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Growth Stimulatory Effects of *Enterobacter* and *Serratia* Isolated from Biofilms on Plant Growth and Soil Aggregation

Rida Batool and Shahida Hasnain
Department of Microbiology and Molecular Genetics,
University of the Punjab, Q.A. Campus, Lahore, 54590, Pakistan

Abstract: Twelve bacterial strains of family Enterobacteriaceae were isolated from different biofilms. Total nine biofilms were collected from polluted as well as from non-polluted environments. Isolates were purified and characterized both morphologically and biochemically. Total eleven strains were affiliated with *Enterobacter* while only one showed affinities with *Serratia*. Isolated strains of *Enterobacter* and *Serratia* were used to inoculate the seedlings of *Zea mays* in mono and mixed culturing. The growth parameters of *Zea mays* seedling and soil aggregation were recorded. Monoculture inoculations caused an increase in all length parameters i.e., shoot length, root length, seedling length of *Zea mays* seedlings. Number of roots decreased and number of leaves remained constant. When all these strains were used as mixed culture, a decrease in all length parameters i.e., shoot length, root length, seedling length was observed while number of roots increased and number of leaves decreased. Monoculture inoculation resulted in increase in weight of aggregates on roots and causing decrease in weight of aggregates in soil. In mixed culture inoculation, weight of aggregates on roots as well as in soil was decreased over control.

Key words: *Enterobacter, Serratia, Zea mays*, length parameters, inoculation

INTRODUCTION

Biofilms are complex communities of microorganisms attached to surfaces or associated with interfaces. Their association with surfaces involved synthesis of extracellular homo or hetero polymers of sugars called exopolysaccharides (EPS)[1]. Biofilms include bacteria, fungi, yeast, protozoan and other microbes. It can be composed of population that develops from a single species or a community derived from multiple microbial species. They can form on a vast variety of biotic and abiotic surfaces[2]. The major component in biofilm matrix is water upto 97%[3]. Biofilms are involved in drug resistance and provide ant microbial activities[4]. Further microbial biofilms serve as biobarriers in oil industry and also used in treatment of wasted water[5]. Microbial biofilm formation with the plant roots protect the plant against the soil borne diseases and improves crop productivity and physico-chemical characteristics of soil[6]. Aggregate formation is an important factor, in controlling germination and plant root growth[7]. According to Amellal *et al.*[8] inoculation of seeds with bacteria promotes plant growth of Wheat. The main effects of bacterial inoculation were the increase of RAS (root adhering soil) mass, macropore volume of RAS and nitrogen uptake by the plant and finally plant growth. According to Alami *et al.*[9] significant increase in RAS mass around the roots of sunflower plantlets inoculated with *Rhizobium* sp. strain YAS34 could be the result of either an increase in soil adhesion to roots or a higher aggregate stability or both. All of this may be due to EPS production. Keeping this in a view we are concerned with the isolation of gram negative *Enterobacter* and *Serratia* sp. from different biofilms obtained from different sources. Further the effects of mono and mixed culturing were studied on plant growth promotion and soil aggregation.

MATERIALS AND METHODS

Bacterial strains and culture conditions: Twelve different bacterial strains of *Enterobacter* and *Serratia* were isolated from nine biofilm samples collected from different sources (Table 1). Ten microliter inoculum of each biofilm sample was plated on nutrient as well as on Eosine Methylene Blue Agar (EMB). EMB agar was used as a selective media for family Enterobactraceae. Pinkish

Corresponding Author: Dr. Shahida Husnain, Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore, 54590, Pakistan Tel: 92-42-9231238 Fax: 92-42-9230481

Table 1: Isolation of bacterial strains from different biofilms obtained from different sources

