ISSN: 2395-5775

Available Online at http://www.journalijcir.com

International Journal of Current Innovation Research
Vol. 09, Issue, 05, pp. 08-12, May, 2023

International Journal of Current Innovation Research

DOI: 10.24327/IJCIR

Research Article

IN VITRO EFFECT OF HERBICIDE GLYPHOSATE ON THE ACTIVITIES OF *RHIZOBIUM* ISOLATED FROM GROUNDNUT FIELD

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ARTICLE INFO

Article History:

Received 15th March, 2023
Received in revised form 17th April, 2023
Accepted 16th May, 2023
Published online 28th May, 2023

Key words:

Rhizobium, Herbicide resistance, Glyphosate,

ABSTRACT

The bacterium *Rhizobium* is s symbiotic nitrogen fixer and is commonly used as Nitrogenous biofertilizer over the legume fields. The agrochemical application has considerable negative effects over the performance of Biofertilizer microbes. In the present research, four *Rhizobium* species were isolated from groundnut field which is frequently exposed to the herbicide glyphosate using YEMA medium and their plant growth promoting activities *viz*., production of Ammonia, IAA, HCN and the solubilization efficiency to solubilize Phosphorus and Zinc were tested. All the four isolates could produce significant amount of Ammonia, HCN and IAA and the herbicide caused moderate effects on the efficiency of production of these compounds. Whereas, no *Rhizobium* isolate were able to solubilize Zn and Phosphorus with and without the presence of herbicide Glyphosate.

INTRODUCTION

The problems with the weeds and controlling them by the farmers are being one of the important practices in agriculture from the earlier days to increase the yield. Within a very short period, herbicides use shore up by manifolds. Increase in the consumption of herbicide is likely to be at least two to three times more in the years to come. The chemical properties of herbicides determine their retention and transport in soils (Kearney and Wauchope, 1998). A herbicide can reach groundwater if its water solubility is greater than 30mg/L; its adsorptivity, Koc (Koc= partition coefficient between soil organic matter and water), is less than 300-500 ml/g; its soil half-life is longer than about 2-3 weeks; its hydrolysis half-life is longer than approximately 6 months and its photolysis is longer than 3 days (Barcelo, 1991). The microbes which are present in the land which is continuously exposed to a particular chemical like herbicides develops resistance against that particular chemical compounds and became effective utilizers of that compounds for its energy and carbon source. Isolating of native strains adapted to the environment and their study may contribute to the formulation of inoculants to be used in region crops. On the other hand, characterization and identification of these bacteria are necessary for wide ecological studies of the plant rhizosphere.

MATERIALS AND METHODS

Sample collection

Root nodules were collected from groundnut filed which was frequently exposed to the herbicide glyphosate. The collected nodules were taken into laboratory for the isolation of *Rhizobium* species

Isolation of *Rhizobium* from collected root nodules

The nodules collected were thoroughly washed in tap water to remove the adhered soil particles. After, the nodules were immersed in 0.1 % mercury chloride for surface sterilization followed by repeated washing with distilled water to remove the traces of mercury chloride. The sterilized nodules were cut using sterile blade and crushed with the use of sterile forceps to get the fine paste of nodules. Then the nodule solution was serially diluted by following standard procedure upto concentration of 10^{-6}. Then, 1 mL of serially diluted samples from 10^{-4} concentration was transferred to sterile Petri plates and evenly distributed throughout the plates and sterile unsolidified YEMA medium was poured and it was allowed to solidify for the selective isolation of *Rhizobium*. Then the plates were incubated at room temperature for 48 – 72 hrs. After incubation period, the plates were observed for the growth of *Rhizobium*.

Characterization and identification of the isolated *Rhizobium* species

For cell shape, arrangement and motility, Gram staining and hanging drop technique were performed and the results were noted. For identification, the biochemical tests Indole, Voges-Prauskauer, Urease, Citrate, TSI, Oxidase test, Catalase test were performed and the results were noted

Screening of *Rhizobium* isolates against different concentration of herbicide glyphosate

To test glyphosate resistant level for all the four isolates against the herbicide glyphosate, different concentration in ppm from 10, 000 ppm to 19,000 ppm were prepared and amended with nutrient agar and the cultures were streaked over the plates. The plates were incubated and the growth of the isolate in the plates containing herbicide glyphosate

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indicated its resistance against that concentration of herbicide glyphosate

Effect of herbicide glyphosate on the plant growth promoting activities of *Rhizobium* isolates

Screening of *Rhizobium* for IAA production (Gorden and Paleg , 1957)

