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Evaluation of Persistence and Plant Growth Promoting Effect of Bioencapsulated Formulation of Suitable Bacterial Biofertilizers

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Formulation is a major step in the successful commercialization of microbial inoculants used as biofertilizers and biocontrol agents.Although formulation research is progressing slowly, several developments including liquid and granular formulations have contributed to the ease of use at the farm combined with the economic benefits of increased crop yield levels. In the present study, the bacterial biofertilizers; *Rhizobium, Azotobacter and Azospirillum* were formulated with Biogel matrix and the formulated biofertilizers were evaluated for seedling emergence of respective biofertilizers treated width Green gram(*Vigna radiata*) and Black gram(*Vigna mungo L.*). The persistence and soil enzyme activity such as Alkaline phosphatase, Urease , total N,P and K content and Nitrate reductase assay was also studied. Biogel matrix formulation of all the tested biofertilizers showed improved persistence and plant growth parameters. The study suggest the possible utilization of formulation of biofertilizers with Biogel matrix.

Key words: Biofertilizers, Formulation, Encapsulation,Persistence,Biogel matrix.

Biofertilizers are microorganisms that help plants to grow by increasing the quantity of nutrients. Since these fertilizers contain living microorganisms, it increases or promotes the supply of important nutrients crucial for the overall productivity of the soil. An increasing number of farmers and agriculturists are turning to the use of biofertilizers as these are gentler on the soil as against chemical fertilizers[1,2].It is easier to fully appreciate the importance of biofertilizers when we know how harmful chemical fertilizers can get for the soil and the crop. Chemical fertilizers are meant to boost the growth of plants and increase the fertility of the soil; however they cause significant damage to the environment. These chemical based fertilizers also make use of nitrogenous fertilizers or chemicals, are expensive and not as conveniently available. The value of biofertilizers has further increased in an increasingly eco-conscious world. Since these fertilizers are eco-friendly they can be used generously to promote healthy crops. The quality of the soil is also improved thanks to these environmentally friendly fertilizers[3,4].

Formulation is a crucial aspect for producing inoculants containing an effective bacterial strain and can determine the success or failure of a biological agent[5]. Formulation typically consists of establishing the active ingredient (i.e., microorganism) in a suitable carrier together with additives that aid in the stabilization and protection of the microbial cells during storage and transport, and at the target site. Whether a product is new or improved, it is imperative that the formulation be stable during production, distribution, storage, and

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transportation[6].The formulation should also be easy to handle and apply so that it is delivered to the target in the most appropriate manner and form, protects the agent from harmful environmental factors, and maintain or enhance activity of the organism in the field[7,8].Another important consideration is the cost-effectiveness of the formulation. Therefore, several critical factors including user preference have to be considered before delivery of the final product. Commercial inoculant formulations are available as powder, granule, and liquid. Generally, peat has been the preferred carrier in powder form. The rhizobial cells in the inoculant are metabolically active and continue to grow and multiply as long as favorable nutrient and environmental conditions are maintained [9].In the present study, formulation of bacterial biofertilizers has been studied.

MATERIALS AND METHODS

Bacterial biofertilizers

The bacterial biofertilizers i.e, *Rhizobium, Acetobacter and Azospirillum* were obtained from Krishna Agro Biotech, Chennai and the cultures were maintained on Nutrient Agar slants.

