Universal Journal of Environmental Research and Technology
 eISSN 2249 0256
Available Online at: www.environmentaljournal.org
Volume 2, Issue 4: 293-299

Open Access **Research Article**

Effect of Different Herbicides on the Nodulation Property of Rhizobial Isolates

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Abstract:

The soil bacteria *Rhizobium* species in association with leguminous plants play vital role in agriculture. The amount of nitrogen (N) supplied by fixation depends on the ability of the inoculant rhizobia to fix nitrogen and also on the ability of the plant to provide energy to the rhizobia in the nodules. Many factors that influence either the rhizobia directly or the ability of the plant to send energy to the nodules have a negative impact on nitrogen fixation and ultimately, crop yield. Application of pesticides both in crop and soil is known to affect plant growth and microbial activity. Attempt was made to isolate Rhizobia from the roots of wild legumes and studying their nodulation property in presence of most commonly used herbicides. Three herbicides namely 2,4 D amine salt, Round Up & Atrazine were used for this experiment. We found as the herbicide conc. is increased from below MIC,MIC level and Above MIC level, the shoot length of plants, dry wt. of plant shoot, total root nodule number of plants and dry wt. of nodules was decreased. There was no development of nodules on side root system of plants and the decreased vigor in plants was observed.

Keywords: Rhizobium, nitrogen fixation, herbicides

1. Introduction:

Rhizobium-legume symbioses are the primary source of fixed nitrogen in land based systems and can provide well over half of the biological source of fixed nitrogen (Tate, 1995).Atmospheric nitrogen fixed symbiotically by the association between *Rhizobium* species and legumes represents a renewable source of nitrogen for agriculture (Peoples et al,1995). *Rhizobia* forms symbiotic association with legumes. The organisms belonging to genus *Rhizobium* are Gram negative, short rods, 0.5 -0.9 µ wide & 1.2 -3.0 µ long, motile by polar or subpolar flagella, nonspore forming, & aerobic with opt. temp.25-30ºC. Most strains produce gum (extracellular Polysaccharide slime) of varying composition (Alexander, 1983). Members of the genus Rhizobium, upon infection of the appropriate legume, can cause the formation of nodules and participate in the symbiotic acquisition of nitrogen (Alexander,1983).

Nodule is outer growth like tuber, slightly pinkish due to presence of haemoglobin only a small proportion of the invaded root hairs develop nodules, usually less than 5 % of the infections ultimately resulting in nodules. There are significant differences among legumes in the morphology of the nodules. Red & white clovers have club shaped & lobed structures, the nodules of alfalfa are more branched & longer while those of cow pea, peanut, & lime bean exhibit a spherical shape. Nodule size is a low as several millimeters in diameter to the size of a baseball. Legumes with fibrous roots frequently have a greater abundance of nodules than plants with well farmed tap roots, and plants bearing large nodules often have a few, whereas roots with smaller structures have them in greater numbers. Nodules are not found on all of the genera & species of *Leguminoceae* (Alexander, 1983)

Typical environmental stresses faced by the legume nodules and their symbiotic partner (*Rhizobium*) may include photosynthate deprivation, water stress, salinity, soil nitrate, temperature, heavy metals, and biocides (Walsh, 1995).The most problematic environments for rhizobia are marginal lands with low rainfall, extremes of temperature, acidic soils of low nutrient status, and poor water-holding

capacity.The legume-*Rhizobium* symbioses and nodule formation on legumes are more sensitive to salt or osmotic stress than are the rhizobia. Salt stress inhibits the initial steps of *Rhizobium*-legume symbioses. High soil temperatures in tropical and subtropical areas are a major problem for biological nitrogen fixation of legume crops (Michiels, 1994). High root temperatures strongly affect bacterial infection and N2 fixation in several legume species. High (not extreme) soil temperatures will delay nodulation or restrict it to the subsurface region (Graham, 1992).

Use of herbicides for weed control in legume fields has contributed to increased yield and improved quality (Knott, 1985)**.** Frequently, herbicides not only affect plant growth but have a detrimental effect on soil microorganisms, growth and metabolism (*Sawicka et al,1996*). Some studies have evaluated the effect of different herbicides on *Rhizobium* growth and nitrogen fixation activity. The effect depends on the herbicide, its concetration, and different weather conditions. Applied research methodology also may depend on the *Rhizobium* or *Bradyrhizobium* species and even the strain used (*Sprout et al*, 1992). Attempt was made to isolate Rhizobia from the roots of wild legumes and study their nodulation property in presence of different herbicides.