Samples	Source/location	Material	Bacterial strains isolated
Punjab University New Campus Lahore			
A	Dry area near canal water	Soil	NIL
B	Canal water	Floating leaf	NIL
Botanical garden Punjab University New Campus, Lahore			
C	Pond	Soil on shopping bag	C_1, C_2, C_4
D	Pond	Dry feather	NIL
E	Pond	Dry leaf	NIL
F	Pond	Straw on mud	F_1, F_2, F_3
G	Pond	Mud	G_1, G_2, G_3
Industries along Sheikhupura Road			
A`	Effluents dumped outside the cement industry	Muddy water	A`
B`	Industrial effluents	Muddy Water	$B`_1$, $B`_2$

and bluish black colonies appeared on EMB agar were *Enterobacter* and *Serratia*, respectively. Isolated strains were designated as C_1, C_2, C_4, F_1, F_2, F_3, G_1, G_2, G_3, A`, $B_1`$ and $B_2`$. Colonies obtained were further purified on L-agar.

Strains characterization: Isolated strains were characterized morphologically, biochemically and physiologically following Gerhardt *et al.*[4] and Cappuccino and Sherman[2].

POST GERMINATION INOCULATION (MONO-MIXED CULTURE) EXPERIMENTS ON THE GROWTH OF *ZEA MAYS* SEEDLINGS AND ON SOIL AGGREGATION

Bacterial Growth Conditions: Bacterial cultures were grown on L-agar at 37°C for 24 h.

Experimental Setup: Effects of mono and mixed culturing of *Enterobacter* and *Serratia* on plant growth promotion (*Zea mays*) and soil aggregation were studied[10]. Randomly selected healthy seeds of *Zea mays* (obtained from Punjab seed corporation, Lahore) were surface sterilized with 0.1% $HgCl_2$. Twelve monoculture (C_1, C_2, C_4, F_1, F_2, F_3, G_1, G_2, G_3, A`, $B_1`$ and B_2) and 4 mixed culture (C, F, G, B`) combinations of *Enterobacter* and *Serratia* strains were used in these studies. Bacterial cultures were grown on L-agar at 37°C for 24 h. For preparation of bacterial suspensions, 24 h old bacterial cultures from L-agar were suspended in sterilized distilled water and bacterial population was adjusted to 10^8cells mL^{-1}. Mixed cultures were prepared by mixing all the strains, which were inhabitant of one common biofilm and a single suspension was prepared. Mixed suspensions were designated as C, F, G and B`. For making suspension C, 10^8 bacterial cells of each of strains C_1, C_2 and C_4 were mixed and same procedure was followed for making rest of the suspensions i.e., all strains isolated from one biofilm were mixed together in equal proportion. Surface sterilized seeds of *Zea mays* were aseptically and uniformly spreaded to sterilized petriplates lined with double layer of filter paper. Ten microliter of sterilized distilled water were supplied to each petriplate in order to moisten the filter paper. Petriplates were placed in the dark at 25±2°C for three days for germination. After on germinating seeds were dipped for 15 to 20 min in mono and mixed culture bacterial suspensions for inoculation. For control, germinated seeds were soaked in sterilized glass distilled water for 15 to 20 min. Thirty nine pots incase of monoculture and 15 pots incase of mixed culture (3 replicates for each strain and three for control) were taken and filled with 120 g sieved soil. Inoculated and non-inoculated germinated seeds were uniformly transferred to the respective labeled pot at the rate of 4 to 5 seedlings per pot. All the pots were kept in light (±10 K lux and 25±2°C) and arranged in Completely Randomized Block Design.

Harvest: After 10 days, growth parameters, weight of aggregates in soil as well as on roots were determined.

Statistical analysis: Data was collected and standard errors of the means and LSD were calculated by Steel and Torrie[11].

