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Evaluation of Physiological Properties of Winged Bean *Rhizobium* and Its Comparison with Other Rhizobia

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A B S T R A C T

A study was carried out in the Department of Soil Science, DRPCAU, Pusa, Bihar during 2014-15 to evaluate physiological properties of *Rhizobium* isolated from winged bean, pea, gram, lentil and moong. Production of indole acetic acid (IAA), resistance to antibiotics and tolerance to variable pH are some important key concerns for its adaptability to various soil conditions as inoculants. Rhizobia, in general, followed a similar trend towards pH tolerance in terms of growth (measured as OD) *i.e.*, their growth was higher around neutral pH. The per cent increase or decrease in original pH of the broth due to growth of rhizobia was maximum at extremity (at pH 4.0 or 9.0). At pH 7.0 all rhizobia grew profusely. Growth of rhizobia was either positively or negatively influenced by the change in pH. However, moong showed better adaptability in between pH 5.0 to 7.0. IAA production by winged bean *Rhizobium* was highest among the tested rhizobia. Production of IAA increased with the age of the culture. Moong *Rhizobium* produced lowest amount of IAA. Both winged bean and lentil rhizobia were resistant to Vancomycin (30µg) while pea and moong rhizobia were resistant to both Ampicillin (10µg) and Nalidixic acid (30µg) as measured using bio-dices. Gram *Rhizobium* was observed to be resistant to both Chloramphenicol (30µg) and Clarithromycin (15µg). Varying degree of susceptibility to antibiotics was observed for all five rhizobia and all of them were highly susceptible to Tetracycle (30µg).

Keywords

Rhizobium, Antibiotic, IAA, pH, Winged bean

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Introduction

Rhizobium species are soil bacteria, which display symbiotic interaction with specific legume hosts and most of these are sensitive to fluctuations in the quality and quantity of chemicals in the rhizosphere and hence affect the growth and productivity of whole plant (Miller and Wood, 1996). Most crops are sensitive to relatively high levels of salinity and acidity. In the case of legumes, there are additional problems because it is not only the plants but also the symbiotic *Rhizobium* bacteria, which are sensitive or act differentially both at the free-living stage as well as during the symbiotic relationship (Lloret *et al.*, 1995). *Rhizobium* species appear to be varying in their symbiotic efficiency under acidic or alkaline conditions (Ali *et al.*, 2009). Extreme pH affects nodulation by reducing infection by rhizobia. Highly acidic soils and highly alkaline soils (pH<4.0, pH

>8.0) affect survival, the growth of both partners, and thus reduce nitrogen fixation (Bordeleau and Prévost, 1994). The effect of varying pH levels on the growth of *Rhizobium* sp. in pure culture and *Rhizobium* inoculated plants have been recorded by some workers (Elizabeth *et al.*, 2000). Rhizobia are known to produce significant levels of IAA both in free living conditions and also symbiotically in nodules (Ernstsen *et al.*, 1987). Indole acetic acid (IAA) is one of the most physiologically active auxins. The majority of rhizobial strain (74%) associated with different crop could produce IAA (Halda-Aliza, 2003). Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake (Datta and Basu, 2000). The *Rhizobium* is also known to possess resistance or susceptibility towards known antibiotic substances (Prasuna, 2014). Resistance of nodule-forming bacteria (rhizobia) refers to their intrinsic resistance to antibiotics in terms of normal growth. Hartman and Amarger (1991) and Tas *et al.*, (1996) emphasized the intrinsic resistance of different rhizobial strains to antibiotics as a significant phenotypic characteristic. A broad variation regarding susceptibility of individual rhizobial strains to antibiotics was found with respect to different rhizobial species.

Materials and Methods

An experiment was conducted in the Department of Soil Science, Dr. Rajendra Prasad Central Agricultural University, Pusa, Bihar during 2014-15 to evaluate physiological properties of *Rhizobium* isolated from winged bean, pea, gram, lentil and moong.

Growth at different pH

Tolerance to change in pH was evaluated in terms of rhizobial growth. An equal aliquote (40ml) of pre-sterilized YEM broth was taken in six different 100ml conical flasks. pH of the broth was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0 with the help of 0.5M NaOH or HCl wherever required. An equal volume of liquid culture (1.2ml @3%) of *Rhizobium* was added to each flask and was incubated at 25-30^{o}C. Separate sets were employed for all five different isolated rhizobia (winged bean, pea, gram, lentil and moong). Growth was measured in terms of optical density at 620nm after 24, 48 and 72hrs. After 72hrs., content of each flask was centrifuged at 10,000rpm for 10min. Supernatant was used to measure change in pH of the medium due to rhizobial growth(Mensah *et al.*, 2006).

IAA production by *Rhizobium*

Production of indole acetic acid (IAA) was estimated following the method suggested by Ehmann, 1977. A loop full of *Rhizobium* culture was inoculated in 25ml of Luria Berteni (LB) broth (tryptone, NaCl and yeast extract @10, 10 and 5gl^{-1}) amended with 50µgml^{-1} tryptophan. Inoculated medium was incubated at 30±2^{o}C in rotatory shaker for 24, 48 and 72hrs. Thereafter the content of conical flask was centrifuged at 10,000rpm for 15min. Two ml of supernatant was taken in culture tubes, 2-3 drops of O-phosphoric acid and 4ml of Salkowski reagent containing 1ml of 0.5M $FeCl_3$ in 50ml of 35% $HClO_4$ was added; left for 30min. in dark at room temperature and absorbance was recorded at 530nm. Auxin was quantified by preparing a calibration curve (10-100µg ml^{-1}) using IAA as standard.

