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Biofilm Formation of Zinc Solubilizing, Potassium Releasing Bacteria on the Surface of Fungi

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A B S T R A C T

Attachment of soil bacteria to plant cells is supposedly the very early step required in plant–microbe interactions. Attachment also is an initial step for the formation of microbial biofilms on plant roots. For the rhizobia–legume symbiosis, various mechanisms and diverse surface molecules of both partners have been proposed to mediate in this process. A biofilm is an aggregate of microorganisms in which cells are stuck to each other and/or to biotic/abiotic surface. These adherent cells are frequently embedded within a self-produced matrix of Extracellular Polymeric Substance (EPS) is a polymeric jumble of DNA, proteins and polysaccharides. The high population density achieved in biofilms provides the opportunity to perform enhanced biochemical reactions than that of single isolates and dual cultures. In the present investigation we aimed towards the development of biofilms under *in vitro* conditions, using a combination of agriculturally important potential microorganisms like zinc solubilizing, potassium releasing bacteria on the fungal hyphosphere. All the isolates were screed for their zinc solubilization as well as potassium releasing capacity along with biochemical characterization. Biofilm formation of zinc solubilizing and potassium releasing isolates has considerably enhanced the release of potassium as well as zinc. Four hyphobacteria has exhibited high potential for solubilization of organic Zn to inorganic Zn as well as potassium (K) released considerably high when biofilm is used.

Introduction

Biofilms are the common life strategy for bacteria in natural environments. Biofilms are composed of populations or communities of microorganisms embedded in self-produced polymeric matrix (mainly extracellular polysaccharides) that have adhered to environmental surfaces in which sufficient moisture is present (Costerton *et al.*, 1995). These three-dimensional microbial communities may be formed in all environments colonized by bacteria, such as on solid substrates in contact with moisture or on tissue surfaces in living organisms. The mutualistic association between microbial communities and plant roots, the so-called rhizosphere, form an environment that fulfils the requisites for biofilm formation: sufficient moisture and a supply of nutrients, which are provided by the plant. Most researchers working with rhizospheric bacteria have not described the formation of biofilms on plant roots. In the past, however, different reports

have indicated that rhizospheric bacteria (such as *Rhizobium, Azospirillum* and *Pseudomonas*) associated with root surfaces are embedded in the root mucigel and might also be encased in a self-produced extracellular matrix.

Transmission electron microscopy has shown the presence of fibrillar material around rhizobia attached to the root surface (Fujishige *et al.,* 2006). These observations further support the proposal that root colonizing bacteria are capable of forming biofilms, it is reasonable to suppose that the molecular mechanisms operating in bacterial attachment to roots also might be relevant for biofilm development. The dynamic processes that characterize relationships between plants and microbial communities are complex. Soil microorganisms have an important influence on soil fertility and plant growth (Andrade *et al.,* 1997; Miransari, 2011).A recent study showed that biofilm formation of rhizobia with common soil fungi is a plausible strategy for the survival (Seneviratne and Jayasinghearachchi, 2003). These biofilms can be used to successfully introduce bacterial inoculants into soil because they can protect the inoculants against adverse environmental conditions and the competition by native soil populations.

This study on zinc solubilization by bacteria has an immense importance in zinc nutrition to plants in the world. The rhizospheric microorganisms play a pivotal role in the enhancement of crop production by the solubilization of unavailable form of metal into available form. This metal solubilization was due to the production of organic acids and pH drop by organisms.

For optimal nutrition of a crop, the replenishment of a K depleted soil solution is affected predominantly by the release of exchangeable K from clay minerals. Consequently, for maximal crop growth, soil solution and exchangeable K need to be replenished continually with K through the release of non-exchangeable K through the weathering of K reserves (i.e. micas and feldspars) (Sparks and Huang, 1985) or the addition of K fertilizers. Many microorganisms in the soil are able to solubilize 'unavailable' forms of K-bearing minerals, such as micas, illite and orthoclases, by excreting organic acids which either directly dissolves rock K or chelate silicon ions to bring the K into solution (Groudev, 1987; Friedrich *et al.,* 1991; Ullman *et al.,* 1996; Bennett *et al.,* 1998). Biofilm formation of this zinc solubilizers and potassium releasers has an immense importance in agriculture.