RESULTS

Total twelve different bacterial strains of *Enterobacter* and *Serratia* were isolated from nine biofilm samples collected from different sources of canal and pond in the vicinity of Punjab University, Quaid-e-Azam campus, Lahore as well as from industrial effluents along Sheikhupura road (Table 1). The isolated strains were designated as C_1, C_2, C_4, F_1, F_2, F_3, G_1, G_2, G_3, A`, $B_1`$and $B_2`$. For initial and primary isolation as well as for calculating the percentage of *Enterobacter* and *Serratia*, both nutrient and EMB agar were used. In different biofilm samples i.e; A, B, C, D, E, G, A` and B` the percentage occurrence of *Enterobacter* and *Serratia* was 0, 1.89, 30.93, 2.38, 0, 45, 83.19 and 2686.57, respectively. While biofilm sample F had 1.78% *Serratia* strains. Colonies of *Enterobacter* and *Serratia* were purified on L-agar at 37°C for further characterization. For morphological characterization 24 h old bacterial cultures incubated at 37°C were used and colony and cell morphology were observed. Colonies of all isolates were circular with entire

margins. Colonies of all isolates were convex except C_1, G_3, A`, $B_1$` and B_2` which have colonies with raised elevation. The colors of colonies were transparent shiny ($C_1$, $C_2$), off-white ($C_4$, $F_1$, $F_2$, $F_3$, $G_1$, $G_2$, $G_3$, A`, B_1`, $B_2$`) and pink (F_2). The colony size of all isolates varied from 0.01 to 0.6 mm. All isolates had motile, gram negative and rod shaped cells. Biochemical tests were conducted to check the differences between *Enterobacter* and *Serratia* strains obtained from different sources. All the bacterial isolates showed positive results for catalase, Voges-proskauer and nitrate reduction test while negative results for oxidase, methyl red and hydrogen sulfide gas production test. In oxidation fermentation test, acid and gas was produced by all strains except F_1, F_2 and G_2, which showed no gas production. All bacterial isolates except C_2, C_4, F_2 and G_1 were unable to hydrolyze the starch. Gelatin was hydrolyzed by all bacterial isolates except C_1, C_2, F_1 and G_3.

Effects of Post Germination Mono and Mixed Culture Inoculation of *Enterobacter* and *Serratia* on Growth of 10 Days Old *Zea mays* Seedlings and Soil Aggregation

Shoot length: In monoculture inoculation, generally with all bacterial inoculations a significant increase in shoot length over respective non-inoculated treatment was observed. Maximum increase (76.88%) in shoot length was observed with the inoculation of strain F_3 while minimum increase (13.44%) was observed with the inoculation of strain C_2 (Table 2 and Fig. 1a). In mixed culture, generally with all mixed bacterial inoculations a significant decrease in shoot length over respective non-inoculated treatment was observed. Maximum decrease (38.49%) in shoot length was observed with the inoculation of mixed culture of isolates of biofilm sample F while minimum decrease (14.13%) was observed with the inoculation of mixed culture of isolates of biofilm sample B` over respective control (Table 3 and Fig. 1c).

Root Length: In monoculture inoculation, generally with all bacterial inoculations a significant increase in root length over respective non-inoculated treatment was observed. Maximum increase (117.23%) in root length was observed with the inoculation of strain A` while minimum increase (44.40%) was observed with the inoculation of strain F_3 (Table 2 and Fig. 1a). In mixed culture, generally with all mixed bacterial inoculations a significant decrease in root length over respective non-inoculated treatment was observed. Maximum decrease (61.19%) in root length of *Zea mays* seedlings was observed with the inoculation of mixed culture of isolates of biofilm sample B` while minimum (51.52%) decrease was observed with the

Table 2: Effect of post germination monoculture inoculation on growth parameters of *Zea mays* seedlings

