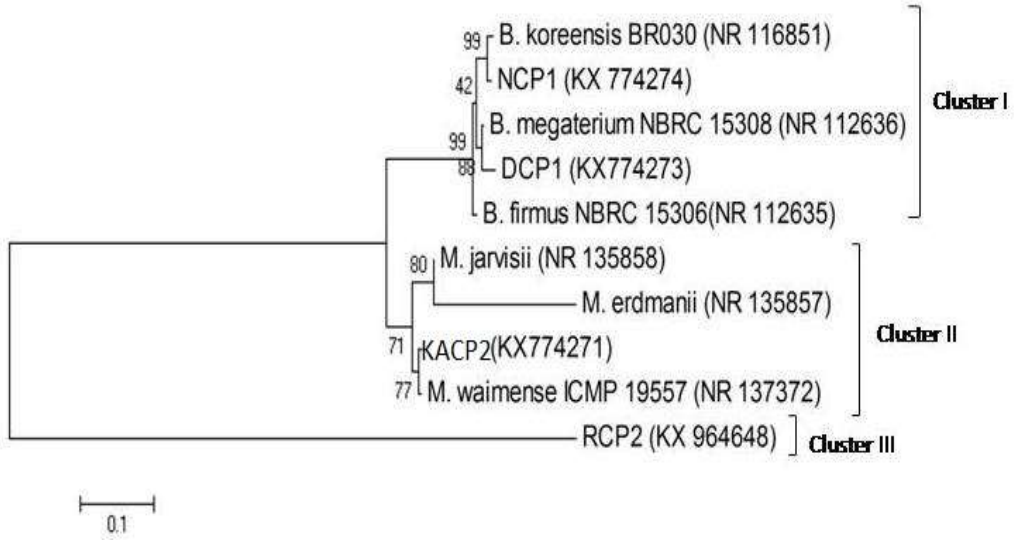

Figure 60: Phylogenetic (neighbor-joining) tree depicting the relationship ofisolates obtained from *C. pallida* and closest reference strains retrieved from NCBI. The species nameis followed by their GenBank accession numbers. The numbers shown next to each bifurcation are bootstrap percent values based on 1000 replications.

4.11.4. 16S rDNA sequencing analysis of isolates obtained from *M. pudica*:

4 isolates obtained from *M. pudica* (SMP2, HMP1, DMP2 and KMP1) representing 3 clusters of PCR-RFLP analysis were selected for 16S rDNA gene sequencing. NCBI-BLAST analysis of the sequences revealed that SMP2, HMP1 and DMP2 were closely related to *Burkholderia mimosarum* PAS44. The isolate KMP1 showed closest similarity with *Mesorhizobium loti* MAFF303099. The phylogenetic tree constructed by aligning the 16s rDNA sequences of isolates with the reference

sequence was in full agreement with blast analysis results (Figure 61). In the phylogenetic tree the isolates were distributed in 2 separate clusters. Cluster I consisted of 3 isolates (SMP2, HMP1 and DMP2) and grouped with the reference strains of *Burkholderia* genus. The isolate KMP1 form a separate cluster and grouped with the reference strains of *Mesorhizobium* genus. The acquired 16S rDNA sequences of the 4 isolates were deposited in the GenBank database through Sequin submission format. The GenBank accession number of the isolates is provided in table 35.

Table 35: Isolates, GenBank accession number and their closest match in NCBI

Isolate	PCR-RFLP cluster type	GenBank accession no.	Closest Match in NCBI	Similarity
SMP2	Type I	KJ_939616	***Burkholderia mimosarum* PAS44 (NR_752958)**	99%
HMP1	Type I	KJ_939618	***Burkholderia mimosarum* PAS44 (NR_752958)**	99%
DMP2	Type II	KJ_939619	***Burkholderia mimosarum* PAS44 (NR_752958)**	99%
KMP1	Type III	KJ_939617	***Mesorhizobium loti* MAFF303099 (NR_074162)**	99%

