Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.3097

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **5 (4):** 117-122 (2017)

Research Article

International Journal of Pure & Applied Bioscience

OPEN ACCESS

Evaluation of Persistance and Efficiency of Different Biofilmed Biofertilizers with Chickpea Crop

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Received: 7.06.2017 | Revised: 15.06.2017 | Accepted: 18.06.2017

ABSTRACT

Soil is considered to be a storehouse of microbial activity, though the space occupied by living organisms is estimated to be less than 5% of the total space. Soil samples were collected from the waste lands of Parthenium rhizosphere soils in college farm, Rajendranagar, Hyderabad and isolated different Rhizobacteria namely Bacillus subtilis, Pseudomonas flourescens and Trichoderma viride isolate from soil by serial dilution and plate count method. Rhizobium leguminosarum strain is collected from Biofertiliser unit, Agriculture Research Institute (ARI) Rajendranagar for the preparation of a Biofilm. A Biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to a surface. Plant-associated microorganisms fulfill important functions for plant growth and health. Such beneficial biofilms can be developed in vitro and be used as biofertilizers (Biofilmed Biofertilizers, BBs) and biocontroling agents for different crops, when applied at high cell densities. The research studies conducted so far in this field with special attention into development of biofilms of N2-fixing, P-solubilizing, and disease control bacteria and fungi. Four types of biofilms namely B. subtilis –T. viride, P. flourescense – T.viride, R. leguminosarum –T.viride B. subtilis –P. flourescense – R. leguminosarum –T. viride biofilms were prepared. Biofilm formation is a dynamic process involving quorum sensing for successful attachment and colonization. Regarding the persistence studies of biofilms in soil; the four types of biofilms were incubated in soil. Initial microbial populations were recorded. After 90 days of incubation, there is a decline in the counts of biofilm partners ranging from 50-73% under pot cultures were observed and recorded.

Key words: *Bacillus subtilis, Pseudomonas flourescens, Rhizobium leguminosarum, Trichoderma viride.*

INTRODUCTION

For many years' microbes in nature have been viewed as simple life forms growing as individual cells. This has enabled the characterization of the microorganisms. However, planktonic growth is not the natural situation for microorganisms and care. During the last decades an intensive research has been conducted in the area of biofilms: medical-industrial and plant associated biofilms.

Cite this article: Nissipaul, M., Triveni, S., Reddy, R.S. and Sushma, R.D., Evaluation of Persistance and Efficiency of Different Biofilmed Biofertilizers with Chickpea Crop, *Int. J. Pure App. Biosci.* **5(4):** 117-122 (2017). doi: http://dx.doi.org/10.18782/2320-7051.3097

Usually biofilms are defined as complex microbial communities attached to the surface or interface enclosed in an extracellular matrix of microbial and host origin to produce a spatially organized three dimensional structure. Biofilm is a normal common existence in bacterial ecosystems. Within the biofilms bacteria have cooperative behavior and they may be susceptible to harsh environmental conditions. It is the preferred state of existence because bacterial community adds defenses and multiple mechanism of bacterial survival and enhances its fitness. Biofilms represents complex communities of multiple microbial species which remain attached to surfaces or at the interfaces[3], and possess the capacity to maintain the metabolic activity under adverse environmental conditions, exhibiting increased survival in a competitive environment[7]. Microorganisms also gain access to resources and niches that require critical mass and cannot effectively be utilized by isolated cells. Acquisition of new genetic traits, nutrient availability and metabolic cooperation have also been suggested as means for optimization of population survival in biofilms.

Plant biotechnology contributed to the development of numerous new crop varieties with enhanced disease resistance, greater drought and salt tolerance, and better nutritional value. Unfortunately, the beneficial plant–microbe interaction was often ignored in breeding strategies although plant-associated microorganisms fulfill important ecosystem functions for plants and soils[6]. This includes the effects of plant-associated microorganisms on plant health and growth; they enhance stress tolerance, provide disease resistance, aid nutrient availability and uptake and promote biodiversity[2,4]. Furthermore, plant-associated microbial communities show, due to specific secondary metabolism and morphology, a certain degree of specificity for each plant species[1]. This knowledge has yet to be exploited in agricultural biotechnology.

In this article we highlight themes regarding the diversity and persistance of the bacterial biofilms and elucidate their potential as a rich source of novel biologically active compounds. The underground resources of plant rhizosphere could provide insights associated with global climate change. So far these resources have been neglected to large extent but hopefully with the help of new technologies we will be able to understand and employ the natural potential of biofilms for our agro-ecosystems.

MATERIAL AND METHODS

Soil Sample Collection & Isolation of Rhizobacteria

Soil samples were collected from the waste lands of *Parthenium* rhizosphere soils in college farm, Rajendranagar, Hyderabad and isolated different Rhizobacteria.

