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The application of Biofilm based biofertilizer to increase the productivity of Chick pea (*Cicer arietinum*)

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Abstract

With the introduction of green revolution, the modern agriculture is getting more and more dependent on the steady supply of synthetic inputs *i.e.* Chemical fertilizers. The excessive use of chemical fertilizers by farmers in agriculture has resulted in several environmental problems. To overthrow these problems application of biofilm based biofertilizers has been found effective. Generally the biofertilizers are beneficial microorganisms involve in breakdown of organic matter, nitrogen fixation in a symbiotic manner, and secretion of growth promoting substances. They also supply nutrients to the plants, control soil borne diseases and maintain the physical structure of soil in cultivable fields. Intensive research has been done to find out the usefulness of this aspect in the leguminous plants like *Cicer arietinum.* In natural environment, the *Cicer arietinum* plants had symbiotic association with nitrogen fixing bacteria *i.e., Rhizobium leguminosarum* in the root nodules.

Current population of India is 1.34 billion. At present India has a capacity to produce about 259 million tonnes or little more of food grains every year, which is barely sufficient to feed the current population of the country. The population of the country is increased rapidly at about 1.34% per annum and is expected to overtake the population of China[11] by the year of 2030. Estimated food requirement will be more than 350 million tonnes by 2025 and about 500 million tonnes by 2050. To meet the demand of the food supply at large scale, application of fertilizer is essential in modern agriculture. With green revolution role of fertilizers has already been proven by many countries and by obtaining food self-sufficiency within short period of time[4].

The modern agriculture is getting more and more dependent on the steady supply of chemical fertilizers. The excessive use of chemical fertilizers by farmers in agriculture has resulted in several environmental problems like depletion of ozone layer, degrading the physical structure of soil, leading to a lack of oxygen in the plants root zone besides being quite expensive, sudden decrease in natural micro flora and acidification of water. To overthrow these problems application of biofertilizers has been found effective. Generally the biofertilizers are beneficial microorganisms involve in breakdown of organic matter, nitrogen fixation in a symbiotic manner, and secretion of growth promoting substances. They also supply nutrients to the plants, control

soil borne diseases and maintain the physical structure of soil in cultivable fields. Intensive research has been done to find out the usefulness of this aspect in the leguminous plantations[7].

The main purpose of our research is to isolate bacteria (*Rhizobium*) as a nitrogen fixing bacteria from root nodules of chick pea (*Cicer arietinum*) and its identification, characterization and hence the production of biofertilizer from it after growing it in the selective media and finally check the biofilm forming capacity of rhizobium and its estimation. Chickpea (*Cicer arietinum* L.) is a high proteinaceous leguminous plant grown in India. It is grown an about 9.21 million ha with production of 8.88 million ton with productivity of 995 kg/ha.

Process of biofilm formation can also be defined as an assemblage of microbial cells that are irreversibly associated with a biotic or abiotic surface and confined in a matrix of primarily polysaccharide material allowing survival and growth in sessile environment. Scientists have realized that more than 99% of bacteria exist in nature as biofilms. They have also investigated that survival mechanisms for rhizobia subjected to stress by studying the formation of biofilms and describing many of the factors that mediate biofilm formation. The biofilm formed by non-spore forming bacteria protect its community from the fluctuation and often severe conditions of the rhizosphere, such as desiccation, extreme pH levels[2]. UV radiation, temperature, salt, nutrient availability, as well as tolerance against antibiotics, protection from protozoa predation and production of secondary metabolites and exoenzymes[10].

Study area :

This study was conducted in district Bhopal, the capital city of Madhya Pradesh, India located at 23°15'N 77°25'E and comprises approximately 285.88 km^2. The city is situated at an average elevation of 527 meters above the sea level.

Collection and extraction of root nodules from chickpea plants (Cicer arietinum):

The experimental material for the present study was collected from different areas of Bhopal, M.P. The sample was collected in two consecutive years 2015-2016. The main aim behind this way of sample collection was to check whether both isolates of 2015-2016 show same effect or not.