	Shoot Length (cm)	Root length (cm)	Seedling length (cm)	No. of roots	No. of leaves	Weight of aggregates on roots (g)	Weight of aggregates in soil (g)
Strains	X̄±SE						
Cont.	18.0±0.98	5.63±0.41	23.64±0.95	4.77±0.59	3±0	0.46±0.02	4.25±0.85
C_1	20.73±0.91	8.41±0.58	32.48±0.67	3.33±0.63	3±0	1.10±0.08	2.61±0.45
C_2	20.42±0.86	9.12±0.32	29.54±0.18	5.10±0.25	3±0	0.60±0.11	4.87±0.22
C_4	27.13±0.23	10.12±0.49	37.24±0.54	5.22±0.37	3±0	1.19±0.26	6.07±0.70
F_1	26.41±0.65	11.48±0.33	37.89±0.82	5.11±0.22	3±0	1.49±0.16	4.80±0.58
F_2	27.34±0.38	8.24±0.65	35.58±0.72	4.88±0.79	3±0	1.17±0.14	3.71±0.04
F_3	31.84±0.82	8.13±0.39	39.97±0.74	4.33±0.68	3±0	1.31±0.08	7.36±0.63
G_1	25.83±0.21	11.03±0.63	36.86±0.23	4.66±0.13	3±0	0.87±0.26	4.94±0.59
G_2	28.53±0.29	9.36±0.46	37.89±0.99	3.11±0.08	3±0	2.73±0.14	6.8±0.83
G_3	29.19±0.80	10.57±0.59	43.10±0.34	2.99±0.31	3±0	0.55±0.07	4.76±0.35
A`	28.96±0.92	12.23±0.96	41.19±0.80	3.44±0.24	3±0	1.65±0.09	4.12±0.24
$B`_1$	22.60±1.78	11.58±0.4	34.46±0.82	5.55±0.39	3±0	0.73±0.08	5.25±0.18
$B`_2$	26.07±0.62	10.1±0.13	36.8±0.60	4.66±0.56	3±0	1.31±0.17	3.93±0.35
LSD at p = 0.05	0.795	1.90	3.65	2.21	0	0.072	0.814

Table 3: Effect of post germination mixed culture inoculation on growth parameters of *Zea mays* seedlings

	Shoot length (cm)	Root length (cm)	Seedling length (cm)	No. of roots	No. of leaves	Weight of aggregates on roots (g)	Weight of aggregates in soil (g)
Strains	X̄±SE						
Cont.	26.03±0.44	20.11±0.91	46.14±0.33	4.10±0.33	2.71±0.05	0.90±0.007	14.30±0.19
C	19.48±0.30	7.86±0.55	27.34±0.80	4.99±0.15	2.66±0	0.91±0.02	6.90±0.27
F	16.01±0.36	8.09±0.59	24.11±0.80	5.11±0.55	2.55±0.08	0.76±0.04	12.63±0.58
G	17.93±0.51	9.75±0.73	27.68±0.82	4.44±0.39	2.66±0	0.58±0.03	8.38±0.22
B`	22.35±0.20	7.66±0.63	30.01±0.64	5.99±0.81	2.66±0.15	0.89±0.019	8.94±0.47
LSD at p = 0.05	1.072	0.627	0.636	0.757	0.913	0.092	1.22

