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Novel Biofilm Biofertilizers for Nutrient Management and Fusarium Wilt Control in Chickpea

M. Nissipaul*, Sodimalla Triveni, R. Subhashreddy and B. Suman

Department of Agricultural Microbiology and Bioenergy, College of Agriculture, Ragendranagar, Hyderabad, India
**Corresponding author*

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A B S T R A C T

Wilt caused by *Fusarium oxysporium f. spciceris* is a devastating disease of chickpea. It occurs in 2 stages; seedling stage (0-30%) and reproductive stage (0-57%). Annual chickpea yield loss due to *Fusarium* wilt was estimated to be 10% in India. So, our objective is to control chickpea wilt disease by using Trichoderma based biofilms as an alternative to chemical fungicides. *Trichoderma viride* is a potential antagonistic fungi which prevents diseases like wilt, brown rot, damping off, charcoal rot etc. We have isolated different strains of PGPR bacteria from waste lands of *Parthenium* rhizosphere soils to prepare a biofilm. A biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to biotic/abiotic surface. Our work was aimed towards the development of biofilms under in vitro conditions, using a combination of agriculturally important potential microorganisms *like Bacillus subtilis, Pseudomonas flourescens* and *Rhizobium leguminosarum* with the fungus *Trichoderma viride* as the matrix and screened for various biochemical traits like Antifungal activity, Ammonia production, HCN production, IAA production, Protein content, Siderophore production and Phosphate solubilization; and when compared to individual treatments, coinoculations and biofilms the biofilm performed well in all the biochemical properties. These biofilms were evaluated for their disease management and crop production in chickpea. A field experiment which comprised of 9 treatments were conducted. The synergism in terms of the PGP traits in the biofilms revealed their promise as superior PGP inoculants hence this in vitro experiment is to be carried out under field conditions to show better results.

Introduction

The chickpea or chickpea (*Cicer arietinum*) is a legume of the family Fabaceae, subfamily Faboideae. It is also known as gram or Bengal gram, Garbanzoor garbanzo bean and sometimes known as Egyptian pea, ceci, cece or chana. Its seeds are high in protein. It is one of the earliest cultivated legumes: 7,500-year-old remains have been found in the Middle East. The fungus *Fusarium oxysporium* enters the vascular system of the infected plant via the roots. It produces enzymes that degrade the cell walls so that gels are formed that block the plant's transport system. Discolouration of the internal tissues progresses from the roots to the aerial parts of the plant, yellowing and wilting of the foliage occur, and finally there is necrosis. Biofilms represents complex communities of multiple microbial species which remain attached to surfaces or at the

interfaces (Lynch *et al.*, 2003), and possess the capacity to maintain the metabolic activity under adverse environmental conditions, exhibiting increased survival in a competitive environment (Stewart, 2002). Biofilms comprise layers of prokaryotic or eukaryotic cells, which can also play a key role in plant-microbe interactions, promote plant growth and reduced.

Materials and Methods

Phosphate solubilization

Sterilized Pikovskaya's agar medium was poured as a thin layer in the sterilized Petri plates and allowed for solidification. The Pikovskaya's plates were spot inoculated with 11 isolates of *Bacillus spp., Pseudomonas spp., Rhizobium spp.*, incubated at 28±2^{0}C for 2-3 days. Formation of a clear zone around the colonies was considered as positive result for phosphate solubilisation. It was calculated by following formula.

PSE (Phosphate Solubilization Efficiency) = Z⁄C x 100

Z - Clear zone including bacterial growth
C - Colony diameter

Ammonia production

The isolates were tested for Ammonia production by inoculating the isolates in to 10 ml of sterilized peptone water in the test tubes. The tubes were incubated for 48-72 h at 36±2°C. After that Nessler's reagent (0.5 ml) was added in each tube. Change in colour of the medium from brown to yellow colour was taken as positive test for Ammonia production.

Indole Acetic Acid Production

Indole acetic acid production was tested according to Gorden and Weber (1951). The active culture of each test isolate was raised in 5 ml respective broth tubes and incubated at determined temperature and time. After incubation these cultures were centrifuged at recommended rpm and time. Two drops of O-phosphoric acid was added to 2 ml of supernatant to develop the colour. Development of pink colour considered as positive test for IAA production.

