Original Article

Talcs And Beneficial Bacteria Teaming Up To Shield Sunflowers From Root Rot

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Abstract

A talc-based formulation of biocontrol agents, Pseudomonas aeruginosa (PGPR-11 and PGPR-27), Bradyrhizobium sp., (NFB-1) and Sinorhizobium sahelens (NFB-30) was developed supplemented with 1% chitin. Their suppressive effect was promising against root infecting fungi, like Rhizoctonia solani, Macrophomina phaseolina, Fusarium oxysporum and F.solani, different growth parameters of sunflower plants were also observed in screen-house and field experiments at different time intervals. Seeds were treated by talc chitin-based formulation of NFB-1, NFB-30, PGPR-11, and PGPR-27, effectively reduced the incidence of M. phaseolina F. solani, F. oxysporum and R. solani, as compared to untreated plants (control). Sunflower seeds treated with bioformulation mixture, significantly increased growth parameters and suppressed root rot pathogens. During the storage of antagonistic bacteria viz, P. aeruginosa (PGPR-11 and PGPR-27) and Bradyrhizobium sp., (NFB-1) and S.sahelens (NFB-30) strains maintained their population level upto 6months. The study reveal that talc base formulation can be the best for the maintaining the shelf life of plant growth promoting bacteria.

Keywords: Antagonist; chitin; biocontrol formulation; shelf life.

Introduction

Excessive use of chemical pesticides and inorganic fertilizer causes harmful effects on soil microorganisms, affects soil fertility and pollutes the atmosphere [1]. Increasing chemical pollutants the use of synthetic fertilizers and pesticides is the biggest reason and will further harm the environment and posing serious threat to the environment [2]. To reduce these threats an alternative eco- friendly method is needed to achieve our targeted in agriculture [3]. Rhizospheric microorganisms such as PGPR and rhizobia have emerged as an effective tool for protecting plants from diseases [4, 5] They participate in many biological activities of the soil ecosystem, dynamically transforming them into nutrients and bringing sustainability to crop production [6] Recently, scientists have paid attention to PGPR and their use as an alternate replacement of pesticides

and fertilizers to enhance the growth of plants. [7]. The effective application of PGPR relies upon its ability to live in the soil, its co-existence with crops, its ability to interact with native soil microbes, and surrounding mineral elements [8]. In addition, the industrial success of PGPR strains requires economically feasible market requirements, consistent and wide-scale effects, care and steadiness, extended life, lesser expenses, and accessible professional resources [7]. Transfer of microbial cells in soil or rhizosphere using an inoculant formulation involving a carrier material is an attractive option [9]. Many organic, synthetic and inert substances were evaluated, such as vermiculite, phosphate rock powder, calcium sulphate, polyacrylamide gels, and alginates as carrier materials [10]. A talc based formulation was also developed for the field application of PGPR [11] Rhizospheric bacteria can survive a maximum 2 month on talc based formulation while adding 20% xanthan gum and keeping it at 4°C can maintain the shelf life of bacteria up to 180 days on talc based formulation [12]. The use of a talc-based carrier material of P. fluorescens (Pf1) gradually reduce blister blight disease and increase the growth of tea plant and yield and also demonstrated that P. fluorescent (Pf1) effectively controls dry root rot in mungbean plants [13]. Biological control agent formulation based on Talc chitin is an effective way to control root rot fungi [14]. Chitin when blended with soil, it activates microorganisms and mineralized stable compounds in a short period of time, which are inhibitory to fungi causing root rot and also suppressed parasitic nematodes [14]. Formulation containing chitin would enhance plant growth and biological activity of Bacillus subtilis [15]. In a previous study, we have reported suppression of root rotting fungi of chili under field condition by the PGPR and rhizobia [16]. The current report describes the development of a talc based formulation for the field application of PGPR and rhizobia using sunflower as test plant.

Materials and Methods

Talc based formulation

For mass multiplication commercial talc powder (1 g/kg) was mixed with carboxy-methyl-cellulose adjusted the PH (7) with calcium carbonate. Prawn chitin (1% w/w) was added and this talc powder was transferred in polythene bags at 250g per bag; sealed and sterilized at 15 psi for 20 minutes. One week old aqueous bacterial suspensions of P.aeruginosa strains PGPR-11, 27, Bradyrhizobium spp (NFB-1), and Sinorhizobium sahelens (NFB-30) were injected in each bags at 100 ml per 250 g carrier having 2.3 x 10^9cfu/ml of PGPR-11; 5.3 x 10^9cfu/ml of NFB-1 3.1 x 10^9 cfu/ml of PGPR-27and 4.4 x 10^9cfu/ml of NFB-30. Two sets were prepared of each bacterium and kept at room temperature (28 ±5 °C) and the other at refrigerated temperature (6°C). Formulation without inoculation of bacteria was used as control. The shelf life of the inoculums was examined at 0 day and after 3 and 6 months of interval by dilution plate method [17].