2.0: Materials and Methods:

2.1 Collection of Sample:

Three different leguminous plants were collected from farms from agriculturally developed Kale village, 8 km south to Karad city. These samples were labeled as 1, 2 & 3. Each leguminous plant was identified by specific characteristics appearance of family – *Leguminoseae (*Yadav and Sardesai, 2002*).* The sickle was used to dig into the soil around the plant. A plant was uprooted along with soil around them in such a way that entire root system was obtained intact. The plant along with its rhizospheric soil was taken in plastic bags & brought to laboratory for isolation purpose (Bergersen, 1980).

2.2 Processing of Plant Specimen:

The plant roots were held below running tap water to remove soil around roots followed by immersing root system in tap water with gently shaking to clean the root system. Further it was used for isolation of *Rhizobium* (Subba Rao, 2006).

2.3 Isolation of *Rhizobium*:

Healthy, pink nodules from legume roots were selected and subjected to surface sterilization with 95% ethanol and H_2O_2 followed by successive washes with sterile distilled water. Suspension was made by crushing single nodule in a sterile petriplate containing few drops of sterile normal saline. A loopful of suspension was streaked onto Congo Red Yeast Extract Mannitol Agar (CRYEMA) by four quadrant method & incubated at 25º C for 3 days. A well isolated colourless colony was restreaked onto same medium & incubated at 25º C for 3 days for purification. Isolated colonies were transferred onto slant of sterile Yeast Extract Mannitol agar and incubated at 25ºC for 3 days. Then they were placed in refrigerator at 4^0 C for preservation. After every two months, transfer on fresh slant was given.

2.4 Confirmation of *Rhizobium*:

Four confirmatory tests were performed viz. Congo Red Dye Absorption Test (Skinner & Lovelock, 1979),Ketolactose test (Subba Rao, 2006), Nile Blue Reduction Test (Bergersen, 1980) and Growth on Glucose Peptone Agar (Skinner & Lovelock, 1979) t o confirm isolates as *Rhizobium* & not the *Agrobacterium* or other bacteria, which frequently come as contaminant.

2.5 Characterization of Isolates:

Colony Characteristics, Gram Staining and motility by hanging drop technique (Cruickshank et al, 1975) was studied. The utilization of carbohydrate was studied using the basal medium_Yeast Extract Broth with 1 % carbohydrate & Bromothymol blue (BTB) indicator incorporated in it. Arabinose, xylose, Rhamnose, Glucose, galactose & fructose, Sucrose, Maltose and Raffinose sugars were tested. The suspension of rhizobial isolate was inoculated in tubes & all were incubated at 25º C for 3 days & change in colour of the fermentation medium due to acid production was observed to detect ability of the isolate to utilize carbohydrate.(Stower & Eaglesham, 1983).

Enzymatic Properties such as *Gelatin Liquefication Test* (Cruickshank et al, 1975), *Starch Hydrolysis Test* (Cruickshank et al, 1975) and *Litmus Milk Test* (Bradshaw, 1979) were studied.

2.6 To visit agrochemical shops from Karad city to know the dominating herbicides sold from the market and used by the farmers:

There are about 28 agrochemical shops in Karad city. Survey was done in total of 11 agrochemical shops from different locations of Karad city. It was aimed to collect the information regarding the name and quantity of herbicides sold from the agrochemical shops.

2.7 To study herbicide sensitivity pattern of rhizobial isolates to the selected herbicides:

From the list of 5 herbicides obtained after survey, 3 were selected for study. Effect of these selected herbicides on the growth of isolate was observed. Yeast Extract Mannitol Agar was used in which different concentrations of these 3 herbicides were incorporated. The isolate suspension was streaked and incubated at 25°C for 3 days and observed for growth. The resulting herbicide sensitivity pattern was noted.

2.8: To study the effect of selected herbicides on nodulation of cowpea (*Vigna unguiculata*)–

This experiment involved following steps:

1. ***Preparation of Pots*** :

Sieved sand was first washed 3-4 times to remove clay particles & taken in a disinfected tray and sterilized in hot air over for 2 successive days. Then it was filled in round bottom plastic bags of 13 cm length & 17.5 cm width under aseptic conditions. These plastic bags had four holes created initially at the bottom. The experiment was run in six sets, each containing four pots lebelled as A (positive control), B, C, D. Twenty fifth pot was lebelled as negative control.

