Iron scavengers PGPR and their role as soil health enhancer in rice fields of Bihar

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ABSTRACT

PGPR from rhizospheric soil grip nutrient there by reducing need for chemical fertilizers and revitalizing soil quality. The rice fields of Bihar are abundantly rich in PGPR belonging to *Pseudomonas, Azotobacter, Serratia* and *Arthrobacter* genus. Weeds grow in abundance along with rice crop which enhances crop productivity and soil fertility through multifarious biochemical approaches. *Azospirrillum* and *Azotobacter* are influential Fe-chelating PGPR which assists siderophore production in the weed rhizosphere that scavenge iron from insoluble Fe- compounds present in soil and trigger soil health and nutrient availability in the rice fields.

Key words: Rhizosphere, Fe- chelating PGPR, weeds.

INTRODUCTION

Rhizosphere is a unique nutrient rich locus associated with large number of microbes which interact with plant and have capability to influence growth of plant and promote soil fertility. Microbes and crop plant in holobiant association in rhizospheric zone have immense potential for improving soil health (**Parades and Lebias,2016**) maintain the macro and micro nutrients (P, N,F) to enhance soil fertility ultimately enhancing crop productivity. Those beneficial micro-organisms which are living into the rhizospheric region and support plant growth and development and promote soil health and fertility are known as **PGPR** (Plant Growth Promoting Rhizobacteria). PGPR are heterogeneous group of soil bacteria that are naturally occur in a form of colonies in rhizospheric soil region which help to improve plant development and maximize crop yields(**Wu et al.,2005**) and promote soil health (**Dazza et.al.,2008).** PGPR such as *Burkholderia , Klebsiella, Enterobacter,Pseudomonas, Serratia, Azotobacter, Azospirrillum* and *Bacillus* are dominant driving forces in recycling soil nutrients, diminishing the dependence on chemical fertilizers and consequently crucial for soil fertility (**Glick et.al.,2012**). However, weed rhizospheric soil have a pivotal role in agriculture as various PGPR belonging to *Serratia, Pseudomonas, Azotobacter, Burkholderia* have been reported from the agricultural fields of Bihar where weeds grow in abundance alongwith main crop .Rice is indigenous cereal crop of Bihar and is the most important daily dietary staple food for nearly 3 billion people. Rice is the dominant crop with 3550 Kg/ha productivity. On the basis of huge productivity, it plays an important role in socio- cultural life and economy of Application of chemical fertilizers has always been the main approach to promote cultivation of rice in agricultural field of Bihar. If the consumption trend continues with rising human population, demand for rice production also increases. To meet the demand of rice yield, overuse of fertilizers is applied by the farmers which can cause unanticipated environmental impacts by deterioration of the soil health and diminished microelements(Fe, S, Zn) in the soil. Iron is the fourth most abundant mineral nutrient after N, P, K in the earth's crust; physiologically indispensable most limiting element for Rice production as well as growth and development but readily unavailable in the soil. Iron bioavailability in soil and plant surface boost rice productivity and enhance proper growth and development of Rice plant. Rice require higher concentration (10-6) of iron content and when plant cells detect concentration below this threshold, (**Miethke et.al.,2007**). Micro-organisms play a pivotal role to fulfil the supply of micronutrients and reducing the dependence on chemical fertilizers (**Adesemoye et.al.,2009**). Micro-organisms have been intentionally introduced into crop soil and rhizospheric region in attempts to provide nutrient supply and improvement of soil health(**Lynch et.al.,1981**). Of this, Siderophore production is an important phenomenon in the rhizosphere that enhance nutrient availability to the host plant by sequestration of Fe3+ ion from the soil by