Evaluation of improved persistence of bacterial biofertilizers with semi synthetic formulation

Inoculum preparation

The respective biofertilizers was inoculated into SYG Medium (2% Peptone, 1% Glucose, 0.2% Yeast extract, 0.1% K_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4$-$7H_20$, 0.02% $MnCL_2$, 0.02%$ZnSO_4$-$7H_20$, 0.02% $FeSO_4$-$7H_2O$) and incubated at 37ºC for 48 hours under shaking conditions. After incubation the cells were harvested by centrifugation, 1000 rpm for 15 minutes and the collected cell pellets were washed in phosphate buffer saline and the washed cell suspension was used as source of inoculu

Formulation with biogel

The media with the following composition was prepared (100gm rice powder, 100gm soyabaen powder, 2gm glucose, 1gm CaCO3, 1gm yeast extract, 20gm soil, 50mg FeSO4, 10mg MnCl2, 10gm cotton seed powder) and was kept for sterilization in a water bath for 1 hour. Then the washed centrifuged cells was mixed and spread on glass plate and kept at room temperature for drying for 4 days. After incubation period the mixture was grinded into fine powder and sprinkled over the soil, kept in pots**.**

Formulation with Chitosan

Chitosan (Analytical grade-ä) was dissolved at different concentrations(0.1, 0.25, 0.50, and 0.75) with glacial acetic acid under sterile conditions for half an hour. After stirring, the reaction mixture was kept overnight in hot air oven to obtain fine powder. The dried powder(1g) was mixed with the respective bacterial inocula, kept at room temperature for 24 hours. Later the cells coated chitosan was sprinkled over the soil kept in pots

Persistence Study

After 10 days of the treatment, the treated soil was evaluated for the occurance of Bacterial Biofertilizers. After the successive biogel and chitosan treatment the treated soil sample (10g) was suspended in 90 ml of sterile distilled water, kept under shaking conditions for 10 mins. The suspended sample was serially diluted, 0.1ml of the aliquote was spread plated on Yeast Extract Mannitol Agar (YEMA), nitrogen free minimal media for *Rhizobium*, *Acetobacter* and *Azospirillum*.The inoculated plates was incubated at 37C for 48 hours. After the incubation the colonies were counted and recorded.

Seed Treatment

The healthy and mature seeds of green gram and black gram were purchased from Agriculture Department and used for further studies. Both the seeds were soaked overnight in both the formulation containing respective bacterial biofertilizers. After overnight incubation the seeds were sown in respective pots. Seedling emergence was recorded and plant growth parameters such as shoot length, number of new branches emerged, leaf surface area, chlorophyll content, Nitrate reductase assay in respective treatment.

RESULT AND DISCUSSION

A key constraint to successfully commercializing beneficial microorganisms is overcoming difficulties in formulating a viable, cost-effective, and user-friendly final product[10,11]. The live nature of the active ingredient (i.e., the microbial agent) underscores the importance of formulation in maintaining the microbial cells in a metabolically and physiologically competent state in order to obtain the desired benefit when applied[13].

The development of new microbial formulations is a challenging task and requires greater effort in terms of funding and research towards making significant advances in this field.Worldwide, Cote[14] reported that, Persistence of insecticidal activity of novel bioencapsulated formulations of *Bacillus thuringiensis* var. *kurstaki* against *Choristoneura rosaceana*. DiPel™, a registered *Bacillus thuringiensis* var. *kurstaki (Btk)-base6* formulation, and experimental bio-encapsulated *Btk* formulations were sprayed in an apple orchard. Their persistence was assessed in the laboratory against obliquebanded *eairo\\er {Choristoneura rosaceana)* larvaeforthree consecutive years.

Table 1. Total count of formulated biofertilizers

Treatment		CFU/g								
		Rhizobium			*Azotobacter*			*Azospirillum*		
		Days			Days			Days		
		10	20	30	10	20	30	10	20	30
1	Biogel matrix	35×10^4	13×10^6	14×10^7	40×10^2	5×10^4	21×10^6	89×10^2	116×10^4	135×10^5
2	Chitosan									
	(Concentration) 0.1	17×10^3	21×10^7	41×10^8	21×10^3	17×10^7	36×10^8	21×10^3	10×10^6	31×10^8
	0.25	21×10^3	5×10^{10}	27×10^9	27×10^3	7×10^8	27×10^9	42×10^3	27×10^8	31×10^9
	0.50	4×10^4	17×10^8	47×10^9	51×10^4	21×10^8	51×10^9	15×10^4	34×10^8	109×10^9
	0.75	14×10^5	21×10^9	27×10^9	11×10^5	26×10^8	7×10^9	7×10^5	19×10^7	11×10^8
3	Control	17×10^2	21×10^4	10×10^5	11.3×10^3	19×10^4	41×10^4	10×10^3	19×10^4	$7x10^5$