Table 36: Reference 16S rDNA sequences retrieved from NCBI for phylogenetic study

Reference strain from NCBI	GenBank accession no.	Source
Burkholderia mimosarum PAS44	NR_752958	NCBI
Mesorhizobium loti MAFF303099	NR_074162	NCBI
Burkholderia nodosa Br3437	AY_773189	NCBI
Burkholderia tropica Ppe8	AJ_420332	NCBI
Burkholderia denitrificans KIS3044	AY_773184	NCBI
Burkholderia sabie Br3047	AY_773186	NCBI
Burkholderia seminalis R2419	NR_042635	NCBI
Bradyrhizobium japonicum USDA6	AB_231927	NCBI
Rhizobium leguminosarum bv viciae USDA2370	JQ_085246	NCBI
Mesorhizobium thiogangeticum YJ-RT5	KF876855	NCBI
Mesorhizobium ciceri NBRC100389	NR_113894	NCBI

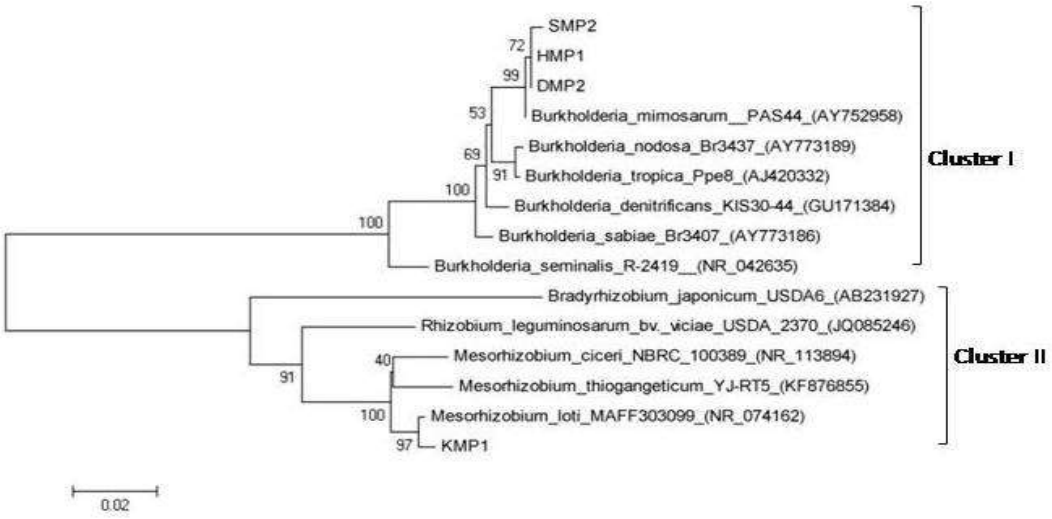

Figure 61: Phylogenetic (neighbor-joining) tree depicting the relationship ofrhizobia isolates obtained from *M. pudica* and closest reference strains retrieved from NCBI. The species nameis followed by their GenBank accession numbers. The numbers shown next to each bifurcation are bootstrap percent values based on 1000 replications.

4.12. **Nodulation study and assessment of plant growth**

4.12.1. Nodulation study of isolates obtained from *L. purpureus*:

Seeds of *L. purpureus* plants were treated with their respective isolates to investigate their ability of nodulation and effect on vegetative parameter. The results clearly revealed that all 4 isolates successfully induced root nodules on their host plant (Figure 62). Inoculation of HIDL1 significantly increased the shoot dry weight and inoculation of DDL2 significantly increased root length of *L. purpureus* when compared with un-inoculated control (Table 37). However, isolates SDL1 and SIDL1 have no significant effect on the vegetative parameters of the host plant. Reference strain *R. leguminosarum* MTCC-99 significantly increases shoot dry weight and root dry weight of host plant (Figure 63).

