ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Isolation and Production of Biofertilizer from of Rhizospheric Bacteria and Their Effect on *Hordeum Vulgare* Development

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Abstract

Biofertilizers are the inoculants of bacteria, fungi and algae which increase the soil fertility by balancing the nutrients content in the soil. They are cost effective renewable sources of plant nutrients. Some bacteria increase the soil fertility by addition of nutrients due to their biological activity. These bacteria biofertilizer may be symbiotic or free-living. They decrease the use of chemical fertilizers thus minimizing the cost. The aim of present study is to formulate a simple method for the isolation of *Rhizobium* and *Azotobacter* from root nodules of leguminous plants and soil taken from District Kangra Tehsil Indora Himachal Pradesh and cost effective production of biofertilizer. Soil sample were collected from Indora region district Kangra .Serial dilutions were performed. It is found that under specific conditions inoculation with *Azotobacter* and *Rhizobium* has positive effects on the growth of *Hordeum vulgare*. Growth in case of *Azotobacter* is maximum when inoculated in *Hordeum vulgare*. Mixture of *Azotobacter* and *Rhizobium* shows average growth of plants

Keyword: Biofertilizer; *Rhizobium*; *Azotobacter*; Himachal Pradesh; Symbiotic; Nitrogen fixation.

Introduction

Agriculture sector is important sector as it provides largest employment opportunities [1], [2]. It is the predominant occupation of two-third of working population for their livelihood. For decades, it had been associated with production of basic food crops [3]. But due

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

to increase in population demand of food has been increased. This has led to the use of chemical fertilizer to meet the demand [4], [5]. Use of chemical fertilizer in agriculture results in the deterioration of soil condition and environment. The harmful effect of chemical fertilizer has led to alternative fertilizer research [6]. Biofertilizers are one such approach which can be used to minimise the environmental pollution [6], [7]. Biofertilizers are termed as the inoculants made from bacteria, fungi and algae which increase the nutrient contents in the soil by fixing nitrogen, phosphorous content in the soil [1], [8], [9]. Biofertilizers are of two types mainly- Nitrogen biofertilizer and Phosphorous biofertilizer. Nitrogen biofertilizers increases the nitrogen content by fixation of atmospheric nitrogen and phosphorous biofertilizer increases the phosphorous content by solubilising the soil phosphorous [1].

For the first time *Rhizobium* was prepared in USA in 1930s [10]. It is a gram negative bacterium which forms symbiotic association with the leguminous plants and fixes the atmospheric nitrogen symbiotically [11]. This association begins with the attachment of bacteria in the soil to the root hairs. The cells divide and forms a nodule required for fixation [12]. They also possesses PGPR activity such as, Secrete phytohormones, shows phosphate solubilisation activity, siderophore production [13]. Rhozbia fall in to two classes of Proteobacteria the alpha proteobacteria and beta proteobacteria. Use of inoculation of legume in agriculture has been in use from many years. The legume-*Rhizobium* is an example of mutualism. Rhizobia provide amino acids to the plants and in return get carbohydrates, fats and many other compounds [14], [15].

Azotobacter is a motile, oval shape bacterium, having thick walled cyst [16]. Shape of the cell is affected by amino acid glycine which is present in the nutrient media. *Azotobacter* produces pigments example *Azotobacter chrococcum* forms a dark brown water soluble pigment melanin. Many other species produces pigments from yellow- green to purple colour. *Azotobacter* is found in neutral and alkaline soil. Its distribution is independent of the type of plant [17]–[19]. *Azotobacter* species have several types of nitrogenise. The basic one is molybdenum-ion nitrogenase. An alternative type contains vanadium. *Azotobacter* plays a crucial role in plant development and is widely found in different environment. *Azotobacter* has the potential to be used as biofertilizer [20]r. *Azotobacte*r is a free living nitrogen fixing bacterium with add-on ability to liquefy inorganic phosphate in plant utilizable form, and due to these PGP traits, it can be applied in aquaculture systems [21] and vermicompost production [22]. It was reported earlier [13], [23]–[25] that *Azotobacter chrococcum* have beneficial effect

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

on the growth, development, and yields of corn in lab condition, similar results were obtained when inoculation of *Azotobacter chrococcum* was given to wheat seed in-vitro [22], [26].
The present research deals with the isolation of two bacteria which are used for the production of biofertilizers.