Materials and Methods

Soil sampling

In order to isolate ZnSB, KSB a total of fifteen rhizospheric soil samples were collected at a depth of 10-30 cm, from different sites near Rajendranagar, Hyderabad, Telangana, India. From each site, three subsamples were collected from adjacent plants and well mixed to form a composite sample. All tools used in soil sampling were surface disinfected using 70% ethanol and soils were placed in sterile sample bags, stored at 4 ^{0}C and processed within a week. Each sample was homogenized in sterile saline (0.85 % NaCl, w/v), serially diluted and used.

Screening of isolates for zinc solubilizing and potassium releasing ability

The isolates were inoculated into modified TRIS medium (ingredients g L^{-1}), (Glucose- 10.0 g; Zinc phosphate- 1 g; Ammonium sulphate- 0.5 g; Potassium chloride- 0.2 g; Yeast extract- 0.5 g; Ferrous sulphate- 0.01 g; Manganese sulphate- 0.01 g; Di-potassium

hydrogen phosphate- 0.25 g; Agar- 20 g and Double distilled water- 1000 ml) containing 0.1 % insoluble zinc compound ($ZnPO_4$). The test organisms were inoculated on these media and incubated at 28 ± 2 ºC for 48-72 h. Subsequently, isolates were inoculated on the modified Alexondrov's medium (Glucose- 5 g; Magnesium sulphate- 0.5 g; Ferric chloride- 0.005 g; Calcium carbonate- 0.1 g; Tri-calcium phosphate- 2g; Potassium alumino silicate- 2 g; Double distilled water- 1000 ml) containing 0.2 % potassium alumino silicate as a potassium source.

The test organisms were inoculated on the media and incubated at 28 ± 2 ^{0}C for 48-72 h. The diameter of the colony and clearing zones around the colonies were measured.

To standardize the procedures for biofilm formation of efficient Zinc (Zn) solubilizing and Potassium (K) releasing bacteria and fungi

Biofilm formation involves in the selection of efficient zinc solubilizers and potassium releasers based on qualitative data along with fungal partner to use as a substrate. For this initially compatibility between the pure isolates of fungi and bacterial isolates checked on the common media (TRIS-minimal medium). After that the pure isolates of fungi and bacterial isolates were inoculated aseptically in the common 500 ml broth which contains both insoluble zinc and potassium sources.

The broth was then kept in the incubator at 28 ± 2 ^{0}C for 15 days without disturbing it along with control. In between after 7 days of inoculation pH of the broth and counts of both bacterial and fungal spores were taken, fluctuation (acidic) in the pH was corrected with addition of a pinch of $CaCo_3$to the medium.

Microscopic observation of biofilms

Compound microscope

The biofilms were observed for every 2 days interval by visual and microscopical observations at 100X magnification. After harvesting, the biofilm was washed with sterile water to remove the non adherent cells on biofilm. A loopful of biofilm was spread on the slide, air dried and heat fixed. Two drops of Lactophenol cotton blue stain was added and after 1 min washed and then safranine was added. After 1 min, the slide was washed and covered with cover slip and observed under microscope at 10, 40 and 100X objectives (oil immersion). Microscopical pictures were taken.

In vitro screening of biofilms

Initially all the individual isolates were screened biochemically characterized thereafter finally biofilms were confirmed by using same biochemical tests. Biochemical tests like starch hydrolysis, IMVIC tests, catalase and oxidase were used for screening (Table 1).

Results and Discussion

Zinc solubilizing capacity

The isolates evaluated for their efficiency of zinc solubilization on TRIS minimal medium. Results revealed that the isolate ZnSF-4 has showed the maximum solubilization zone of 54 mm and least solubilization zone observed in ZnSB-8 (7 mm). No solubilization was observed in KSB-2, KSB-3 and KSF-1. The solubilization efficiency ranges from a maximum of 315 % to a minimum of 10 %. The maximum solubilization efficiency was observed with ZnSF-4 having 315 % whereas minimum solubilization efficiency was found in ZnSF-3 (10 %). So far, only bacterial

species belong with species of *Bacillus* spp and *Pseudomonas* spp were reported to be zinc solubilizer as they form a clear halo zone (Simine *et al.,* 1998 and Saravanan *et al.,* 2003) (Table 3).

