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Influence of Persistent Biofilms Enzymatic Activity in Mung Bean Crop for Improved Yields

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ABSTRACT

The enzymatic activity in the soil is mainly of microbial origin, being derived from intracellular, cell-associated or free enzymes. The present study on microbiological investigations, biochemical and enzymatic analysis of plants and pot experiments were carried out in the Department of Agricultural Microbiology and Bioenergy, PJTSAU, Rajendranagar, Hyderabad, on phosphate solubilizing bacteria and with these phosphate solubilizing bacteria (PSB) in association with Trichodema fungi different combinations of biofilms and coinoculations were prepared. The experiment was conducted in Mung bean crop. Three enzymes (Dehydrogenase, Phosphatase and Urease) activity was observed at all the three plant growth stages, and flowering stage being the highest for dehydrogenase (87.71 µg of TPF/g of soil/day), acidic phosphatase (130 µg p-nitro phenol/g of soil/day) and alkaline phosphatase (148 µg p-nitro phenol/g of soil/day) activity as a result of high persistency of microorganisms at this stage in T4 treatment. Since chemical fertilizers are not frequently used with Mungbean crop, use of PSB, biofilms, coinoculants as biofertilizers would help in improving better crop stand and crop yields.

Key words: *Biofertilizers, Biofilms, Coinoculants, Enzymes.*

INTRODUCTION

Healthy soils are essential for the integrity of ecosystems to recover from disturbances, such as drought, climate change, pest infestation, pollution, and human exploitation including agriculture[4]. The biological activity in soil is largely concentrated in the topsoil, the type of enzyme and enzyme levels in soil systems vary in amounts primarily due to the fact that each soil type has different amounts of organic matter content, composition, and activity of its living organisms and intensity of biological processes. Soil enzyme activities can be used as measures of microbial activity, soil productivity and inhibiting effects of pollutants[16]. Plays an important role in agriculture by catalyzing several vital reactions necessary for the life processes of micro-organisms in soils and the stabilization of soil structure, the decomposition of organic wastes, organic matter formation and nutrient cycling[3].

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Microorganisms also respond quickly to environmental stress compared to higher organisms, as they have intimate relations with their surroundings due to their high surface to volume ratio.

This study was focused on three types of enzymes Dehydrogenase, Phosphatase and Urease. Dehydrogenase enzyme is high in soils polluted with pulp and paper mill effluents[7] but low in soils polluted with fly ash[10]. When there is a signal indicating P deficiency in the soil, acid phosphatase secretion from plant roots is increased to enhance the solubilization and remobilization of phosphate, thus influencing the ability of the plant to cope with P-stressed conditions [6]. Urease enzyme is responsible for the hydrolysis of urea fertilizers applied to the soil into NH3 and CO2 with the concomitant rise in soil pH. Urease activity in soils is influenced by many factors. These include cropping history, organic matter content of the soil, soil depth, soil amendments, heavy metals, and environmental factors such as temperatures[15,19]. Generally, urease activity increases with increasing temperature. Microorganisms respond quickly to changes; hence they rapidly adapt to environmental conditions, and thus they can be used for soil health assessment, and changes in microbial populations and activities may therefore function as an excellent indicator of change in soil health. Soil microorganisms participate in the processes that are crucial for long-term sustainability of agricultural systems[8].

MATERIAL AND METHODS

Soil samples were collected from the Mungbean rhizosphere soils in NBPGR, Rajendranagar, Hyderabad and isolated different rhizobacteria (Bacillus, Pseudomonas and Rhizobium).

Protocol for biofilm formation

The 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. 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. 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/pot conditions. And these treatments were used as biofertilizers.

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)

DEHYDROGENASE

Dehydrogenase is an enzyme that occurs in all viable microbial cells. The dehydrogenase enzyme activity is commonly used as an indicator of biological activity in soils[1]. This enzyme is considered to exist as an integral part of intact cells but does not accumulate extracellularly in the soil. The Method

followed was described by Casida *et al*.[2]. The enzyme activity in 1g soil was determined by adding 0.05 g of $CaCO_3$, 2.5 ml of distilled water and by using 1 ml of 3 % TTC (Try phenyl Tetrazolium Chloride) where it is reduced to light pink TPF (Tri Phenyl Formazon) on the incubation for 24 h. Later it was dissolved in 10 ml of methanol and finally made up to 25 ml. The intensity of the red color was measured on a spectrophotometer at 485 nm.