Antibiotic resistance test

The susceptibility or resistance of rhizobia to an antibiotic was assayed with the help of antibiotic disc, a method adopted by Cole and Elksan (1979). After plating and solidification of the YEM agar medium on Petri plates, 0.1ml of bacterial suspension culture was

spreaded allover YEM agar surface. Antibiotic impregnated bio-discs were placed on the surface of solidified medium. The antibiotic discs (HiMedia) used were of amoxyclav (AMC) 30µg, ampicillin (AMP) 10µg, azithromycin (AZM) 15µg, chloramphenicol (C) 30µg, clarithromycin (CLR) 15µg, erythromycin (E) 15µg, gentamycin (GEN) 10µg, kanamycin (K) 30µg, nalidixic acid (NA) 30µg, streptomycin (S) 10µg, tetracycline (TE) 30µg, vancomycin (VA) 30µg. The Petri plates were then incubated at 30±2^{o}C for 48hrs. to observe the growth of rhizobia. Resistance to an antibiotic was detected by the formation of inhibition zone around the antibiotic discs used which was measured with the help of a scale.

Results and Discussion

Effect of pH on growth of *rhizobial* strain isolated from leguminous crops *viz*., winged bean, pea, gram, lentil and moong, IAA production ability and antibiotic susceptibility of these *rhizobial* strain were presented and discussed in the present study.

pH tolerance

Growth of winged bean *Rhizobium* along with pea, gram, lentil and moong rhizobia at different pH ranging from 4.0 to 9.0 recorded at 24, 48 and 72hrs in term of optical density are presented in (Table 1 and Fig. 1-3). The study showed all tested rhizobia a wide diversity in tolerance to their growth at different pH. However rhizobia in general, followed a similar trend towards pH tolerance in term of growth measured as optical density (OD). Their growth was superior around neutral pH. Least growth was recorded at pH 4.0 and an increase in growth was observed up to neutral pH *i.e.*, pH 7.0 but thereafter decreased at pH as high as pH 9.0. This might be related to less saline nature of the soil from which the isolates were recovered. Such observations were also made by many workers at different institutions time to time. In a study of rhizobial strains isolated from root nodules of chickpea carried out by Maatallah *et.al.*, (2002) showed 90 to 100 per cent of isolates to grow in lightly acid and neutral pH as also observed in present study. In an another study carried at North Ethiopia by Keneni *et al.*, (2010) revealed that native rhizobial strains were able to survive well in the various soil adjusted to pH 4.0 up to 7.0, while the exotic strains were unable to survive pH up to 5.5 of the soil. Findings of work done by Jida and Assefa (2011) is in support to our study where they found that all thirty isolated lentil nodulating rhizobial isolates were ability to grow mildly in YEMA medium set at pH value 5.5 to neutral and slightly alkaline pH (8.0). Also at low pH, 26.7 per cent of the isolates exhibited an acid tolerant character whereas only 13.3 per cent of isolates grew at pH 8.5 and none of the isolates grew at pH 9.0. Bacterial strains (*Rhizobium*) isolated from root nodules of gram also showed similar growth response with respect to different pH adjusted growth medium in a study carried by Gauri *et al.*, (2012). Also, growth was increased with increase in incubation period *viz.*, from 24 to 72 hrs irrespective of pH. In case of winged bean *Rhizobium* growth in terms of OD was found least (0.759) at pH 4.0 while the highest OD (1.069) was recorded at pH 7.0 after 24hrs of incubation whereas at 72hrs, OD was 1.168 at pH 4.0 while it was 1.452 at pH 7.0. Isolates grew at lowest pH 4.0indicating high level of tolerance to acid conditions. Alkaline pH has also a little antagonistic effect on growth of isolates as they grew at pH 9.0. The result of the effects of different pH values on the growth of *Rhizobium* species in culture shows that the *Rhizobium* strains grew well at pH values of between 6.0-8.0 with optimal growth density at pH 7.0 in accordance with Mensah *et al*., 2006, who recorded the optimum pH of growth of *Rhizobium* is between 6.0 and 7.0.