Efficacy of PGPR and rhizobia on talc-chitin based formulation after three months

After three months of interval, bacterial formulations (kept at refrigerator and at room temperature) were evaluated for their efficacy. Sunflower (Helianthus annuus) seeds (25 seeds per row) treated with talc based formulation of PGPR and rhizobia mixed in 1% gum Arabic were sown in the trial field plots (2x2 meter) having 04 replicates and in complete randomized block design at Pakistan Agricultural Research Council (PARC), Crop Disease Research Institute, University of Karachi. The field was infested naturally with Rhizoctonia solani (4 - 9 % colonization), Fusarium sp. (3000 cfu g^{-1} soil), and M. phaseolina (3-8 sclerotia g^{-1} soil), as assessed via using method of Wilhelm [18, 19, 20]. Devoid of bacteria talc-chitin based formulation treatment were used to treat the sunflower seeds and considered as control. After 45 days, plants

were uprooted (@ 04 plants per replicate) and observations were recorded; under tap water roots were washed and blot dried. Plant growth parameter like fresh weight and height of plant was noted. To find out the occurrence of fungi that cause root rotting; plant root were cut into 1cm small pieces and roots were surface sterilized with 1 % commercial bleach and placed onto potato dextrose (PDA) plates supplemented with streptomycin (0.2 g/Liter) and penicillin (10^5 units/Liter), incubated for 5-7 days at 28 ºC, and identified the fungi emerging from each piece of root (infection percentage and colonization percentage) were determined as styled by [21].

Efficiency of PGPR and rhizobia on talc-chitin based formulation after six months

After six months, the efficacy of talc based formulation was evaluated in clay pots, field plots and at farmers' fields using sunflowers as test plants.

Clay pot experiment

Clay pots of 15cm diameter were used to performed this experiment included sandy loam soil (pH- 8.0). Each contain 01 Kg soil of field plot having the same population of root rot pathogens as mentioned above. Sunflower seeds (6 seeds per pot) treated with each biocontrol formulation and control were sown in each pot. Each pot was thinned @ 4 seedlings after development of 4 leaves stages and excess were removed. Data were noted after 45 days.

Filed plot experiment

Field plot experiment was piloted in the same field plot as described with similar treatments and conditions.

Farmer's field Experiment

A Farmers fields experiment was performed at Malir, Karachi, where ¼ acre was selected for the experiment in a two acres field. The sandy loam soil (pH 8.08) showed propagules of different fungi that cause root diseases i.e. M. phaseolina (7-8 sclerotia g^{-1} soil), Fusarium species (3400 cfu/g) and R. solani (7-8% colonization of sorghum seeds used as bait). Sunflower seeds (10 seeds per m) treated with talc-chitin based formulation of NFB-1, NFB-30, PGPR-11, and PGPR-27 were sown. Plants without any treatment or seeds treated with talc formulation without bacteria considered as control. Experiment performed in randomized block design with 4 replicates and watered as per requirement. After 45 and 90 days, different growth parameters included, length of plants and weight of plant and the treatment effect against the root rotting fungi were observed and recorded.

Statistics Analysis

The data were analyzed by using analysis Varience (ANOVA) and the level of significant was findout according to Gomes & Gomes [22].

Results

Population of antagonist bacteria on talc chitin-base formulation

During the storage period of the antagonistic bacteria viz, P. aeruginosa (PGPR-11, PGPR-27), Bradyrhizobium sp., (NFB-1) and S.sahelens (NFB-30) showed slight increase in population till 3 months but after 6 incubation period bacterial population showed a decline, but still maintained their population up to 10^8 cfu/g (Table-1).

Table.1. Population of Pseudomonas aeruginosa and rhizobia on talc-chitin based formulation at different time intervals.

Bacterial strains	**Population (cfu/g)**	
At 0 days	At Refrigerator Temperature	At Room Temperature
PGPR-11	-	$7.2 X10^8$
PGPR-27	-	$6.5 X10^8$
NFB-1(Bradyrhizobium sp.)	-	$6.2 X10^8$
NFB-30(S.sahelens)	-	$7.0 X10^8$
After 3 months		
PGPR-11	$2.1X10^9$	$3.7X10^9$
PGPR-27	$7.2X10^9$	$3.2X10^9$
NFB-1(Bradyrhizobium sp.)	$2.2X10^9$	$4.1X10^9$
NFB-30(S.sahelens)	$2.2X10^9$	$2.3X10^9$
After 6 months		
PGPR-11	$1.1X10^9$	$2.4X10^9$
PGPR-27	$4.3X10^9$	$2.5X10^9$
NFB-1(Bradyrhizobium sp.)	$1.6X10^9$	$3.3X10^9$
NFB-30(S.sahelens)	$1.4X10^9$	$2.8X10^9$