The results for all microbial counts are reported as per gram of oven-dried soil. All the treatments were incubated at 28°C for 10 days. The microbial counts were done by spread plating technique. A dehydrogenase activity measurement test using triphenyl tetrazolium chloride (TTC) was successfully developed. TF yield (µmol/mL) was related to the number of cells measured by optical density (OD_{600}).

PHOSPHATASE

Phosphatase enzymes are believed to play critical roles in P cycles and are correlated to P stress. The procedure followed was of Tabatabai and Bremner[13] (Acid phosphatases) and Eivazi and Tabatabai[15] (Alkaline phosphatases). The principle in the estimation of phosphatase enzyme activity is that the soil extract from 1 g of soil was allowed to react with para nitro phenol, which was estimated colorimetrically.

Phosphatase activity was estimated by taking 1g of soil sample and mixed it with 0.2 ml of toluene 4 ml of MUB (Modified Universal Buffer) and 1ml of disodium para nitrophenol solution. This solution was kept in an incubator at 37 ^{0}C for 1 h. Later 1 ml of 0.5 M $CaCl_2$ and 4 ml of 0.5 M NaOH were mixed properly and the intensity of the yellow color of filtrate was read on spectrophotometer at 420 nm.

UREASE

As described by Tabatabai and Bremner[14]. Urease activity in soil can be assayed by quantifying the ratio of release of NH^{4+} from the hydrolysis of urea. To estimate the urease activity 5 g of soil was taken and 9 ml of distilled water along with 1 ml of 0.2 M urea solution was added and incubated by swirling in BOD for 2 h. Then 15 ml of KCl - $AgSO_4$ solution was added and shaken in mechanical shaker. Later transferred 1 ml of the supernatant solution into a 50 ml volumetric flask and 1 ml of 6% EDTA, 2 ml of phenol nitroprusside and 8 ml of buffered hypochlorite was added. After mixing thoroughly, the flasks were kept at 40^{0}C for 30 min for color development. Then brought it to room temperature and measured the color intensity in spectrophotometer at 636 nm.

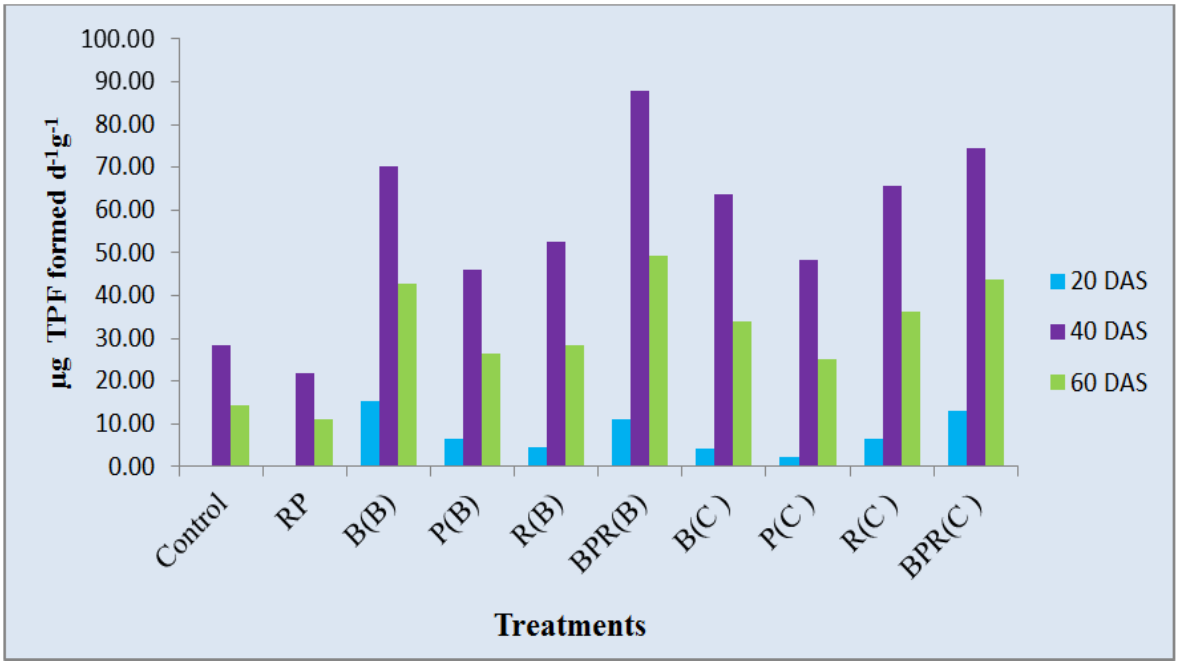
RESULTS AND DISCUSSION

In organic systems, plant production depends primarily on nutrient cycling in soils that are controlled by microbes and soil enzymes. Soil enzymes correlate with soil fertility, microbial activity, biochemical cycling of various nutrient elements like C, N, S.