For isolation of rhizobacteria, the method proposed by Vlassak *et al*[9]., was followed. In this procedure 10 g of soil from each soil sample was taken in a conical flask to which 90 ml of normal saline was added. The sample was agitated for 15 minutes on a vortex and serial dilutions of soil suspensions were prepared.

0.1ml of respective samples were spread on sterilized Petri plates containing specific media *i.e. B. subtilis* on Nutrient agar, *P. flourescens* on Kings B, *R. leguminosarum* on Yeast Extract Mannitol Agar with Congo red and *T. viride* on Potato Dextrose agar. The Petri plates were incubated at room temperatures ($28^{\circ}C \pm 2^{\circ}C$) for 24-72 h. All the strains were studied for their cultural and morphological characterization and the best performed strains were used for Biofilm preperation.

Biofilm preparation

The inocula used for the preparation of different biofilms were five days incubated culture of Fungi (3 ml) and two days old culture of Bacteria (5 ml) in 250 ml broth. Three sets of each for all the biofilms were prepared. Initially 5 ml of the bacterial culture was inoculated and then incubated for one days in a shaking incubator at 110 rpm and then inoculation of *Trichoderma viride* (5 ml). The flasks were incubated under static conditions at 30 for 16 days until a thick film of culture is observed on

the surface of the liquid medium. The growth of the biofilm was observed for every two days' interval. The progressive growth of biofilm was observed under microscope. The population counts were done using serial dilution- plate count method. After 16 days' incubation the biofilm is harvested and washed repeatedly with sterile water for 2-3 times to remove the non-adherent cells from biofilm, then centrifused and vortexed on a cyclomixer for 10 min, with the use of sterilised glass beads to make it as a uniform suspension. The biofilm is a liquid suspension which is ready to apply under field conditions and also for taking the population counts, Fresh weights and Dry weights (65^{o}C oven dried culture for 24h).

***In Vitro* Screening of Isolates for various Plant Growth Promoting and Biocontrol Attributes.**

For biochemical tests single, dual and biofilm partners were used. The single cultures with a population CFU of *R. leguminosarum* (12×10^6), *B. subtilis* (18×10^7), *P. fluorescens* (22×10^7) and *T. viride* (31×10^5) were used for biochemical tests. For dual cultures the same counts mentioned above of the bacteria and fungi were used in the ratio of 1:1 each partner mixed and further used. The population counts of *T. viride-B. subtilis* film had the populations in CFU of *T. viride* (11.8×10^6) and *B. subtilis* (86×10^8), *T. viride- R. leguminosarum* film had the population of *T. viride* (99×10^6) and *R. stolinifer* (52×10^8) in *T. viride- P. flourescens* film had the population counts in CFU was *T. viride* (12.6×10^6) and *P. flourescens* (94×10^8) respectively.

Pure cultures of single isolates, dual cultures of biofilm partners were isolated by streaking on respective media plates and screened for following Plant growth promotion properties in terms of Protein estimation, NH_3 production, HCN Production, Siderophore production, IAA production, Phosphate solubilization, and Antagonism towards selected phyto-pathogenic fungi.

RESULTS AND DISCUSSION

Population of different partners in biofilms in different growth media

After preparation of biofilms the viable counts were taken to know the population load in biofilms and given in the Table (4.4). Different media was used to study the population. The *P. fluorescence - T. viride* biofilms (*T. viride* - 12.6×10^6 and *P. fluorescence* - 61×10^8) followed by *B. subtilis – T. viride* with a population counts of *B. subtilis* - 58×10^8 and *T. viride* - 10.7×10^6) showed highest population in Pikovskaya's medium when compared to nutrient medium and YEM broth. But the *R. leguminosarum –T. viride* biofilm recorded highest population (*R. leguminosarum* - 52×10^8 and *T. viride* - 9.9×10^6) in YEM broth when compared to Pikovskaya's broth and nutrient broth.

Persistence of Biofilms in Soil

The Initial population counts and population counts at 30 DAI, 60 DAI, & 90 DAI of the *B. subtilis–T. viride, R. leguminosarum–T viride, P flourescens-T viride biofilms* were presented in the Table 4.15

Persistence of *B. subtilis – T. viride* biofilm in soil

Initial microbial population in *Bacillus subtilis - T. viride* biofilms were *Bacillus* (86×10^8) and *T. viride* (11.8×10^6) which is represented in the Table 4.15. After 90 d of incubation, with 55 % reduction in *Bacillus* population and around 51 % decline in the counts of *Trichoderma. viride*. The population counts of *Bacillus* and *Trichoderma viride* were 39×10^8 and 58×10^5 respectively.

Persistence of *P. flourescens -T viride* biofilm in soil

Initial microbial population in *P. fluorescence –T. viride* biofilms were *P.* (94×10^8) and *T. viride* (126×10^5). After 90 days after incubation, with 56 % reduction in *P. fluorescence* population and around 49 % decline in the counts of *T. viride*. The population counts of *P.* and *T. viride* were 41×10^8 and 65×10^5 respectively.