Table.1 Effect of varying growth medium pH on growth of rhizobia

Rhizobia / pH	24 hrs.					48 hrs.					72 hrs.				
	WB	P	G	L	M	WB	P	G	L	M	WB	P	G	L	M
4.0	0.76	0.66	0.50	0.65	0.39	1.06	1.08	0.95	0.83	0.78	1.17	1.20	0.93	1.04	1.01
5.0	0.88	0.79	0.52	0.70	0.67	1.20	1.12	0.98	1.04	1.13	1.21	1.37	0.95	1.11	1.29
6.0	0.93	0.99	0.56	0.70	0.80	1.24	1.32	1.16	1.06	1.29	1.26	1.50	1.46	1.14	1.33
7.0	1.07	1.11	0.68	0.75	0.92	1.32	1.49	1.46	1.20	1.40	1.45	1.52	1.60	1.39	1.56
8.0	1.01	1.10	0.61	0.74	0.82	1.29	1.42	1.21	1.20	1.36	1.30	1.50	1.59	1.32	1.43
9.0	0.98	1.09	0.58	0.71	0.81	1.26	1.21	1.00	1.13	1.34	1.27	1.30	1.01	1.23	1.42

WB: Winged bean; P: Pea; G: Gram; L: Lentil; M: Moong

Table.2 Effect of different rhizobial growth on growth medium pH

Rhizobia / pH	Change in pH					% increase or decrease in pH				
	WB	P	G	L	M	WB	P	G	L	M
4.0	4.94	4.89	5.17	4.84	4.18	24	22	29	21	5
5.0	5.91	5.58	5.99	5.95	5.02	18	12	20	19	0.4
6.0	6.23	6.17	6.45	6.48	5.58	4	3	8	8	-7
7.0	6.97	6.95	6.90	6.99	6.36	- 0.4	-0.7	-1.4	-0.1	-9
8.0	7.05	6.68	6.93	7.04	5.08	-12	-17	-13	-12	-36
9.0	7.28	7.22	7.26	7.27	4.67	-19	-20	-19	-19	-48

WB: Winged bean; P: Pea; G: Gram; L: Lentil; M: Moong

Table.3 IAA (µg ml^{-1}) production during growth of five different rhizobia

Rhizobia	24 hrs.	48 hrs.	72 hrs.
Winged bean	53.2	58.5	64.6
Pea	35.0	44.4	53.5
Gram	32.4	45.5	57.0
Lentil	44.3	47.0	50.2
Moong	28.5	35.2	43.6

Table.4 Effect of antibiotics on susceptibility or resistance of different rhizobia

Antibiotic	Zone of inhibition (mm)				
	Winged bean	Pea	Gram	Lentil	Moong
Amoxyclav (AMC) – 30µg	20	12	25	17	20
Ampicillin (AMP) – 10µg	16	- (r)	22	16	- (r)
Azithromycin (AZM) – 15µg	16	34	14	16	28
Chloramphenicol (C) – 30µg	14	20	- (r)	16	14
Clarithromycin (CLR) – 15µg	16	25	- (r)	13	26
Erythromycin (E) – 15µg	15	25	16	18	30
Gentamycin (GEN) – 10µg	13	18	28	14	17
Kanamycin (K) – 30µg	12	18	18	14	14
Nalidixic Acid (NA) – 30µg	22	- (r)	14	18	- (r)
Streptomycin (S) – 10µg	12	16	16	14	18
Tetracycline (TE) – 30µg	20	30	32	22	36
Vancomycin (VA) – 30µg	- (r)	16	17	- (r)	18