Effectiveness of formulation of talc-chitin of rhizobia and PGPR after storage of three months

Bacterial formulation kept at room temperature

Maximum inhibition of F. solani caused by PGPR-11, PGPR-27 and S.sahelens (NFB-30) (6.2%) than control (43.7%) while PGPR-27 and S.sahelens (NFB-30) caused completely inhibition of F. oxysporum than control (18.7%). M. phaseolina infection was significantly ($p<0.05$) inhibited by Bradyrhizobium sp., (NFB-1) and PGPR-27(0%) than control plants (12.5%). Plants treated with Bradyrhizobium sp., (NFB-1) and PGPR-27 showed no infection of R. solani (Table-2). Maximum fresh shoot weight and plant height were found in plants treated with PGPR-11, Bradyrhizobium sp., (NFB-1), and S.sahelens (NFB-30) (Table 2 & Figure.1).

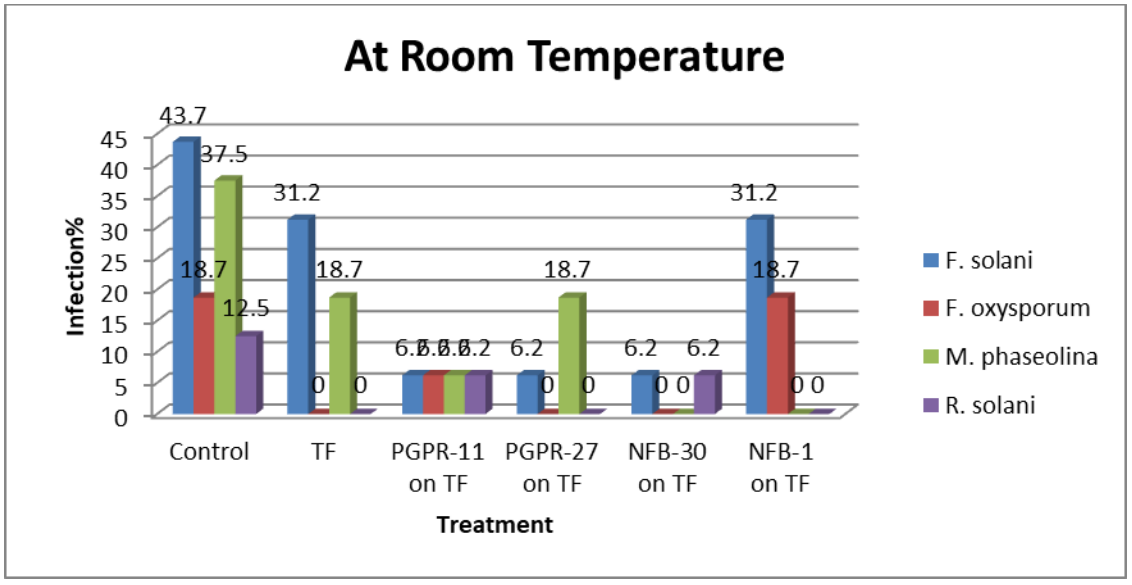
Bacterial formulation kept at refrigerated temperature

PGPR-27 and S.sahelens (NFB-30) caused complete inhibition of F. oxysporum than control plants (18.7%). M. phaseolina was significantly ($p<0.05$) inhibited by Bradyrhizobium sp. (NFB-1) and PGPR-2 as compared

to control plants. Similarly R. solani infection was not found in plants treated with Bradyrhizobium sp., (NFB-1) and PGPR-27 (Table-2). Significantly greater weight and height of plant were recorded in PGPR-11, Bradyrhizobium sp., (NFB-1), and S.sahelens (NFB-30) treated plants than control plants (Table 2 & Figure.1).

Table. 2 Effect of Pseudomonas aeruginosa and rhizobial isolates on talc-chitin based formulation after 3 months storage on the growth of sunflower plant under field condition.