Chickpea plants were uprooted carefully so that intact roots can be obtained. The roots of the plants were rinsed in tap water to remove loosely adhering soil. Five to ten nodules were removed from each plant with forceps. Plants possessing healthy nodules with pink colour were selected and transported to the lab without any delay. Root nodules of chickpea plant *(Cicer arietinum)* were used as study material for isolation and further morphological and physiological characterisation of Rhizobium strains.

Biofertilizer preparation:
Production of broth culture :

Once the pure culture of Rhizobium has been established and confirmed for its various activities, the next step was production of the Rhizobia broth culture into a form which is easily used by farmers. Rhizobia are

relatively easy to grow in liquid medium. Since rhizobia are not competitive with other microorganisms, it is very important to sterilize the whole of growth vessel and medium as well as ensuring inoculation of the fermenter with rhizobial starter culture under sterile environment. The purpose of the production is to have high density of rhizobia in the broth culture. This can be influenced by culture medium, rhizobial strain, temperature and aeration. Rhizobia are aerobic bacteria and need oxygen for growth. Optimum temperature for rhizobial growth is 28-30^{o} C.

The medium supplies energy, nitrogen, certain mineral salts and growth factor. General yeast extract mannitol (YEM) medium is used in rhizobial broth culture. Compositions of YEM broth are as follows: K_2HPO_4: 0.5gl^{-1}; $MgSO_4.7H_2O$: 0.1gl^{-1}; NaCl: 0.2gl^{-1}; Mannitol: 10.0gl^{-1}; Yeast extract: 0.5gl^{-1}; Distilled water: 1 litre; pH: 7.0. For production of broth culture, flasks of different sizes are often used. It is important that all equipment's must be sterilized. Flasks are filled with media to one-third to two-third and sterilized in an autoclave at 121^{o} C. After the medium was cooled to room temperature, a pure culture of Rhizobium was taken with the help of loop and transferred into the flask. Flasks were plugged in to allow the microbes to grow and incubated for 5-7 days at 28 ± 2^{o} C. This method was simple by which microorganisms were transferred from a purified culture to the medium for their multiplication. To avoid any kind of contamination, the process of inoculation was done under laminar air flow chamber aseptically.

Fermentation

The sterilized medium was inoculated with a pure culture of the desired microorganisms under aseptic conditions as described above. This pure culture is called broth, and will be used for further multiplication of the microbes and their commercial production. The broth was put in conical flasks of necessary size depending on the amount of broth required to be used in biofertilizer production. The flasks were fixed on a rotatory shaker and shaken continuously for an hour and then kept in an incubator. Same process was repeated for 5-7 days in order to achieve the same population of microbes as in steel fermenters. When the number of microbes reaches up to 10^7 to 10^9 cells per ml, the broth was ready to mix with seeds or directly into the soil.

Inoculation of chickpea seeds :

For inoculation the seeds of chickpea were treated with Rhizobium broth culture. Eight broth cultures were used separately. From each broth culture 10 ml of broth culture was taken in a test tube. The seeds of chickpea (Cicer arietinum) were surface sterilized by 70% ethanol and then treated with 1% sodium hypochlorite for 2 minutes followed by repeated washing with sterile water. The seeds were then soaked directly into the broth culture for at least 2 minutes. Finally, the seeds were grown in the trays with two planting rows. The cups of trays were marked properly, so as to identify the samples properly. One of the cups of tray was marked as control. The seeds of control were not soaked with broth culture. These trays were constantly given water. Seed germination and per cent seedling emergence was calculated by using the below formula:

$$\% \text{ Emergence} = \frac{\text{Number of emerged seedlings}}{\text{Number of seeds sown}} \text{ X } 100$$

The growth was checked regularly and the results were noted in the note book. After few weeks the chickpea plants were removed from the trays and the shoot length, root length, leaf size, branch size were noted.

A total of 12 isolates were recovered from root nodules of chick pea (*Cicer arietinum*), collected from different regions of Bhopal. All of the isolates were gram negative and rod shaped non-spore forming. The authentication test confirmed that each isolate has ability to nodulate the host plant. The plants treated with control did not show any nodule formation, instead show chlorosis and wilting after first two weeks of the experiment. The 12 isolates that nodulate the host plant confirms themselves after the authentication test as *Rhizobium leguminosarum*.