Dehydrogenase enzyme is known to oxidize soil organic matter by transferring protons and electrons from substrates to acceptors. These processes are the part of respiration pathways of soil microorganisms. Here the results recorded for dehydrogenase activity was highest i.e., 87.71 µg TPF formed/day/gm at flowering stage in T4 (*Trichoderma* + *Bacillus* + *Pseudomonas* + *Rhizobium* - Biofilm). Treatments T1 (*Trichoderma* + *Bacillus subtilis* – Biofilm) and T5 (*Trichoderma viride* + *Bacillus subtilis* – Coinoculation) of *Bacillus* formulations were showed on par results with dehydrogenase activity i.e., 30.70 µg TPF formed/ day/gm at flowering stage. The conclusion is that the DHA was high in T4 treatment. These It concludes that high enzyme activity at flowering stage and low at harvest stage showed with increase in the organic levels. i.e., organic carbon which acts as energy source for proliferating the microbial population in T4. Thus it is inferred that higher rates of N, P and K fertilization enhanced the activities of soil dehydrogenase enzymes and the effect was more pronounced with dehydrogenase avtivity in T4.

Effect of biofilms and coinoculations on dehydrogenase activity in different stages of plant growth

Treatments	Dehydrogenase activity (µgTPF formed d^{-1} g^{-1})		
	20 DAS	40 DAS	60 DAS
Control	0.00	28.50	19.48
RP (Rock Phosphate)	0.00	21.92	13.10
T1 : Trichoderma viride + Bacillus subtilis (Biofilm)	**15.35**	70.17	30.70
T2 : Trichoderma viride + Pseudomonas fluorescence (Biofilm)	6.57	46.05	26.30
T3 : Trichoderma viride + Rhizobium leguminosarum (Biofilm)	4.38	52.63	24.16
T4 : Trichoderma viride + Bacillus subtilis + P. fluorescence + R. leguminosarum (Biofilm)	10.96	**87.71**	**32.87**
T5 : Trichoderma viride + Bacillus subtilis (Co-inoculation)	4.37	63.59	30.70
T6 : Trichoderma viride + Pseudomonas fluorescence (Co-inoculation)	2.19	48.24	28.50
T7 : Trichoderma viride + Rhizobium leguminosarum (Co-inoculation)	6.57	65.78	21.92
T8 : Trichoderma viride + Bacillus subtilis + Pseudomonas fluorescence + R. leguminosarum (Co-inoculation)	13.15	74.56	26.36
CD	0.16	2.00	0.69
SE(m)	0.05	0.67	0.22
CV	1.45	2.09	1.47