Protien estimation

One ml of the sample was taken and cells were pelleted by centrifuging at 10,000 rpm for 8 min. Spectrophotometric measurement of colour development was done using the method of Lowry *et al.*, (1951). Intensity of blue colour was measured at absorbance maxima of 660 nm.

Siderophore production

Siderophore production was estimated qualitatively. By taking 0.5% of cell free culture supernatant and added to 0.5 ml of 0.2% aqueous Ferric chloride solution. Appearance of orange or reddish brown colour indicated the presence of Siderophore (Yeole and Dube 2000).

Hydrogen cyanide production

The HCN production was done by the method of Castric and Castric (1983). Medium plates*i.e*. Nutrient agar for *B. subtilis*, Kings B for *P. flourescens*, YEMA for *R. leguminosarum*, were prepared separately and incubated for 24 h. One ml of culture of each test isolate was inoculated on respective media plates separately. A disc of Whatman filter paper No.1 of the diameter equal to the Petri plate size, impregnated with alkaline picric acid solution (0.5% picric acid (w/v) in 1% sodium carbonate) was placed in the upper lid of the inoculated Petri plates under aseptic condition. The control plate did not receive the inoculum. The plates were

incubated at 28±2 °C for 48-72 h. Change in colour from yellow to light brown, moderate or strong reddish brown was taken as an indication of HCN production.

Antagonistic activity

Antagonistic activity was studied by following dual culture technique (Skidmore and Dickinson, 1976). First, the bacterial cultures were streaked on respective media plates and incubated at respective temperature and time. Then take a loopful of each bacterial culture and streak on the Nutrient agar plate at one end, and place 5 mm mycelial disc of test pathogen at the other end. Control plate was maintained by placing only pathogen mycelial disc on the plate without bacteria.

The assay of plates were incubated at 28 ± 2 °C for 5 days and observations were made on inhibition of mycelial growth of the test pathogens. For each bacterial isolate three replications were maintained with suitable controls. The per cent growth inhibition over control was calculated by using the formula:

Percent Inhibition =

$$\frac{\text{Growth of Pathogen in control (mm)} - \text{Growth of Pathogen in treatment (mm)}}{\text{Growth of Pathogen in control(mm)}} \times 100$$

Note: The percent inhibition in control is taken as zero percent.

Results and Discussion

Biochemical attributes of Biofilms related to PGP activity

All the 7 *B. subtilis* and 2 *P. flourescence* individual isolates/dual cultures/biofilms were able to form clear zone of phosphate solubilisation on agar plate ranged from 10-19 mm with highest zone of solubilisation efficiency (170%) efficiency is observed in T8 (*Trichoderma viride*+ *Rhizobium leguminosarum* + *Pseudomonas fluorescence*+ *Bacillus subtilis* (Biofilm) (Table 1).

All the cultures used were found to be Ammonia producers and based on the development of yellow colour they were classified as weak, moderate, and strong. Except *T. viride*, B1, and B4, all the individual cultures/ dual cultures and biofilms. It is able to produce HCN. All the individual isolates, dual cultures, biofilms had shown the IAA production and based on the intensity of pink color development they are classified.

Protein estimation was done by spectrophotometric measurement of blue colour development at absorbance maxima of 660 nm. The highest values for proteins were recorded in T_4 (*Trichoderma viride* + *Pseudomonas fluorescence* (Biofilm), (0.41 mg ml^{-1}) and the lowest was recorded with T_9 (*Trichoderma viride* + *Rhizobium leguminosarum* + *Pseudomonas fluorescence* + *Bacillus subtilis* (Coinoculation) (0.28 mg ml^{-1}) and B2 of *B. subtilis*. Protein estimation was not observed in *T. viride* individual isolate.