Persistence of *R. leguminosarum - T. viride* biofilm in soil

From the Table 4.15 the initial microbial populations in *R. leguminosarum –T. viride* biofilms were *R. leguminosarum* (52×10^8) and *T. viride* (99×10^5). After 90 d of incubation, with 73 % reduction in *Bacillus* population and around 55 % decline in the counts of *T.*

viride. The population counts of *R. leguminosarum* and *T. viride* were 14×10^8 and 45×10^5 respectively.

Persistence of *B. subtilis - P. flourescens - R. leguminosarum -T. viride* biofilm in soil

Initial microbial populations in *B. subtilis –P. fluorescence – R. leguminosarum –T. viride* biofilms were *B. subtilis* (65×10^8) *P.* (70×10^8) *R. leguminosarum* (52×10^8) and *T. viride* (112×10^5). After 90 d of incubation, with 57% reduction in *B. subtilis* population, 54% in *P.* population, 59% reduction in *R. leguminosarum* population and around 55% decline in the counts of *T. viride.*

Table 1: *In vitro* screening of biofilms for various Plant growth promoting attributes

S.No.	Treatments	Phosphate solubulisation			Ammonia production	IAA production	Protein estimation ($mgml^{-1}$)
		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	+++	++

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

T1 : Control
T2 *: Trichoderma viride + Rhizobium* (Biofilm)
T3 : *Trichoderma viride + Bacillus subtilis* (Biofilm)
T4 *: Trichoderma viride + Pseudomonas fluorescence* (Biofilm)
T5 : *Trichoderma viride + Rhizobium* (Co-inoculation)
T6 : *Trichoderma viride + Bacillus subtilis* (Co-inoculation)
T7 : *Trichoderma viride + Pseudomonas fluorescence* (Co-inoculation)
T8 : *Trichoderma viride + Rhizobium + Pseudomonas fluorescence+ Bacillus subtilis* (Biofilm)
T9: *Trichoderma viride + Rhizobium + Pseudomonas fluorescence+ Bacillus subtilis* (Biofilm)

Table 3: Population counts of different biofilms in soil to study their persistance

Biofilms	Initial microbial population in Biofilms		30 DAI		60 DAI		90 DAI	
	Bacteria $\times 10^8$	Fungi $\times 10^6$	Bacteria $\times 10^8$	Fungi $\times 10^6$	Bacteria $\times 10^8$	Fungi $\times 10^6$	Bacteria $\times 10^8$	Fungi $\times 10^6$
Bs- Tv	86	11.8	78	9.7	63	7.4	39	5.8
Pf- Tv	94	12.6	82	10.9	79	8.7	41	6.5
Rs- Tv	52	9.9	43	7.8	35	6.2	14	4.5

Bs- Tv: *Bacillus subtilis* + *Trichoderma viride* (Biofilm)
Pf- Tv: *Pseudomonas fluorescence* + *Trichoderma viride* (Biofilm)
Rl- Tv: *Rhizobium leguminosarum*+ *Trichoderma viride* (Biofilm)

Table 4: Population counts of *T. viride* + *R. leguminosarum* + *P.*

Bs- Pf-Rs-Tv Biofilm	Initial microbial population in Biofilm	30 DAI	60 DAI	90 DAI
Trichoderma $\times 10^6$	11.2	9.2	7.1	5.8
Bacillus $\times 10^8$	65	57	48	28
Pseudomonas $\times 10^8$	70	59	51	32
Rhizobium $\times 10^8$	39	31	28	16

Tv-Bs-Pf-Rs : *T. viride* + *B. subtilis* + *P. fluorescence*+ *R. leguminosarum* (Biofilm)

A thick mat of biofilm is formed on the surface.

Harvesting and washing of the biofilm under aseptic conditions

Vortexed with sterilized glass beads on cyclomixer to form as uniform suspension.

Fig. 1: Preperation of Biofilm

CONCLUSION

After 90 days of incubation, in *B. subtilis –T. virride* biofilms with 55 % reduction in *Bacillus* population and around 51 % decline in the counts of *Trichoderma.* In *P. flourescense –T.viride* biofilms with 56 % reduction in *Pseudomonas* population and around 49% decline in the counts of *Trichoderma*. In *R. leguminosarum –T.viride* biofilms with 73% reduction in *Rhizobium* population and around 55 % decline in the counts of *Trichoderma*, respectively were recorded.

The live nature of the active ingredient (i.e., the microbial agent) emphasizes the need of a formulation to maintain the microbial cells in a metabolically and physiologically competent state, so that the desired benefit is observed, when applied[5].

Similar results were reported by Triveni *et al*[8]., who showed the persistance of *Bacillus- Trichoderma viride* biofilm in

different formulations by taking its population counts at 30, 60 and 90 days of incubation at room temperature. Composite: Vermiculite (1:1) proved to be the most promising carrier in terms of high inocula load of partners at 30, 60 and 90 days of incubation,that illustrated the promise of the selected carrier.

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