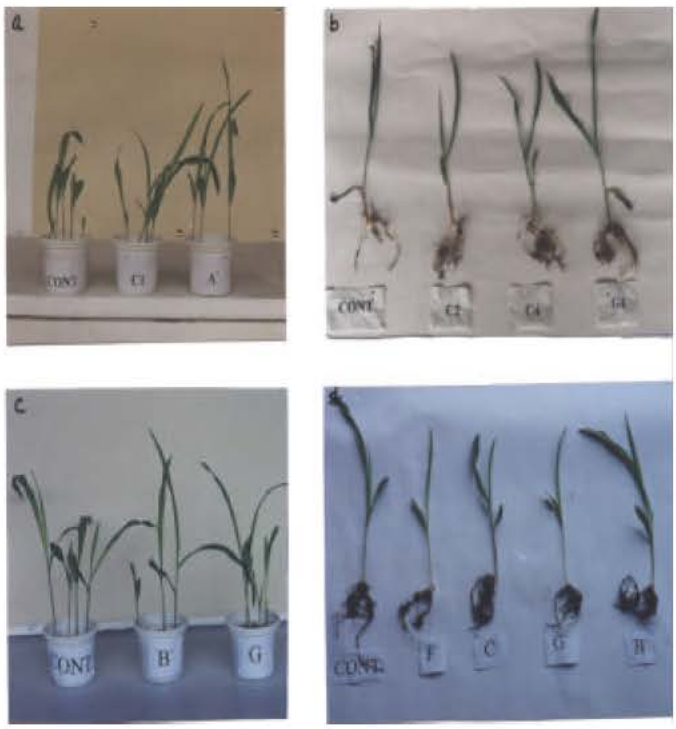

Fig. 1: Effect of bacterial mono and mixed cultures on growth and soil aggregates in *Zea mays* seedlings. a: Effect of bacterial mono cultures on growth of *Zea mays* seedlings. b: Effect of bacterial mono cultures on soil aggregates of *Zea mays* seedlings. c: Effect of bacterial mixed cultures on growth of *Zea mays* seedlings. d: Effect of bacterial mixed cultures on soil aggregates of *Zea mays* seedlings.

inoculation of mixed culture of isolates of biofilm G over control (Table 3 and Fig. 1c).

Seedling length: In monoculture inoculation, generally with all bacterial inoculations a significant increase in seedling length over respective non-inoculated treatment was observed. Maximum increase (82.32%) in seedling length was observed by the inoculation of strain G_3 while minimum increase (24.95%) was observed with the inoculation of strain C_2 (Table 2 and Fig. 1a). In mixed culture, generally with all mixed bacterial inoculations a significant decrease in seedling length over respective non-inoculated treatment was observed. Maximum decrease (47.79%) in seedling length was observed with the inoculation of mixed culture of isolates of biofilm sample F while minimum decrease (35.02%) was observed with the inoculation of mixed culture of isolates of biofilm sample B` over control (Table 3 and Fig. 1c).

Number of roots: In monoculture inoculation, generally with all bacterial inoculation a significant decrease in number of roots over respective non inoculated treatment was observed except incase of inoculation with strains C_2, C_4, F_1, F_2 and B_1` which cause increase in number of roots. Maximum decrease (37.31%) in number of roots was observed with the inoculation of strain $G_3$ while minimum decrease (2.30%) was observed with the inoculation of strain $B_2$`. Maximum increase (16.35%) was observed with the inoculation of strain B_1`while minimum increase (2.30%) was observed with inoculation of strain F_2 over control (Table 2). In mixed culture, generally with all mixed bacterial inoculations a significant increase in number of roots over respective non-inoculated treatment was observed. Maximum increase (46.09%) in number of roots was observed with the inoculation of mixed culture of isolates of biofilm sample B` while minimum increase (8.29%) was observed with the inoculation of mixed culture of isolates of biofilm sample G over non inoculated treatment (Table 3).

Number of leaves: In monoculture, generally with all bacterial inoculations, number of leaves were neither increased nor decreased over respective non-inoculated treatment (Table 2 and Fig. 1a). In mixed culture, generally with all mixed bacterial inoculations a significant decrease in number of leaves over respective non-inoculated

treatment was observed. Maximum decrease (14.02%) in number of leaves was observed with the inoculation of mixed culture of isolates of biofilm sample G while minimum decrease (1.84%) was observed with the inoculation of mixed culture isolates of biofilm sample B` over control (Table 3 and Fig. 1c).

Weight of aggregates on roots: In monoculture inoculation, generally with all bacterial inoculations a significant increase in weight of aggregates on roots over respective non-inoculated treatment was observed. Maximum increase (493.47%) in weight of aggregates on roots was observed with the inoculation of strain G_2 while minimum increase (19.56%) was observed with the inoculation of strain G_3 as compared with control (Table 2 and Fig.1b). In mixed culture, generally with all mixed bacterial inoculations a significant decrease in weight of aggregates on roots over respective non-inoculated treatment was observed except mixed culture of biofilm sample C that showed 1.11% increase in weight of aggregates on roots over control. Maximum decrease (35.55%) in weight of aggregates on roots was observed with the inoculation of mixed culture of isolates of biofilm sample G while minimum decrease (15.55%) was observed with the inoculation of mixed culture of all isolates of biofilm sample B` over control (Table 3 and Fig. 1d).