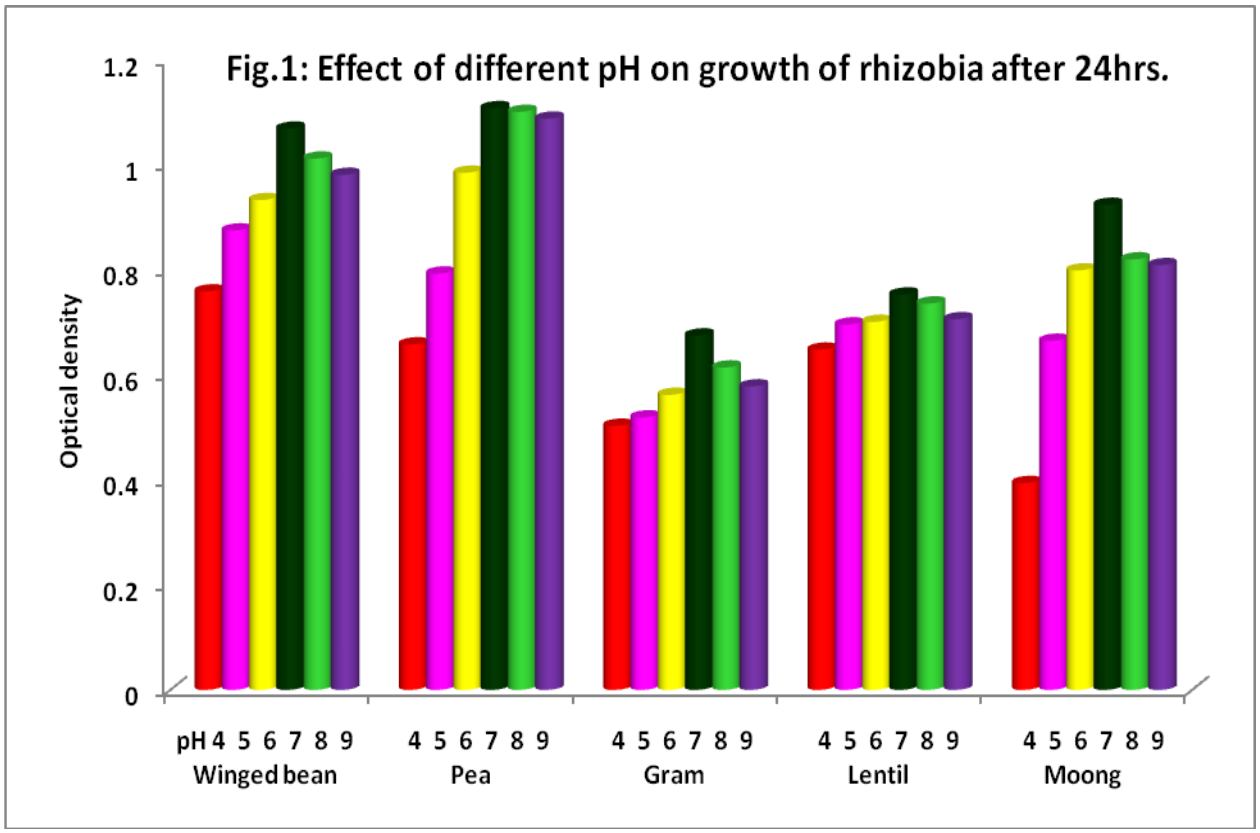


Fig.1: Effect of different pH on growth of rhizobia after 24hrs.
Optical density
1.2
1
0.8
0.6
0.4
0.2
0
pH 4 5 6 7 8 9
Winged bean
4 5 6 7 8 9
Pea
4 5 6 7 8 9
Gram
4 5 6 7 8 9
Lentil
4 5 6 7 8 9
Moong

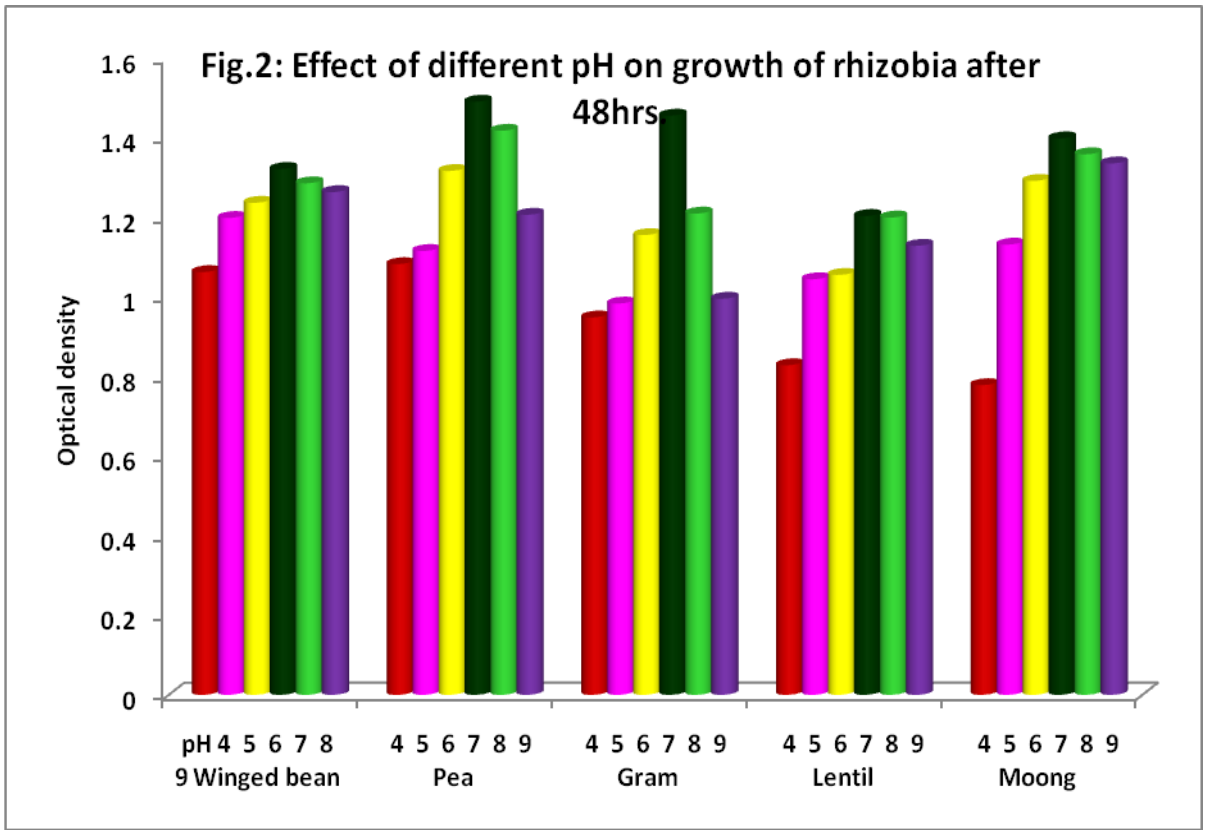


Fig.2: Effect of different pH on growth of rhizobia after 48hrs
Optical density
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pH 4 5 6 7 8 9 Winged bean
4 5 6 7 8 9
Pea
4 5 6 7 8 9
Gram
4 5 6 7 8 9
Lentil
4 5 6 7 8 9
Moong