Treatment	Plant height(cm)	Fresh Weight(g)	Plant height(cm)	Fresh Weight(g)
	At Room Temperature		At refrigerated Temperature	
Control	11.66	1.42	11.66	1.42
Talc- chitin based formulation (TF)	13.77	1.61	12.37	1.53
PGPR-11 on TF	18.5	1.925	14.8	1.975
PGPR-27 on TF	14.04	1.78	12.72	0.75
NFB-30 (S.sahelens) on TF	17.46	2.12	15.39	1.85
NFB-1 (Bradyrhizobium sp.) on TF	16.27	1.84	15.79	1.75
$LSD_{0.05}$	5.631	0.961	5.851	0.951



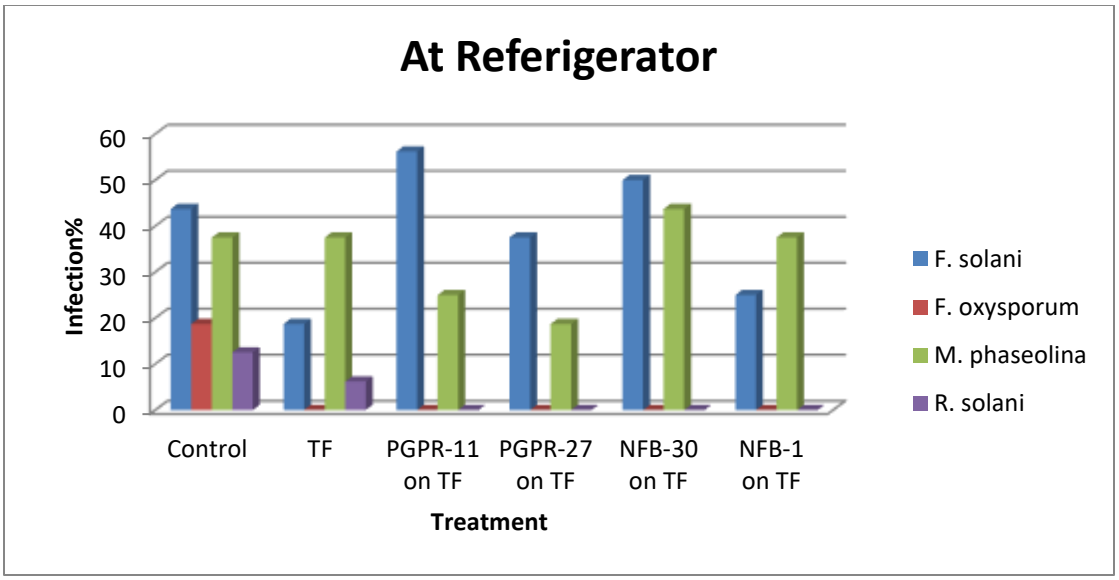


Figure. 1. Effect of Pseudomonas aeruginosa and rhizobial strains multiplied on talc- chitin based formulation after 3 months on the infection of root rotting fungi Fusarium solani, F. oxysporum, Macrophomina phaseolina, Rhizoctonia solani on sunflower roots under field condition

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

[2] Mean values for pathogens in rows showing differences greater than the LSD value are significantly different at $p<0.05$

$LSD_{0.05}$ Treatments 13.51, 7.01 Pathogen 8.12 1

Effectiveness of formulation of talc-chitin of rhizobia and PGPR after storage of six months

Clay pot experiment

At room temperature

Application of NFB-1, NFB-30, PGPR-11, and PGPR-27, formulation, inhibited infection in all 20 and 50 days old plants of Fusarium. oxysporum F.solani significantly ($p<0.05$). However NFB-1 (Bradyrhizobium spp), NFB-30 (S.sahelens) and PGPR-27, were found best on 20 days old plants, whereas all the test bacterial strains were effective on 50 days old plants (Figure.2).

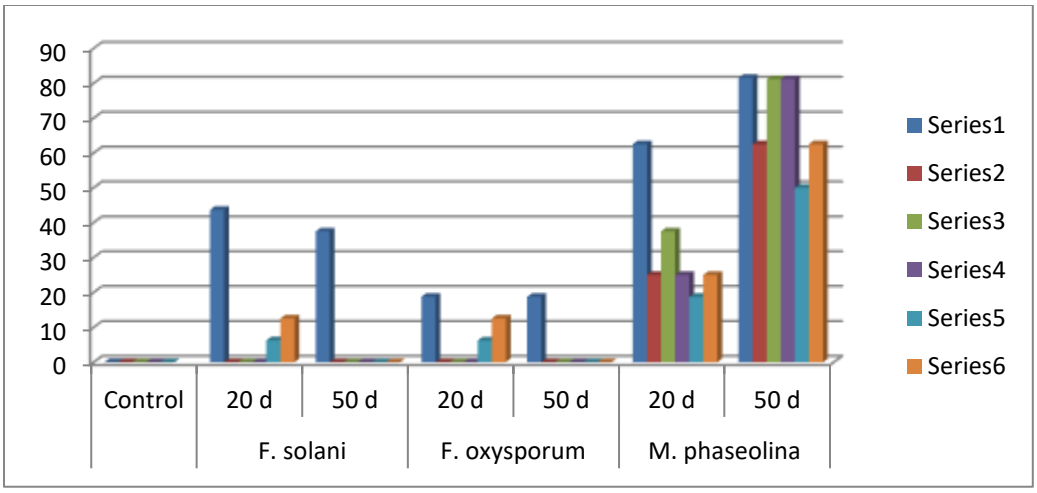
Larger height of plant were recorded in 20 days old plants by PGPR-11, similarly NFB-1 (Bradyrhizobium spp) and PGPR-27 produced large tallness in 50 days old plants (Table 3).

