Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(3): 845-849
Received: 10-03-2017
Accepted: 11-04-2017

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Role of persistency of different biofilms in effective crop growth of Mung bean (*Vigna radiata*)

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Abstract
Plants support a diverse array of bacteria, including parasites, mutualists, and commensals on and around their roots, these microbes have a profound influence on plant health and productivity. Biofilms are communities of microorganisms adhering to abiotic/biotic surfaces. This experiment was conducted in College of Agriculture, PJTSAU, Rajendranagar. There is a growing appreciation that the intensity of growth and yields of mung bean in the pot culture study are significantly influenced by the high persistency of adherent microbial populations (Biofilms). At the end of the experimental period, the *invitro* pot culture study among the T1–T8 treatments, T4 (*Trichoderma viridae* + *Bacillus subtilis* + *Pseudomonas fluorescence* + *Rhizobium leguminosarum*) treatment recorded the high activity in all the parameters at all the three plant growth stages due to high persistency of biofilms. Hence, the yield was recorded high in T4 treatment. The remaining treatments showed significant results in all the parameters. The response of biofilms (T1-T4 treatments) was more pronounced than that of coinoculation (T5-T8) and control. At different stages of crop growth the persistency of biofilms was checked under Scanning electron microscope. Positive responses such as plant growth hormone production, mineral nutrients solubilization, high enzyme activity in the soil and biocontrolling effects due to high persistency had contributed to the increased plant growth with Biofilms. These interactions between bacteria and fungi and their biodiversity had the effect on microbial ecology in soils of pot culture and therefore have the potential to sustain modern agriculture systems with the use of microbial community of biofilms as biofilmed biofertilizers.

Keywords: Persistency, biofilms, effective crop growth, Mung bean (*Vigna radiata*)

Introduction
Root biofilm initiation and development is complex and not well understood due to the dynamic nature of plant root surfaces. In addition to physico-chemical variations throughout the root surface, it is likely that other abiotic factors such as nutrient availability, temperature and relative humidity influence root biofilm associations (Stanley & Lazazzera, 2004) [10]. Bacterial species have adapted to these ever-changing conditions and are capable of starting colonization by forming
Micro colonies on different parts of the roots from tip to elongation zone. Interestingly, root exudates serve as a major plant-derived factor responsible for triggering root colonization (Lugtenberg *et al*., 1999) [7] and biofilm associations (Walker *et al*., 2004) [13]. It is estimated that plants secrete between 10% and 44% of their photosynthates as root exudates. This interaction becomes more complicated when more than one bacterium is involved. Biofilms involved in multitrophic interactions are economically important for several agricultural crops. So that biofilms and their environmental interactions will greatly benefit the scientific community.

Materials and Methods
Study site: The experiment was carried out at College of Agriulture, PJTSAU, Hyderabad.
Isolation of native rhizobacteria (rhizosphere) are carried out on different media like nutrient agar, potato dextrose agar and yeast extrat agar among the isolated bacteria identified Phosphate Solubilising Bacteria like Bacillus, Pseudomonas, Rhizobium and Trihoderma fungi on pikovaskaya medium. The selected bacterial colonies were purified by repeated subculturing (usually 2-3 times). The isolated bacteria were tested for properties such as nitrogenase activity (Hardy *et al*., 1968) [3], phosphate-solubilizing activity (Sundara Rao and Sinha, 1963) [11] and all the bio hemial tests and their survival at lower temperatures. Preliminary identification of the selected isolates was carried out using microscopic and biochemical procedures (Collins and Lyne, 1980) [2].

Preparations of biofilms

The above screened best phosphate solubilizing bacteria were selected for biofilm formation. A biofilm is an aggregate of microorganisms in which cells are struck to each other and/or to a surface. *B. subtilis* + *T. viride* and *P. fluorescenes* + *T. viride* biofilms were prepared in pikovskaya medium, while yeast extract mannitol broth was used for *Trichoderma* + *Rhizobium* and nutrient broth for *B. subtilis* + *P. fluorescenes* + *Rhizobium* + *T. viride* biofilms. The inocula used for the preparation of different biofilms were five days old 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 day in a shaking incubator at 110 rpm and then inoculation of *Trichoderma viride* (5 ml). The flasks were incubated under static conditions at 30 ^{o}C 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 was harvested and washed repeatedly with sterile water for 2 - 3 times to remove the non adherent cells from biofilm, then centrifuged and vortexed on a cyclomixer for 10 min, with the use of sterilized glass beads to make it as a uniform suspension. The biofilm is a liquid suspension which was ready to apply under field conditions and also for taking the population counts, fresh weights and dry weights (65 ^{o}C oven dried for 24 h).

