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BIO-CONTROL ACTIVITY OF PLANT GROWTH PROMOTING RHIZOBACTERIA ON *SCLEROTIUM ROLFSII*

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ABSTRACT

Crop productivity and crop improvement are colloidal components as the demand of the increasing population, worldwide for the provision of food from crops require dedicated agricultural strategies that tend to lean on natural, available and, beneficial, easily reproducible means of products. In general, the soil components rich in organic matter that can avail rich microbial community initiates agricultural productivity in abundance and in the way to deduce it. But, commercially available chemical pesticides, pollution in the environment, biotic and abiotic constituents are found to be the key components that stress the crop productivity. This can be overtaken by the microbes that can function as both "bio-fertilizer" and "antagonistic" agents, mentioned as Plant growth-promoting rhizobacteria(PGPR), as they present satisfactory, advantageous impact when ever required, due to their presence in the rhizospheric region, by providing nutrients uptake from soil and controlling the unnecessary hazardous bio-impact on plants .Present study relies on sustainable agricultural development that utilizes the bacteria from the rhizospheric region thereby recommending bio-formulation in the future to mobilize the unaware farmer for better productivity, free of devastating chemical components that enter the food chain via crop produced by using chemicals, and also by easy means without affecting the surrounding environment and human health. In this context, *Sclerotium rolfsii*, a deleterious pathogen that affects groundnut crops predominantly, how best can be prevented and can be suppressed by using beneficial PGPR is been studied.

Keywords: Bio-inoculants, Rhizosphere, Pathogen, Bio-control, Antagonist.

INTRODUCTION

PGPR colonize root region, boosting development of plant by improvising the intake of the required nourishment and micronutrients from soil. These microbes fix the atmospheric nitrogen, solubilize phosphorus, produce siderophores for iron, organic acids, ammonia, etc. In many cases, these are involved in the release of antibiotics, HCN (Glick, 2012) that suppress the expansion of pathogens within the surrounding environment. Some commonly reported PGPR genera exhibit plant growth-promoting activities are *Bacillus, Pseudomonas, Azospirillum, Burkholderia, Azotobacter, Enterobacter, Erwinia, Rhizobium, Mycobacterium, Pseudomonas syringae, Flavobacterium* (Maurhofer *et al.,* 1994). PGPR produce signals that communicate with other cells within themselves and with other forms of microbes of that community, called quorum-sensing (QS) signals. They participate in expressing gene coordination until particular quorum is obtained and also to monitor their density (Fuqua *et al.,* 1994).Studies on this context have shown that PGPR can change activities of enzymes in the root that help in the production of flavonoids and other metabolites, that bring forth alterations to root exudation chemical nature. PGPR reduce disease incidence by triggering defense in-plant has been reported by several workers. PGPR can suppress the disease in plants by triggering induced systemic resistance (ISR) (Bhattacharyya and Jha, 2012). It is denoted by a term called ISR proposed by Kloepper and later supported by (Pieterse *et al.,* 1996). ISR requires essential endogenous signal molecules, Ethylene (ET), Jasmonic acid (JA) which expresses the grouping of defense-related enzymes and defense-substances for a structural and chemical barrier against the bacterial pathogens, other than PR-proteins (Van Loon, 1999).

ISR is named as Jasmonic acid Ethylene (JA-ET) dependant pathway. PGPR mediate ISR which is subjected to the expression of enzymes that are completely related to defense and defense chemicals in the host plant against pathogen. (Kloepper et al 2004). While, SAR is termed as 2-hydroxybenzoic acid (SA) dependant pathway, necrotizing pathogen-mediated systemic acquired resistance which is liable for expression of pathogenesis related proteins (PR-proteins) playing a direct defensive role against a pathogen. Some of the biotic and abiotic inducers/elicitors are reported by several workers that induce both the kinds of signal transduction in numerous crops. So, for management of diseases in plants now it is possible and is important for early detection of pathogen and early delivering signal information for ISR (intracellularly/ intercellularly) to plant for activation of defense machinery, against the pathogen (Shibuya and Minami, 2001); (Rasool *et al.,* 2020).