Formulation stored at refrigerated

NFB-1 (Bradyrhizobium spp), NFB-30 (S.sahelens), PGPR-11, and PGPR-27, significantly inhibited the infection of F. solani and F. oxysporum on both sunflower roots of 20 and 50 days old (Figure.2).

Greater plant height was recorded with the application of NFB-1 (Bradyrhizobium sp.) on 50 days old plants (Table 3).

At Refrigerator



At room temperature

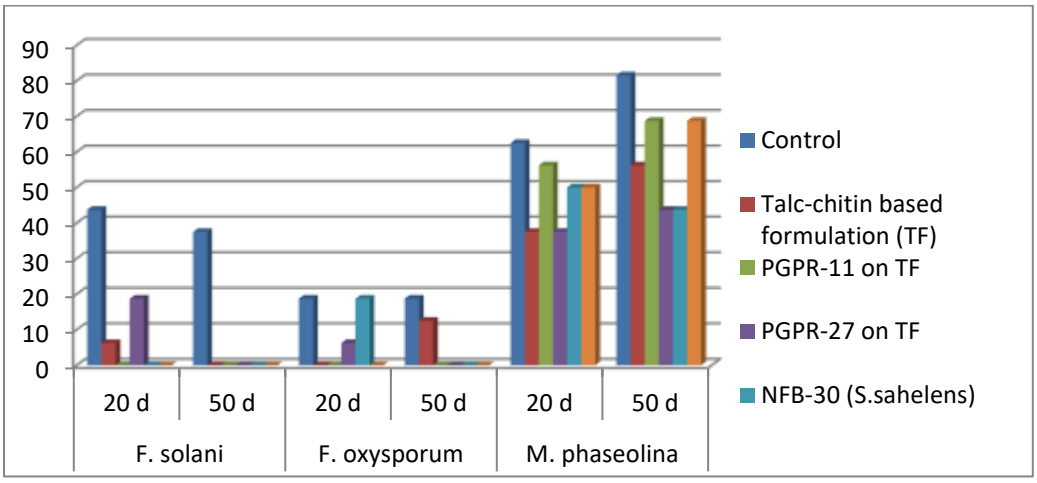


Figure.2. Effect of Pseudomonas aeruginosa and rhizobial strains multiplied on talc-chitin based formulation after 6months on the Infection of Fusarium solani, F. oxysporum and Macrophomina phaseolina on sunflower root under screen condition

$LSD_{0.05}$ = Treatment = 10.9[1] 9.49[1] Pathogen = 7.76[2] 6.71[2] Time = 6.34[3] 5.48[3]

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

[2] Mean values for pathogens in rows showing differences greater than the LSD value are significantly different at $p<0.05$

[3] Mean values for time in rows showing differences greater than the LSD value are significantly different at $p<0.05$

Table 3. Effect of Pseudomonas aeruginosa and rhizobial strains multiplied on talc-chitin based formulation after 6months on the growth of sunflower plant under screen house condition.

Treatments	At refrigerated temperature	At room temperature

	Plant height		Fresh shoot weight		Plant height		Fresh shoot weight	
	20d	**50d**	**20d**	**50d**	**20d**	**50d**	**20d**	**50d**
Control	7.0	16.1	1.07	3.0	7.01	14.6	1.44	2.46
Talc-chitin based formulation (A)	8.7	18.5	1.32	2.87	8.8	17.3	1.04	3.47
PGPR-11 on A	10	17.4	1.43	3.37	8.65	16.7	1.07	2.27
PGPR-27 on A	7.6	20.4	1.12	2.67	9.59	17.4	1.85	2.44
NFB-30 (S.sahelens) on A	8.6	19.6	1.13	2.56	7.78	15.0	1.11	1.875
NFB-1 (Bradyrhizobium spp) on A	8.1	20.6	1.66	3.04	7.43	18.0	1.15	2.05
$LSD_{0.05}$ =	2.2	7.8	0.63	1.66	2.39	3.74	0.51	5.68