Table 2. Effect of *Rhizobium* formulation on soil N,P and K level

S. No.	Treatment	Soil Nutrients(mg/kg)		
		Nitrogen(N)	Phosphorus(P)	Potassium(K)
1	Control	570.0	427.0	701.0
2	Biogel matrix	650.0	467.0	727.0
3	Chitosan formulation	613.0	457.0	711.0

Table 3. Effect of *Azotobacter* formulation on soil N,P and K level

S. No.	Treatment	Soil Nutrients(mg/kg)		
		Nitrogen(N)	Phosphorus(P)	Potassium(K)
1	Control	572.0	430.0	710.0
2	Biogel matrix	672.0	470.0	729.0
3	Chitosan formulation	625.0	460.0	722.0

Table 4. Effect of *Azospirillum* formulation on soil N,P and K level

S. No.	Treatment	Soil Nutrients(mg/kg)		
		Nitrogen(N)	Phosphorus(P)	Potassium(K)
1	Control	575.0	431.0	715.0
2	Biogel matrix	670.0	468.0	730.0
3	Chitosan Formulation	630.0	465.0	725.0

Table 5. Enumeration of total heterotrophic bacterial population in respective biofertilizer treated soil

S. No	Treatment	CFU/g											
		Rhizobium				*Azotobacter*				*Azospirillum*			
		Days				Days				Days			
		0	10	20	30	0	10	20	30	0	10	20	30
1	Bacteria Control	31×10^4	77×10^4	55×10^5	56×10^5	30×10^4	50×10^4	60×10^7	62×10^7	27×10^4	62×10^4	51×10^5	63×10^4
2	Biogel Matri×	17×10^4	16×10^6	11×10^7	11×10^7	18×10^4	27×10^6	32×10^7	42×10^7	20×10^4	31×10^6	35×10^7	50×10^7
3	Chitosan	21×10^4	47×10^6	37×10^7	41×10^7	20×10^4	50×10^6	47×10^7	50×10^7	22×10^4	29×10^6	57×10^7	57×10^7

Table 6 . Enumeration of total heterotrophic fungal population in respective biofertilizer treated soil

S. No	Treatment	CFU/g											
		Rhizobium				*Azotobacter*				*Azospirillum*			
		Days				Days				Days			
		0	10	20	30	0	10	20	30	0	10	20	30
1	Fungi Control	20×10^4	71×10^4	42×10^5	60×10^5	21×10^4	42×10^4	53×10^7	35×10^7	32×10^4	70×10^4	48×10^5	52×10^4
2	Biogel Matri×	21×10^4	25×10^6	15×10^7	19×10^7	30×10^4	35×10^6	39×10^7	56×10^7	32×10^4	37×10^6	65×10^7	50×10^7
3	Chitosan	27×10^4	52×10^6	49×10^7	51×10^7	28×10^4	50×10^6	48×10^7	39×10^7	20×10^4	47×10^6	75×10^7	67×10^7

Table 7 . Enumeration of total heterotrophic actinomycetes population in respective biofertilizer treated soil