the help of *Azotobacter* and *Azospirillum* isolates. ***Azospirillum*** and ***Azotobacter*** are gram negative dynamic rhizobacteria present in the cereal plant and also were exploited from weed rhizosphere. Weeds are undesirable, persistent and competitive grass grow with main plant that hinders optimal crop productivity and quality by making soil nutrient deficient. *Marsilea quadrifolia, Cynadondactylon, Eclipta alba* and *Cyprus iria* were major weeds in Bihar agricultural field of rice that affect the growth and yield of rice cultivar. However, weed rhizospheric soil is hub of *Azotobacter, Azospirillum* and *Pseudomonas sps* which exhibit a significant role in agriculture as they have been reported from agricultural fields of Bihar and promote growth of rice plant. Most efficient and common PGPR in the weed rhizospheric soil of Rice field is Azotobacter and *Azospirillum* which stimulate development and growth of rice plant and enhance nutrient availability to the host plant (rice) by various biochemical process like Biological Nitrogen Fixation, Phosphate Solubilisation, Siderophore production, Phytohormones synthesis (**Bhardwaj et.al.,2014).** *Azospirrillum and* Azotobacter are gram negative PGPR contribute to promote the growth of rice cultivar by sequestration of iron through Siderophore production. Siderophore are specific iron chelating compound with low molecular weight (below 2000Da) **(Budzikiewicz.2010**). Siderophore are water soluble organic compound produced by PGPR which chelates iron to acquisite Fe2+ for plants.Siderophore are specific iron chelating compound with low molecular weight (below 2000Da) (Budzikiewicz.2010). Siderophore are water soluble organic compound produced by PGPR which chelates iron to acquisite Fe2+ for plants.

To satisfy the iron necessity in rice fields, isolates of ***Azotobacter*** and ***Azospirrillum*** are found to be Iron chelating PGPR. Isolates like *Azotobacter* and *Azospirrillum* are found to be reputed/ putative assistant of Siderophore production that can shift virgin land to fruitful land by replenishment of Fe level in rice field. Siderophore forms soluble Iron bound **Siderophore complex [Ferri- Siderophore Complex]** from insoluble Fe-compounds and makes it available to the root of rice plant. Ferri-Sidero complex promote growth and development of Rice plants as well as provide nourishment to the agricultural soil for soil health and intensive fertility under limited environment in rice cultivation.

As a consequence of aforementioned asset, Weed grass has been selected as its growth is untied of any extrinsic supply of chemical fertilizers and its rhizospheric zone is crowded can with vigorous Fe-Chelating PGPR isolates. Such isolates also exhibit probable PGP attributes like Potassium-Solubilisation, Phosphate-Solubilisation, Nitrogen fixation and IAA production. Considering that, present analysis explores the new dimension for isolation of Fe- chelating PGPR with Siderophore producing ability from weed rhizospheric soil grown in rice fields of Bihar and figure out their effects on Fe improvement and PGP promotion in agricultural field.

METHODOLOGY

{1} **Rhizospheric sample collection**:

In the present study a total of 9 rhizospheric soil samples were collected from weed grass grown in the different Rice fields of gaini village, Aurangabad district of Bihar. Each sample were collected randomly during month of November-December from rhizospheric zone of about 0-10cm depth. All samples were kept in aseptic plastic baa, brought to the lab and stored in 4 degree celcius or further processing. Samples were also analysed for physicochemical test for pH, soil, chemical & texture**(Table 1.0)**

{2} Isolation of Fe chelating PGPR strains:

Different isolation methods was used to isolate Iron chelating bacterial isolates particularly *Azotobacter & Azospirrillum* strain from weed rhizosphere.

{a} Serial dilution technique:

10 gm of rhizospheric soil samples were weighted out and dissolved in 90ml of physiological saline(0.85% NaCl solution) shaked vigorously to homogenized soil suspension. Also made a subsequent 8 fold serial dilution upto10-8 of soil suspension.

{b} Enumeration of Fe chelating isolates (Spread Plating method)

About 1ml successive dilutions of 10-3,10-5, 10-6 and 10- n selective media plaes seeded with Azotobacter agar media & Azospirrillum media for isolation of *Azotobacter* & *Azospirrillum* isolates respectively.The plates were incubated for 5-7 days at room temperature(28+_2degree c) for growth. (**Subba Rao, 1994**). Bacterial culture were repeaed thrice for pure Siderophore producing bacteria isolates. All the pure ***Azospirrillum*** & ***Azotobacter*** isolates were isolated on Azotobacter agar media and Azospirrillum agar media and appeared as a individual colony that were maintained as a pure culture in their respective slant at 4degree c and in 25% glycerol stock solution at-80degree c for identification of islates.

{c} Qualitative estimation of Siderophore production:

Isolates were tested for production of Iron chelating compound(Siderophore) qualitativel.