Germination test with water agar

Mungbean seeds (variety LGG- 406) were surface sterilised by 70% ethanol for 30 sec, followed by 0.1% mercuric chloride for 3min and then rinsed the seeds several times with sterile distilled water. These seeds were air dried in laminar flow chamber. The percent germination of seeds was tested on 0.8 % water agar for 48 to 96 h with different coinoculants and biofilms.

A pot culture experiment was conducted to investigate the persistency of biofilms to improve nutrient management in Mungbean.

Treatments

Control (RDF)

Rock Phosphate

T1 : *Trichoderma viride* + *Bacillus subtilis* (Biofilm)

T2 : *Trichoderma viride* + *Pseudomonas fluorescence* (Biofilm)

T3 : *Trichoderma viride* + *Rhizobium leguminosarum* (Biofilm)

T4 : *Trichoderma viride* + *B. subtilis* + *P. fluorescence* + *R. leguminosarum* (Biofilm)

T5 : *Trichoderma viride* + *Bacillus subtilis* (Co-inoculation)

T6 : *Trichoderma viride* + *Pseudomonas fluorescence* (Co-inoculation)

T7 : *Trichoderma viride* + *Rhizobium leguminosarum* (Co-inoculation)

T8 : *Trichoderma viride* + *B. subtilis* + *P. fluorescence* + *R. leguminosarum* (Co-inoculation)

In vitro developed biofilms were applied in pot culture to study their persistency.

Population counts of Bacteria and Fungi at different intervals of crop growth period (0, 20, 40, 60 DAS) were noted down

Results

The microbial counts were taken at different (0, 20, 40, 60 days) growth intervals. It was found that the crop growth period influence the microbial population during vegetative and flowering stage due to less availability of nutrients to microbes by the root exudation.

The data regarding population counts and persistency of biofilms was mentioned in tables. Initial population counts in Table 4.8 and at different stages of crop growth in Table. 4.9.

1. Persistence of *B. subtilis* – *T. viride* biofilm in soil (in cfu g^{-1})

There was an increase in population counts upto the flowering stage and after flowering stage there was a decrease in microbial count at harvesting stage. Initial microbial population in *Bacillus subtilis* - *T. viride* biofilm was *Bacillus* (6.40×10^{7}) and *T. viride* (5.03×10^{4}). At the vegetative stage the population of *Bacillus* 12.12×10^{7} and *T. viride* 5.33×10^{4} increased respectively. At flowering stage the highest population counts of *Bacillus* and Fungi 17.93×10^{7} and 11.33×10^{4} were observed. After 60 days of sowing, there was 23.10 % reduction in *Bacillus* population and around 26.47 % decline in the counts of *Trichoderma viride*. The population counts of *Bacillus* and *Trichoderma viride* were 8.15×10^{7} and 6.33×10^{4} respectively at 60 DAS.

Initial bacterial population of *Bacillus subtilis* - *T. viride* coinoculation was *Bacillus* (8×10^{7}) and *T. viride* (6.5×10^{4}). At vegetative stage the populations increased to the less extent when compared to at the time of inoculation i.e., *Bacillus* (9.14×10^{7}) and *T. viride* (7.33×10^{4}). At flowering stage the highest population of *Bacillus* and Fungi was 15.03×10^{7} and 13.33×10^{4} found. After 60 days of sowing, there was 20.35 % reduction in *Bacillus* population and around 30 % decline in the counts of *Trichoderma viride* when compared to flowering stage.

In consortium the fungal population was augmented because of individual growth, but in all the biofilms the bacterial population was increased, It might be due to good colonization of fungal hyphae and bacteria. When compared to the dual cultures, biofilms registered more no. of bacterial population this will influence the biofilm survival and activity in soil.