MATERIALS AND METHODS

Isolation from Rhizospheric soil

Soil from the root regions of the groundnut plants, wild plants, and marine plants was collected by carefully

Table 1 . Screening methods applied

Screening tests	Media used	Incubated at 30-40°C	Reagent used	Reference
Nitrogen fixation	Jensens media	2-3 days	-	Rokhzadi A et al, 2008)
Phosphate Solubilisation	Pikovskaya's Agar Media	4 days	-	(T. Karpagam et al, 2014)
Ammonia Production	Peptone Water	72 hr	Nessler's	(Cappuccino Sherman, 1992)
Organic acid Production	Pikovskaya's Agar Media	4 days	Methyl Red Indicator	(T. Karpagam et al, 2014)
HCN Production	NM amended with Glycine	4 days	-	Lorck (1948).
IAA Production	NB amended with Tryptophan	48 hshaker	Salkowski	(Etesami *et al.,* 2014)
Siderophore	CAS agar media	5-7 days	-	Kadam etal 2010)

Fig 1 Antagonistic activity of Bacillus sps against Sclerotium

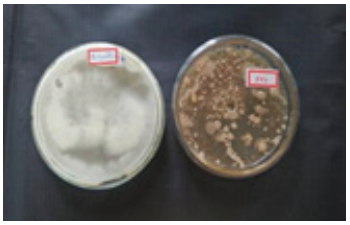

Fig 1.1 Effect of ZMRJ5 on Sclerotium (right): control with Sclerotium alone(left)

Fig 1.2 Effect of ZMRJ9 on Sclerotium (right): control with Sclerotium alone(left)

Fig 1.3 Effect of ZMRJ5+ ZMRJ9 on Sclerotium (right): control with Sclerotium alone(left).

Fig 2 PGPR strains on growth promotion of Groundnut plant pot trials

Fig 2.1 Pots from left to right-Control(Negative),Control(Positive using commercial PGPR formulation), ZMRJ5 inoculated, ZMRJ9 inoculated, ZMRJ5+ZMRJ9 inoculated.

Fig 2.2 From left to right Control(Negative), Control(Positive using commercial PGPR formulation), ZMRJ5 inoculated, ZMRJ9 inoculated, ZMRJ5+ZMRJ9 inoculated.

separating the soil particles and were subjected to serial dilutions. Later, were spread on sterilized Nutrient agar media plates, and then incubated at 30°C for 24-48 hrs.

Screening *in-Vitro* for PGPR potency

The following screening tests were conducted to evaluate the PGPR potency of the isolates *in-vitro*, using the standard methods employed by many researchers in the field.

Identification using 16S RNA sequencing

Based on 16S RNA gene sequencing of the bacteria along with morphological analysis and biochemical tests, potent PGPR isolates were identified

Molecular characterization

Identification of bacteria using 16S rRNA analysis method was accomplished by 3b biotech solutions, Gujarat. First, the bacterial genome was isolated using an insta isolation kit. Using 16S r RNA Universal primers, the full-length gene was amplified, and then purified PCR product was sequenced. Sequencing reactions were performed. Aligned sequence data were analyzed using BLAST technology, using NCBI BLAST similarity search tools. Multiple alignment sequences were obtained. Phylogeny tree was obtained using Tree dyn 1983. The bacterial strain was deposited in GenBank, NCBI, and accession numbers were obtained.

Bio-control assessment *in-vitro*: Bacillus Vs Pathogen

Before the bio-control activity assessment, compatibility between both the identified isolates was assessed. After compatibility check, bio-control activity of the potent PGPR against pathogens of groundnut was tested in-vitro. Consistent with the well diffusion method (Magaldi et al 2004) 1mL of *Sclerotium* pathogenic suspension is dispersed and is spread on the PDA agar surface; holes of 1 cm were aseptically made with a sterile corn borer. The bacterial suspension having (cfu $2x10^{-8}$ /mL) was poured into the wells. Control with pathogen is maintained as positive control. Then, for five days, incubated at 25 ± 10^0C, the bacterial bio-agent diffuses within the agar medium and inhibits the expansion of the pathogen.

Pot trails

For growth promotion

Inoculum preparation

Table 2 Impact of PGPR strains on growth promotion of Groundnut plant, while in experiment

PGPR strains	Plant Tallness (cm)	Shoot size (cm)	Root size (cm)	No of leaves	Shoot Wt fresh (cm)	Root Wt Fresh (cm)	Shoot Wt dry (cm)	Root Wt dry (cm)	Chlorophyll (Spad units)
Control -ve	20.20^{a}	11.20^{a}	9.00^{a}	11.67^{a}	9.50^{a}	7.73^{a}	1.30^{a}	0.91^{a}	20.42^{a}
Control +ve	30.50^{b}	20.50^{b}	10.10^{ab}	16.00^{b}	13.57^{b}	11.50^{b}	2.50^{b}	1.23^{b}	34.68^{bc}
ZMRJ5	31.97^{bc}	20.47^{b}	11.63^{bc}	21.33^{c}	14.73^{b}	11.87^{b}	2.47^{b}	1.29^{bc}	36.23^{d}
ZMRJ9	33.30^{c}	20.80^{b}	12.33^{c}	21.33^{c}	16.37^{c}	13.23^{c}	2.80^{b}	1.37^{c}	35.25^{bc}
ZMRJ5+ZMRJ9	36.50^{d}	22.30^{b}	14.37^{d}	23.33^{d}	19.17^{d}	17.97^{d}	3.27^{c}	1.75^{d}	37.28^{d}