{3} Identification of Fe chelating isolates:

{a} Morphological Identification of isolates: Fe chelating bacterial isolates were morphologically identified through colony characteristics; shape, size, consistency(texture), opacity,pigmentation & Gram's reaction as per the standard procedures.(**Barthalomew & Mittewar;1950**).

{b} Biochemical Identification of isolates:

Selected Bacterial isolates were bochemically identified by performing different activities like citrate utilization test, catalase test, MR test, VP test, Indole test. (**Cappuccino & Sherman,1992**)

{4} In Vitro screening of isolates for PGP traits;

{a} Ability of Siderophore production by Fe chelating isolates: For detection of Sideropore producing ability of PGPR , Universal Blue CAS agar method were used.(**Schwyn & Neilands 1987**) Bacterial isolates were spot-inoculated on **CAS(Chrome-Azurole Sulphonate) agar** media and incubated in incubator at room temperature for 5-7 days andthen observed for Halo zone around colony growth. This test was done in tripilicates and the diameter of halozone was measured in cm.

{b} Ability for Tricalcium phosphate (TCP) Solubilization by isolates:

Ability of P-Solubilization by Fe chelating bacterial isolates were done spot-ioculation of **PVK MEDIA** ON THE AGAR PLATES, Having bacterial isolates. The plates were incubated for 4-5 days under room temperature and were observed for production of clear Solubilization zone around the bacterial colonies.(**Pikovaskaya, R.E.,1948**). This test was also done in triplicates and the diameter were measured in mm and calculated by PSE(Phosphate Solubilization Efficiency) formula;

PSE= S/Tx 100

Where, S= Solubilizationzone and total colony diameter of bacteria

T=Total diameter of Bacterial colony.

Results and discussion

In the rice field of Bihar, rhizospheric zone of weed grass provide a novel substitute for isolation of some Fe chelating PGPR with Siderophore producing ability which were *Azotobacter*and *Azospirrillum*. These PGPR were confirmed as Fe chelating PGPR on the basis of enumeration of isolates on selective Azotobacter agar media and Azospirrillum agar media and were recognised by morphological and biochemical identification and were further screened for PGP attributes to acquisite iron necessity in the rice field.

1) Rhizospheric Sample Collection

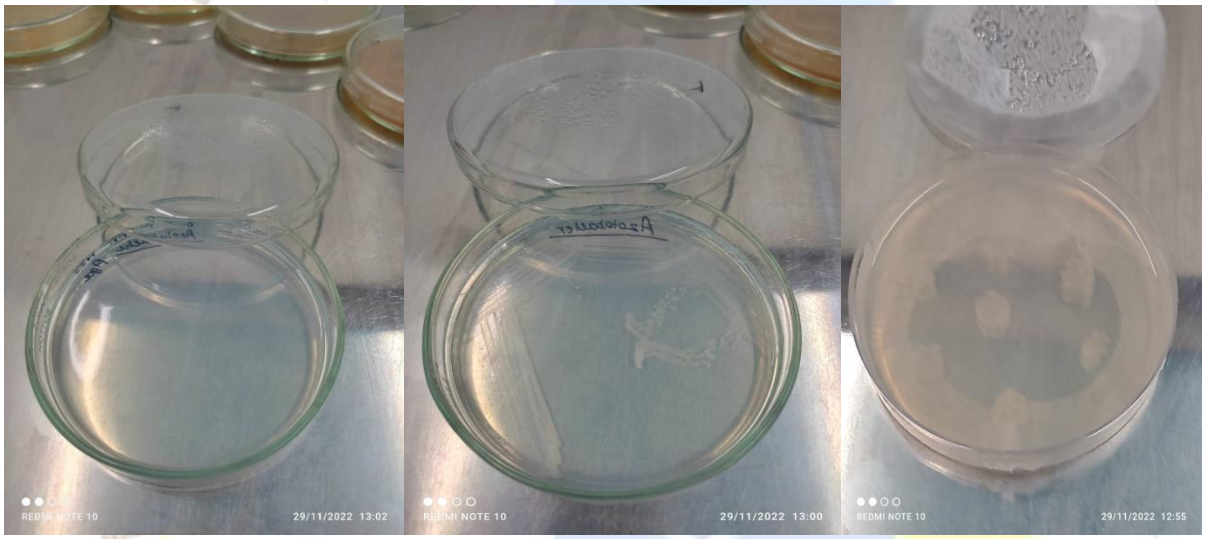
Overall 9 rhizospheric soil samples of common weed grass were selected randomly that grown ubiquitously in the different Rice fields of Gaini village, Aurangabad district (Bihar). All soil samples were isolated at the depth range of 0-10cm mixed thoroughly to make composite sample and kept in the laboratory conditions within aseptic plastic bags. Physiological analysis of all the 9 samples were done.(Table 1.0)