Figure 62: Nodulation test on *L. purpureus*. A) SDL1 (Pot); B) HIDL1 (Pot); C) SIDL1 (Pot); D) Control; E) HIDL1; F) SDL1; G) SIDL1; H) DDL2; I) *R. leguminosarum*

Table 37: Effect of inoculation of isolates on vegetative parameters of *L. purpureus*

Isolate	Shoot length (cm)	Root length (cm)	Shoot Dry Weight (mg)	Root Dry Weight (mg)
Control	16.22 ^{b}± 1.786	4.89 ^{b}± 0.772	59.00 ^{b}± 7.150	29.56 ^{b}± 4.474
HIDL1	17.22 ^{b}± 1.698	4.89 ^{b}± 0.351	**85.65 ^{a}± 11.433**	35.12 ^{b}± 5.586
SDL1	16.33 ^{b}± 1.826	4.67 ^{b}± 0.726	69.32 ^{b}± 7.128	35.71 ^{b}± 4.446
SIDL1	18.67 ^{b}± 2.494	5.33 ^{b}± 0.527	79.18 ^{b}± 7.854	28.55 ^{b}± 2.146
DDL2	15.67 ^{b}± 0.943	**7.00 ^{a}± 0.764**	71.36 ^{b}± 7.316	31.60 ^{b}± 4.128
R. leuminosarum	18.56 ^{b}± 0.948	5.11 ^{b}± 0.512	**85.51 ^{a}± 4.406**	**40.25^{a}± 1.055**
F	0.549	1.855	1.774	1.237

#Values are the means of nine replications and means followed by different letters are significantly different according to Least Significance Difference (LSD) test at P=0.05.

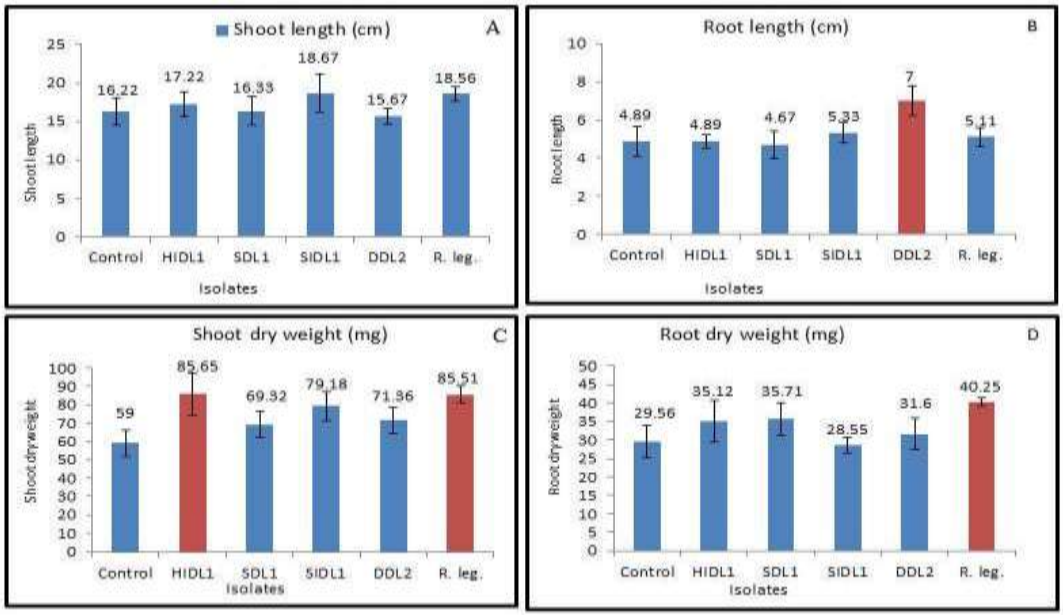

Figure 63: Effect of inoculation of isolateson vegetative parameters of *L. purpureus*. A) Shoot length; B) Root length; C) Shoot dry weight; D) Root dry weight