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

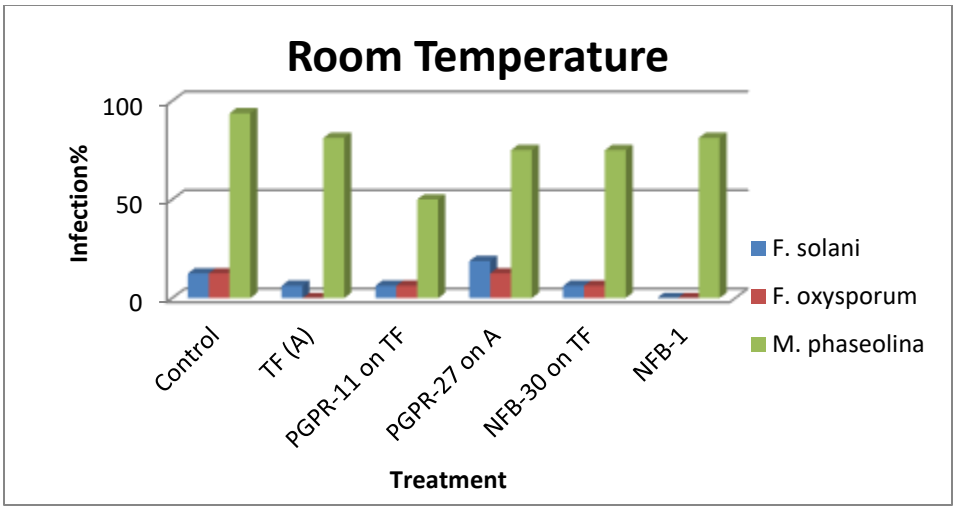
Field plot experiment

Bacterial formulation kept at room temperature

The seed treatment with Bradyrhizobium spp., (NFB-1), S.sahelens (NFB-30), and PGPR-11 inhibited infection of R. solani , M. phaseolina and F. solani, where plants treated with NFB-1 showed complete inhibition of F. solani and R. solani (Figure.3). PGPR-11 treated plants showed significantly better fresh shoot weight and plant height as compared to control plants (Table 4).

Bacterial formulation kept at refrigerated temperature

PGPR11, PGPR-27 and Bradyrhizobium sp., (NFB-1) inoculation completely inhibited of F. solani and F. oxysporum (Figure.3). NFB-30 showed maximum height and weight of plant (Table 4).



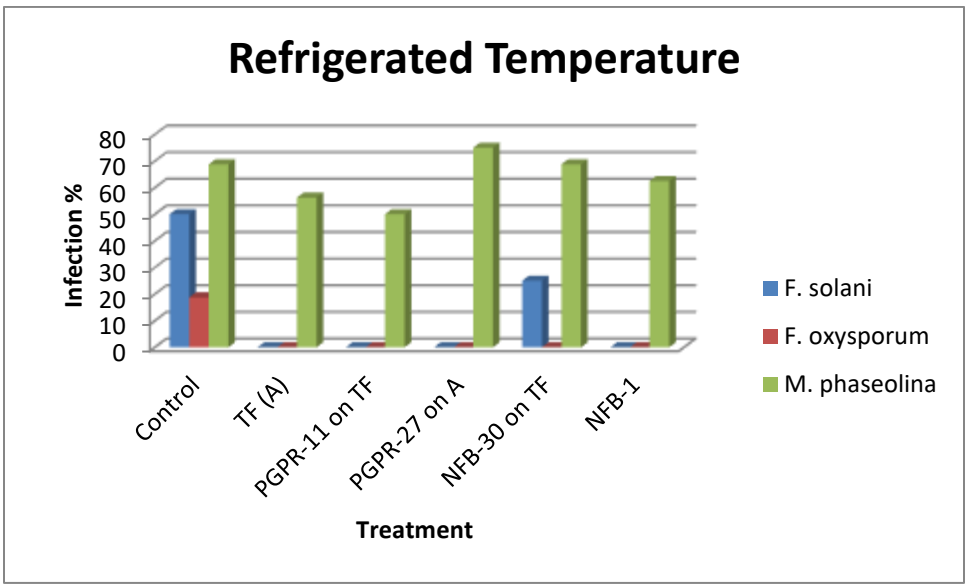


Figure.3 . Effect of Pseudomonas aeruginosa and rhizobial strains multiplied on talc-chitin based formulation after 6months on the infection of Fusarium solani, Rhizoctonia solani and Macrophomina phaseolinaon sunflower root in field plot experiment.

LSD0.05	Treatment	15.43	Treatment	18.94
	Pathogen	10.91	Pathogen	13.39

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

[2] Mean values for pathogens in rows showing differences greater than the LSD value are significantly different at $p<0.05$

Table 4. Effect of Pseudomonas aeruginosa and rhizobial strains multiplied on talc-chitin based formulation after 6months on the growth of sunflower plants under field condition.