Potassium releasing capacity

All isolates were screened for their potassium releasing ability. The isolated potassium releasing bacteria and fungi had showed solubilization zone ranging from a maximum of 58 to a minimum of 8 mm. The isolate KSF-2 had shown the maximum solubilization zone of 58 mm followed by KSF-1 (43 mm), KSB-1 (12 mm), KSB-4 (12 mm), KSB-3 (11 mm), KSB-5 (10 mm) and least solubilization zone was observed in ZnSB-7 (5 mm). The maximum solubilization efficiency was showed by KSB-1 with the efficiency of 150 % followed by KSB-4 (140 %), KSB-3 (120 %), KSB-1 (100 %), KSB-2 (60 %), KSF-1 (34 %) and with minimum solubilization efficiency in KSF-2 (28 %). The solubilization process of minerals may be due to the production of various organic acids such as acetic, formic, gluconic, oxalic and succinic acids Lal (2002). Adeleke *et al.,* (2010) have reported the ability of ectomycorhizal fungi in mobilization of P and K sources from insoluble ore. Greater release of K from muscovite has been recorded in *B. muciloginosus* (Sugumaran and Janarthanam, 2007) (Table 4).

Biofilm formation of efficient zinc solubilizing and potassium releasing bacteria and fungi

Compatibility of bacteria with fungi for biofilms formation

Compatibility deals with the degrees of intimacy that are exhibited between the two partners (Bacteria and Fungi) in terms of their different physical associations. The physical association between them was highly specific symbiotic associations of fungal hyphae and bacterial cells. This colonization in biofilms differs from consortium or dual cultures.

Observation of the effects of fungi on bacterial development is clear that fungi can promote distinct differences in bacterial development by contributing to a distinctive ecological niche, within which bacteria exhibit physiological differences, such as resistance to antibiotics, stress, etc, the observations were clear that the compatibility between the bacteria and fungi was very high in *in vitro* conditions of biofilms (Table 2).

Wide range of microorganisms found considerable variation in their ability to form a biofilm. The bacterial and fungal organisms synergistically associated and play key role in persistence and show good compatibility. These bacterial-fungal interactions often result in changes to the nutritional influence of one or both partners towards plants. They can also result in unique contributions to biogeochemical cycles and biotechnological processes. Microorganisms occupying such structures typically showed enhanced resistance and record best results towards plants when inoculated.

Among all isolates the most compatible combinations found to be ZnSF-2 with ZnSB-2; ZnSF-4 with ZnSB-8; KSF-2 with ZnSB-2, moderately compatible combinations are ZnSF-2 with ZnSB-8, ZnSB-10 and KSB-4; ZnSF-4 with ZnSB-2; KSF-2 with ZnSB-8 and weakly compatible combinations were ZnSF-2 with ZnSB-9 and KSB-2; ZnSF-4 withZnSB-6, KSB-2 and KSB-4; KSF-2 with ZnSB-3, ZnSB-6, ZnSB-10, KSB-2 and KSB-3.

The best combinations ZnSF-2 with ZnSB-2 and ZnSB-2 with KSF-2 is selected for biofilm formation based on their solubilization as well as compatibility data.

In vitro development of biofilms

Microorganisms are capable of growing in both a freely (planktonic) or as biofilms attached to solid surfaces. Biofilms in distinct settings form different structures comprising different microbial consortia dictated by biological and environmental parameters. The conditions were provided for *in vitro* development of biofilms for 15 days in TRIS minimal broth with the best screened isolates (ZnSF-4 + ZnSB-2 and ZnSB-2 + KSF-2). Successfully the biofilm mats were developed with different microbial consortium (Patel *et al.,* 2013).

Salma *et al.,* (2015) reported the best PSB strongly attached to the hyphae of Ri-26 isolates belonged to *Burkholderia spp*. and one was identified as *Rhizobium miluonense.* Triveni *et al.,* (2015) reported that *Trichoderma* based biofilms with *Azatobacter chroococcum, P. fluorescens* and *B. subtilis.* While *A. torulosa* biofilms were prepared by using *B. subtilis* and *Trichoderma.* Heleen *et al.,* (2014) discussed the three mechanisms that play an important role in biofilm survival. The process of cellular chaining, the biomass stickiness also strongly hinders the reorganization of cells within the biofilm. Radha *et al.,* (2014) reported that the *Anabaena -T. viride* biofilmed formulations proved to be the most promising for Soybean, recording 12–25 % enhanced yield and microbial activity.