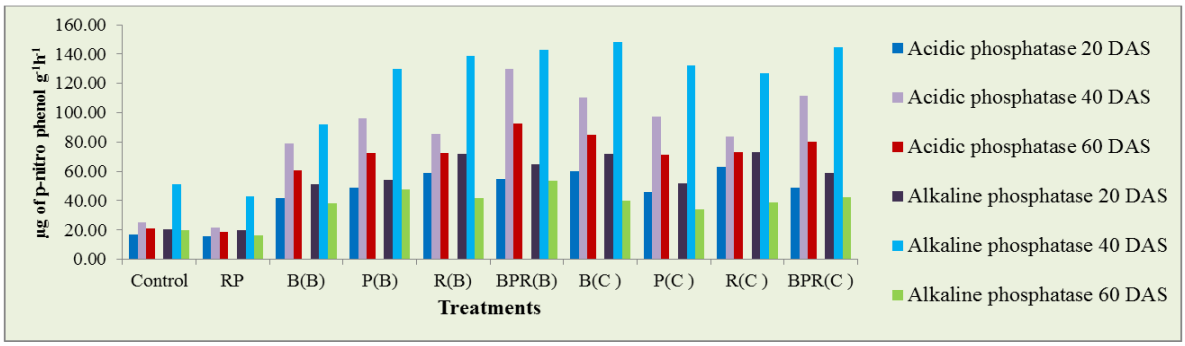


Acidic and alkaline phosphatase activity was maximum at flowering stage i.e., 130 µg p-nitrophenol/g/h and 148 µg p-nitrophenol/g/h in T4 (*Trichoderma* + *Bacillus* + *Pseudomonas* + *Rhizobium* - Biofilm) and T5 (*Trichoderma* + *Bacillus* – Coinoculation) treatments respectively, when compared to harvesting stage 77.00% and 63.90% decline in acidic and alkaline phosphatase activity respectively in T4 treatment towards harvesting stage from flowering stage.

Results of the present study revealed that the activity of soil enzyme phosphatase was significantly influenced by root colonization due to the acidity in root zone by the activity of microbes present in the form of biofilms. Low soil phosphorus availability increases acid phosphatases activities and affects P partitioning in nodules, seeds and rhizosphere of mung bean.

Effect of biofilms and coinoculations on acidic and alkaline phosphatase activity at different stages of plant growth period

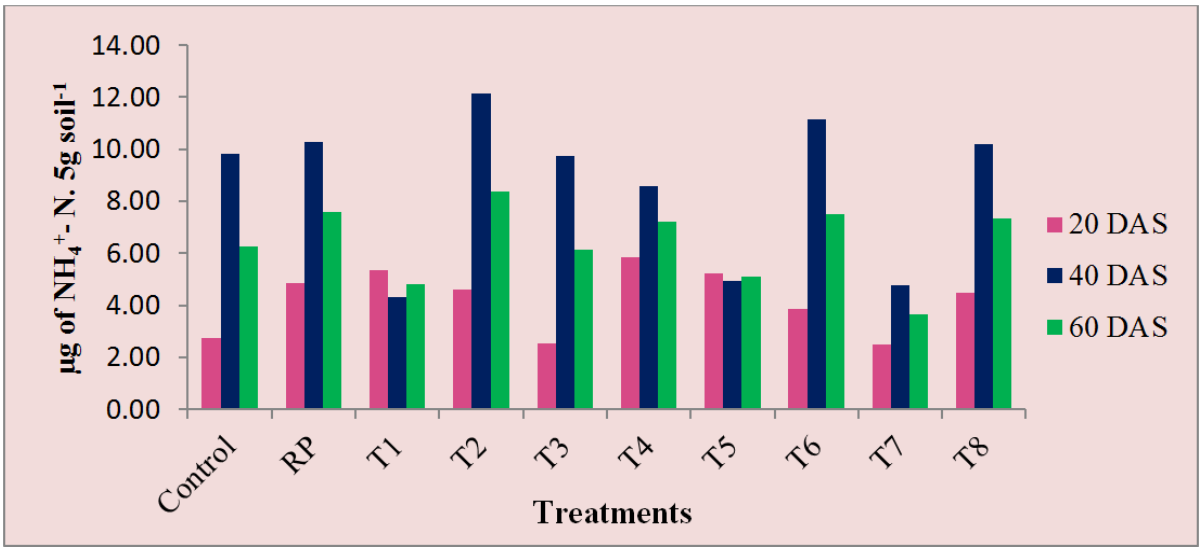
Treatments	Phosphatase activity (µg p- nitrophenol g^{-1} h^{-1})					
	20 DAS		40 DAS		60 DAS	
	Acidic	Alkaline	Acidic	Alkaline	Acidic	Alkaline
Control	17.00	20.70	25.02	51.33	10.80	20.07
Rock Phosphate	16.00	20.00	21.47	43.00	11.80	16.60
T1 : Trichoderma viride + Bacillus subtilis (Biofilm)	42.00	51.00	79.27	92.23	23.27	38.33
T2 : viride + Pseudomonas fluorescence (Biofilm)	49.00	54.00	96.30	130.00	23.03	47.77
T3 : T. viride + Rhizobium leguminosarum (Biofilm)	59.00	72.00	85.67	139.00	27.03	42.00
T4 : Trichoderma viride + Bacillus subtilis + P. fluorescence + R. leguminosarum (Biofilm)	55.00	65.00	**130.00**	143.00	**29.77**	**53.43**
T5 : Trichoderma viride + Bacillus subtilis (Co-inoculation)	60.00	72.00	110.3	**148.00**	21.00	39.70
T6 : Trichoderma viride + P. fluorescence (Co-inoculation)	46.00	52.00	97.10	132.00	19.67	34.27
T7 : Trichoderma viride + R. leguminosarum (Co-inoculation)	**63.00**	**73.00**	83.67	127.00	29.43	39.00
T8 : Trichoderma viride + Bacillus subtilis + P. fluorescence+R. leguminosarum (Co-inoculation)	49.00	59.00	111.30	144.30	21.00	42.33
CD	2.29	1.88	5.06	3.28	1.48	1.36
SE(m)	0.77	0.63	1.70	1.10	0.49	0.46
CV	2.92	2.04	3.51	1.66	3.89	2.12