S. No	Treatment	CFU/g											
		Rhizobium				*Azotobacter*				*Azospirillum*			
		Days				Days				Days			
		0	10	20	30	0	10	20	30	0	10	20	30
1	Actinomycetes Control	$28x10^4$	$50x10^4$	$31x10^5$	$45x10^5$	$33x10^4$	$47x10^4$	$67x10^7$	$52x10^7$	$38x10^4$	$44x10^4$	$72x10^5$	$53x10^4$
2	Biogel Matrix	$15x10^4$	$20x10^6$	$17x10^7$	$14x10^7$	$32x10^4$	$37x10^6$	$40x10^7$	$65x10^7$	$49x10^4$	$57x10^6$	$56x10^7$	$69x10^7$
3	Chitosan	$35x10^4$	$27x10^6$	$50x10^7$	$48x10^7$	$36x10^4$	$62x10^6$	$55x10^7$	$72x10^7$	$24x10^4$	$47x10^6$	$75x10^7$	$67x10^7$

As in control 100% seedling emergence was recorded in both tested seeds.But the time taken to emerge varied in respective treatments (Figure 1,2). In case of Black gram *(Vigna mungo L.*) 30%, 60%, 100%; 20%, 64%, 100%; 25%, 70%, 100% of seedling emergence was recorded in *Rhizobium, Azotobacter and Azospirillum* formulated with biogel matrix.In case of Green gram(*Vigna radiata*) 20%, 44%, 100%; 22%, 45%, 100%; 26%, 56%, 100% of seedling emergence was recorded As in control 100% seedling emergence was recorded in both tested seeds. But the time taken to emerge varied in respective treatments. In case of Black gram(*Vigna mungo L.*) 15%, 25%, 100%; 17%, 24%, 100%; 19%, 25%, 100% of seedling emergence was recorded in *Rhizobium, Azotobacter and Azospirillum* formulated with biogel matrix.In case of Green gram(*Vigna radiata)* 17%, 22%, 100%; 19%, 23%, 100%; 18%, 22%, 100% of seedling emergence was recorded

Total count of respective bacterial biofertilizer was increased in respective test periods.$35x10^2$, $13x10^6$, $14x10^7$, $40x10^2$, $05x10^4$, $21x10^6$, $89x10^2$, $116x10^4$ and $135x10^6$ CFU/g of *Rhizobium, Azotobacter and Azospirillum* was recorded in biogel matrix formulation at 10, 20 and 30 days of treatment. But the untreated control reveals $17x10^2$, $21x10^4$, $10x10^5$, $11.3x10^3$, $19x10^4$, $41x10^4$, $10x10^3$, $19x10^4$, $07x10^5$ CFU/g of *Rhizobium*, *Azotobacter* and *Azospirillum* (Table 1)

Effect of formulation on plant growth parameters of black gram *(vigna mungo l.)*

The formulated black gram *(Vigna mungo L.*) plants recorded higher value in all the parameters measured than untreated control. There were significant differences in shoot length, and leaf surface area count per plant. The length of shoot was found to be increased in all the tested time period in the formulated soil with Biogel matrix in *Rhizobium* viz; 12.2cm, 142.9cm, 14.5cm on 5^{th}, $10^{th}$and 15^{th} days but in control, the length of shoot at respective time period was 9.5cm,10.6cm and 12.5cm.The leaf surface area in Biogel matrix of *Rhizobium* was found to be increased from 1.5cm, 2.7cm and 5.1cm whereas in control it was found as 1.2cm, 2.6cm and 5.5cm. Similarly the soil treated with Chitosan reveales significant differences in the length of shoot; 11.3cm, 11.1cm

Table 8. Effect of temperature (ºC) on formulated biofertilizers

S. No.	Treatment	Biofertilizer	Temperature (°C)		
			40°C	50°C	60°C
1	Control	*Rhizobium*	17×10^2	21×10^4	10×10^5
2	Biogel matrix		17×10^5	27×10^6	20×10^7
3	Chitosan formulation		10×10^5	19×10^6	17×10^7

Table 9. Effect of temperature (ºC) on formulated biofertilizers

S. No.	Treatment	Biofertilizer	Temperature (°C)		
			40°C	50°C	60°C
1	Control	*Azotobacter*	11.3×10^3	19×10^4	41×10^4
2	Biogel matrix		17×10^5	21×10^6	42×10^7
3	Chitosan formulation		11×10^5	18×10^6	39×10^7