Effect of rhizobial broth culture on chickpea seed :

The seeds of chickpea were inoculated separately with each rhizobial broth culture, which are formed with different biofilm forming Rhizobium strains. Some of the seeds of chickpea were sown in the soil without inoculating them and taken as control. Both the control and the inoculated seeds emerge out of the soil. But there is variation in growth of chickpea plants. The difference in shoot and

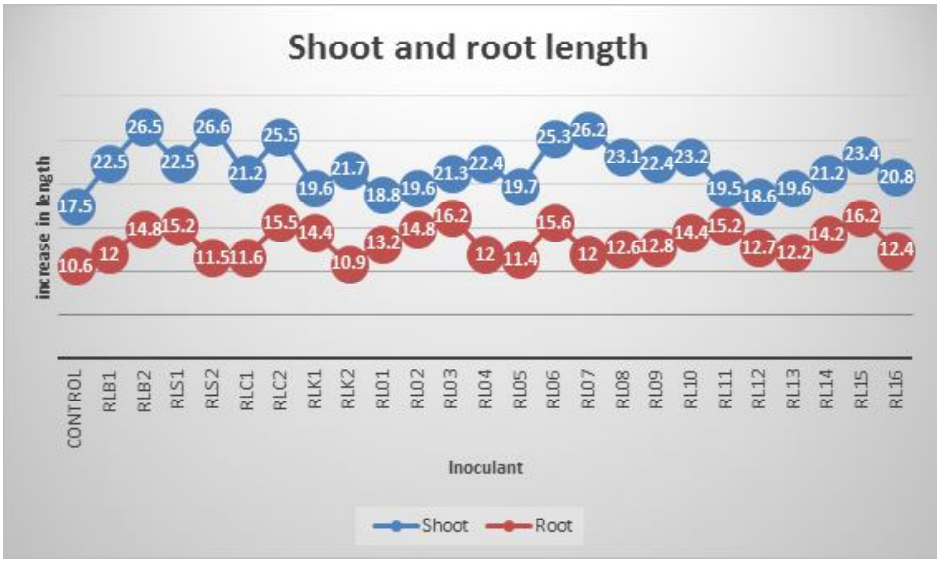
Table 1. Per cent increase of germinated seeds (values showing number of germinated seeds)

Treatments	Shoot length(cm)	% increase	Root length (cm)	% increase	Total length	% increase
Control	17.5± .14		10.6± .14		28.1	
RLB1	22.5± .85	25.57	12.0± .08	13.2	34.5	18.5
RLB2	26.5± .87	51.42	14.8± .78	39.6	41.3	46.97
RLS1	22.5± 1.1	25.57	15.2± .02	43.39	37.7	34.16
RLS2	26.6± .91	52.00	11.5± .83	8.49	38.1	35.58
RLC1	21.2± 1.2	21.14	11.6± .23	9.43	32.8	16.72
RLC2	25.5± .44	45.7	15.5± .74	46.2	41.0	45.9
RLK1	19.6± .17	12.0	14.4± .23	35.8	34.0	20.99
RLK2	21.7± .85	24.0	10.9± .59	2.83	32.6	16.01
RL01	18.8± .36	7.43	13.2± .29	24.5	32.0	13.87
RL02	19.6± .23	12.0	14.8± .89	40.9	34.4	22.42
RL03	21.3± .57	21.7	16.2± .12	52.8	37.5	33.45
RL04	22.4± 1.2	28.0	12.0± .89	14.28	34.4	22.42
RL05	19.7± .19	12.57	11.4± .76	7.54	31.1	10.67
RL06	25.3± .66	44.57	15.6± .12	47.16	40.9	45.55
RL07	26.2± .91	49.7	12.0± .12	13.2	38.2	35.94
RL08	23.1± .17	32.0	12.6± .34	18.86	35.7	27.04

root size, branch length and number of leaves are given in table 1. The present investigation report clearly shows that the length of root and shoot of the control and all other plants of chickpea treated with rhizobial biofertilizers/ isolates increased progressively with the period of time.