Composite Rhizospheric Soil Sample	PH	Electrical Conductivity	Organic Carbon (%)	N (Kg/Ha)	P (Kg/ha)	Fe ppm	Zn ppm	Cu ppm	Mn ppm
1 to 9	6.06	0.200	0.34	246	32	9.33	0.64	1.49	7.08

Table 1.0 :Physiochemial analysis of composite soil sample of weed rhizosphere

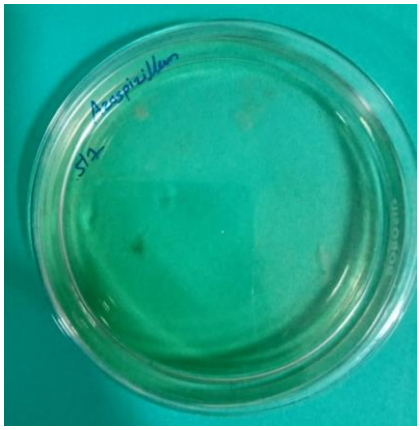
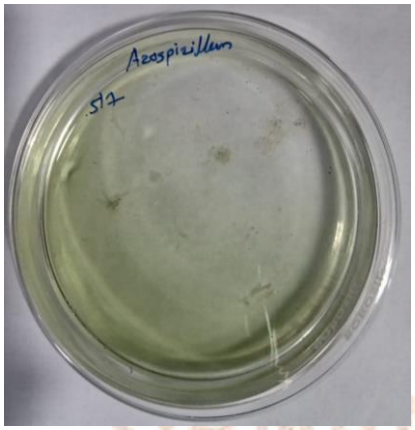
2) Isolation of Fe chelating PGPR isolates:

Total of **23** bacterial isolates were obtained from 9 composite rhizospheric sample by Serial dilution (upto 10-8 folds) and pour plating method on NA media. All the 23 Isolates were designated as SWR-1, SWR-2, SWR-3.....SWR-23. For isolation each *Azotobacter* and *Azospirrillum* isolates were transferred successively to selective Azotobacter agar media and Azospirrillum agar media on separate culture plates respectively using spread plating technique.



Azotobacter Agar *Azotobacter* Organism on Media Azotobacter showing watery colony

Bacterial isolates culture were repeated thrice for pure isolation and incubated for 4-6 days. After 4-6 days of incubation, a total **11** pure isolates were observed on media plates. Out of 11 isolates, 5 strains were segregated on Azospirrillum agar media plates and 6 isolates were confined on Azotobacter agar plates which were identified as *Azospirrillum* isolates (**5**) and *Azotobacter* isolates **(6**) based on morphological and biochemical test.


Azospirrilum Agar Media *Azospirrilum organism on media* *Colony growth on Azospirrilum Agar*

All the *Azospirrillum* and *Azotobacter* isolates were designated by a specific code.(**Table 2.0**)

Isolate Code	**Name of Selective Media**	**Code for bacterial Isolates**	**Indicated isolates**
SWR – 1	Azotobacter agar media	AZTBR-1	*Azotobacter* spp.
SWR – 2			
SWR – 3	Azospirrilum agar media	AZSPM – 3	*Azospirrilum* spp.
SWR – 4	Azospirrilum agar media	AZSPM – 4	*Azospirrilum* spp.
SWR – 5			
SWR – 6			
SWR – 7			
SWR – 8	Azotobacter agar media	AZTBR - 8	*Azotobacter* spp.
SWR – 9	Azotobacter agar media	AZTBR – 9	*Azotobacter* spp.
SWR – 10			
SWR – 11			
SWR – 12			
SWR – 13	Azospirillum agar media	AZSPM – 13	*Azospirillum* spp.
SWR – 14			
SWR – 15			
SWR – 16			
SWR – 17	Azotobacter agar media	AZTBR-17	*Azotobacter* spp.
SWR – 18			
SWR – 19	Azotobacter agar media	AZTBR-19	*Azotobacter* spp.
SWR – 20	Azospirillum agar media	AZSPM – 20	*Azospirillum* spp.
SWR – 21			
SWR – 22	Azotobacter agar media	AZTBR – 22	*Azotobacter* spp.
SWR – 23	Azospirillum agar media	AZSPM – 23	*Azospirillum* spp.