The fresh bacterial cultures were inoculated in 10 ml of nutrient broth containing L-tryptophan (5 µg/ml) and incubated at room temperature for 5 to 7 days. The media were prepared with herbicide glyphosate at the rate of 1000 ppm and without glyphosate to compare the effect of herbicide on IAA production. After incubation, broth cultures were centrifuged at 3000 rpm for 5 minutes. Supernatant was taken and two drops of phosphoric acid was added followed by addition of Salkowaski reagent (50 ml of 35 % perchloric acid and 1 ml of 0.5 M $FeCl_3$). The absorbance of reaction mixture was measured at 530 nm after incubation in dark for 30 min.

Screening of *Rhizobium* isolates for ammonia production (Cappuccino and Sherman, 1992)

The isolated *Rhizobium* bacteria were tested for their capacity to produce ammonia. Fresh cultures of bacteria was inoculated in 10 ml of peptone water in tubes and incubated for 48 to 72 hrs at room temperature. The media were prepared with herbicide at the rate of 1000 ppm and without herbicide to compare the effect of herbicide on ammonia production After 72 hrs, Nessler's reagent (0.5 ml) was added to bacterial suspension. Development of brown to yellow color indicated ammonia production

Screening of the *Rhizobium* isolates for HCN production (Bakker and Schippers, 1987)

Cultures of strains were streaked on solid agar plates supplemented with 4.4 g glycine $litre^{-1}$, with simultaneous addition of filter paper soaked in 0.5 % picric acid in 1% Na_2CO_3 on the streaked region along with the un-inoculated control.

The media were prepared with herbicide at the rate of 1000 ppm and without herbicide to compare the effect of glyphosate herbicide on HCN production. The plates were incubated at room temperature. Development of color from yellow to light brown, moderate brown or strong brown indicated the HCN production

Screening of the *Rhizobium* isolates for phosphate solubilization

All the *Rhizobium* isolates were screened for their efficiency in phosphate solubilization on Apatide's medium. *Rhizobium* isolates, isolated from root nodule of groundnut spot inoculated on Apatide medium plate and incubated at 37°C for 4-5 days. The media were prepared with glyphosate at the rate of 1000 ppm and without glyphosate to compare the effect of herbicide on phosphate solubilization. Phosphate solubilization is observed by forming clear zone around the bacterial colony

Screening of the *Rhizobium* isolates for Zinc solubilization

All the for *Rhizobium* isolates were screened for their efficiency in Zinc solubilization on nutrient agar amended with insoluble zinc source (ZnO). *Rhizobium* isolates, isolated from root nodule of groundnut spot inoculated on the medium and the plates were incubated at 37°C for 4-5 days. The media were prepared with herbicide at the rate of 1000 ppm and without herbicide to compare the effect of herbicide on Zn solubilization. Zinc solubilization is observed by forming clear zone around the bacterial colony

RESULTS AND DISCUSSION

Four *Rhizobium* isolates were isolated from root nodule of groundnut and designated as RH_1, RH_2, RH_3, and RH_4. The morphological and biochemical characterization of the isolates were studied and presented in the Table-1. All the isolates were gram negative motile rod. Similar result was found with Mohammad Shahid, Mohammad Saghir Khan, (2022). They found that among the total isolates from the rhizospheric region, 87.5% isolates were Gram negative rod shaped while 12.5% were Gram positive with small rods.

Screening of *Rhizobium* isolates against different concentration of herbicide Glyphosate

The tolerance level of herbicide glyphosate was tested and the results were given in the table- 2. The results showed that all the four isolates were able to grow up to 16,000 ppm concentration of herbicide glyphosate. No isolates were able to grow beyond 16,000 ppm concentration of herbicide glyphosate. Further increase in herbicide retard the growth of all the four isolates. Juneja and Dogra, (1978) and Jayaprakash and Hemavathy, in their results of their researches that gram negative bacteria have shown greater tolerance to the agrochemicals than gram positive bacteria. Tolerance or resistance in microorganisms against agrochemicals including herbicides is a complex process which is regulated both at physiological/genetic level of microorganism. The temporary resistance (tolerance) against agrochemicals in general, is attributed to physiological changes that induce the microbial metabolism for the

Table1 Morphological and Biochemical characterization of the *Rhizobium* isolates