Materials and Methods

Isolation of *Rhizobium* from root nodules of leguminous pea plant

Soil sample were collected from Indora region district Kangra. YEMA media was prepared and were poured into plates. Serial dilutions were performed .100µl culture was taken from 10^{-6} dilution and was spread on plates with the help of spreader. Plates were incubated at 37°C for 3-7 days.

Isolation of *Azotobacter*

Rhizospheric soil samples were taken from wheat fields of Indora region District Kangra. Ashyby's media was prepared and were poured into plates. Serial dilutions were performed .100ul culture was taken from 10^{-6} dilution and was spread on plates with the help of spreader. Plates were incubated at 37ºC for 3-7 days.

Microscopic and Biochemical assay [27]

Gram staining: Gram staining is preliminary used to differentiate bacterial spp. on the basis of their cell wall and cell membrane composition. In present study this procedure was used to determine the types of Bacterial isolates. One or two drops of 24 h old culture were evenly speeded on non waxy glass slide and culture was fixed by gentle heat.1-2 drops of primary stain i.e. crystal violet of was applied on the fixed bacterial smear to react exactly for 1 min followed by gentle wash by sterilized water. Gram's iodine was applied and allowed to react with smear for 1 min. later the iodinated alcohol was applied and allowed to react for 1min. Finally treated with a counter strain stain i.e. safranin after 5 min washed with sterilized water and air dried. The smear was examined under oil emersion in light microscope the gram positive cell appears violet while gram negative turned pink to red.

Starch hydrolysis test: Starch agar media was prepared and were poured into petri plates and allowed to solidify. Single colony was picked and were streaked on the plates and incubated at 37ºC for 24 hrs.

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Catalase test: Picked up bacterial colony from the plate and transfer the colony on glass slide in a drop of water place few drops of 3% H_2O_2 (dilute 30% commercial solution by 1:10) over the culture.

Production of Biofertilizer

For production of biofertilizer culture was first transferred to liquid broth of selective media of both *Azotobacter* and *Rhizobium* (Ashby's and YEMA) and were rotated on the rotator shaker for 4 days.. Later starter culture was remained in the same condition for two more days for proper growth. Broth was then mixed with carrier material (coal powder) having coal powder: unsterile soil with in ratio of 1:3. This mixture (bacteria + carrier) was left for four to ten days at 22 to 24^{o}C to multiply *Rhizobium* and *Azotobacter* under covered condition with polythene sheet. The microbial inoculants were ready to be used as biofertilizer. Similar steps were performed for production of biofertilizer from mixture of *Rhizobium* and *Azotobacter.*

Application 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 Azotobacter* was added into the soil and 100 seeds were sown.

Results and Discussion

This research work aimed at Isolation of Rhizospheric microbes and production of Biofertilizer and comparing their effect on plant. We collected the soil samples from two different locations one from roots of leguminous pea plant and another from rhizospheric soil of wheat in Tehsil Indora District Kangra**.** Serial dilution was performed. Colonies of *Rhizobium* were found on YEM agar medium. The colonies were sticky in appearance. Analysis of colony morphology indicated round colonies, white colored. Earlier researchers [12], [28], [29] reported the presence of rhizobia and other plant growth promoting bacteria in and nearby soil of the root nodules of the Leguminosae family plants. Various scientific reports documented the presence of various genera of bacteria inside and outside of legume tissues such as, Pseudomonas, Enterobacter, Erwinia, Aerobacter, Bacillus, Chryseomonas, Sphingomonas, Flavimonas, Agrobacterium, and Curtobacterium [2], [30]–[34]. *Azotobacter* were characterised by forming, shinning colonies, which turns brown color in old age if the