4.12.2. Nodulation study of bacterial isolates obtained from *C. cajan*:

Seeds of *C. cajan* plants were treated with their respective isolates to investigate their ability of nodulation and effect on vegetative parameter (Table 38). ICC3 and SICC1 were excluded from the study as they were known human pathogens. The results revealed that the isolates ICC1, DCC2 and the reference strain *R. leguminosarum* MTCC-99 successfully induced root nodules on their host plant (Figure 64). Inoculation of DCC2 to the host plant significantly increased shoot length and shoot dry weight compared to the un-inoculated control. However, there is no significant change in root height and root dry weight. No significant change was observed in vegetative parameters of *C. cajan* when treated with ICC1. Reference strain *R. leguminosarum* MTCC-99 significantly increases shoot length, root length and shoot dry weight compared to the un-inoculated control (Figure 65).

Figure 64: Nodulation test on *C. cajan*. A) DCC2; B) Control; C) Control; D) ICC1; E) DCC2; F) *R. leguminosarum*

Table 38: Effect of isolates on vegetative parameters of *C. cajan*

Isolate	Shoot length (cm)	Root length (cm)	Shoot Dry Weight (mg)	Root Dry Weight (mg)
Control	15.56[b]± 1.215	3.44 [b]± 0.377	167.11 [b]± 22.576	36.00 [b]± 6.984
DCC2	**19.00[a]± 1.312**	3.56 [b]± 0.242	**235.67 [a] ± 18.788**	44.11 [b]± 4.208
ICC1	16.78[b]± 1.256	3.33 [b]± 0.333	192.00 [b]± 22.061	31.22 [b]± 2.253
R. leguminosarum	**21.12[a]± 0.904**	**4.44 [a]± 0.338**	**241.22 [a]± 13.796**	44.89 [b]± 4.234
F	**4.315***	2.425	**3.285***	1.941

#Values are the means of nine replications and means followed by different letters are significantly different according to Least Significance Difference (LSD) test at P=0.05.

* Significant at 5% ANOVA.