Table 10. Effect of temperature(ºC) on formulated biofertilizers

S. No.	Treatment	Biofertilizer	Temperature (°C)		
			40°C	50°C	60°C
1	Control	*Azosprillium*	$10x10^3$	$19x10^4$	$7x10^5$
2	Biogel matrix		$12x10^5$	$21x10^6$	$9x10^7$
3	Chitosan formulation		$11x10^5$	$15x10^6$	$8x10^7$

Table 11. Alkaline phosphatase and Urease activity of soil treated with formulated biofertilizers

S. No	Treatment	Enzyme Activity			
		Alkaline Phosphatase Activity Days		Urease Activity Days	
		30	60	30	60
1	Control	+	+	+	+
2	*Rhizobium* Biogel matrix	+	+	+	+
3	Chitosan formulation	+	+	+	+

Table 12. Alkaline phosphatase and Urease activity of soil treated with formulated biofertilizers

S. No	Treatment	Enzyme Activity			
		Alkaline Phosphatase Activity Days		Urease Activity Days	
		30	60	30	60
1	Control	+	+	+	+
2	*Azotobacter* Biogel matrix	+	+	+	+
3	Chitosan formulation	+	+	+	+

and 13.0cm was recorded on 5th, 10th and 15th days and the leaf surface area was observed as 1.0cm, 2.1cm and 4.7cm.

In *Azotobacter,* the shoot length reveals 12.0cm 13.7cm and 16.5cm in soil treated with Biogel matrix, in control 10.6cm 11.5cm and 13.9cm with Chitosan 11.4cm 13.1cm and 15.0cm was measured on 5th, 10th and 15th days; the leaf surface area of control was observed as 1.3cm, 2.9cm and 5.0cm; 1.8cm 2.0cm and 4.2cm in Biogel matrix and in Chitosan 1.6cm 2.0cm and 4.2cm was measured. In *Azospirillum*, the shoot length reveals 13.5cm 14.4cm and 14.9cm in soil treated with Biogel matrix and in control 11.8cm 12.6cm and 13.6cm with Chitosan 12.3cm 14.1cm and 13.2cm was measured on 5th, 10th and 15th days;

Table 13. Alkaline phosphatase and Urease activity of soil treated with formulated biofertilizers

S. No	Treatment	Enzyme Activity			
		Alkaline PhosphataseActivity Days		Urease Activity Days	
		30	60	30	60
1	Control	+	+	+	+
2	*Azospirillum* Biogel matrix	+	+	+	+
3	Chitosan formulation	+	+	+	+

Table 14. Chlorophyll content of respective formulated biofertilizer plants Black gram (*Vigna mungo L.*) Green gram (*Vigna radiata*)

S. No	Treatment	Chlorophyll content	
		Black gram(mg/g)	Green gram(mg/g)
1	Control	121.0	128.0
2	*Rhizobium*(Biogel matrix)	176.0	147.0
3	*Rhizobium* (Chitosan formulation)	135.0	129.0
4	*Azotobacter* (Biogel matrix)	141.0	152.0
5	*Azotobacter(*Chitosan formulation)	129.0	148.0
6	*Azospirillum*(Biogel matrix)	169.0	130.0
7	*Azospirillum*(Chitosan formulation)	145.0	137.0

Table 15. Nitrate reductase activity of soil treated with formulated biofertilizers

S. No	Treatment	Nitrate reductase activity			
		Days 30	Days 60	Days 30	Days 60
1	*Rhizobium*(Control)	+	+	+	+
	Rhizobium(Biogel matrix)	+	+	+	+
	Rhizobium (Chitosan formulation)	+	+	+	+
2	*Azotobacter*(Control)	+	+	+	+
	Azotobacter (Biogel matrix)	+	+	+	+
	*Azotobacter(*Chitosan formulation)	+	+	+	+
3	*Azospirillum*(Control)	+	+	+	+
	Azospirillum(Biogel matrix)	+	+	+	+
	Azospirillum(Chitosan formulation)	+	+	+	+

the leaf surface area of control was observed as 1.4cm, 2.6cm and 5.3cm; 1.9cm 2.3cm and 4.2cm in Biogel matrix and in Chitosan 1.7cm 2.2cm and 4.0cm was measured.