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

species are *A. chroococcum*. Biofertilizers are environment friendly naturally produced fertilizers bearing a diversity of plant growth promoting microorganism which induce the plant development through various mechanisms such as Biological nitrogen fixation, liquefaction of insoluble phosphate, Phyto hormones production, biosynthesis of vitamins, growth regulators [35]–[37]s. Further these microorganisms were used for the production of biofertilizer. Biofertilizer was added to the soil. The effect of biofertilizer was observed on Barley (*Hordeum vulgare).* The 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 3).

Isolation of *Rhizobium*: Soil samples were collected from Indora region district Kangra. YEMA media was prepared and were poured into plates. Serial dilutions were performed. 100µl culture was taken from 10^{-6} dilution and was spread on plates with the help of spreader and plates were incubated at 27^{o}C for 3-7 days. *Rhizobium* produced creamish white semi-transparent, sparkling and comparably tinny colonies on selective media (Table 1).

Isolation of *Azotobacter* from soil sample: Rhizospheric soil samples were taken from wheat fields of Indora region District Kangra. Ashby's media was prepared and were poured into plates. Serial dilutions were performed. 100ul culture was taken from 10^{-6} dilution and was spread on plates with the help of spreader. Serial dilution was performed and the bacterial inoculated petri plates were placed in a incubator for incubation at 37^{o}C for 3-7 days. *Azotobacter* formed slimy, glistening colonies also its presence was revealed by the appearance of green, yellow pigment (Table 2).

Table1. Biochemical characterisation test results for *Rhizobium*

Test	Effect	Result
Gram staining	Pink colour	Gram negative
Starch Agar test	Starch hydrolysed	+ve
Catalase	Evolution of gas	+ve
Oxidase	Pink colour	+ve

Table2.Results of Biochemical characterisation tests for *Azotobacter*

Test	Effect	Result
Gram staining	Pink colour	Gram negative

ISSN: 0971-1260
Vol-22-Issue-17-September-2019

Starch agar test	Starch hydrolysed	+ve
Catalase test	Evolution of gas	+ve

Biofertilizer production and effect on plant growth:

Further these isolated microorganisms were used for biofertilizer production. Biofertilizers increases the soil fertility by increasing the nutrient content in the soil [35]. Biofertilizer application in soil or at plant seeds, and roots is enhanced the availability of nitrogen, phosphorous and potassium nutrients in nearby area of plant which helps to enhance soil health and supply of different kinds of nutrients in the soil [2], [8]. To determine the effect of biofertilizer several trials had been done in lab on plant *Hordeum vulgare* and it was found that the sterile soil containing *Azotobacter*, *Rhizobium*, and mixture of both was compared with control (Table 3). The growth of plant was found to be maximum in case of soil inoculated with *Azotobacter* (Table 3). The minimum growth of plant was seen in case of control. Similar results were documented by [34], [38], [39] when the inoculation of *Rhizobium* significantly increased the growth and yield parameters with the potato and pea seeds. It was observed [40], [41] that inoculation of cluster beans and French beans seeds with *Rhizobium* spp. primarily responsible for effective development of growth traits such as, plant height, number of leaves and branches, fresh and dry weight and dry matter %, compared to the un-inoculated plants.

Table.3. Effect of Biofertilizers on growth of *Hordeum vulgare*

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

Conclusion

Fertilizers are those substances which contain all nutrients required for the plant growth. Continuous use of land leads to the loss of element which is essential for plant growth. This leads to the infertility. Therefore it has become necessary to maintain the balance of nutrients in the soil. Biofertilizers prepared from microorganism increases nutrients availability to crop plants. 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.

Acknowledgment

I am very thankful to Bhojia Institute of Life Sciences, Budh, Baddi, H.P., India for technical support to complete this study and unlimited help in all steps.

Competing Interest's Statement

The author(s) declare(s) that there is no conflict of interest.

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