Weight of aggregates in soil: In monoculture inoculation, generally with all bacterial inoculations a significant increase in weight of aggregates in soil over respective non-inoculated treatment was observed except incase of inoculation with strains C_1, F_2, A` and $B_2$` which cause decrease in weight of aggregates in soil over control. Maximum increase (73.18%) in weight of aggregates in soil was observed with the inoculation of strain F_3 while minimum increase (12.0%) was observed with the inoculation of strain G_3 as compared with control. Maximum decrease (38.58%) in weight of aggregates in soil was observed with the inoculation of strain C_1 while minimum decrease (3.06%) was observed with the inoculation of strain A` as compared with control (Table 2 and Fig. b). In mixed culture, generally with all mixed bacterial inoculations a significant decrease in weight of aggregates in soil over respective non-inoculated treatment was observed. Maximum decrease (51.74%) in weight of aggregates in soil was observed with the inoculation of mixed culture of isolates of biofilm sample C while minimum decrease (11.76%) was observed with the inoculation of mixed culture of all isolates of biofilm sample F over control (Table 3 and Fig. 1d).

DISCUSSION

Biofilms are broadly defined as assemblage of microorganisms and their associated extracellular products at an interface, typically attached to an abiotic or biotic surface. Different biofilms were collected from different sources of pond and canal in the vicinity of Punjab University, Quaid-e-Azam campus, Lahore as well as from industrial polluted areas along Sheikhupura road (Table 1). At the time of collection, only biofilm sample 'A' showed photosynthetic activity while biofilms present on bird's feather and straw (sample D and F) were dry and rough. Both nutrient agar and Eosine Methylene Blue (EMB) agar plates were used for initial and primary isolation as well as for calculating the percentage of *Enterobacter* and *Serratia* strains present in biofilms. *Enterobacter* strains appeared pink and *Serratia* strains appeared bluish black on EMB agar[12]. The percentage occurrence of *Enterobacter* strains was 0, 1.89, 30.39, 2.38, 0, 45, 83.19 and 2686.5, respectively in biofilm sample A, B, C, D, E, G, A` and B`.While the percentage occurrence of *Serratia* strain was 178% in biofilm sample F. As compared to total number of colonies present in biofilm samples, the percentage of *Enterobacter* is relatively less except incase of biofilm sample F and B`. No *Enterobacter* and *Serratia* strains were recovered from biofilm sample A and E. Sample B` whose biofilm was collected from industrial polluted area have highest number of *Enterobacter* strains and sample F whose biofilm was collected from straw have highest density of *Serratia* strains. *Pseudomonas* strains are able to form biofilm under all environmental conditions that allow growth but *E. coli* and other members of Enterobacteriaceae will not form biofilms under most conditions unless amino acids are supplemented[13,14] It becomes apparent that percentage of *Enterobacter* was (more in polluted areas, hence polluted areas have more *Enterobacter* diversity. Members of Enterobacteriaceae are reported as pollution indicator. They are involved in many bioremedation/biodegradation processes[5]. Bacterial diversity depends upon temporal and spatial distribution of bacteria[15]. Colonies of *Enterobacter* and *Serratia* strains were further purified on L-agar at 37°C. Total twelve bacterial strains (*Enterobacter* and *Serratia*) were isolated from nine different samples (Table 1). These strains were designated as C_1, C_2, C_4, F_1, F_2, F_3, G_1, G_2, G_3, A`, $B_1$` and B_2`.