Table.1 Biochemical characters of all Zinc solubilizing and Potassium releasing isolates

S. No	Isolates	Indole production	Oxidase test	Catalase test	Starch hydrolysis	MR test	VP test	Citrate utilization	Gelatin liquefaction
1	ZnSB-1	+	+	+	-	-	+	-	+
2	ZnSB-2	-	+	+	-	-	+	+	+
3	ZnSB-3	-	+	+	-	-	+	+	-
4	ZnSB-4	-	+	+	-	-	+	+	+
5	ZnSB-5	+	+	-	-	-	+	+	+
6	ZnSB-6	-	+	+	+	-	+	+	+
7	ZnSB-7	+	+	+	-	-	+	-	-
8	ZnSB-8	-	+	+	-	-	+	+	+
9	ZnSB-9	-	+	+	-	-	+	-	+
10	ZnSB-10	-	+	+	-	-	+	-	+
11	ZnSF-1	-	+	+	-	-	-	+	+
12	ZnSF-2	-	+	+	-	-	+	+	-
13	ZnSF-3	-	+	+	+	-	+	+	+
14	ZnSF-4	-	+	+	-	-	-	+	+
15	ZnSF-5	-	+	+	+	-	-	+	+
16	KSB-1	-	+	+	+	-	+	-	+
17	KSB-2	-	+	+	-	-	+	+	-
18	KSB-3	+	+	+	-	-	-	-	+
19	KSB-4	-	+	+	+	-	+	+	-
20	KSB-5	-	+	+	+	-	-	-	+
21	KSF-1	-	+	+	-	-	-	+	+
22	KSF-2	-	+	+	-	-	-	+	-

Table.2 Compatibility study of Zinc solubilizing bacteria and Potassium releasing bacteria for biofilm preparation

S.No	Bacterial isolates	Zinc solubilizing fungal isolates					Potassium releasing fungal isolates	
		ZnSF-1	ZnSF-2	ZnSF-3	ZnSF-4	ZnSF-5	KSF-1	KSF-2
1.	ZnSB-1	-	-	-	-	-	-	-
2.	ZnSB-2	-	+++	-	++	-	-	+++
3.	ZnSB-3	-	-	-	-	-	-	+
4.	ZnSB-4	-	-	-	-	-	-	-
5.	ZnSB-5	-	-	-	-	-	-	-
6.	ZnSB-6	-	-	-	+	-	-	+
7.	ZnSB-7	-	-	-	-	-	-	-
8.	ZnSB-8	-	++	-	+++	-	-	++
9.	ZnSB-9	-	+	-	-	-	-	-
10.	ZnSB-10	-	++	-	-	-	-	+
11.	KSB-1	-	-	-	-	-	-	-
12.	KSB-2	-	+	-	+	-	-	+
13.	KSB-3	-	-	-	-	-	-	-
14.	KSB-4	-	++	-	+	-	-	+
15.	KSB-5	-	-	-	-	-	-	-

+ Weakly Compatible ++ Moderately Compatible
+++ Highly Compatible - Not Compatible

Table.3 Zinc solubilization by isolates

Isolates	Solubilization zone (mm)	Solubilization efficiency (%)
ZnSB-1	0	0
ZnSB-2	0	0
ZnSB-3	0	0
ZnSB-4	0	0
ZnSB-5	0	0
ZnSB-6	6	50
ZnSB-7	5	66
ZnSB-8	6	100
ZnSB-9	0	0
ZnSB-10	0	0
ZnSF-1	0	0
ZnSF-2	0	0
ZnSF-3	0	0
ZnSF-4	0	0
ZnSF-5	0	0
KSB-1	12	100
KSB-2	8	60
KSB-3	11	120
KSB-4	12	140
KSB-5	10	150
KSF-1	43	34.3
KSF-2	58	28.8

Table.4 Potassium relesed by the isolates

Isolates	Solubilization zone (mm)	Solubilization efficiency (%)
ZnSB-1	10	42
ZnSB-2	9	50
ZnSB-3	9	28
ZnSB-4	12	33
ZnSB-5	15	25
ZnSB-6	11	37.5
ZnSB-7	8	33.3
ZnSB-8	7	16
ZnSB-9	15	50
ZnSB-10	13	62.5
ZnSF-1	36	63
ZnSF-2	30	66.6
ZnSF-3	21	10.5
ZnSF-4	54	315
ZnSF-5	42	162
KSB-1	12	300
KSB-2	0	0
KSB-3	0	0
KSB-4	9	125
KSB-5	9	28
KSF-1	0	0
KSF-2	43	138.8