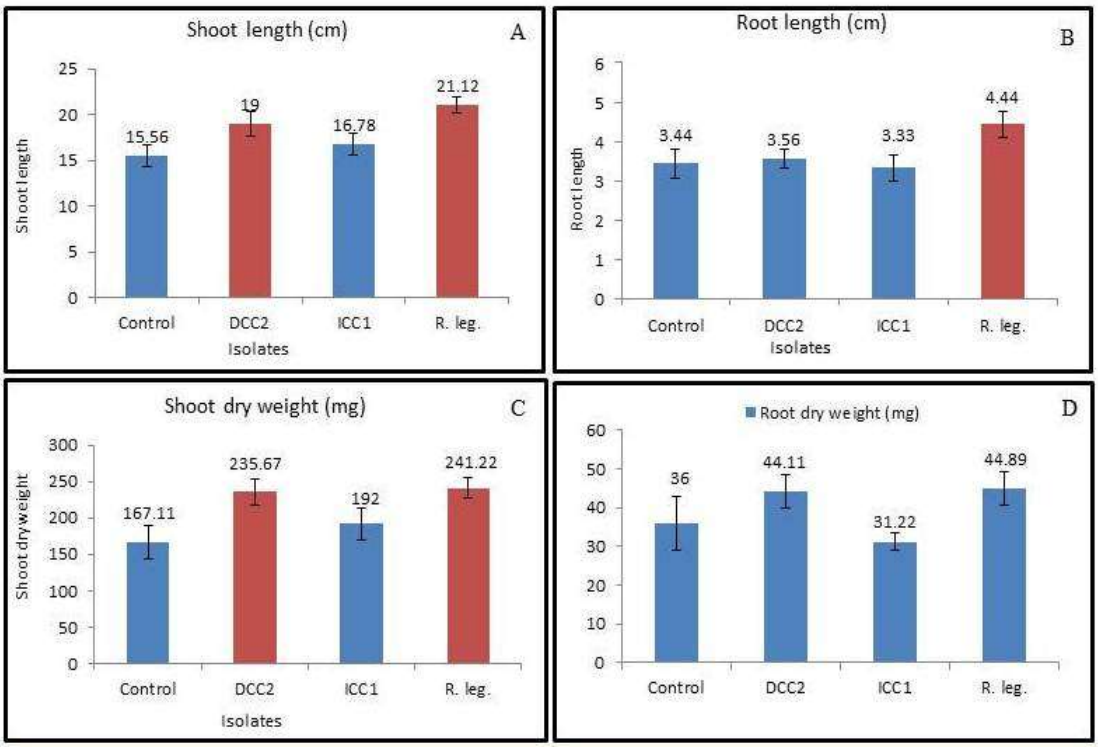

Figure 65: Effect of inoculation of isolates on vegetative parameters of *C. cajan*. A) Shoot length; B) Root length; C) Shoot dry weight; D) Root dry weight

4.12.1. Nodulation study of bacterial isolates obtained from *C. pallida*:

2 isolates (RCP2 and KACP2) of *C. pallida* which were genetically identified as *M. jarvisii* successfully induce root nodules on their host plant (Figure 66). However the other 2 isolates (NCP1 and KACP2) which were identified as *Bacillus megaterium* and *Bacillus koreensis* were unable to induce nodule formation on their host plants. Inoculation of NCP1 significantly increased the shoot dry weight of *C. pallida* plant compared to the un-inoculated control (Table 39). The remaining 3 isolates (RCP2, DCP1 and KACP2) and reference strain *R. leguminosarum* MTCC-99 have no significant effect on the vegetative parameters of the host plant when compared with un-inoculated controls (Figure 67).

Figure 66: Nodulation test on *C. pallida*. A) Control (Pot); B) DCP1 (Pot); C) NCP1 (Pot); D) Control; E) RCP2; F) NCP1; G) DCP1; H) KACP2; I) *R. leguminosarum*

Table 39: Effect of isolates on vegetative parameters of *C. pallida*

Isolate	**Shoot length (cm)**	**Root length (cm)**	**Shoot Dry Weight (mg)**	**Root Dry Weight (mg)**
Control	$4.66^{b} \pm 0.235$	$2.22^{b} \pm 0.222$	$8.44^{b} \pm 0.796$	$2.80^{b} \pm 0.268$
RCP2	$5.11^{b} \pm 0.447$	$2.72^{b} \pm 0.147$	$9.57^{b} \pm 0.856$	$2.97^{b} \pm 0.310$
NCP1	$5.05^{b} \pm 0.679$	$2.88^{b} \pm 0.217$	**$10.26^{a} \pm 0.646$**	$2.82^{b} \pm 0.332$
DCP1	$4.44^{b} \pm 0.444$	$2.33^{b} \pm 0.166$	$6.00^{b} \pm 0.685$	$2.72^{b} \pm 0.310$
KACP2	$4.16^{b} \pm 0.263$	$2.44^{b} \pm 0.242$	$7.37^{b} \pm 0.729$	$2.80^{b} \pm 0.300$
R. leguminosarum	$4.38^{b} \pm 0.217$	$3.22^{b} \pm 0.391$	$7.64^{b} \pm 0.683$	$3.04^{b} \pm 0.480$
F	0.839	2.395	**4.443***	0.130

#Values are the means of nine replications and means followed by different letters are significantly different according to Least Significance Difference (LSD) test at P=0.05.

* Significant at 5% ANOVA.