The production of siderophores was observed with all the treatments and is more or less equal. The more production of siderophores was recorded with the treatments T_2 (*Trichoderma viride* + *Rhizobium leguminosarum* (Biofilm) and the lowest were recorded in T5, T6. In the present study, all the PGPR individual isolates/dual cultures and biofilm cultures were examined for the potential to inhibit fungal pathogen *Fusarium oxysporum* under *in vitro* conditions. Each isolate having some percent inhibition, with some inhibition zone. The highest percent inhibition (37.15 %) was recorded in T_8 (*Trichoderma viride* + *Rhizobium*

leguminosarum + *Pseudomonas fluorescence* + *Bacillus subtilis* (Biofilm) with an inhibition zone of (03.00 mm) and the next best is T_9 and T_4 (36.6 %) and inhibition zone of (3.01 mm). The lowest inhibition was recorded in T_2 *R. leguminosarum* + *T. viride* biofilms and its dual culture T_5 with percent inhibition of 31.65 % and 29.95 % respectively (Table 2). Kerkar *et al.*, (2012) reported that out of the 125 bacteria isolated from the biofilms, 16 produced indole-3-acetic acid (IAA). Four isolates consistently produced high IAA concentrations ranging from 9.5 to 14.2 µg mL^{-1} in the presence of 4 mg mL^{-1} tryptophan concentrations in the growth media (Tale 3).

Table.1 *In vitro* screening of biofilms for various plant growth promoting attributes

S.No.	Treatments	Phosphate solubulisation			Ammonia production	IAA production	Protein estimation (mg ml^{-1})
		Zone diameter		Solubulisation efficiency			
		Solubulisation Zone	Culture media				
1	T_1	-	-	-	-	-	-
2	T_2	-	-	-	++	++	0.29
3	T_3	24	13	184.6	+++	+++	0.30
4	T_4	20	11	181	+++	+++	0.41
5	T_5	-	-	-	++	++	0.30
6	T_6	19	14	135.5	++	++	0.29
7	T_7	20	12	166.6	++	+++	0.39
8	T_8	21	11	190	+++	+++	0.29
9	T_9	24	13	184.6	+++	+++	0.28

IAA- Indole Acetic Acid Ammonia production
+ Weak production ++ Moderate production
+++ Strong production − No production

Table.2 *In vitro* screening of efficient biofilms for bio control activity

S. No.	Treatments	Antifungal activity		Siderophore production	HCN production
		Percent inhibition of *Fusarium* (%)	Inhibition zone (mm)		
1	T_1	-	-	-	-
2	T_2	31.65	00	+++	++
3	T_3	34.40	01.00	+++	+++
4	T_4	36.6	03.01	+++	+++
5	T_5	29.95	00	++	++
6	T_6	33.85	01.00	++	++
7	T_7	36.05	03.00	+++	+++
8	T_8	37.15	03.00	+++	+++
9	T_9	36.6	03.01	+++	++

HCN- Hydrogen cyanide Siderophore production
+ Weak production ++ Moderate production
+++ Strong production − No production

Table.3 Effect of Biofilmed biofertilisers on plant growth parameters in chick pea

Treatments	Plant height (cm)		Root length (cm)		Shoot dry Wt (g)	Root dry Wt (g)
	30 DAS	60 DAS	30 DAS	60 DAS	Average	Average
T_1	13.27	23.93	6.53	12.90	2.18	0.35
T_2	14.37	28.23	8.13	16.00	3.55	0.54
T_3	13.83	28.30	7.93	14.47	3.33	0.47
T_4	14.40	**29.43**	8.27	15.73	3.54	0.48
T_5	13.93	27.97	7.87	14.33	3.55	0.51
T_6	13.37	27.40	7.33	13.50	3.08	0.44
T_7	14.27	28.53	7.87	14.33	3.27	0.46
T_8	**14.77**	**29.43**	**8.43**	**17.40**	**4.12**	**0.61**
T_9	14.67	28.67	8.37	16.30	4.09	0.57
SEm	0.27	0.72	0.26	0.41	0.19	0.03
CD (P= 0.05)	0.84	2.17	0.80	1.26	0.57	0.11

Table.4 Effect of Biofilmed biofertilisers on disease suppression (*Fusarium* wilt) in chickpea

Treatments	Initial plant population	Final plant population	Wilt Incidence (%)
T_1	360	285	**20.7**
T_2	385	349	9.3
T_3	382	352	8.0
T_4	386	381	1.3
T_5	380	329	13.7
T_6	372	337	9.3
T_7	380	363	4.7
T_8	**400**	**397**	1.1
T_9	389	380	2.2
SEm	6.52	8.56	2.06
CD (P = 0.05)	2.96	4.20	45.51

