Original Research Article

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

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Abstract

Wilt caused by *Fusarium oxysporium f. spiceris* 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.

Keywords

Biofertilizers, Nutrient Management and Chickpea

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, Garbanzooor 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°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) = \[ \frac{Z \cdot C}{\bar{C}} \] 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.

**Protein 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:

\[
\text{Percent Inhibition} = \frac{\text{Growth of Pathogens in control (mm)} - \text{Growth of Pathogens in treatment (mm)}}{\text{Growth of Pathogens in control (mm)}} \times 100
\]

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. fluorescense* 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 fluorescense* + *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 T4 (*Trichoderma viride + Pseudomonas fluorescense* (Biofilm), (0.41 mg ml⁻¹) and the lowest was recorded with T9 (*Trichoderma viride + Rhizobium leguminosarum + Pseudomonas fluorescense + 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 T2 (*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 T8 (*Trichoderma viride + Rhizobium
leguminosarum + Pseudomonas fluorescence + Bacillus subtilis (Biofilm) with an inhibition zone of (03.00 mm) and the next best is T9 and T4 (36.6 %) and inhibition zone of (3.01 mm). The lowest inhibition was recorded in T2 R. leguminosarum + T. viride biofilms and its dual culture T5 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             | Solubilisation efficiency |                 |                               |
|       |            | Solubilisation Zone       | Culture media       |                 |                               |
| 1     | T1         | -                        | -                   | -              | -                             |
| 2     | T2         | -                        | -                   | ++             | ++                            | 0.29                          |
| 3     | T3         | 24                       | 13                  | 184.6          | +++                           | 0.30                          |
| 4     | T4         | 20                       | 11                  | 181            | +++                           | 0.41                          |
| 5     | T5         | -                        | -                   | ++             | ++                            | 0.30                          |
| 6     | T6         | 19                       | 14                  | 135.5          | +                             | 0.39                          |
| 7     | T7         | 20                       | 12                  | 166.6          | +                             | 0.39                          |
| 8     | T8         | 21                       | 11                  | 190            | +++                           | 0.29                          |
| 9     | T9         | 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      | T1         | -                   | -                      | -              | -               |
| 2      | T2         | 31.65               | 00                     | +++            | ++              |
| 3      | T3         | 34.40               | 01.00                  | +++            | +++             |
| 4      | T4         | 36.6                | 03.01                  | +++            | +++             |
| 5      | T5         | 29.95               | 00                     | +              | ++              |
| 6      | T6         | 33.85               | 01.00                  | +              | ++              |
| 7      | T7         | 36.05               | 03.00                  | +++            | +++             |
| 8      | T8         | 37.15               | 03.00                  | +++            | +++             |
| 9      | T9         | 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 |
| T1         | 13.27            | 23.93           | 6.53            | 12.90          | 2.18    | 0.35    |
| T2         | 14.37            | 28.23           | 8.13            | 16.00          | 3.55    | 0.54    |
| T3         | 13.83            | 28.30           | 7.93            | 14.47          | 3.33    | 0.47    |
| T4         | 14.40            | 29.43           | 8.27            | 15.73          | 3.54    | 0.48    |
| T5         | 13.93            | 27.97           | 7.87            | 14.33          | 3.55    | 0.51    |
| T6         | 13.37            | 27.40           | 7.33            | 13.50          | 3.08    | 0.44    |
| T7         | 14.27            | 28.53           | 7.87            | 14.33          | 3.27    | 0.46    |
| T8         | 14.77            | 29.43           | 8.43            | 17.40          | 4.12    | 0.61    |
| T9         | 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 (%) |
|------------|--------------------------|------------------------|--------------------|
| T1         | 360                      | 285                    | 20.7               |
| T2         | 385                      | 349                    | 9.3                |
| T3         | 382                      | 352                    | 8.0                |
| T4         | 386                      | 381                    | 1.3                |
| T5         | 380                      | 329                    | 13.7               |
| T6         | 372                      | 337                    | 9.3                |
| T7         | 380                      | 363                    | 4.7                |
| T8         | 400                      | 397                    | 1.1                |
| T9         | 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) |
|------------|-------------------------------|----------------|---------------------|
| T1         | 13.7                          | 18.9           | 780                 |
| T2         | 16.7                          | 19.8           | 1255                |
| T3         | 16.3                          | 19.4           | 1181                |
| T4         | 17.0                          | 19.9           | 1278                |
| T5         | 15.7                          | 19.2           | 1138                |
| T6         | 15.0                          | 19.1           | 1020                |
| T7         | 16.7                          | 19.3           | 1158                |
| T8         | 17.7                          | **20.6**       | **1409**            |
| T9         | 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 Sclerotium 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 T8 (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 T8 (T. viride + R. leguminosarum + P. fluorescence+ B. subtilis (Biofilm) i.e. (1.1 %) which is on par with T4 (1.3 %) and then followed by T9 (T. viride + R. leguminosarum + P. fluorescence+ B. subtilis (Coinoculation) (2.2 %). The highest percent of wilt occurrence was observed in T1 (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 T8 (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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