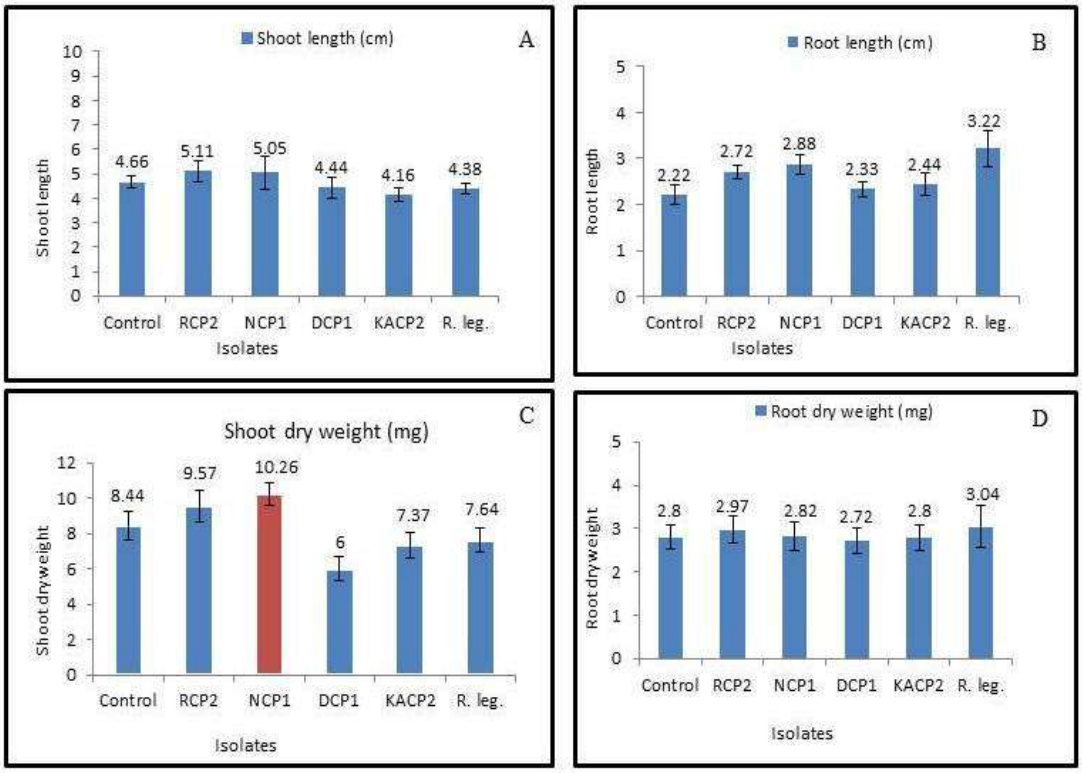

Figure 67: Effect of inoculation of isolateson vegetative parameters of *C. pallida*. A) Shoot length; B) Root length; C) Shoot dry weight; D) Root dry weight

4.12.2. Nodulation study of bacterial isolates obtained from *M. pudica*:

Similarly, seeds of *M. pudica* plants were treated with their respective isolates to investigate their ability of nodulation and effect on vegetative parameter (Figure 68). The 4 isolates of *M. pudica* induce root nodules on their host plant. Inoculation of KMP1 to the host plant significantly increased shoot dry weight compared to the un-inoculated control (Table 40). However the other 3 isolates (SMP2, DMP2 and HMP1) has no significant effect on the vegetative parameters of the host plant when compared with un-inoculated controls. Reference strain *R. leguminosarum* MTCC-99 also significantly increases shoot dry weight of the host plant (Figure 69).

Figure 68: Nodulation test on *M. pudica*. A) Control (Pot); B) DMP2 (Pot); C) *R. leguminosarum* (Pot); D) Control; E) KMP1; F) HMP1; G) DMP2; H) SMP2; I) *R. leguminosarum*

