THINK INDIA JOURNAL

ISSN: 0971-1260

Vol-22-Issue-17-September-2019

Isolation of plant growth promoting bacteria from soil samples taken from Kangra region of Himachal Pradesh and attempt to produce biofertilizer formulation

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Abstract

Global population explosion has increased the food demand worldwide. This has led to the development of modern agricultural practises including equipments and increased chemical fertilizers and pesticides usage to meet the demand. Use of chemical fertilizer in agriculture results in the deterioration of soil condition and environment. This has led to search for sustainable alternatives. Biofertilizer technology is one among all that can be used in a scientific way to avoid environmental pollution. Therefore present sudy have been devised to look for the development of microbial inoculants from the unexplored sites of Himalayan state Himachal Pradesh in India.

Keywords: Chemical fertilizers, *Azotobactor*, *Rhizobium*, Biofertilizers, Kangra

Introduction

The worldwide human population is increasing on an exponential rate. This has led to the global food demand and development of modern agricultural practises. The use of pesticides and

ISSN: 0971-1260

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chemical fertilizers has many adverse effect on soil qualities and environment as well. To achieve the goal of global food demand along with environmental restoration and conservation, alternatives to chemical fertilizers have to be devised to achieve sustainable agriculture. The microorganisms having agronomic importance are very unique place and contribute towards sustainable agro biodiversity. They are performing the important tasks of nutrient and micro element acquisition for plants. The term biofertilizer appropriately called as 'microbial inoculants 'are commercially prepared from microorganisms by using which nitrogen, phosphorous level and growth of plants increases [1]. Some other important properties of biofertilizers are their use as low cost, renewable sources of plant nutrients.

Bacteria as biofertilizer: Some bacteria increase the soil fertility by addition of nutrients due to their biological activity. These bacterial biofertilizer may be symbiotic or free-living. These microbes hold a mutual relationship with the plants. Some with leguminous plants and some with non-leguminous plants. One of the most important and well-studied symbiotic bacteria is *Rhizobium* forms interactions with leguminous plants. On the other hand free-living nitrogen fixing bacteria are able to absorb molecular nitrogen from air and convert it into nitrogen salts. Examples- *Azotobacter, Clostridium.*

Fungi as biofertilizer: Fungi which lives as symbiotic association with roots of higher plants known as mycorrhiza. No root hairs or root caps are found in mycorrhizal association. Mycorrhizal can be categorised into two categories:

Ectomycorrhiza: Fungal hyphae absorb nitrogen, phosphorous, potassium, calcium from the soil and convert it into simple form which can be easily absorbed by plants.

Endomycorrhiza: fungi send hyphae tips into the cortex of roots. These are important in phosphate nutrition of plants.

The biofertilizer thus can reduce the input cost of agricultural production, and can be pragmatic in revegetation of marginal and low commercial sites, such as metal tailings ponds [2]. Biofertilizers can be applied in different ways including coating on seeds, administration in soil at root system

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of plant, mixing with soil, by dissolving into water. Soil biological and physico-chemical properties can be improved by using chemical fertilizers in a balanced and reduced manner along with organic manures and bio-fertilizers, this also improves the efficiency of applied fertilizers.

The first biofertilizer identified were *Rhizobium* species that are capable of atmospheric nitrogen fixation and root nodulation in leguminous plants and are in commercially use as inoculants for many years [3]. Researchers have shown that enhanced nitrogen fixation and its availability to plant by *Azotobacter* has promotory effect on plant vigour under certain environmental conditions [4].

Therefore, present study has been undertaken to optimize the isolation and characterization of *Rhizobium* and *Azotobacter* from root nodules of leguminous plants and soil taken from different location of Indora region, District Kangra (HP) and cost effective production of biofertilizer.

MATERIALS

Glass ware & Chemicals: All the Glass wares (test tubes, conical flask, petri plates, measuring cylinder, spreader, inoculation loop, burner, and stirrer) used were of Borosil company. The chemicals used in the present study were of Qualigens, ThermoFisherScientific, NICE, HiMedia Laboratories, Rankem.

Media: YEMA (Yeast Extract Mannitol Agar) media was used for isolation of *Rhizobium* [5]. The media used for isolation of *Azotobacter* was Ashby's media [6]. According to media composition, the reagents were weighted by electronic balance. One thousand ml distilled water was measured by volumetric flask and taken in a conical flask. All the constituents were mixed in 1000 ml distilled water and were autoclaved at 121^{o}C at 15 lbs pressure for 15-20 min. Media was poured into plates under aseptic conditions and allowed to solidify.