Results shown in table 1 and 2 clearly depict that the inoculated plants gave significantly higher number of nodules, root length and shoot length as compared to un-inoculated plants. It was also observed that un-inoculated seeds after germination gave lesser biological yield as compared to inoculated seeds (table 2). After this study we can easily say that inoculation of *Cicer arietinum* seeds with *Rhizobium leguminosarum* as a liquid biofertilizer was very significant in case of production of nodules, length of root shoot ratio and germination of seeds. The inoculated seeds after germination give significantly higher root shoot length at a percentage of about 50% as compared to the un-inoculated control (Table 1).

The above results are closely related with the findings of Khalequzzaman and Hossain[6], Ali *et al.*,[1], who also reported that length of root shoot ratio increased in plants which are already inoculated with rhizobium culture as compared to the un-inoculated plants. Yadegari *et al.*,[12] reported increase in the number of root nodules and also plant yield after inoculation. Rashid *et al.*,[9] reported the increase in number of root nodules by the application of biofertilizer in combination with nitrogen fertilizer. Khanam *et al.*, and Bhuiyan *et al.*,[3] reported increase in the number of nodules and seed yield of the rhizobial inoculation. Fatima *et al.*,[5] stated that the effect of plant growth was highly significant with an increase in length of both root shoot.



Graph - 1: Increase in length of root/shoot ratio after inoculation in comparison with culture.

Table 2. Effect of *Rhizobium leguminosarum* on growth and yield contributing characters of chick pea:

Varieties	Control	RLB1	RLB2	RLS1	RLS2	RLC1	RLC2	RLK1	RLK2	RL01	RL02	RL03	RL04	RL05	RL06	RL07	RL08
No. of Nodule/plant	12	30	32	29	30	30	30	24	26	23	30	25	28	22	18	31	27
No. of Pods/plant	05	34	36	32	34	28	35	26	20	24	31	22	25	27	34	32	19

Effect of *Rhizobium leguminosarum* on yield of *Cicer arietinum* varieties is clearly visible in table 2. Significantly, higher yield was obtained from RLB2 which is statistically similar to RLC2, RL07 and RLS2. The lowest yield was observed in RL01 and RL08 which is not statistically identical to any other variety. The control also shows nodule formation which is an indication of presence of pre-existing native rhizobia in the soil.

The above figure shows the seed germination of chickpea after inoculation. The seeds which are inoculated with Rhizobium inocula in each cup shows hundred per cent germination as compared to the control, which does not show germination of all seeds. The inoculated seeds germinate much faster than the uninoculated ones. Same results were obtained by Pawar *et al.,* 2014. They found that when different legumes treated with rhizobial inocula shows a greater amount of growth as compared to the control.The *Rhizobium* legume symbiotic relationship is highly specific and most legume plants form association with only a limited number of the *Rhizobium* strain. The rhizobacteria involved in such interaction belong to several genera e.g. *Acetobacter, Actinoplanes, Agrobacterium, Alcaligens, Bacillus, Arthrobacter, Pseudomonas, Seretia and Xanthomonas* (Triplett and Sadowsky, 1992).

Practices of modern farming affect badly our environment and soil health. Land degradation, soil erosion, nutrient runoff, loss of biodiversity, water pollution is all because of new techniques of farming practices. Because of these practices, they negatively impact our environment and the change climatic conditions on this planet. The changing climatic conditions in turn directly affect the lives of poor farmers as it directly affects the crop production. The main cause of this is excessive use of chemical fertilizers. However, biofilm based biofertilizers enhance the fertility of soil and is an efficient plant nutrition management which ensure sustainable agricultural production and protect the environment.

Our present study was concluded that the chickpea seeds were well grown in biofilm based biofertilizer as compared to control. The

essential nutrients present in the biofertilizer are in sufficient amounts which are easily available to plants and eco-friendly. This biofertilizer can be recommended as an effective fertilizer which in turn boosts the productivity of chickpea. The biofilm based biofertilizer will definitely increase the efficiency of root and shoot system by providing the essential nutrients for growth and development. This process could be adopted in chickpea growing areas to enhance the rate of growth and productivity in the said fields and also help in reducing fertilizers which are harmful to us and also to our environment.

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