	At room temperature		At refrigerated temperature	
Treatment	Plant height(cm) 30days	Fresh shoot weight(g) 30days	Plant height(cm) 30days	Fresh shoot weight(g) 30days
Control	24.62	5.60	21.43	4.13
Talc-chitin based formulation (A)	28.0	7.43	23.25	3.83
PGPR-11 on A	31.5	7.41	26.56	3.93
PGPR-27 on A	21.87	5.86	26.50	3.9
NFB-30 (S.sahelens) on A	21.87	4.96	27.06	6.11
NFB-1(Bradyrhizobium sp.) on A	25.37	5.53	27.0	4.9
Lsd0.05	11.65	3.70	12.13	3.54

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

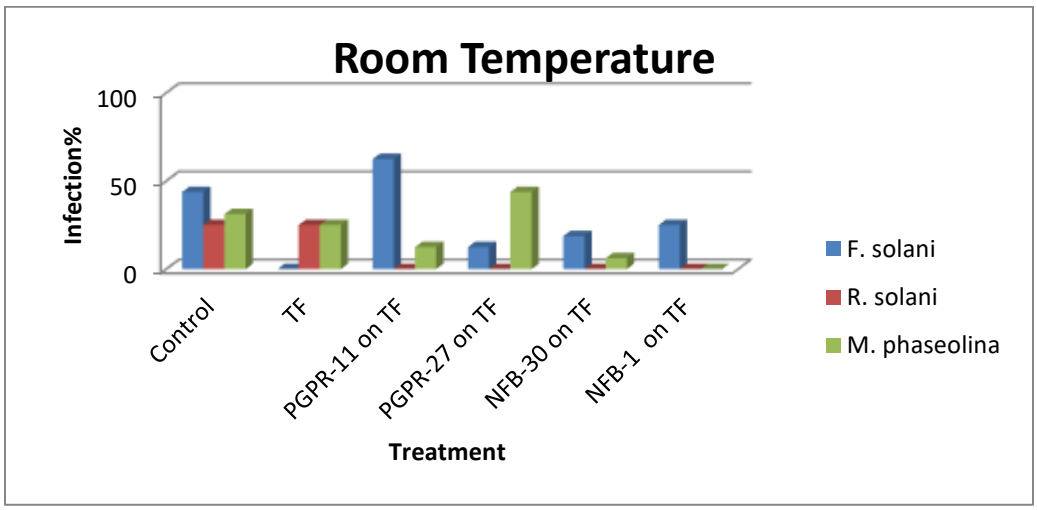
Experiment at farmer's field

Bacterial formulation kept at room temperature

Inhibition of F. solani was observed by PGPR-27, Bradyrhizobium sp., (NFB-1) and S.sahelens (NFB-30). While the infection of R. solani was completely reduced by all treatments. While NFB-1 completely suppressed M. phaseolina (Figure.4). Height of the plant was enhanced when sunflower seeds were treated with S.sahelens (NFB-30) and Bradyrhizobium sp., (NFB-1). PGPR-27 produced maximum fresh weight of shoot. (Table 5).

Storage at refrigerated

The infection of M. phaseolina, R. solani and F. solani was completely suppressed by PGPR-11. While all bacterial strains showed significant reduction against all root rot pathogens. Highest fresh shoot weight was observed in PGPR-11 and NFB-30 bacterial formulations (Table 5 & Figure.4).