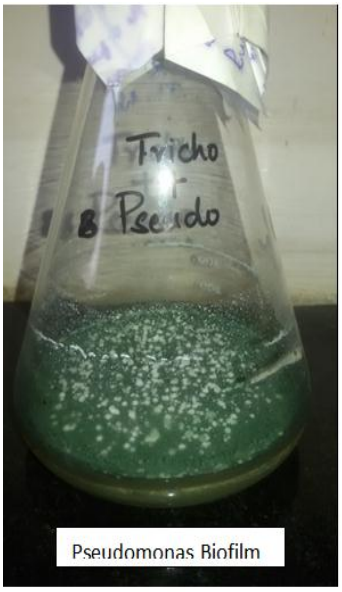


Treatment T2 (*Trichoderma* + *Pseudomonas* – Biofilm) showed maximum urease activity at flowering stage (12.16 µg of NH_4^+- N in 5g soil). There was 64.10% decline in the urease activity of T2 treatment at harvesting stage, followed by T6 (*Trichoderma* + *Pseudomonas* – Coinoculation) i.e., 11.13 µg of NH_4^+- N in 5g soil. Hence we can say that the *Pseudomonas* isolate was best performer of urease activity. Urease enzyme activity is often measured as an indicator of the health of microbial communities in the absence of plants. In the present study urease activity was high in T2 due to the ammonium strongly interfered both with the expression of the urea uptake by *Pseudomonas* and its activity.

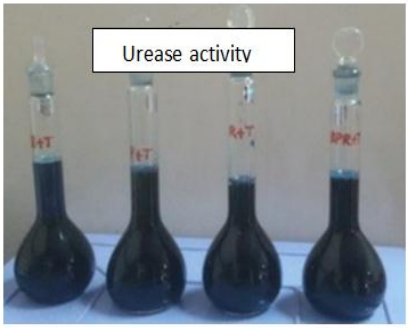
Effect of biofilm and coinoculations on urease activity in different stages of plant growth

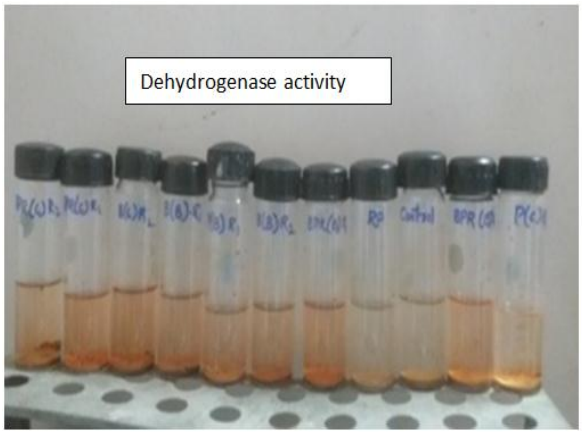
Treatments	Urease activity (µg of NH_4^+-N 5g $soil^{-1}$)		
	20 DAS	40 DAS	60 DAS
Control	2.74	9.82	2.43
Rock Phosphate	4.86	10.29	5.67
T1 : *Trichoderma viride* + *Bacillus subtilis* (Biofilm)	5.36	4.30	5.24
T2 : *Trichoderma viride* + *Pseudomonas fluorescence* (Biofilm)	4.61	**12.16**	4.36
T3 : *Trichoderma viride* + *Rhizobium leguminosarum* (Biofilm)	2.55	9.73	1.93
T4 : *Trichoderma viride* + *Bacillus subtilis* + *P. fluorescence* + *R. leguminosarum* (Biofilm)	**5.86**	8.60	**7.48**
T5 : *Trichoderma viride* + *Bacillus subtilis* (Co-inoculation)	5.24	4.96	6.67
T6 : *Trichoderma viride* + *Pseudomonas fluorescence* (Co-inoculation)	3.86	11.13	3.93
T7 : *Trichoderma viride* + *Rhizobium leguminosarum* (Co-inoculation)	2.49	4.77	2.24
T8 : *Trichoderma viride* + *Bacillus subtilis* + *Pseudomonas fluorescence* + *R. leguminosarum* (Co-inoculation)	4.49	10.19	5.55
CD	0.33	0.76	0.33
SE(m)	0.12	0.24	0.11
CV	4.92	3.93	4.26