Soil Samples for *Rhizobium* isolation: Soil samples were collected from Indora region of District Kangra. YEMA media plates were prepared. Serial dilutions were performed for isolation.100µl culture was taken from 10^{-6} dilution and was spread on plates. The cultures were incubated at 27^{o}C for 3-7 days.

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Soil Samples for *Azotobacter* isolation: Soil samples of root inhabiting area were taken from wheat fields of Indora region, District Kangra (HP). Ashyby's plates were prepared. A serial dilution method was performed for isolation. The cultures were 37^{o}C for 3-7 days.

Biochemical Characterisation: Isolated bacteria were subjected to different biochemical tests for their characterization and identification. Bacterial isolates were subjected to gram's staining to identify whether it is gram positive or gram negative. Starch agar media was prepared and used to test their starch hydrolysis activity as per the method suggested by [7]. Single colony of isolates was taken on slide and few drops of hydrogen peroxide were put on it, then it was detected for the evolution of gas to see the catalase test [6]. A single-line streak inoculation of test organism was done on the agar plate and then incubated. p-aminodimethylaniline oxalate was added and observed for colour change [6] to perform the oxidase test.

Bio fertilizer Production

Biofertilizer production was initiated by preparing starter culture for both the isolates. Starter culture of both *Azotobacter* and *Rhizobium* was prepared by transferring the inoculum to liquid broth of selective media (Ashby's and YEMA respectively) and kept in the rotatory shaker for 4 days. Proper growth was acquired by keeping starter culture in the same condition for two more days. Broth was then mixed with carrier material (coal powder). A set of coal powder: unsterile soil was prepared in the ratio of 1:3. Multiplication of *Rhizobium* and *Azotobacter* was obtained by incubating the carrier containing inoculant for 4-10 days by covering with polythene at 22-24^{o}C. The formulated microbial inoculants were ready to be used as biofertilizer. Similar steps were performed for production of biofertilizer from mixture of *Rhizobium* and *Azotobacter*.

Use of Biofertilizer

350 g of sterile soil was taken and microbial inoculants of *Rhizobium* (Biofertilizer) were added into the soil. 100 seeds of Barley (*Hordeum vulgare*) were sown. Similarly 350 g of sterile soil was taken and microbial inoculant of *Azotobacter* (Biofertilizer) was added into the soil and 100 seeds of *Hordeum vulgare* were sown. 350 g of sterile soil was taken and 100 seeds of *Hordeum vulgare* were sown (control). Similarly, 350 g of soil was taken and mixture of *Rhizobium and*

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Azotobacter was added into the soil and 100 seeds were sown. Control included no addition of biofertilizers.

RESULTS

Isolation of PGP Bacteria: Soil samples were collected from Indora region, District Kangra, H.P. After performing the experiment and requisite incubation *Rhizobium* formed white translucent, glistening and comparatively small colonies on selective media (Fig. 1). Whereas *Azotobacter* formed slimy, glistening colonies also its presence was revealed by the appearance of green, yellow pigment [1] (Fig. 1).