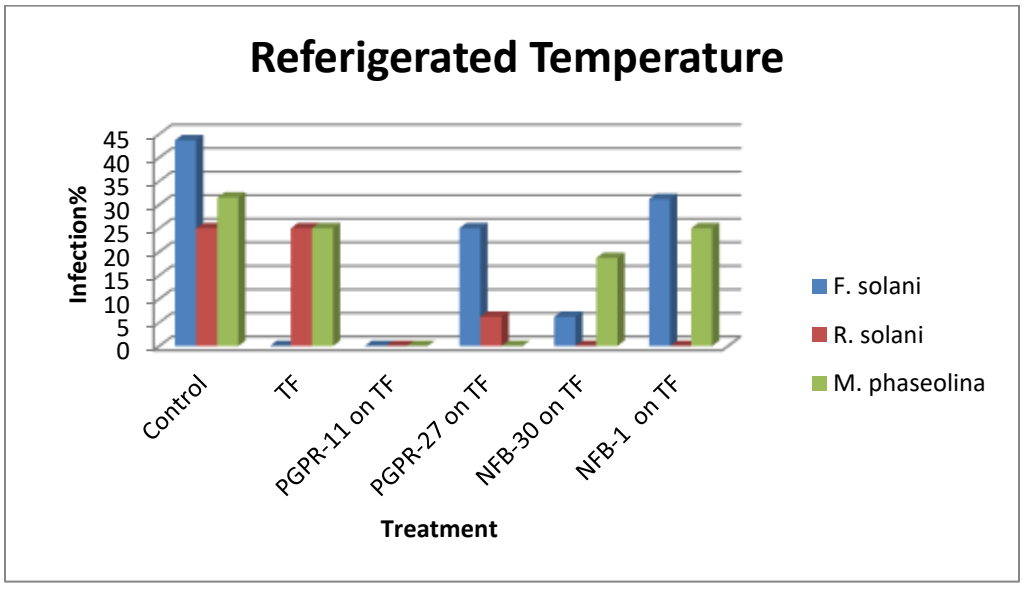


Figure 4. Effect of Pseudomonas aeruginosa and rhizobial strains multiplied on talc-chitin based formulation after 6months on the infection of Fusarium solani, Rhizoctonia solani and Macrophomina phaseolina on sunflower roots at farmer's field.

LSD0.05 = Pathogens= 16 8.6 Treatments = 22.7 12.19

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

[2] Mean values for pathogens in rows showing differences greater than the LSD value are significantly different at $p<0.05$

Table 5. Effect of Pseudomonas aeruginosa and rhizobial strains multiplied on talc-chitin based formulation after 6months on the growth of sunflower plants at farmer's field.

Treatments	**Plant height (cm)**	**Fresh shoot weight (g)**	**Plant height (cm)**	**Fresh shoot weight (g)**
	At room temperature		**At refrigerator temperature**	
Control	109.0	75.0	136.5	202.5
Talc-chitin based formulation (A)	127.7	175	131.5	132.5
PGPR-11on A	129.5	247	130.2	142.5
PGPR-27 on A	119.0	270	152.7	287.5
NFB-30 (S.sahelens) on A	132.0	252.5	132.7	245.0
NFB-1(Bradyrhizobium sp) on A	136.7	230	127.5	177.5
$LSD_{0.05}$ =	26.6	192	26.9	160.5

[1] Mean values for treatments in columns showing differences greater than the LSD value are significantly different at $p<0.05$

Discussion

Discarded shellfish is a main source of chitin, presents 17% in shrimp, 14.5% in prawn [23] and 18.7% in crab [24] In current study, use of Rhizobium spp., and PGPR on talc chitin based formulation enhanced plant growth and reduced fungal infection on sunflower plant in screen house condition. Rhizobium spp., are bacteria found in soil that establish a mutual symbiotic association with leguminous plants [25, 26] Leguminous plant seeds inoculated with

Rhizobium before planting are applied to increase crop production through enhancing the process of nodulation and hence reduce the use of nitrogen fertilizer [25, 27] Number of reports indicate that Rhizobium and Bradyrhizobium act as potential biocontrol agents against phytopathogens. In the current study, similar results were shown by species of Rhizobium and Bradyrhizobium in improving plant growth and suppression of root rotting fungi in each experiment. In our previous study plant growth promoting rhizobacteria were found to produce iron chelating siderophores [21, 28], antibiotics, hydrogen cyanide and these compounds have deleterious effects on pathogenic rhizosphere microorganisms, generating an environment friendly for growth of root [29, 30]. Furthermore, some rhizobacteria are also known to release plant hormones and also solubilize phosphorus [28].

Microorganisms can be stimulated when soil is integrated with chitin [31], as a result, they release toxic substances [32] which may lead to an environment not suitable for these pathogens. This dissolved chitin enhances the propagation of beneficial microbes in soil mixture and around the root zone to help in reducing root infection caused by various fungi. [33, 34] as well as enhances the growth of plants by releasing the waste that may provide food for the soil bacteria, which ultimately enhances the growth of the plant [35]. Similarly, in this study species of PGPR, Rhizobium and Bradyrhizobium used alone and or multiplied on chitin-talc based formulation enhanced disease resistance against root rotting fungi. The chitin-based wastes are abundant in various regions of the world and their utilization requires excess cost. Incorporation of chitin waste in microbial inoculants may provide a food base for biocontrol agents when amended in soil.

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Conflict of interest

The authors declare no conflict of interest.

Funding

None

Conclusion

Effective use of rhizobia or rhizobacteria on a commercial level need to be formulated in such a way that their survival period can be prolonged. Being a natural occupant of the rhizoplane and rhizosphere of different crop plants, the future of rhizobia and PGPR seems enormous as a biocontrol agent contrary to numerous pathogens. Bio-pesticides of PGPR and rhizobia should be developed commercially.

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