Bacteria exhibit great diversity in their biochemical as well as metabolic processes[16]. Keeping in view that bacteria inhabitants of different biofilms, obtained from

different sources might have different morphological and ecological attributes, comparison was made between twelve viable strains of *Enterobacte*r and *Serratia* for their diverse morphological and biochemical characterization. Variation and differences were recorded among the isolates belonging to nine different biofilms In order to check the differences among the isolated strains of *Enterobacter* and *Serratia*, biochemical tests were performed. From biochemical point of view, *Enterobacter* and *Serratia* strains obtained from different sources showed diversity. So according to Starr *et al.*[12], *Enterobacter* and *Serratia* (members of family Enterobacteriaceae) may be defined as, Gram negative, motile, rod shaped, non-acid fast, able to grow both aerobically and anaerobically, oxidase negative, catalase positive bacteria.

Keeping in view the interaction of microbes from biofilms with plants as well as with soil, we are concerned with studying of effects of post germination inoculation (mono and mixed culture) on the growth of plant (*Zea mays*) and soil aggregation. In monoculture inoculation experiment, all length parameters showed over respective control. Number of leaves was not changed over respective non-inoculated treatment (Table 2). With bacterial inoculation, only one parameter i.e., number of roots was decreased when compared with the respective non-inoculated treatment (Table 2). Bacterial inoculation is reported to stimulate plant growth and yield[8,16,17]. Bacteria in the rhizosphere produce Exopolysaccharides (EPS). This not only provides advantage of protection to cells but it also enhances soil aggregation, which in turn improves water stability, which is critical to the survival of plant. As regard the soil aggregation, it was observed that with all bacterial inoculation, both weight of aggregates in the soil as well as on roots increased significantly over respective non-inoculated treatment (Table 2). Bacterial inoculation seems to modify soil structure around the root system by improving infiltration, water supply, aeration, presence of enhanced amount of humic acid/organic compounds, increase in soil macropore volume, increase in water holding capacity and aggregation of soil[8,9,18].

It appeared that through the stimulation of root exudates, *Enterobacter* and *Serratia* have partial or indirect effect on soil aggregation. *Enterobacter* and *Serratia* isolated from biofilms obtained from different sources have a positive effect on plant growth as well as on rhizosphere soil aggregation.

Effects of mixed culture inoculation in stimulating the growth of *Zea mays* were also studied. In mixed culture inoculation experiment, all the length parameters i.e., shoot length, root length and seedling length decreased as compared to control (Table 3). Bacteria can influence the efficiency of one another and can nullify/modify the effects of one another, when they live in association with one another. Generally with mixed culture inoculation number of roots was increased while number of leaves was decreased (Table 3). Both weights of aggregates on roots as well as in the soil decreased incase of mixed culture inoculation (Table 3). Mixed culture inoculation had shown stimulatory as well as inhibitory effects on the growth of *Zea mays*. It appeared that when bacterial strains are inoculated together they either secrete special metabolites or various types of EPS, those interact with each other and give negative impact on growth of plant. So according to Afrasayab *et al.*[10] mixed culture inoculation had curtailed effect on plant growth.

By making comparison between the two experiments i.e., monoculture inoculation and mixed culture inoculation experiments, it became evident that the promotion of plant growth is determined by soil aggregation. In monoculture inoculation, the percentage increase in weight of aggregates in soil was 12.0-73.18% and aggregates on roots i.e., root adhering ability showed 19.56-493.47% increase over respective non-inoculated treatment. In mixed culture inoculation, the percentage decrease in weight of aggregates in soil was 11.67-51.74% and root adhering ability showed 15.55-35.55% decrease over respective, control. The weight of aggregates both in soil and on roots was more incase of monoculture inoculation than mixed culture inoculation. It may be due to the possibility that in the presence of more strains in mixed culture lesser amount of EPS is secreted. When bacterial strains are grown together they compete and interact with one another

Overall it is evident that *Enterobacter* and *Serratia* are able to secrete EPS. EPS on one hand provides protection to bacterial cell against desiccation and other environmental stresses and on the other hand through EPS secretion they promote plant growth, in which soil aggregation plays a vital role. Further studies on soil structure, soil aggregation and soil analysis will present a better picture of interrelationship of soil aggregation and plant growth promotion

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