2. Persistence of *P. flourescens* – *T. viride* biofilm in soil (in cfu g^{-1})

Initial microbial population in *P. fluorescence* – *T. viride* biofilms were *P. fluorescence* (6×10^{5}) and *T. viride* (5.5×10^{7}). When the crop reaches to vegetative stage the population of *P. fluorescence* and *T. viride* was 15.12×10^{7} and 6.33×10^{4}. At flowering stage the highest population counts of *P. fluorescence* – *T. viride* was 19.02×10^{7} and 10.33×10^{4} respectively. After 60 DAS, there was 16.35% reduction in *P. fluorescence* population and around 38.7% decline in the counts of *T. viride*. The population counts of *P. fluorescence* and *T. viride* were 15.91×10^{7} and 6.33×10^{4} respectively.

Initial bacterial population of *P. fluorescence* - *T. viride* coinoculation *P. fluorescence* (7.10×10^{7}) and *T. viride* (7.98×10^{4}). In the vegetative stage the *P. fluorescence* population (10.12×10^{7}) and *T. viride* (9.00×10^{4}). At flowering stage the highest population of *P. fluorescence* and Fungi 15.83×10^{5} and 14.33×10^{4} respectively. After 60 days of sowing, there was 24.38 % reduction in *P. fluorescence* population and around 34.89 % decline in the counts of *Trichoderma viride*.

3. Persistence of *R. leguminosarum* - *T. viride* biofilm in soil (in cfu g^{-1})

The initial microbial populations in *R. leguminosarum* – *T. viride* biofilms were *R. leguminosarum* (5.40×10^5) and *T. viride* (3.90×10^4). In the vegetative stage the *Rhizobium* population increased nearly 33.66% increased i.e., (8.14×10^7) and *T. viride* population was nearly 99% increased i.e., 4.33×10^4. At flowering stage the highest population counts of *R. leguminosarum* – *T. viride* were 13.83×10^7 and 14.33×10^4 respectively. After 60 days of sowing, 33.83 % reduction in *R. leguminosarum* population and around 34.89 % decline in the counts of *T. viride* was found. The population counts of *R. leguminosarum* and *T. viride* were 9.15×10^7 and 9.33×10^4 cfu gm^{-1} respectively.

Initial bacterial population of *R. leguminosarum* - *T. viride* coinoculation *R. leguminosarum* was (3.4×10^5) and *T. viride* (3.57×10^4). At vegetative stage the populations were 4.34×10^5 and *T. viride* 5.33×10^4. At flowering stage the highest population of *R. leguminosarum* and Fungi were 12.16×10^5 and 12.33×10^4 respectively. After 60 days of sowing, there was 47.4 % reduction in *R. leguminosarum* population and around 48.66% decline in the counts of *Trichoderma viride*.

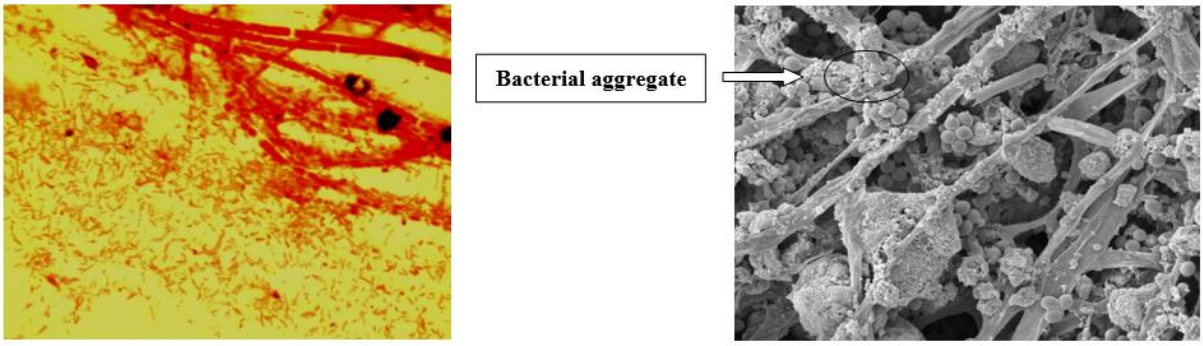
4. Persistence of *B. subtilis* - *P. flourescens* - *R. leguminosarum* - *T. viride* biofilm in soil (in cfu g^{-1})