Pot A (positive control) = pot with cowpea seed & respective rhizobial isolate.

Pot B = Pot with cowpea seed, respective rhizobial isolate & Respective test herbicide in conc. lower than minimum inhibitory concentration (MIC) of respective rhizobial isolate.

Pot C = Pot with cowpea seed respective rhizobial isolate & respective test herbicide in MIC Conc. of respective rhizobial isolate.

Pot D = Pot with cowpea seed, respective rhizobial isolate & respective test herbicide in Conc. higher than the MIC of respective rhizobial isolate.

2. Preparation of nitrogen free plant nutrient solution and test herbicide concentrations:

Arnon's mineral mixture (Arnon DI, 1938) was used as nitrogen free plant nutrient solution. Various herbicide Conc. were prepared in same medium for each herbicide in different bottles (500ml capacity).

3. Preparation of Rhizobial inoculum:

Three rhizobial isolate were inoculated in loopful amount in 3 different sterile Yeast Extract Mannitol broth tubes (each containing 5ml) and incubated at 25°C for 4-5 days to get heavy growth. Then this was used as *Rhizobium* inocula.

4. Effect of selected herbicides on cowpea nodulation caused by test rhizobial isolates:

The large sized, healthy seeds of cowpea were collected and given surface sterilization treatment first in H_2O_2 solution for 5 min. and then washed three times in sterile distilled water. In negative control pot, the surface sterilized seeds were directly transferred to 6 cavities created in the pot with sterile glass rod & it was covered with sand. Surface sterilized seeds were transferred to 6 sterile petri plates where they were coated with respective rhizobial isolate and then transferred to pots A (positive control) B,C & D pots of all 6 sets and covered with sand. Negative control & positive control (A) pots were daily inoculated with Arnon's mineral mixture medium & B, C, D pots of 3 sets with three dilutions of each herbicide containing same medium. After germination of seed, at the end of 6 days the remaining rhizobial isolate culture was poured near the root system in all pots except negative control pot. First pot with R-1 strain was inoculated with 2,4-D amine salt, second pot with R-2 strain was inoculated with Atrazine and third pot with R-3 strain was inoculated with Round Up herbicide. During experiment, drying of pots was prevented. At the end of desired period, data on the appearance of plants in terms of colour & vigour, the number of nodules formed on the root system, dry weight of the stems & nodules are collected (Subba Rao,2006)

3.0 Results and Discussion:

3.1 Sample collection :

Three different leguminous plants were collected from Kale village & they were identified as-*Crotolaria hebecarpa,Desmodium cephalotes and Tephrosia strigosa.*

3.2 Isolation of *Rhizobium*:

Surface sterilized nodules were crushed in sterile Petri plates with sterile saline water aseptically. Bacteroids were observed in microscopic observation of suspension. After initial streaking in four quadrant pattern & purification by repeating the method twice, total 3 isolates were obtained and labeled. *Crotolaria hebecarpa was labeled as* R-1, *Desmodium cephalotes as* R-2 and *Tephrosia strigosa as* R-3.

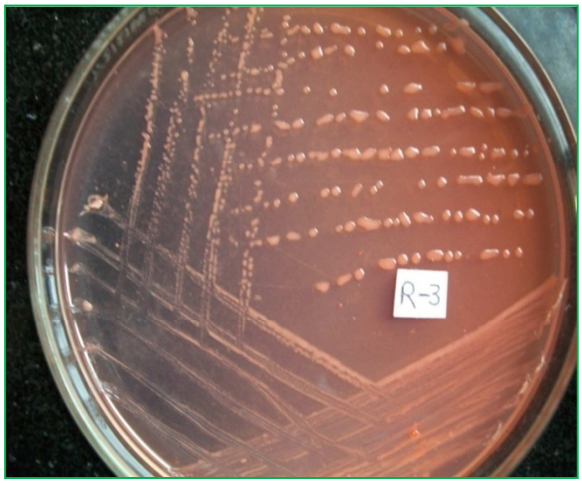

Fig 1: **Growth of Rhizobial isolate No.3(R-3) on CRYEMA after incubation at 25ºC for 3 days.**

3.3 Confirmation of Rhizobium spp:

These isolates were confirmed by performing confirmatory tests & results were as per table no.1. All the isolates were confirmed to be the members of the genus *Rhizobium*.