Table 3 Effect of strains exhibiting biocontrol activity on Groundnut plant under sick pot trials

Isolates	Days	% seed germination	Shoot length in cm	Root length in cm	No. of leaves per plant	Chlorophyll content in mg/g	Vigour index
ZMRJ5	15 days	80	20.43	12.09	22.67	0.36	1646.72
	30 days	80	24.06	13.44	26.67	0.45	1938.5
	45 days	80	26.16	16.61	35.33	0.54	2109.68
	60 days	80	29.09	18.64	42.67	0.57	2345.84
ZMRJ9	15 days	80	20.46	12.65	23.33	0.36	1649.69
	30 days	80	22.45	14.1	31.33	0.45	1810.1
	45 days	80	26.28	17.05	35.33	0.53	2119.44
	60 days	80	28.45	18.85	43.33	0.57	2294.84
ZMRJ5+ ZMRJ9	15 days	80	21.39	12.65	23.33	0.37	1710.4
	30 days	80	23.74	14.1	26.67	0.45	1912.5
	45 days	80	26.58	17.05	34	0.57	2143.46
	60 days	80	33.12	18.85	44.67	0.67	2667.64

Fig 3 Effect of strains exhibiting biocontrol activity on Groundnut plant under sick pot trials (Left-Control with pathogen, Right-ZMRJ5+ZMRJ9 inoculated.

The inocula to be used in pot trials were prepared using suitable culture medium. Then required measure is taken to 250ml screw cap bottles with broth culture and allowed to grow in them on a rotator (95 rpm) at 27ºC for twenty four hours. This suspension of bacteria with 10^{8} CFU ml/1 in sterile H_2O is sufficient. Seeds to be used are now to be sterilized on the surface using 95 percent ethanol and soaked in 0.2 percent $HgCl_2$ solution for 3 min, followed by baths of steril water 5 times.1 mL of overnight developed bacterial culture was applied on each seed.1ml culture (10^{8} cells) of each isolate was delivered as inoculum on the groundnut seeds placed on tissue and were packed in an airtight bag for 3 days and is allowed to germinate in it. A Positive Control (with the commercially available PGPR), and negative control (with sterile water) is maintained similarly. After 3 days, seeds are transferred onto the pots and everyone is supplied with sterilized water daily. After every week, 2-3ml of bioinoculant is introduced again as a booster dose. Data regarding Percentage increase over the control parameters were collected and analyzed statistically (Dipanwita Dasgupta et al 2015).

Evaluation of Growth Performance under Pot Trials

For Pot trials, before filling the pots the soil collected is dried in shade, sterilized repeatedly by autoclaving 3-4 times. The surface-sterilized untreated, treated seeds (control) were transferred to pots at the depth of 5 mm, containing sterile soil. Ensure each pot contains at least of three seeds and likewise in triplicates. These were carefully noticed for any observable changes every day for 15-20 days. After 20 days of duration, the plant was uprooted carefully, packed in polythene bags and is carried to laboratory for further test procedures.

Pot Assay for biocontrol of stem rot

To take a look at the biocontrol potential of bacterial antagonists *in vivo* towards *Sclerotium rolfsii,* a pot assay was set up. Potato Dextrose broth is used for culturing of *S. rolfsii* at room temperature for three weeks (Ordentlich *et al.,* 1988) and then the mycelia of sclerotia produced have been used in poor health pots. This experiment is to be carried out in three pots each as triplicates. All pots had been first disinfected with 5 p.c $CuSO_4$ solution. First set (Positive Control) of three pots, used to be filled with one hundred fifty gm sterilized soil sand (1:1) combination with sclerotia of *S. rolfsii* artificially inoculated at 1 *sclerotia*/ gm soil (Fouzia Yaqub and Saleem Shahzad, 2005). The pots containing inoculum had been incubated for 15 days at room temperature, often stirred and watered

for colonization of a fungus in the soil. Then three surface disinfected untreated seeds of groundnut seeds had been sown in pots. The second set (Negative Control) of three pots, used to be inoculated with one hundred fifty gm of sterilized soil sand combination, and three untreated seeds have been sown in pots. The third set (Test) of three pots, used to be crammed with one hundred fifty gm of sterilized soil sand combination artificially inoculated with sclerotia of *S. rolfsi*i at the rate of 1 sclerotia/ gm of soil. The pots containing inoculum have been incubated for 15 days at room temperature, regularly stirred and watered for colonization of a fungus in the soil and then seeds treated/ soaked with biocontrol agent's for 24 hrs had been sown in pots. All these pots have been stored at room temperature and watered regularly. Percent germination, shoot length, root length, and chlorophyll content material had been recorded after 15, 30, 45, and 60 days. Data is gathered on ailment severity after 60days and is rated thus based totally on the scale provided. Where, 0 for no infection; 1 for 1-25% infection: 2 for 26-50% infection: 3 for 51-70% infection: 4-76-100% infection;

Vigour index of the plant = (Mean shoot size + Mean root size) x Germination (%) Baki et al 1973. Percent Disease control (PDC) used to be calculated by using the following components after 60 days.