Table 2.0 : Isolation & Implication of Isolates on selective media.

* Qualitative estimation of Siderophore production:

All the 11 bacterial isolates; 6 isolates of *Azotobacter* (AZTBR-1,8,9,17,19& 22) and 5 isolates of *Azospirrillum*(AZSPM-3,4,13,20& 23) were assayed for production of Iron chelating compound i.e. Siderophore qualitatively. 0.5% of culture supernatant was suspended to 0.5 ml of FeCl3 solution (0.2%) and incubated for 2 days. Emergence of orange or reddish brown colour change after incubation period estimated the ability of Siderophore formation by *Azotobacter* and *Azospirrillum isolates*.(Yeole&Dube, 2000).

3) Identification of Fe chelating isolates:

Isolated *Azotobacter*(6)& *Azospirrillum* (5) strains were identified as Fe chelating PGPR due to morphological and biochemical analysis using standard protocols.

{A} Morphological Identification of isolates:

11 bacterial isolates obtained from weed rhizospheric soil samples were morphologically identified as *Azotobacter sps*(6) and *Azospirrillum sps*(5) by studying Gram's reaction, Colony Shape, nature, colour, opacity, surface and margin (Table3.0)

SL.NO	**Selected bacterial isolates**	**Code for Isolates**	**Gram's Reaction**	**Colony Shape**	**Colony Nature**	**Colony Colour**	**Opacity**	**Surface**	**Margin**
1	AZTBR – 1	S1	Negative	Spherical	Glistening	Milky White	Untransparent	Smooth	Entire
2	AZSPM – 3	S3	Negative	Irregular		Gray white	Transparent	Raised	Slightly curved
3	AZSPM – 4	S4	Negative	Rod		Gray white	Transparent	Raised	Slightly curved
4	AZTBR – 8	S8	Negative	Spherical	Unglistening	Milky White	Untransparent	Smooth	Entire
5	AZTBR – 9	S9	Negative	Oval	Unglistening	Milky White	Clear Transparent	Smooth	Entire
6	AZSPM – 13	S13	Negative	Rod		Brown	Untransparent	Smooth	Slightly curved
7	AZTBR – 17	S17	Negative	Spherical	Unglistening	Yellow	Clear Transparent	Smooth	Entire
8	AZTBR – 19	S19	Negative	Oval	Glistening	Milky white	Untransparent	Smooth	Entire
9	AZSPM – 20	S20	Negative	Rod		Brown	Transparent	Smooth	Slightly curved
10	AZTBR – 22	S22	Negative	Oval	Glistening	Yellow	Untransparent	Smooth	Entire
11	AZSPM – 23	S23	Negative	Irregular		Brown	Transparent	Flat dense	Slightly curved

Table 3.0 : Morphological Identification of Isolates

B} Biochemical Identification of isolates:

The biochemical test of 5 **isolates of *Azospirrillum* S3, S4,S13,S20 &S23 and** 6 **isolates of *Azotobacter* S1,S8,S9,S17,S19&S22 were performed by IMVIC test, catalase test(Table4.1)**

a) Catalase test: 11 bacterial isolates were transferred with sterile inoculation loop on 11 different slide and 1-2 drop of H2O2 were added. Effervescence were observed on all the 5 **plates of *Azospirrillum* S3, S4, S13, S20 &S23 and** 4 **of *Azotobacter* Isolates S1, S8, S17,& S19 were positive for catalase test while 2 isolates of *Azotobacte*r; S9,& S22 showed negative Catalase reaction.**

(b) MR test: 3 isolates of *Azotobacter*(S17, S19and S22**)& all** 5 ***Azospirrillum* isolates (**S3,S4, S13,S20&S23)**forms red colour exhibited positive result to MR test while no Colour formed by 3 isolates of *Azotobacter* (** S1,S8 & S9**) exhibited negative MR test.**

(c) VP test: 4 *Azotobacter*strain(S9, S17, S19 &S22) and all *Azospirrillum* isolates (S3, S4, S13, S20 &S23) were negative for VP test and 2 Azotobacter strain(S1& S8) were positive for VP test.