Table 1: Results of Confirmation tests for isolates

Confirmatory test	Rhizobial Isolate number		
	R-1	R-2	R-3
Congo red dye absorption	-	-	-
Growth on Glucose Peptone	-	-	-
Nile Blue Reduction	-	-	-
Ketolactose	-	-	-

- = negative test.

3.4 Study of Colony characteristics, morphology & Gram property of isolates:

The isolate suspension was streaked on Congo red Yeast Extract Mannitol Agar & incubated at 25ºC for 3 days. After incubation, colony characteristics, morphology and Gram staining properties were observed. Colonies of all isolates were circular,regular in margine, raised to convex in elevation,mucoid,colourless,Gram negative, short rods and motile.

3.5 Study of sugar fermentation property of Rhizobial isolates-:

This test was performed to check the ability of isolate to utilize various sugars. The results obtained were as shown in table no.2

Table 2: Carbohydrate Utilization by Rhizobial isolate

Sugars	Rhizobial Isolate code		
	R-1	R-2	R-3
Arabinose	+	+	+
Xylose	+	+	+
Rhamnose	+	+	+
Glucose	+	+	+
Galactose	+	+	+
Fructose	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Raffinose	+	+	+

(+)=Acid production (-) = No acid production

All the rhizobial isolate produced acid from Arabinose, Xylose, Glucose, Galactose, Fructose, Sucrose Maltose and Raffinose.

3.6 Study of enzymatic properties of isolates:

The results of enzymatic property along with the litmus milk reduction were as recorded in table number 4. The results of Gelatin liquefication test, Starch hydrolysis test and Litmus milk test are as per table No.3.

Table 3: Results of enzymatic properties of isolates

Test	Rhizobial Isolate Number		
	R-1	R-2	R-3
Gelatin liquefaction test	-	-	-
Starch hydrolysis test	-	-	-
Litmus milk test	+	+	+

3.7 Visit to Agrochemical shops from Karad city & to farmers from nearby village:

On market survey in Karad city and visit to farmers from agriculturally developed villages, the dominating herbicides sold from market & used by the farmers were found to be 2,4 D amine salt, Atrazine, Round UP and Mera-71with the sale of 8.0, 6.5,6.1,4.3(Liters/week/shop) respectively. From these, total of 3 herbicides namely 2,4 D amine salt, Round Up & Atrazine were selected & used to find out sensitivity pattern of rhizobial isolates.

3.8 Herbicide sensitivity pattern of rhizobial isolates to the selected herbicides:

All the isolates were subjected to find out herbicide sensitivity pattern and minimum inhibitory concentration was found to be50 mg/ml for 2,4 D amine salt and 100 mg/ml for both Atrazine and Round Up (Table 4).

Table 4: Herbicide sensitivity pattern of Rhizobial isolates

Sr. no.	Pesticide used	Conc. (mg/ml)	Isolate number		
			R-1	R-2	R-3
1	2,4 D amine salt	10	+	+	+
		25	+	+	+
		50	-	-	-
2	Atrazine	25	+	+	+
		50	+	+	+
		75	+	+	+
		100	-	-	-
3	Round Up	25	+	+	+
		50	+	+	+
		75	+	+	+
		100	-	-	-

(+)=Growth (-)= No Growth

3.9 Study of effect of selected herbicides on nodulation of cow pea:

The pot experiment was carried out on terrace of 'A block', Boys hostel, Yashwantrao Chavan College of Science, Karad. All the pots (4 pots in each set) were arranged in 3 lines and other negative control pot, were put on the cot. It was covered with net to avoid damage due to birds & insects. For this experiment, cow pea plants were allowed to infect by 3 different rhizobial isolates in 3 sets & effect of 3 selected herbicides was seen on rhizobial isolates & in turn on nodulation (Table 5).

Fig 2: Effect of 2,4 D amine salt herbicide on cow pea nodulation by R-1 isolates (1 plant from pot A,B and C respectively).

At the third day of experiment, the germination of cowpea seed started in all positive controls of 3 sets. Seed germination was not observed in D pots of all three sets. It might be due to the unfavourable conditions because of high residual pesticide conc. in sterile sieved sand. After 24 days of experiment, the bags were cut with scissor very carefully & plants were removed. Plant roots were washed gently under tap water. Then the effect of pesticide in terms of appearance of plants, shoot length measurement was noted. Also the nodule morphology, nodule count on both main root & side root system was noted. In case of set I, shoot length of plants from pots A, B & C was found to be 6 cm, 3.7 cm & 2.0 cm respectively. In case of set II, shoot length of plants from pots A ,B and C was found to be 3.2 cm, 1.5 cm and 1.2 cm respectively. In case of set III, shoot length of plants from pots A , B and C was found to be 1.5 cm ,1.0 cm and 0.6 cm respectively.