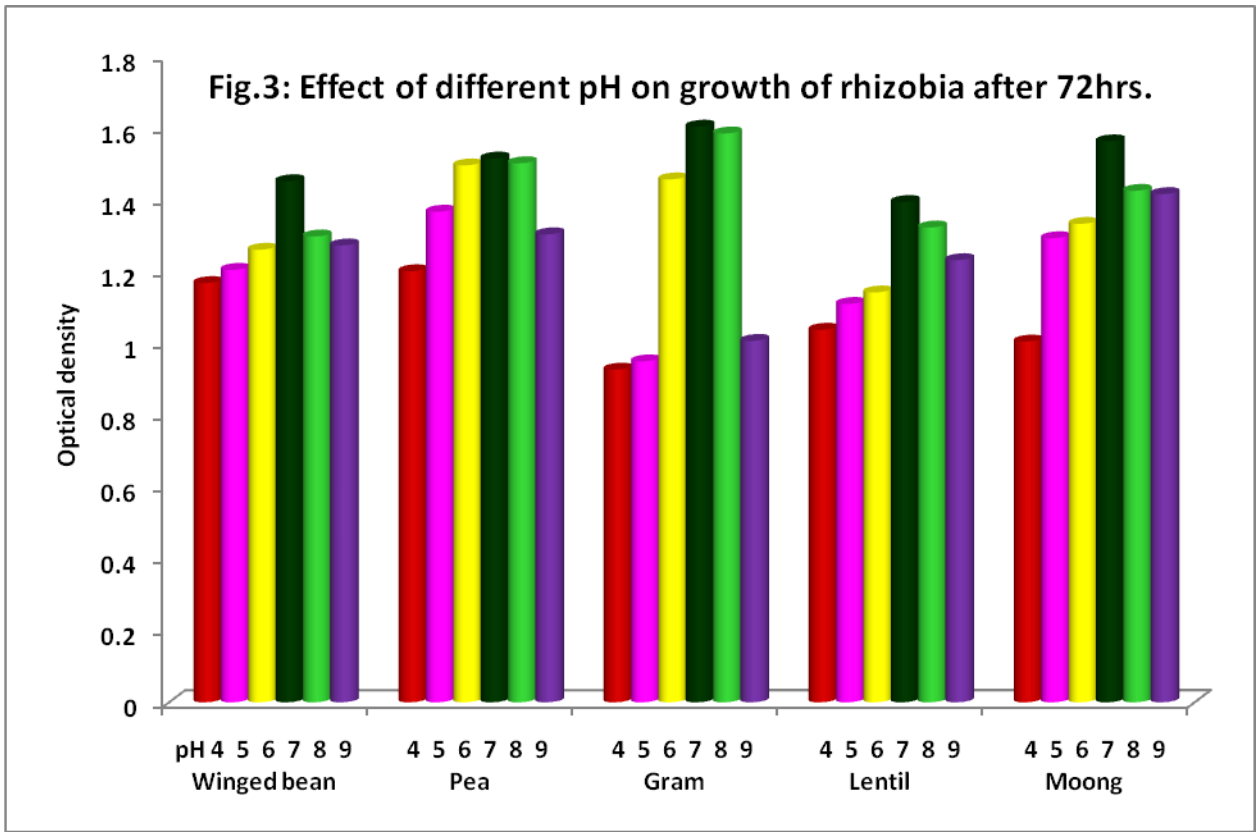


Fig.3: Effect of different pH on growth of rhizobia after 72hrs.
Optical density
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pH 4 5 6 7 8 9
Winged bean
4 5 6 7 8 9
Pea
4 5 6 7 8 9
Gram
4 5 6 7 8 9
Lentil
4 5 6 7 8 9
Moong