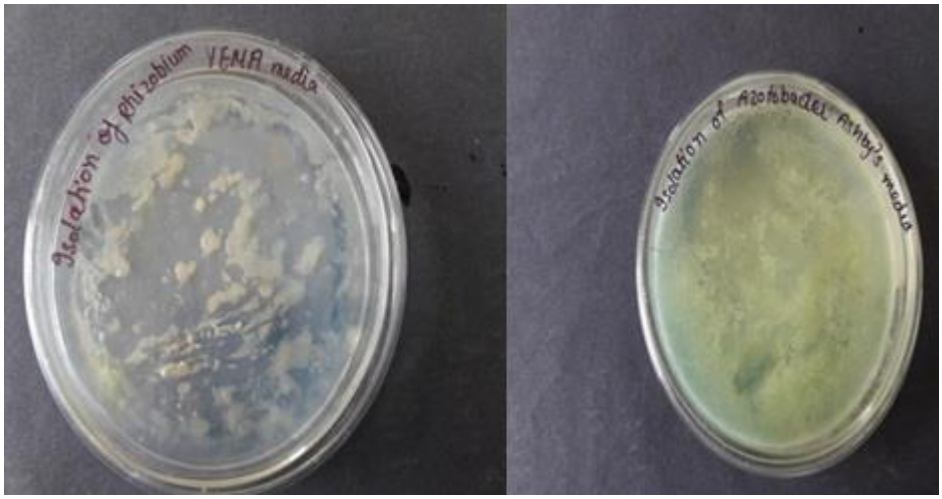

Fig. 1 *Rhizobium* and *Azotobacter* isolates on selective media

Biochemical characterisation (*Rhizobium*): Detailed microscopic examination of microbial isolates showed them to be gram negative rod cells. Isolates showed the positive starch hydrolysis assay. Culture was examined for the presence or absence of bubbling or foaming, bubble formed which indicate the test to be positive. (Table 1).

Biochemical characterisation (*Azotobacter*): Starch hydrolysis test assay was positive. Culture was examined for the presence or absence of bubbling or foaming, bubbles were produced (Table 1).

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Table 1

Test	Effect	Result (*Rhizobium* isolates)	Result (*Azotobacter* isolates)
Gram staining	Pink colour	Gram negative	Gram negative
Starch Agar test	Starch hydrolysed	+ve	+ve
Catalase	Evolution of gas	+ve	+ve
Oxidase	Pink colour	+ve	+ve

Biofertilizer production and effect on plant growth

Further isolates were used for biofertilizer production. To determine the effect of biofertilizer several trials had been done in lab on plant *Hordeum vulgare* and sterile soil containing *Azotobacter*, *Rhizobium*, and mixotrophic culture was compared with control. The growth of plant was found to be maximum in case of soil inoculated with *Azotobacter*. The minimum growth of plant was seen in case of control (Fig.2 & 3). The effect of biofertilizer on Barley (*Hordeum vulgare)* was as follows: seeds started germinating on third day. It was observed that on third day 11 seed were germinated in case of *Azotobacte*r, 10 in case of *Rhizobium + Azotobacter*,7 in case of *Rhizobium* and 5 were seen in control (Table 2)

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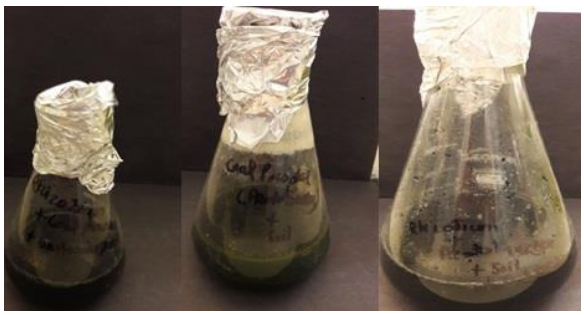

Fig.2 Microbes activated with carrier (coal powder) as biofertilizer formulation.

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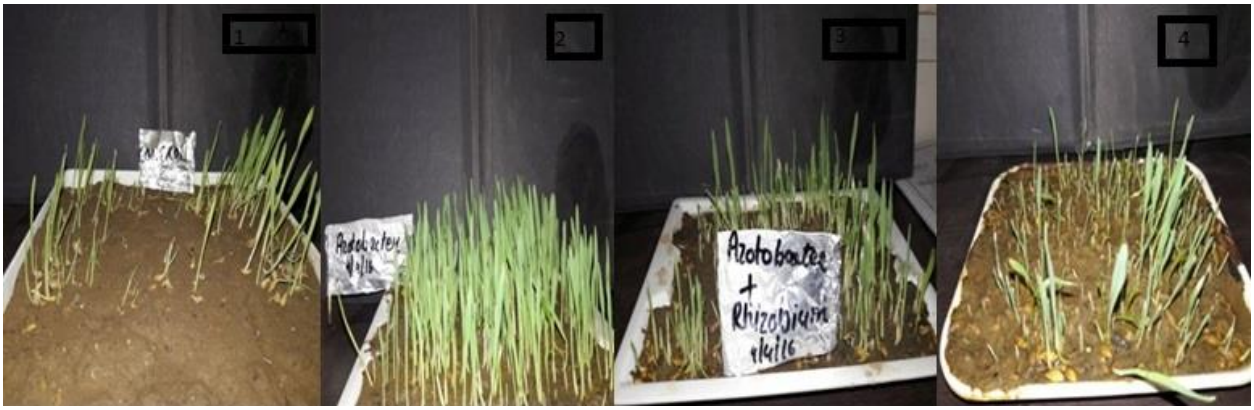