After 30th days of respective formulated biofertilizer treatment the total N, total P and total K level was increased than untreated control (Table 4). Biogel matrix formulated *Rhizobium* reveals 650mg/kg of total N and 613mg/kg of total N Chitosan formulation.But control reveals 570mg/kg of total N, but no distinct difference in K and P.Total P and total K was recorded in respective biogel matrix and chitosan formulation as 467mg/kg, 457mg/kg and 727mg/kg, 711mg/kg (Table 2,3,4). As in formulated *Azotobacter* and *Azospirillum* treatment 672mg/kg, 625mg/kg and 670mg/kg , 630mg/kg of total N was recorded in biogel matrix and chitosan formulation. 470mg/kg , 460mg/kg of total P and 729mg/kg, 722mg/kg of total K and 468mg/kg , 465mg/kg of total P and 730mg/kg, 725mg/kg of total K was recorded in respective biogel matrix and chitosan formulation

All the microbial population was significantly increased in respective biogel matrix formulation and chitosan treatment (Table 5,6,7). 17x10^4, 16x10^6, 11x10^7, 11x10^7, 18x10^4, 27x10^6, 32x10^7, 42x10^7, 20x10^4, 31x10^6, 35x10^7, 50x10^7 CFU/g and 21x10^4, 47x10^6, 37x10^7, 41x10^7, 20x10^4, 50x10^6, 47x10^7, 50x10^7, 22x10^4, 29x10^6, 57x10^7, 57x10^7 CFU/g of bacterial population was recorded in biogel matrix and chitosan formulation (Table 5).Similarly the Fungal population in respective treatment was 21x10^4, 25x10^6, 15x10^7, 19x10^7, 30x10^4, 35x10^6, 39x10^7, 56x10^7, 32x10^4, 37x10^6, 49x10^7, 60x10^7 CFU/g and 27x10^4, 52x10^6, 49x10^7, 51x10^7, 28x10^4, 48x10^6, 39x10^7, 65x10^7, 17x10^4, 32x10^6, 54x10^7, 43x10^7 CFU/g. Actinomycetes population respective treatment was 15x10^4, 20x10^6, 17x10^7, 14x10^7, 32x10^4, 37x10^6, 40x10^7, 65x10^7, 49x10^4, 57x10^6, 65x10^7, 68x10^7 CFU/g and 35x10^4, 27x10^6, 50x10^7, 48x10^7, 32x10^4, 62x10^6, 25x10^7, 72x10^7, 24x10^4, 47x10^6, 75x10^7, 67x10^7 CFU/g

The effect of temperature reveals that the formulated biofertilizers retained viability at all the tested temperatures.17x10^5, 27x10^6, 20x10^7 and 10x10^5, 19x10^6, 17x10^7 CFU/g was counted in biogel matrix and chitosan treated *Rhizobium*

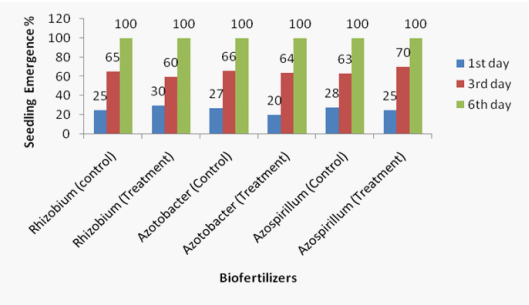

Fig. 1. Seedling emergence % of Black gram(*Vigna mungo L.*) with Biogel Matrix formulated Biofertilizers