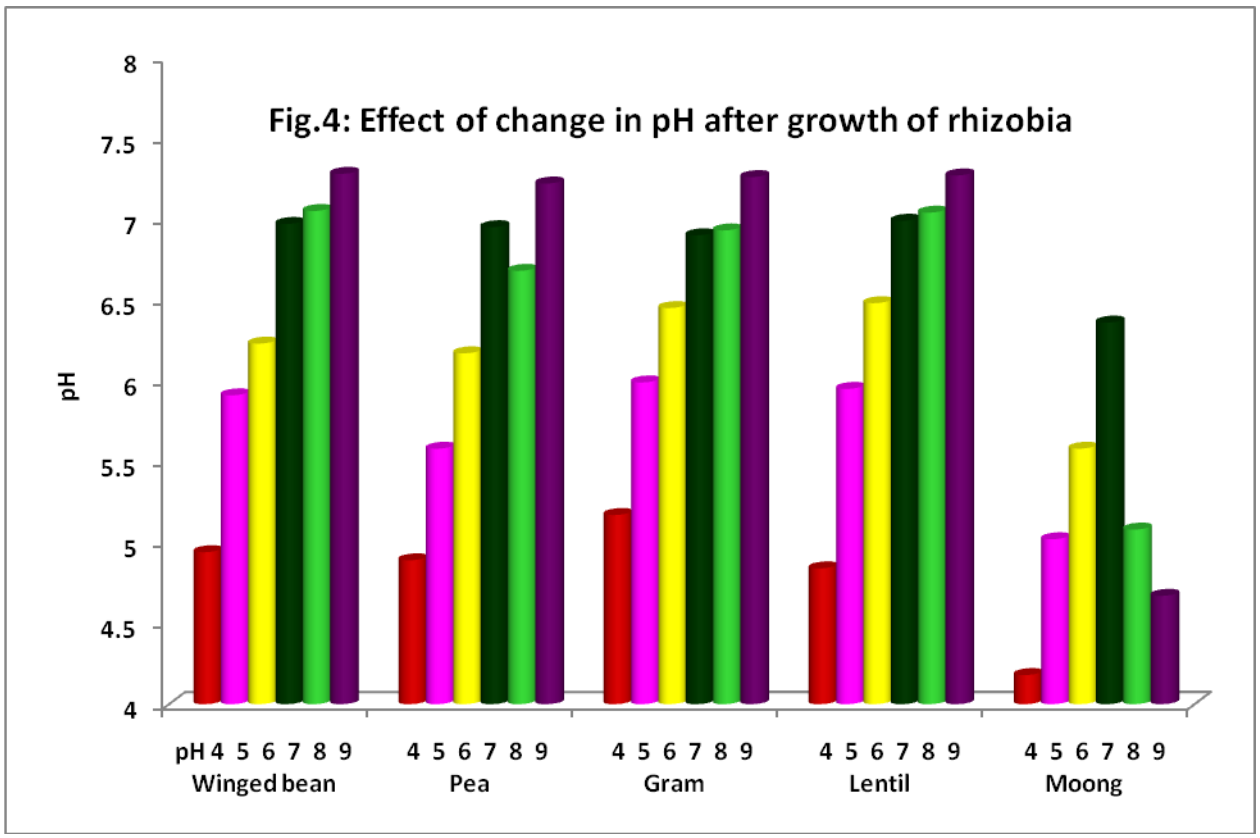
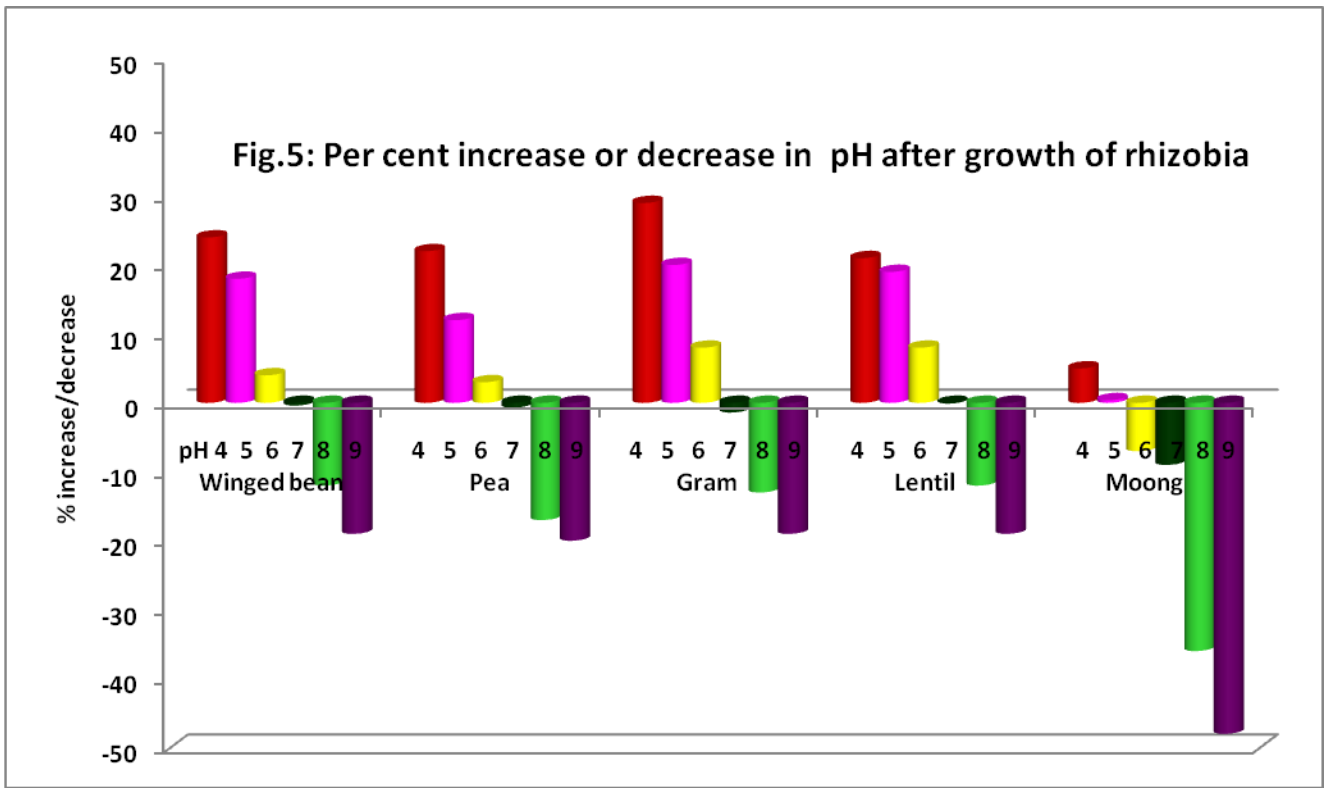
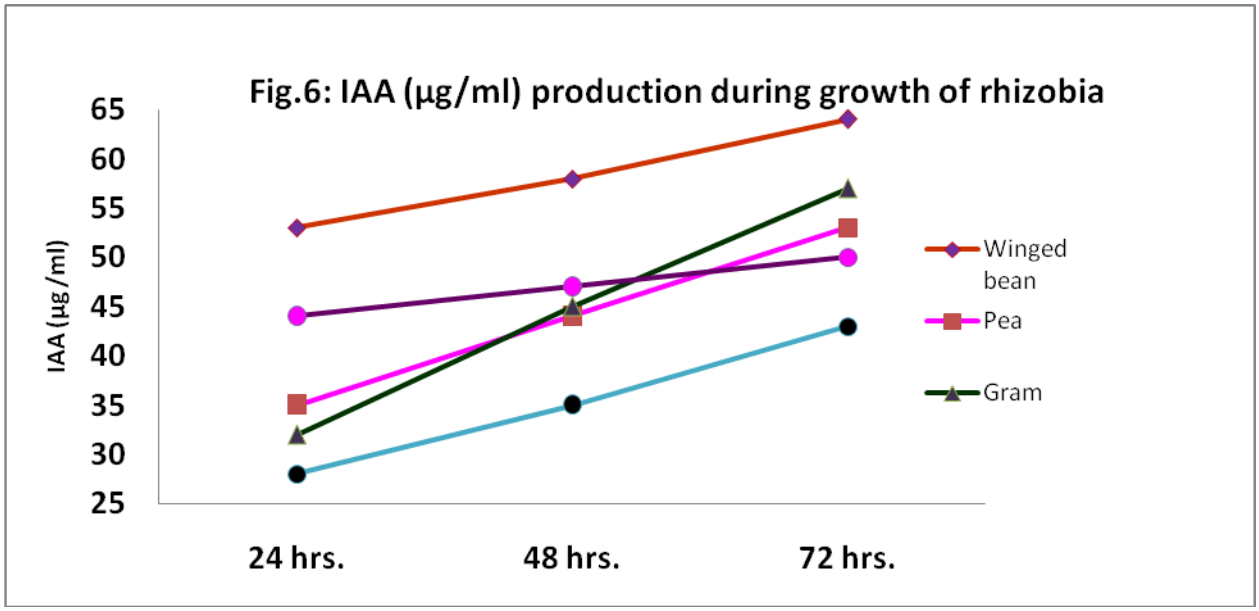