Initial microbial populations of bacteria was $6.02\times10^7$and *T. viride* was 5.99×10^4. At vegetative stage persistency of bacteria and *Trichoderma* was 21.22×10^7 and 5.33×10^4. At flowering stage the highest population of bacteria was 28.97×10^7 and *T. viride* 17.33×10^4. After 60 days of incubation, there was 15.25 % reduction in bacteria population, 51.93% decline in the counts of *T. viride.*

Initial microbial populations of bacteria 7×10^7, *T. viride* 6.49×10^4 in coinoculation. At vegetative stage the bacteria was 19.35×10^7 and *T. viride* 4.33×10^4. At flowering stage the highest population was recorded and at there was 14.21 % reduction in bacteria population and around 51.93 % decline in the counts of *T. viride.*

Biofilm persistency may indicate that microorganisms were capable of surviving in the environment in the form of biofilm. Biofilms were found to be considerably more resistant to stress, including heat and desiccation. As with environmental effects, biofilmed cells were significantly more resistant than individual cells.

As per the results obtained we can conclude that the presence of a subpopulation of persisters in the biofilm may account for the observed broad resistance. The difference between coinoculation and biofilm communities is that the frequency of persisters is much higher in the biofilm population when compared to co inoculations in the above mentioned results. The best persistency was observed with T4 (*Trichoderma viride* + *Bacillus subtilis* + *Pseudomonas fluorescence* + *Rhizobium leguminosarum* - Biofilm) next followed by T8 (*Trichoderma viride* + *Bacillus subtilis* + *Pseudomonas fluorescence* + *Rhizobium leguminosarum* – coinoculation). This might be due to high indigenous populations within the biofilm and as the crop growth stage increases soil physical, chemical and biological properties, elevated levels of readily available nutrients, phytohormones, enzymes were also improved. So that, the population of the bacteria and fungi in the biofilm was increased accordingly. This study was conducted to exploit the synergistic effect of fungi and bacteria along with varying levels of nutrition in improving the population/persistency and growth of organisms in the form of biofilms.