Table 5: Results of effect of selected herbicides on Nodulation of cow pea

Rhizobial Isolate No.	Pot no.	Plant Characteristics			Nodule characteristics				Dry wt. of nodule (mg)
		Appearance of plants	shoot length (cm)	Dry wt. of shoot (mg)	Nodule Morphology	Nodule count			
						On main root system	On side root system	Total	
R-1 (in presence of 2,4 D amine salt)	A	Healthy &Green but stunted	6.0	101	Club shaped & elongated	48	254	302	61
	B	Healthy & Green	3.7	85	Round & Large sized	32	0	32	39
	C	Dried & Shrinked leaves	2.0	31	-	-	-	-	-
	D	No seed germination	-	-	-	-	-	-	-
R-2 (in presence of Atrazine)	A	Healthy & Green	3.2	95	Club shaped & elongated	45	113	158	28
	B	Dried & Shrinked leaves	1.5	36	Elongated but dried	18	0	18	11
	C	Dried & Shrinked leaves	1.2	27	-	-	-	-	-
	D	No seed germination	-	-	-	-	-	-	-
R-3 (in presence of Round Up)	A	Healthy & Green	1.5	64	Club shaped & elongated	27	62	89	21
	B	Dried & Shrinked leaves	1.0	15	Round shaped	8	0	8	18
	C	Dried & Shrinked leaves	0.6	09	-	-	-	-	-
	D	No seed germination	-	-	-	-	-	-	-

In case of set I, total nodule count of plant from pot A & B was found to be 302 & 32 respectively. In case of set II, total nodule count of plants from pot A & B was found to be 158 & 18 respectively. In case of set III, total nodule count of plants from pot A & B was found to be 89 and 8. Here, total nodule count decreased with increase in pesticide concentrations. There was no development of nodules on side roots of plants in presence of pesticide. In case of set I, root nodules of plants from A pots were fresh & white in colour, club shaped & elongated, and measuring 1.5 mm × 2 mm in size. Whereas the root nodules from plants of B pot were large sized & round shaped, measuring 4 mm in diameter. No nodule was seen on root of plants from C pot. In case of second and third sets, root nodules of plants from A pots were fresh, white coloured, club shaped & elongated, and measuring 1.5mm × 2 mm in size. The root nodules of plants from B pots of set II were of same morphology & of 1.3 mm ×1.7 mm size, but they were shrinked & were of dark coloured. Whereas the root system of plants from B pots of III was very seriously damaged, dried & Shrinked.

The shoots of legume plants & also root nodules from plants of all III sets were incubated at 37º C for 2 days & then dry wt. in terms of milligrams (mg) was noted. In case of set I, the dry wt. of shoot of plants from pot A, B & C was found to be 101 mg, 85 mg & 31 mg respectively. The dry wt. of root nodules of plants from pot A & B was 61 mg & 39 mg respectively. In case of set II, the dry wt. of shoot of plants from pot A , B and C was found to be 95 mg , 36 mg and 27 mg respectively. The dry wt. of root nodules of plants from pot A & B was 28 mg & 11 mg respectively. In case of set III, the dry wt of shoot of plant from pot A,B and C was found to be 64 mg, 15 mg and 09 mg respectively. The dry wt. of root nodules of plant from pot A and B was found to be 21 mg and 18 mg.

In summary, following were the observations from pot experiments -as herbicide conc. is increased from below MIC,MIC level and Above MIC level, the shoot length of plants was decreased, dry wt. of shoot of plants was decreased, total root nodule number of plants was decreased, dry wt. of nodules was also decreased, there was no development of nodules on side root system of plants and the decreased vigour in plants was observed. In some cases, though Rhizobia were able to tolerate the pesticide concentration the plants couldn't tolerate it & got damaged.

This is similar to the reports on the similar work done on Rhizobial isolates from soyabean. Herbicides induced the reduction in nodulation. Reductions in the nodulation can be the indirect result of herbicide injury to the plant or from direct action of the herbicide on the Rhizobia or reductions can result from action against both the partners in the symbiosis.

4.0 Acknowledgement:

The authors are thankful to Principal, Yashwantrao Chavan College of Science, Karad-415110, M.S., India for the facilities and help rendered for this work.

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