The suppression of the disorder used to be calculated primarily based on the ailment severity index.(PDC= (% Disease in check) - (% Disease in Treatment)/ (% Disease in check) x 100)

Statistical analysis

Data collected is analyzed using ANOVA (analysis of variance). Dunkan's multiple range test (DMRT) differentiates the average of the treatment in growth promotion experiments employing SPSS software version 21.0.

RESULTS AND DISCUSSION

Bacterial isolates-Screening and identification results

Nearly fifteen isolates were isolated. all these isolates were obtained from root soil,Among the fifteen isolates, four isolates were found to be potent, after screening. (ZMRJ5 and ZMRJ9) Two of them were the most potent PGPR. These were identified and GenBank has provided the following accession number(s) for nucleotide sequence(s) of the submitted organisms:

Bacillus zhangzouensis-Seq1- MH612945

Bacillus velezensis-Seq2- MH612946

Means are taken from a unique attempt using three commonly treated pots, in which a single pot is represented three plants. The identical alphabet(s) presented in column's each are not significantly different at $p \leq 0.05$. upon testing using DMRT.

Evaluation of Bio-control activity

PGPR, influence plant growth promotion by providing necessary nutrient availability and suppressing the pathogenic community in the rhizospheric zone. The soil region near the root called rhizosphere area is rich with the microbial community as they are attracted towards the root by the root exudates. The beneficial microbes promote the growth of the plant naturally by promoting the uptake of the nutrients, also by solubilizing the unavailable forms of the nutrients and make them available to the plant and combat the deleterious organisms by releasing lytic enzymes, antibiotics, etc. thereby killing them. So, a suitable PGPR formulation must be recommended to reduce pollution by hazardous chemical fertilizers.

In this case, *Bacillus zhangzouensis, Bacillus velezensis*. When used as mixed culture their antagonistic activity is noted to be very effective and inhibition of the *Sclerotium* took place at its earliest In-vitro and is proved to be potent PGPR based on the screening tests. But when used individually, either of the *Bacillus sps* could not promote the plant growth to the extent they can promote when used as mixed culture, the same is the condition with the antagonistic activity.

In pot trials for plant growth

Bacillus zhangzouensis, Bacillus velezensis the mixed culture of both these microbes can promote plant growth to a maximum in a short period. A positive control, which is a commercially available PGPR is maintained for comparison. The extreme growth was noticed in the positive control in the first few days, and also in the 15-20 days. There was a constant growth day by day in the pots of the bio-inoculants used.it reached the maximum height very earlier than the others. However, the growth in the pots of the bio inoculants could compete with PGPR positive control within a short period and reach its maximum by the end of the pot trails experiment than the positive control.

Pot trials for bio-control activity

Later on, the antagonistic activity of these microbes was studied against plant pathogens of groundnut in pots. leaves started wilting easily in the sick pots. But those seeds which are treated with the bio inoculants before planting did not allow the pathogen to proliferate thus, no impact of *sclerotium* was noticed in the sick pots where they were used.in this case, mixed bio inoculants could eliminate the pathogen to a maximum extent than the individual bioinoculants.

The in-vitro results of biocontrol activity have shown more than 60% potency to destroy the myceliaof *Sclerotium.* The bacterial bio inoculants reduced disease symptoms on foliage ZMRJ5+ZMRJ9 proved the declination of disease rate to +controlplants. Statistical analysis proves, there is a significant difference in the means of the variables. It was revealed by data analysis that variations in values % germination, shoot length, root length No. of leaves and

chlorophyll content of the treatment by using *Bacillus sps* as an antagonist on *Sclerotium rolfsii,* that cause stem rot disease in groundnut, was found to be significant at 5% level because the calculated F values (101.189) was found to be more than the Table Value (2.27) which indicates that the results obtained, are not merely by chance but due to the effective treatment.

Disease suppression rate was also noticed in the plants where bio inoculants were used individually. The present study proves that the selected PGPR support in increasing the shoot and root size, fresh wt, and dried wt, chlorophyll content in the plants that are treated.

It can be concluded from the above discussion that the synergistic effect of *Bacillus* sps isolated from soil and identified are capable to promote plant growth simultaneously inhibiting the growth of phytopathogen. Therefore, the isolates can be utilized as a biocontrol agent and biofertilizers for groundnut crops in agricultural systems.

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