Fig.4: Effect of change in pH after growth of rhizobia
pH
8
7.5
7
6.5
6
5.5
5
4.5
4
pH 4 5 6 7 8 9
Winged bean
4 5 6 7 8 9
Pea
4 5 6 7 8 9
Gram
4 5 6 7 8 9
Lentil
4 5 6 7 8 9
Moong





Tolerance to alkaline pH could be related to the calcareous and dry soils from which these isolates were isolated. Growth of different isolated rhizobia grown in YEM broth medium adjusted at varying pH under the study either positively or negatively influenced the change of pH value (Table 2 and Fig. 4-5). The per cent increase or decrease in original pH of the broth due to growth of rhizobia was maximum at extremity *i.e.*, at pH 4.0 or 9.0 and minimum at neutral. Maximum increase (29%) in pH (from 9.0 to 5.17) was observed due to growth of gram *Rhizobium* while maximum

decrease (48%) in pH (from 9.0 to 4.67) was observed due to growth of moong *Rhizobium*. Least increase (3%) and decrease (0.1%) in pH (from 6.0 to 6.17 and from 7.0 to 6.99) were recorded by growth of pea and lentil rhizobia respectively. However, moong showed better adaptability in pH between 5.0 and 7.0. Investigation carried out by different workers on *Rhizobium* isolated from root nodules of different crop *viz.*, *Rhizobium trifolii*, *R. phaseoli*, *R. leguminosarum* and *Bradyhizobium japonicum* revealed the fact close to observation made in the present study (Bhatt *et al.,* 2013; Dutta *et al.,* 2015; Rai and Sen, 2015 and Kapembwa *et al.,* 2016).