Fig.3 Comparison of different biofertilizer formulations on growth stimulation of *Hordeum vulgare*. (1) Control +sterilised soil +seed (2) *Azotobacter* +sterilised soil +seed (3) *Azotobacter* +*Rhizobium*+ sterilised soil +seed (4) *Rhizobium* +sterilised soil+seed

Biofertilizer	**Concentration**	**No. of plants (Day 3)**	**No. of plants (Day 5)**	**No. of plants (Day 9)**
Control	-	**5**	7	20
Azotobacter	30 ml	11	25	**50**
Rhizobium	30 ml	7	13	30
Rhizobium+Azotobacter	30 ml	10	18	45

Table 2 Effect of biofertilizer.

Discussion

For centuries it has been known that N_2 fixing symbiotic bacteria are contributing in soil nutrient enrichment in leguminous plants. This research work was aimed at isolation of rhizospheric microbes and production of biofertilizer and comparing their effect on plants. Rhizobium colonies have been isolated on YEM agar medium. The isolates were sticky and showed lower level mucous

ISSN: 0971-1260

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production. Morphologically colonies were round and white in colour. On the other hand, appearance of slimy and glistening colonies revealed the presence of *Azotobacter*.

The present study clearly shows the plant growth promoting potential of the isolated microbes. As the isolated have been characterized as *Rhizobium* and *Azotobacter* and there are many reports suggesting their possible role in plant growth promotion. *Rhizobium* in addition to atmospheric nitrogen fixation, play other roles also including hormones production and solublization of organic and inorganic phosphate [8]. The effect of biofertilizer on growth and yield of Tomato has been reported by Ramakrishan and Selvakumar in year 2012 [9]. Significant vegetative growth has been reported in the treatments of single or combined inoculation than control plants in tomato, similar trends of biofertilizer effect has been established in this study. The enhanced effect of plant growth promotion in comparison to other treatments is also in accordance with the previous studies done by Mirzakhani in 2009[10] where they found the increased effect of *Azotobacter* in enhancing the availability of soil nitrogen that could enhance flowering yield. Selvakumar in 2012 [11] reported that the biofertilizers had improved the chlorophyll content in black gram and chilli. Dubey and Mishra observed that the combined inoculation of gladiolus corn with *Azotobacter* & PSB was found best for corn weight, cormel weight and increased propagation coefficient [12]. *Azotobacter* was found to be more effective in improving the spikes quality among tested biofertilizers. Shrivastav and Govil in 2007 has observed the significant improvement in vegetative and floral characters by biofertilizers (most effectively by *Azotobacter*) administration as compare to control [13]. The mechanism of plant growth promotion mediated by *Azotobacter* includes N2 fixation, synthesis and secretion of vitamin B, biotin, nicotinic acid, heteroxins, pantothenic acid and

ISSN: 0971-1260

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gibberelins etc. which initiate plant rhizogenesis. The superiority of *Azotobacter* as biofertilizer over other tested isolates in growth stimulation has also been established in this study.

Conclusion

Biofertilizers have no adverse effect on soil health and environment as these are naturally occurring microorganisms of rhizosphere or soil. They promote plant growth directly by fixing atmospheric nitrogen into nitrates and phosphorous solubilisation, plant growth hormones production, providing better nutrient uptake and increased tolerance towards drought and moisture stress and indirectly by biocontrol i.e. displacement of fungi and plant pathogenic bacteria. Biofertilizers has several advantages such as cost effective, nontoxic, increases the soil fertility and productivity of crops. Rhizobia varies greatly among host plant species and bacterial strains for their nitrogen fixing capability [14]. Therefore, selection of best strains must take rhizobia and host compatibility into account for selection of biofertilizer. In the above research Rhizospheric microorganisms were isolated from two different locations one from root nodules of leguminous pea plant and another from Rhizospheric soil of wheat of Indora region District Kangra (H.P.) and were used for biofertilizer production using carrier (coal powder). It has been concluded that plants show maximum growth in soil in which *Azotobacter* biofertilizer is added and shows minimum growth in case of control. Mixture of *Azotobacte*r and *Rhizobium* shows average growth of plants. Use of *Azotobacter* as biofertilizer is more effective than *Rhizobium* and mixture of both. Further investigation of the identified isolates upto species level and their specific effect on particular events of plant development can be assessed by following the in-vitro plant co-culture [15] methodology for specific development of new age biofertilizers.

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