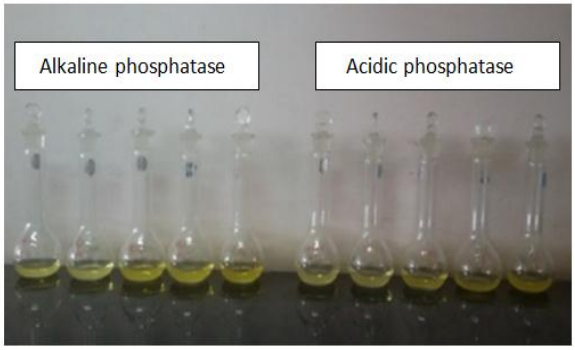

14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
µg of NH4+- N. 5g soil-1
20 DAS
40 DAS
60 DAS
Control
RP
T1
T2
T3
T4
T5
T6
T7
T8
Treatments

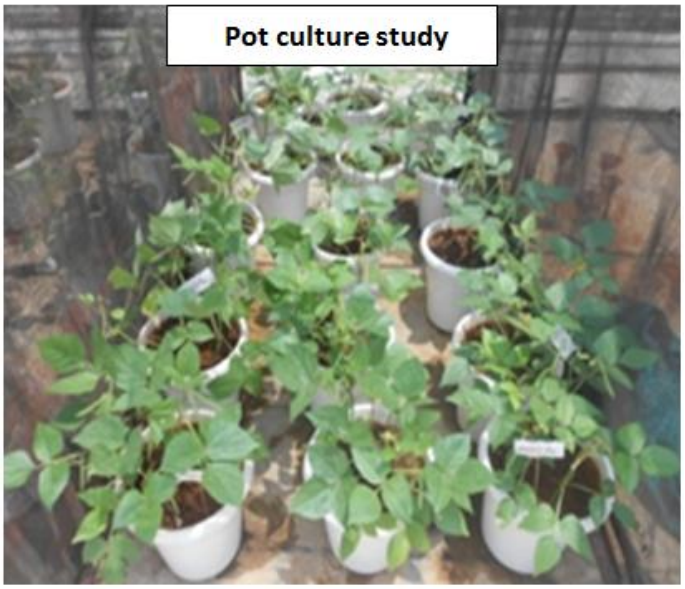

Tricho
Pseudo
Pseudomonas Biofilm


Color development


Urease activity


Dehydrogenase activity


Alkaline phosphatase
Acidic phosphatase



CONCLUSION

The more grain yield (631 kg ha^{-1}) was recorded in treatment T3 (*Rhizobium* + *Trichoderma* - Biofilm) and T4 (*Trichoderma* + *Bacillus* + *Pseudomonas* + *Rhizobium* - Biofilm). The lowest grain yield was (525 kg ha^{-1}) recorded in Control or untreated plants. The above results correlated with an increase in enzyme activity especially acid phosphatase activity actually increases with increased root colonization and there by influenced the plant growth. Application of biofilmed biofertilizers has resulted in improved nodulation in plant roots and resulted in supplying higher amount of nitrogen for growth and yield attributes which in turn given the maximum yields. This is due to availability of nutrients through integration of different inoculants responded better in terms of growth and yield parameters over inorganic consortium, individual and control. Which may be due to balanced availability of nutrients throughout the growth period of crop integrated nutrient management system.

Acknowledgement

A better understanding of the role of this soil enzymes activity in maintaining the soil health will potentially provide a unique opportunity for an integrated biological assessment of soils due to their crucial role in several soil biological activities, their ease of measurement, and their rapid response to changes in soil management.

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