(d) Citrate utilisation test: 5***Azotobacter*strain (**S1, S8, S9, S17 &S22**)** were positive for citrate utilisation while one isolate; S19 was negative for citrate test. No any Azospirrillumstrain were negative for citrate test.

This test confirms the occurrence of Fe chelating PGPR particularly *Azotobacter*(6)&*Azospirrillum*(5) from weed rhizospheric soil samples**.**

Sl.No.	Isolate Code	Catalase	MR Test	VP Test	Citrate Utilization
1	S1	+ VE	-	+	+ VE
2	S3	+ VE	+	-	+ VE
3	S4	+ VE	+	-	+ VE
4	S8	+ VE	-	+	+ VE
5	S9	-VE	-	+	+ VE
6	S13	+ VE	+	-	+ VE
7	S17	+ VE	+	-	+ VE
8	S19	+ VE	+	-	-VE
9	S20	+ VE	+	-	+ VE
10	S22	-VE	+	+	+ VE
11	S23	+ VE	+	-	+ VE

Table 4.1 : Biochemical Identification of Isolates

4) In vitro screening of isolates for PGP attributes:

All the 23 **isolates obtained from 9 composite samples were screened for PGP trait analysis.**

(a) Siderophore production ability:

All the 23 isolates were spot-inoculated on CAS agar media for testing Siderophore production ability. Out of 23 isolates, a total of 11 isolates; 6 of *Azotobacter* designated as S1, S8,S9,S17,S19 &S22 and 5 isolates of *Azospirrillum* designated as S3 ,S4 ,S13 ,S20& S23 were observed to form orange halozone around the bacterial colonies. Out of 6 isolates of*Azotobacter* strain, 3 isolate S9,S19 & S22 represent highest ability (+++) of Siderophore production on CAS (Chrome-azuroleSulphonate) agar media by formation of clear orange halozone of 2-3 cm diameter range around the bacterial colony. 2 isolates; S17 & S20 were seen to have moderate (++) ability of Siderophore production with 1-2 cm diameter of halozone; one isolate coded as S8 have low(+) Siderophore producing ability with diameter range 0.1-0.9cm**.**

Out of 5 isolates of *Azospirillum* , 3 isolated code S4, S13 & S23 formed orange halozone of 2-3 cm around bacterial colonies on CAS agar media representing highest ability (+++) of Siderophore production while 2 isolates S3 & S20 form orange halozone of 2-3 cm representing moderate (++) ability of Siderophore production **(Table 4.2)**

Bacterial Isolates	**Code for Azospirillum Isolate**	**Code for Azotobacter Isolate**	**Code for Siderophore Production**	**Orange halo Zone**	**Dia meter of halozone (cm)**
SWR-1		AZTBR – 1	S1	++	1.60
SWR - 2					
SWR – 3	AZSPM-3		S3	++	1.62
SWR – 4	AZSPM-4		S4	+++	2.30
SWR -5					
SWR -6					
SWR -7					
SWR -8		AZTBR – 8	S8	+	0.25
SWR -9		AZTBR – 9	S9	+++	2.80
SWR -10					
SWR -11					
SWR -12					
SWR -13	AZSPM- 13		S13	+++	2.65
SWR -14					
SWR -15					
SWR -16					
SWR -17		AZTBR – 17	S17	++	1.35
SWR -18					
SWR -19		AZTBR – 19	S19	+++	2.78
SWR -20	AZSPM-20		S20	++	1.38
SWR -21					
SWR -22		AZTBR – 22	S22	+++	2.72
SWR -23	AZSPM- 23		S23	+++	2.68

Siderophore Producing ability:

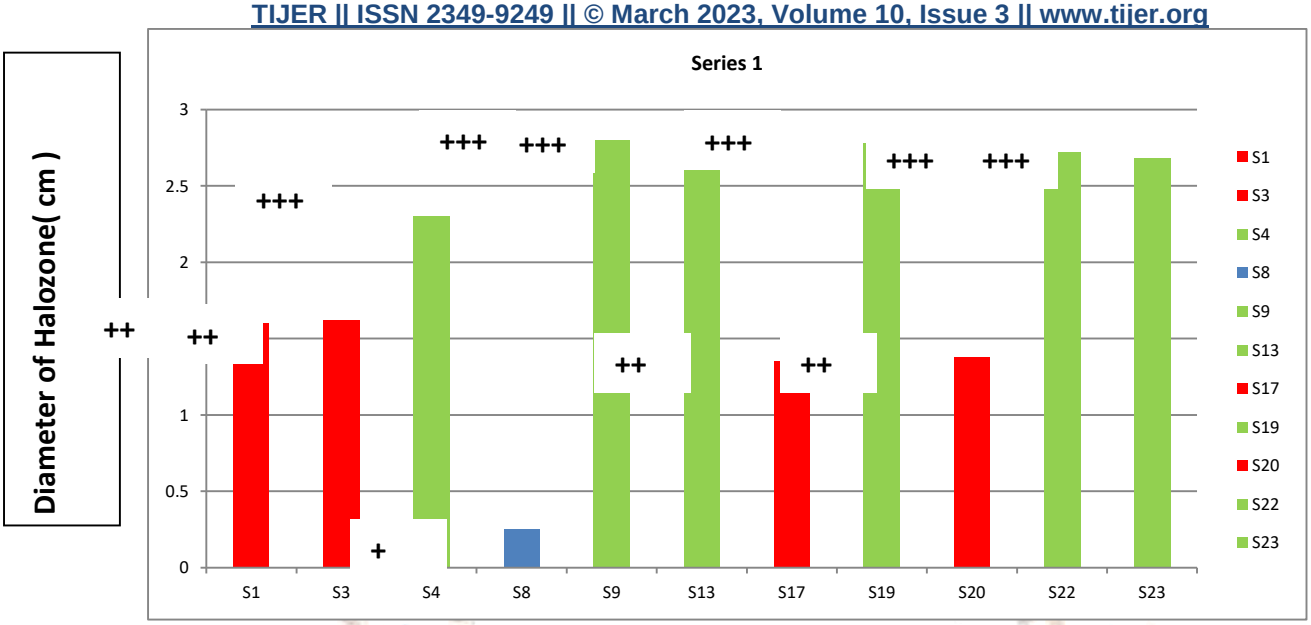
+++ **: Highest Siderophore Production**

++ : Moderate **Siderophore Production**

+ **: Low Siderophore Production**

Table 4.2: Screening of Bacterial Isolates for Siderophore Production

Total 11 isolates of 23 PGPR isolates have a considerable affinity for Fe chelating substance i.e. Siderophore production by forming orange halo zone around bacterial colonies on CAS agar media with diameter range 0.25 to 2.80 cm **(fig.4.3)**



Fe Chelating Isolates code with Siderophore ability

+ - **Hazy halo zone; Low sid. Prod. (0-0.9 cm)**

++ - **Clear halo zone; Moderate sid. Prod. (1.0- 1.9 cm)**

+++ - **Strong halo zone; Maximum sid. Prod. (2.0 – 2.9 cm)**

Table 4.3 : Comparison of Orange Halozone formation by Isolates

(b) TCP solubilization ability shown by Fe chelating isolates:

11 Fe chelating isolates of*Azotobacter* (6; S1, S8, S9, S17, S19 &S22) &*Azospirrillum* (5; S3, S4, S13, S20 & S23) were screened for TCP solubilization ability on PVK agar media. Out of 6 *Azotobacter* strain 2 isolates; S9 & S19 exhibited peak PSB zone(+++) i.e. greator than 5 mm and 2 *Azospirrillum* isolates;S13& S23 often peak PSB zone (+++) greater than 4 mm**.(Table 5.1, fig.5.2)**