S. No	Isolate	Gram staining	Motility	Indole	MR	VP	Urease	Citrate	TSI	Oxidase	Catalase
1	RH_1	Gram (-) rod	Motile	-	+	+	+	+	A/A	+	+
2	RH_2	Gram (-) rod	Motile	-	+	+	+	+	A/A	+	+
3	RH_3	Gram (-) rod	Motile	-	+	+	+	+	A/A	+	+
4	RH_4	Gram (-) rod	Motile	-	+	+	+	+	A/A	+	+

formation of a new metabolic pathway to bypass a biochemical reaction inhibited by a specific Agrochemical (Bellinaso*et al.,* 2003). Permanent resistance, on the other hand, depends upon genetic modifications, inherited by the subsequent generation of microbes (Johnsen *et al.*,2001; Herman *et al.,* 2005). Repeated exposure of microorganisms to herbicide like xenobiotic compounds has resulted in the adaptation and evolution of bacteria capable of metabolizing these man-made compounds (Zhang and Bennett, 2005).

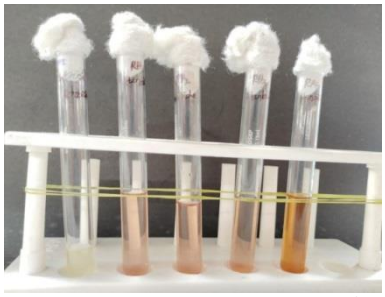

Fig.1 IAA production by *Rhizobium* (First one from right showed maximum IAA production by RH_4 and the last one is control)

Table 2 Tolerance level of *Rhizobium* isolates against herbicide glyphosate

S.No.	Concentration	*Rhizobium* isolates			
		RH_1	RH_2	RH_3	RH_4
1	1000 ppm	+	+	+	+
2	2000 ppm	+	+	+	+
3	3000 ppm	+	+	+	+
4	4000 ppm	+	+	+	+
5	5000 ppm	+	+	+	+
6	6000 ppm	+	+	+	+
7	7000 ppm	+	+	+	+
8	8000 ppm	+	+	+	+
9	9000 ppm	+	+	+	+
10	10000 ppm	+	+	+	+
11	11000 ppm	+	+	+	+
12	12000 ppm	+	+	+	+
13	13000 ppm	+	+	+	+
14	14000 ppm	+	+	+	+
15	15000 ppm	+	+	+	+
16	16000 ppm	+	+	+	+
17	17000 ppm	-	-	-	-
18	18000 ppm	-	-	-	-
19	19000 ppm	-	-	-	-

+:Growth -: No growth

Screening of *Rhizobium* isolates for IAA production

All the four isolates were inoculated into the peptone water containing L-tryptophan, to test the indole acetic acid production amended with and without the addition of herbicide. After completion of the test, results were noted by taking the OD value at 530 nm and the results were presented in the Table-3 and in Fig.-1. All the isolates showed significant quantities of IAA production in the presence and absence of herbicide glyphosate, while the maximum production was recorded by the isolate RH_4 in both in the presence and absence of herbicide glyphosate, remaining isolates showed moderate level of IAA production

Screening of *Rhizobium* isolates for ammonia and HCN production

Ammonia production by the *Rhizobium* isolates in the presence and absence of herbicide was tested and the results were given in the Table- 4 and in Fig.-2. Development of brown colour indicated ammonia production. All the isolates were able to develop brown color, but the dark brown color was developed by the isolate RH_1, followed by RH_3 which indicated the maximum production of ammonia over other isolates.HCN production by the *Rhizobium* isolates was tested and the results were noted and presented in the table – 4 and in Fig.-3.

Color of the filter paper changed into light brown, moderate brown or strong brown indicated the HCN production**.** All the isolates changed the filter paper color into brown when compared with control. The color change was excellent in the filter paper kept over the isolate RH_4. HCN is a toxic compound produced by several beneficial soil bacteria. In the present study, HCN production by the *Rhizobium* isolates was tested and all the isolates produced HCN. Several Rhizobial strains are reported to have the biocontrol properties. The mechanisms of biocontrol by Rhizobia include, competition for nutrients (Arora *et al.,* 2001), production of antibiotics (Bardin *et al.,* 2004; Chandra *et al.*, 2007), production of enzymes to degrade cell walls (Ozkoc and Deliveli, 2001) and production of siderophores (Carson *et al.*, 2000). The production of metabolites such as HCN, phenazines, pyrrolnitrin, viscoinamide and tensin by rhizobia are also reported as other mechanisms (Bhattacharyya and Jha, 2012).