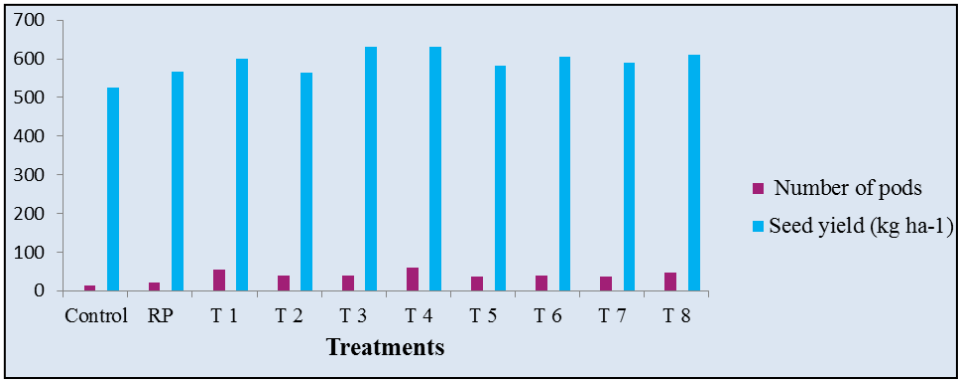
Microscopic view of biofilms

Germination test on water agar

Pot culture studies

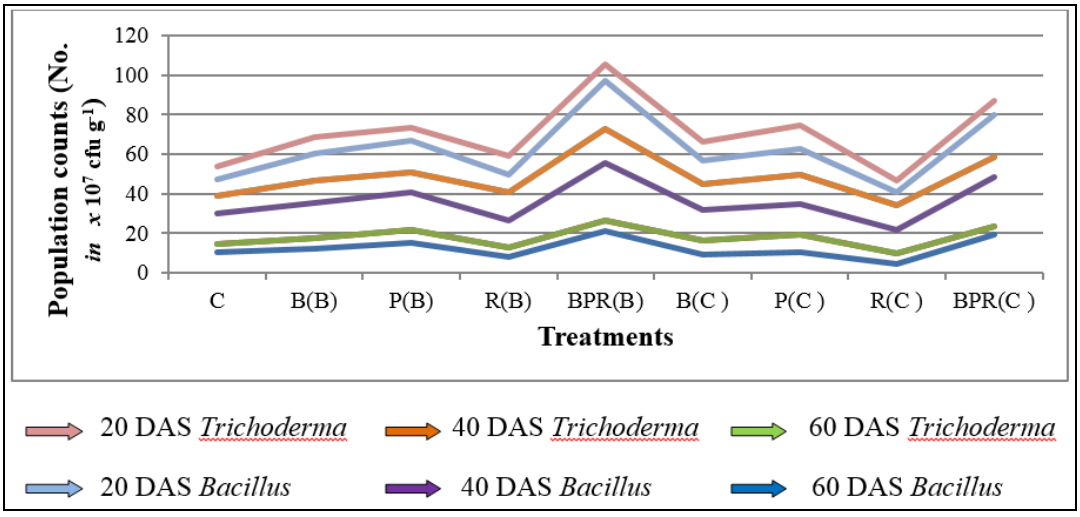
Effect of biofilms and coinoculations on yield attributing characters of Mungbean

Treatments	Number of pods / plant	Seed test weight (g)	Seed yield (kg ha^{-1})
Control	14.67	3.28	525
Rock Phosphate	22.33	3.54	566
T1	53.67	3.74	599
T2	38.33	3.53	565
T3	40.33	3.94	631
T4	60.00	3.94	631
T5	37.33	3.65	583
T6	40.67	3.78	605
T7	38.00	3.68	589
T8	46.33	3.81	610
SE(m)	0.62	0.09	2.39
CV	2.76	4.23	0.70
CD	1.85	0.27	7.10



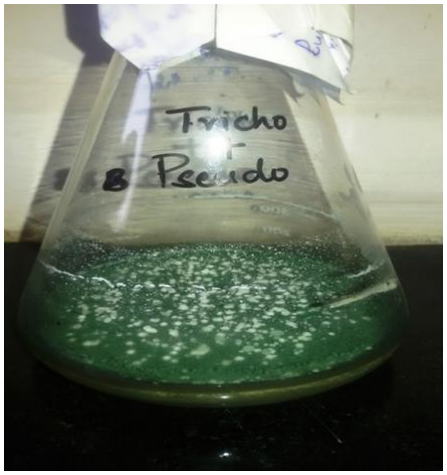
Persistency of population counts of bacteria and fungi at 20, 40, 60 DAS (cfu g^{-1} of soil) in Greengram crop

Treatments	20 DAS		40 DAS		60 DAS	
	Bacteria x10^{7}cfu g^{-1}	*Trichoderma*x10^4 cfu g^{-1}	Bacteria x10^{7}cfu g^{-1}	*Trichoderma* x10^4 cfu g^{-1}	Bacteria x10^{7}cfu g^{-1}	*Trichoderma* x10^4 cfu g^{-1}
Control	10.23	4.33	15.12	9.33	8.15	6.33
T1	12.12	5.33	17.93	11.33	13.78	8.33
T2	15.12	6.33	19.02	10.33	15.91	6.33
T3	8.14	4.33	13.83	14.33	9.15	9.33
T4	21.22	5.33	28.97	17.33	24.55	8.33
T5	9.14	7.33	15.03	13.33	11.97	9.33
T6	10.12	9.00	15.83	14.33	13.64	11.33
T7	4.34	5.33	12.16	12.33	6.39	6.33
T8	19.35	4.33	24.56	10.33	21.07	7.33
CD	0.04	0.94	0.02	0.99	0.07	0.99
SE(m)	0.01	0.31	0.01	0.33	0.02	0.33
CV	0.16	9.48	0.06	4.59	0.31	7.12



Persistency of population counts of bacteria and fungi at 20, 40, 60 DAS (cfu g^{-1} of soil) in Greengram crop

Pot culture yield



Fungal bacterial biofilms

Conclusions

Positive responses such as plant growth hormone production, mineral nutrients solubilization, high enzyme activity in the soil and bio controlling effects due to high persistency had contributed to the increased plant growth with Biofilms. These interactions between bacteria and fungi and their biodiversity had the effect on microbial ecology in soils of pot culture and therefore have the potential to sustain modern agriculture systems with the use of microbial community of biofilms as biofilmed biofertilizers.

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