IAA production

The IAA (indole acetic acid) production by *Rhizobium* isolated from two winged bean germplasm along with four other *Rhizobium* isolated from legumenous crop *viz.,* pea, gram, lentil and moong were shown in Table 3. In this study, all the five *Rhizobium* isolates were found to produce IAA from 24 to 72hrs after inoculation in LB media amended with L-tryptophane. Rhizobia produce significant levels of IAA both in free living conditions and also symbiotically in nodules (Ernstsen *et al.,* 1987). Indole acetic acid (IAA) is one of the most physiologically active auxins. The majority of rhizobial strain (74%) associated with different crop could produce IAA (Halda-Aliza, 2003). Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake (Datta and Basu, 2000). The IAA production increased gradually with increase in incubation period from 24 to 72hrs. *i.e.,* its production was maximum at stationary phase of growth *i.e.*, at 72hrs (Fig. 6). Among the rhizobial isolates studied, a maximum of 64.6 µg ml^{-1} of IAA by winged bean isolate and least of 43.6 µg ml^{-1} in case of moong isolate was produced at 72hrs of incubation. Jida and Assefa (2011) also determined IAA production by 30 rhizobial strains isolated from lentil and found that 36.7 per cent of them were able to produce IAA. All the isolates under present study produced varying concentration of IAA with a gradual increasing trend noticed from 24 to 72hrs. Similar observation were also reported for isolates from moong (Kumar and Ram, 2012; Satyanandum *et al.,* 2013 and Rajput and Panwar, 2013; pea (Kucuk and Cevkeri, 2016) and faba bean (Othman and Tamini, 2016). Kumar and Ram (2016) observed that all the strains of rhizobia isolated from moong crop produced maximum amount of IAA when isolates grown in YEMA medium supplemented with L-tryptophan. They also reported that *Agrobacterium* sp. produced more IAA than *Rhizobium* sp. Dutta *et al.,* (2015) also proposed a view that IAA production increased with increase in incubation period from 24hrs and reached maximum at 72hrs in general irrespective of rhizobial isolates. This could be due to better utilization of medium components for IAA production by winged bean isolate compared to other *Rhizobium* isolate under study. Auxin production by rhizobia is often considered to improve growth and N_2-fixation in many legumes including beans, lentil, chickpea and pea (Yanni, 1992; Huang and Erickson, 2007; Anjun *et al.,* 2011; Zafar-ul-Hye *et al.,* 2013).

Antibiotics resistance

The *Rhizobium* is known to possess resistance or susceptibility towards known antibiotic substances (Gray and Fitch 1983, Trieo-cuot *et al.,* 1987). Varying degree of susceptibility to twelve different antibiotics were observed against all five rhizobia in terms of inhibition zone (mm) is tabulated in Table 4. Out of five different rhizobia, winged bean and lentil rhizobia were resistant to only one antibiotic (Vancomycin 30µg), pea and moong rhizobia were resistant to two antibiotics (Ampicillin

10µg and Nalidixic acid 30µg) while gram *Rhizobium* was observed to be resistant to both Chloramphenicol (30µg) and Clarithromycin (15µg). The results from work of Milicic *et al.,* (2006) with three strains of *Rhizobium galegae* for their intrinsic resistance to different concentration of antibiotics also support the present finding. They observed good growth of *Rhizobium* in presence of Trimethoprim, Clindamycin in addition to Chloramphenicol, but were more susceptible to Tetracycline and Streptomycin as found in present study. Among the different rhizobia studied, maximum zone of inhibition (34mm) was observed in pea rhizobia against Azithromycin 15µg followed by moong rhizobia (36mm) against Tetracycline 30µg. In addition to above Milicic *et al.,* (2006) also found *Rhizobium* to be susceptible to a number of other antibiotics *viz.,* Ampicillin, Gentamycin, Erythromycin, Cephalexin. In support to our study, Maatalah *et al.,* (2002) also found gram *Rhizobium* to be resistant to antibiotics (µg ml^{-1}) *viz*., Ampicillin (50), Chloramphenicol (10). They also found the *Rhizobium* resistant to Kanamycin (10 and 100), Nalidixic acid (50) and Streptomycin (23 and 100) whereas in present study, winged bean rhizobia were found highly susceptible to Nalidixic acid (30µg) and least susceptible to Kannamycin (30µg) and Streptomycin (10µg). All the rhizobia were found highly susceptible to Tetracycline (30µg) except winged bean and pea rhizobia. Similar results also been reported by Datta *et al.,* (2015), who found *Rhizobium phaseoli*, *R. trifolii* and *R. leguminosarum* sensitive to Tetracycline (30µg). In case of winged bean isolate, it was found highly susceptible to Nalidixic acid (30µg) and least susceptible to Kanamycin (30µg), Streptomycin (10µg)resulting in an inhibition zone of 22 and 12mm respectively. Sensitivity of *Rhizobium* isolates to antibiotics may be due to the fact that these bacteria (*Rhizobium*) have not been exposed to these antibiotics in natural environments. Depending on the differences in antibiotic resistance pattern, this technique can be successfully employed in ecological studies particularly in the recovery and enumeration of rhizobia introduced in soil. Resistance to Chloramphenicol (5 and 10µg ml^{-1}) by lentil nodulating rhizobial isolates was reported by Jida and Assefa (2011) supporting the findings of present study where gram rhizobia was found resistant to Chloramphenicol (15µg). They also found *Rhizobium* to be resistant to Nalidixic acid (5 and 10 µg ml^{-1}) unlike winged bean rhizobia to be highly susceptible to Nalidixic acid (30µg) in the present study. *Rhizobium* isolated from different crop root nodules and found resistant to different antibiotics tested in the present study might be due to the intrinsic resistance capacity of different rhizobial strains to antibiotics expressed as phenotypic characteristics. A broad variation regarding susceptibility or resistance of individual rhizobial strains to antibiotics exist in nature as discussed above. The character of resistance of rhizobial strains to antibiotics may serve as a potential tool for monitoring their competitiveness as a 'label' for nodulation and effectiveness.

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