Table 40: Effect of isolates on growth of *M. pudica*

Isolate	Shoot length (cm)	Root length (cm)	Shoot Dry Weight (mg)	Root Dry Weight (mg)
Control	$3.33^{b} \pm 0.250$	$2.11^{b} \pm 0.182$	$2.41^{b} \pm 0.265$	$1.53^{b} \pm 0.124$
KMP1	$2.91^{b} \pm 0.234$	$1.88^{b} \pm 0.161$	**$4.83^{a} \pm 0.513$**	$1.10^{b} \pm 0.148$
HMP1	$3.66^{b} \pm 0.204$	$2.12^{b} \pm 0.283$	$3.22^{b} \pm 0.134$	$1.40^{b} \pm 0.109$
DMP2	$3.44^{b} \pm 0.337$	$1.85^{b} \pm 0.223$	$3.10^{b} \pm 0.560$	$1.41^{b} \pm 0.153$
SMP2	$3.66^{b} \pm 0.322$	$1.92^{b} \pm 0.277$	$2.88^{b} \pm 0.262$	$1.46^{b} \pm 0.216$
R. leguminosarum	$3.88^{b} \pm 0.232$	$2.03^{b} \pm 0.324$	**$4.65^{a} \pm 0.972$**	$1.25^{b} \pm 0.094$
F	1.621	0.216	**3.502***	1.159

#Values are the means of nine replications and means followed by different letters are significantly different according to Least Significance Difference (LSD) test at P=0.05.

* Significant at 5% ANOVA.

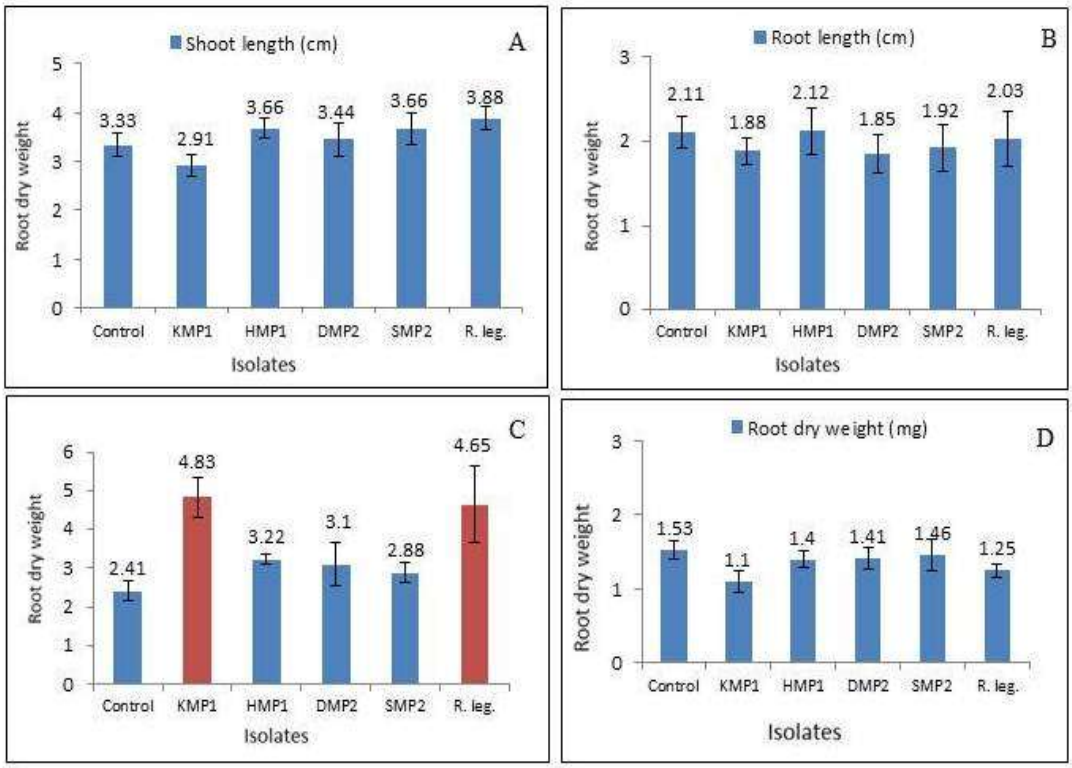

Figure 69: Effect of inoculation of isolateson vegetative parameters of *M. pudica*. A) Shoot length; B) Root length; C) Shoot dry weight; D) Root dry weight