Table.5 Effect of Biofilmed biofertilisers on yield and yield attributing characters of chickpea

Treatments	Number of pods per each plant	Test weight (g)	Seed yield (kg ha^{-1})
T_1	13.7	18.9	780
T_2	16.7	19.8	1255
T_3	16.3	19.4	1181
T_4	17.0	19.9	1278
T_5	15.7	19.2	1138
T_6	15.0	19.1	1020
T_7	16.7	19.3	1158
T_8	**17.7**	**20.6**	**1409**
T_9	17.3	19.8	1334
SEm	0.50	0.14	14.22
CD (P = 0.05)	1.54	1.26	21.10

Shaban and EI- Bramaway (2011) studied the biological control of damping off and root rot causing fungi (*F. oxysporum*, *F. solani*, *Macrophomina phaseolina*, *Rhizoctonia solani* and S*clerotium rolfsii*) with antagonistic organisms (*Rhizobium and Trichoderma* spp). Results revealed that combined effect of both *Rhizobium spp. and*

Trichoderma spp. were found to be beneficial in controlling the fungal diseases of legume crops.

Evaluation of disease and nutrient management of chickpea under field conditions

Effect of biofilmed biofertilisers on different plant growth parameters

Highest plant height (14.7cm and 29.43cm), root length (8.43cm and 17.40cm), shoot (4.12g) and root(0.61g) dry weight of chickpea were analysed at 30 and 60 days after sowing was recorded in T_8 (*Trichoderma viride* + *Rhizobium* + *Pseudomonas fluorescence*+ *Bacillus subtilis* (Biofilm) with when compared to all other treatments.

Karnwal and Kumar (2012) reported that shoot length and dry matter increased up to 43 % of chickpea after inoculation with Plant growth promoting rhizobacteria (PGPR) increased up to 92 % in comparison with control.

Effect of biofilmed biofertilisers on disease suppression (*Fusarium* wilt) in chickpea

Germination percentage of chickpea seeds is 100 % under *in vitro* conditions but under field conditions it is 77-95 %. The difference in the initial population and final population was recorded due to the attack of *Fusarium* wilt during the crop growth. The lowest percent wilt incidence was recorded in T_8 (*T. viride* + *R. leguminosarum* + *P. fluorescence*+ *B. subtilis* (Biofilm) *i.e.* (1.1 %) which is on par with T_4 (1.3 %) and then followed by T_9 (*T. viride* + *R. leguminosarum* + *P. fluorescence*+ *B. subtilis* (Coinoculation) (2.2 %). The highest percent of wilt occurrence was observed in T_1 (control) (20.7 %).

Similar results were reported by Leo *et al.*, (2012) where they conducted on-farm demonstration by using *Trichoderma viride*, PSB and *Rhizobium* to study the effect on wilt incidence, yield and related parameters. Seeds were treated with PSB + *Rhizobium* + *T. viride* followed by soil application of *T. viride* + PSB + *Rhizobium* after 30 DAS (mixed with 200 kg of FYM), wilt incidence was (3.3 %) when compared to the other individual treatments and for control where the wilt incidence was (18.1 %) (Table 4).

Effect of Biofilmed biofertilisers on yield and yield attributing characters of chickpea

At harvest significantly highest number of pods (17.67) per plant, maximum weight of 100 seeds (20.58 g), seed yield (1409 kg ha^{-1}) was recorded in the treatment T_8 (*T. viride*+ *R. leguminosarum* + *P. fluorescence*+ *B. subtilis* (Biofilm) compared to all other treatments (Table 5).

Similar results were reported by Wani *et al.*, (2007). They showed that *Mesorhizobium ciceri* and phosphate-solubilizing rhizobacteria promoted plant growth, grain yield and nutrient uptake by field grown chickpea.

Das *et al.*, (2013) reported that the combined inoculation of *Rhizobium* and PSB significantly enhanced growth, yield attributes, yield, nutrient content and their uptake in seed and straw of chickpea.

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