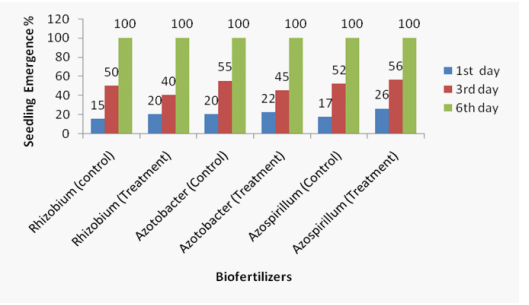

Fig. 2. Seedling emergence % of Green gram *(Vigna radiata)* with Biogel matrix formulated Biofertilizers

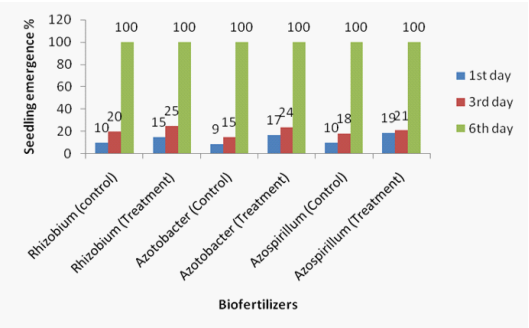

Fig. 3. Seedling emergence % of Black gram (*Vigna mungo L.*) with Chitosan formulated Biofertilizers

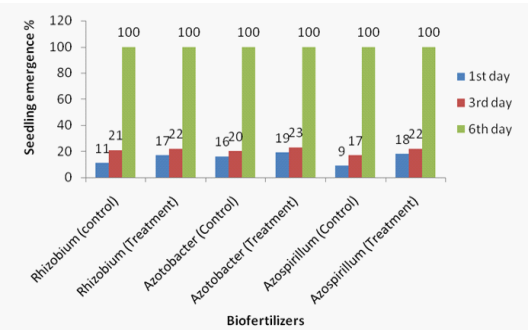

Fig. 4. Seedling emergence % of Green gram *(Vigna radiata)* with Chitosan formulated Biofertilizers

In *Azotobacter* $17x10^5$, $21x10^6$, $42x10^7$ and $11x10^5$, $18x10^6$, $39x10^7$ CFU/g was counted in biogel matrix and chitosan .But in control $11.3x10^3$, $19x10^4$, $41x10^4$ CFU/g was observed .In *Azospirillum* $12x10^5$, $21x10^6$, $09x10^7$ and $11x10^5$, $15x10^6$,$08x10^7$ CFU/g was counted in biogel matrix and chitosan . But in control $10x10^3$, $19x10^4$, $07x10^5$ CFU/g was observed (**Table 8,9,10**).Alkaline Phosphatase and Urease enzyme activity was recorded in all the tested time period n all the formulated Biofertilizers as in control (Table 11,12,13).The Chlorophyll content was significantly increased in all the biofertilizer formulated plants than control.In control 121.0 mg/g and 128.0 mg/g was observed in Black gram(*Vigna mungo L.*) and Green gram(*Vigna radiata)*. Similarly in biogel matrix and chitosan formulated with *Rhizobium, Azotobacter and Azospirillum* 176.0 mg/g, 147.0 mg/g; 135.0 mg/g, 129.0 mg/g; 141.0 mg/g,152.0 mg/g; 128.0 mg/g, 148.0 mg/g;169.0 mg/g, 130 mg/g; and 145.0 mg/g, 137.0 mg/g was observed in Black gram(*Vigna mungo L.*) and Green gram(*Vigna radiata*) (Table 14). Nitrate reductase activity was recorded in all the tested time period in all the formulated Biofertilizers as in control (Table 15**).**The present study would suggests the possible utilization of encapsulated biofertilizers for the sustainable organic agriculture.

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