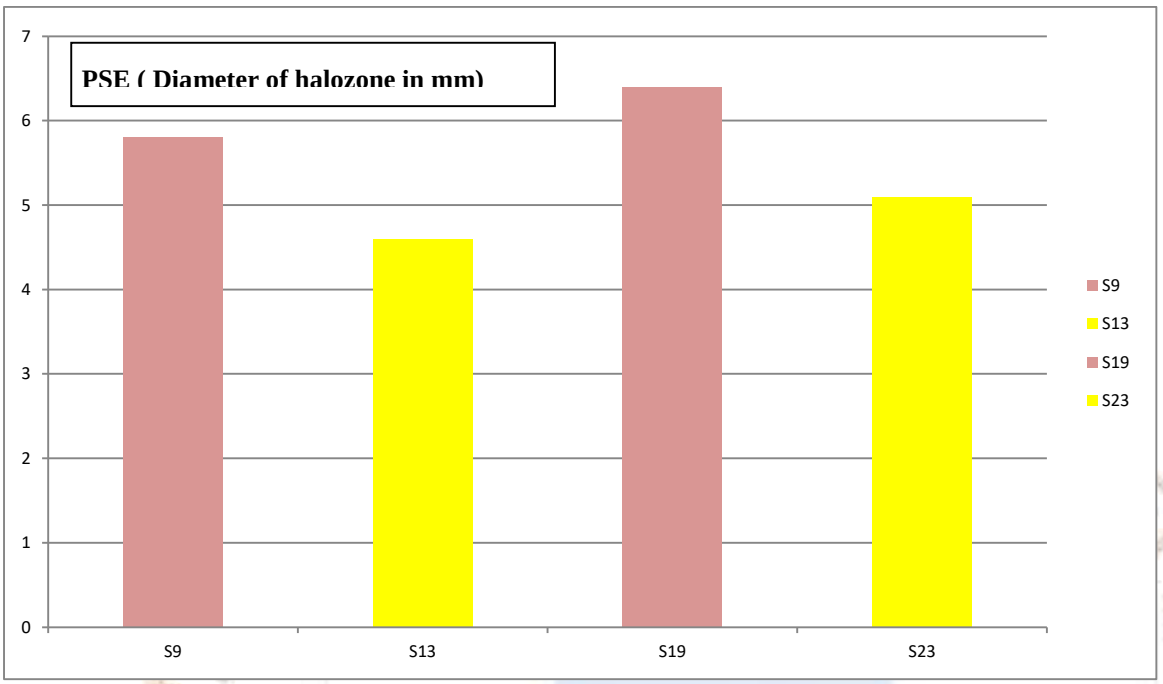
Isolates Name		Code for Isolates	Efficient Halozone Formation	PSE (Diameter of halozone mm)
Azotobacter	Azospirillum			
AZTBR – 1		S1	-	
	AZSPM – 3	S3	-	
	AZSPM - 4	S4	-	
AZTBR – 8		S8	-	
AZTBR - 9		S9	+++	5.8
	AZSPM - 13	S13	+++	4.6
AZTBR - 17		S17	_	
AZTBR - 19		S19	+++	6.4
	AZSPM – 20	S20	-	
AZTBR – 22		S22	-	
	AZSPM - 23	S23	+++	5.1

TCP Solubilizationactivity :

+++ : Peak clear halozone

- : No halozone

Table 5.1 : Screening of Fechelating Azotobacter&Azospirillum isolates for TCP –Soluilization



TCP Solubilizing Isolates Code

: Azotobacter isolate

: Azospirillum isolates

Fig 5.2: Comparison of TCP solubilisations zone formation of Azotobacter & Azospirillum isolates.

Conclusions:

Based on the results, the chemical composition of weed rhizospheric soil contain an average % of organic carbon(0.34%), Nitrogen(2.46%), , Phosphorus(0.32%),Iron(0.933%) and trace amount of cu(1.49%), Zn(0.64%)&Mn(7.08%) that triggers soil health & enhancement and also promotes growth and productivity of rice plant.The Iron content (9.33kg/ha) in the rice field affects other micronutrient in promotion of plant growth by pigment formation, Iron helps the Mg ion in photo synthesis ultimately enhance crop productivity. The successful isolated rhizobacterialstrain from weed rhizospheric zone were ***Azotobacter*** and ***Azospirrillum***which were considered as potent Fe chelating PGPR . Such strain were isolated on their selective media and identified on their qualitative Siderophore assay and morphological, as well as biochemical characterization. Isolated **S9, S13, S19**&**S23** strains were influencing Fe chelating bacteria of which**, S9&S19** strain belongs to *Azotobacter spp*. &**S13 & S23** belongs to *Azospirrillumspp* that scavenge Fe from insoluble Fe compounds of soil and assists Siderophore production in the weed rhizosphere.Consequently provide source of Fe nutrient in the rice field for balancing nutrient availability for rice cultivar and also maintain soil health.

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