Attempts were made by Nissi *et al.,* (2014) to improve the crop production and disease control in chickpea. Isolated 15 bacterial cultures and characterized developed biofilms *in vitro*. Four biofilms and 4 coinoculations were used as biofertilizers in chickpea crop. The results revealed that T_8 (*T. viride*+ *R. leguminosarum* + *P. fluorescence* + *B. subtilis* (Biofilm)) showed best results in all aspects like protein content, PGP activity, yield and yield attributes etc., followed by T_2 (*Trichoderma viride* + *Rhizobium leguminosarum*- Biofilm). Karivaradharajan *et al.,* (2013) reported *Anabaenae-Pseudomonas* biofilm showed highest P uptake, illustrating the interrelationships of nitrogen fixation with increased P uptake by plant. Triveni *et al.,* (2013) highlighted that compost and vermiculite (1:1) was a suitable carrier for the novel biofilmed biofertilizers. Triveni *et al.,* (2012) recorded *Trichoderma* - *Azatobacte r* biofilm was recorded the highest nitrogenase activity

and ACC deaminase activity. Xuan *et al.,* (2012) reported maximum concentration of soluble phosphorus (P) was determined in the mixed culture of *P. chlororaphis* and *A. pascens.* Seneviratne *et al.,* (2011) reported the combined application significantly increased soil organic C by ca. 20 %, and reduced leaf transpiration by ca. 40 %. Prasanna *et al.,* (2011) reported the *Allocasurina torulosa* - *A. chroococcum* and *A. torulosa – Mesorhizobium ciceri* biofilms were able to utilize new saccharides as compared to the individual cultures. Kokare *et al.,* (2008) studied on biofilm importance and application. Thomas and Clay (2007) revealed on biofilm formation by plant-associated bacteria. Shrout *et al.,* (2006) conducted investigation in associations with QS mechanism. Bronwyn *et al.,* (2004) reviewed on surface properties of the plant tissue. Prakash *et al.,* (2003) studied on biofilms a survival strategy of bacteria. Webb *et al.,* (2003) reported that microbial biofilms are communities of microorganisms adhering to abiotic/ biotic surfaces and embedded in an organic matrix of biological origin which provides structure and stability to the community.

Biochemical characterization of biofilms

In vitro formed biofilms (ZnSF-4 + ZnSB-2 and ZnSB-2 + KSF-2) were confirmed for their biofilm formation by following biochemical screening. Biofilm (ZnSF-4 + ZnSB-2) was confirmed based on the positive result for Vogues Proskuers test and biofilm (ZnSB-2 + KSF-2) was confirmed based on the positive result for gelatin liquefaction. (Triveni *et al.,* 2012)

Screening of biofilms for zinc solubilization and potassium releasing characters

Biofilms were screened further for their efficacy in zinc solubilization as well as potassium releasing ability in order to compare over the individual cultures. The *in vitro* formed biofilms were shown comparatively higher amount of zinc solubilization and potassium releasing ability as compared to individual cultures. Zinc solubilization was 35 % and Potassium releasing ability was found to be 23 % more than the individual cultures (when both the individual isolates taken i.e combined value is less than the biofilm value). This is indicating that biofilms has an added advantage over individual isolates. Seneviratne and Jayasinghearachchi (2005) reported that biofilms increased N and P mineralizations of the soil and showed a high nitrogenase activity even under a very high NO_3 concentration in the soil, compared to its member microbes. Salma *et al.,* (2015) trapped phosphate solubilizing bacteria (PSB) on the hyphae of the arbuscular mycorrhizal fungus (AMF) *Rhizophagus irregularis* (Ri). Results showed that increased P solubilization and P mobilization considerably than individual isolates.

In conclusion formation of biofilms has considerably increased the zinc solubilization and potassium releasing as compared to individual isolates. Biofilms also enhances the chances of survival in the soil for long time, both these factors will begin a new era for the biofertilizers production. This is clear that there is no havoc to environment too.

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