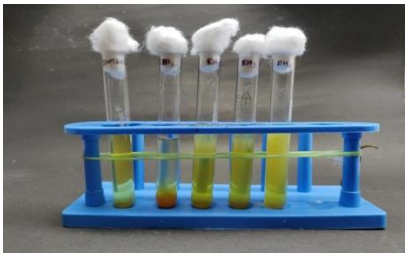

Fig.2 Ammonia production by the *Rhizobium* isolates in the presence of glyphosate herbicide

Table 3 IAA production of the *Rhizobium* isolates

S.No	Isolate	OD value at 530 nm (With herbicide)	OD value at 530 nm (Without herbicide)
1.	RH_1	0.0657	0.167
2.	RH_2	0.0736	0.142
3.	RH_3	0.0756	0.081
4.	RH_4	0.1412	0.390

Table 4 Production of Ammonia and HCN by the *Rhizobium* isolates

S. No	Isolate	In the presence of herbicide		In the absence of herbicide	
		Ammonia production	HCN production	Ammonia production	HCN production
1.	RH_1	+++	++	+++	++
2.	RH_2	+	+	++	+
3.	RH_3	++	++	++	++
4.	RH_4	+	+++	+	+++

-negative, + weak, ++ moderate, +++ strong

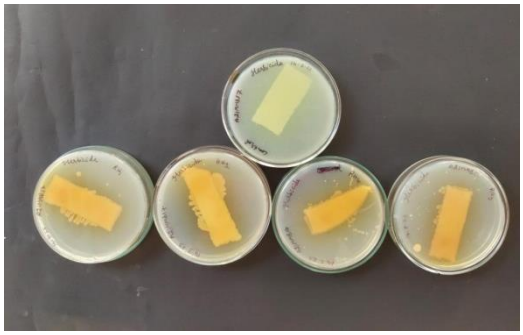

Fig.3 HCN production by *Rhizobium* isolates (on the top one is control)
Screening of *Rhizobium* isolates for phosphate solubilization

Screening of *Rhizobium* isolates for Phosphate and Zinc solubilization

Phosphate solubilization efficiency of the *Rhizobium* isolates was tested using Apatide's medium. After the spot inoculation of the isolates, a clear zone around the colony indicated the phosphate solubilization. Among the four isolates, no isolate was able to solubilize phosphate in in vitro condition in the media containing insoluble phosphate (Table-5). Phosphate solubilization was not found with the plates with no herbicide added also. Zinc solubilization efficiency of the *Rhizobium* isolates was tested using Nutrient agar amended with insoluble ZnO.

Among the four isolates, no isolate was able to solubilize insoluble zinc in in vitro condition in the media containing ZnO (Table-5). In the present study, all the four isolates showed no activity of zinc and phosphate solubilization. In contrast, some findings of *Rhizobium* with the ability of solubilizing minerals were also reported by some researchers. In a study, Consortium of *Rhizobium leguminosarum*-pr-1 and *Pseudomonas* sp. increased the zinc content in shoots was observed by Mishra *et al.* (2012), Gandhi and Muralidharan (2016) also reported highest zinc solubilization in ZnO amended medium up to 36.54 mg mL^{-1} by *Rhizobium* isolated from rhizosphere soil. Sometimes one or more of the Rhizobacteria may not possess a particular plant growth promoting activity. Similar finding was found with Ahmad *et al*. (2008) who screened 72 rhizobacterial isolates and found none of strains positive for chitinase activity which control the pathogenic fungi.

In this study all the four isolates of *Rhizobium* were screened for their ability of producing HCN, ammonia, IAA and solubilization of Zn, P in the presence and absence of herbicide and the results showed that the plant growth promoting activity of the isolates was inhibited by the herbicides. When compared with the treatment with herbicides, without herbicides produced lower quantities of ammonia, HCN, and IAA.

Table5 Solubilization of P and Zn *Rhizobium* isolates

S.No	Isolate	In the presence of herbicide		In the absence of herbicide	
		Solubilization of 'P'	Solubilization of 'Zn'	Solubilization of 'P'	Solubilization of 'Zn'
1.	RH_1	-	-	-	-
2.	RH_2	-	-	-	-
3.	RH_3	-	-	-	-
4.	RH_4	-	-	-	-

+ solubilization- no solubilization

After the spot inoculation of the isolates, a clear zone around the colony indicated the Zinc solubilization.

Many researchers also isolated the *Rhizobium* from groundnut field and tested their plant growth promoting traits (Dardanelli *et al.*, (2009); Dey and Pal (2014);Badawi *et al.* (2011);Gunri

et al. (2014);Didagbé *et al.* (2014); Kumar and Suganya (2017).

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