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Screening of Potential Plant Growth Promoting Properties of *Bacillus* Species Isolated from Different Regions of Nepal

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Abstract

The deleterious effects of intensive use of chemical fertilizers and pesticides in agriculture has led to the substantial research efforts on finding the alternatives to these agrochemicals. This study was aimed to isolate *Bacillus* species from soil of different regions of Nepal and screen for their ability to promote plant growth directly or indirectly by testing their ability to produce plant growth hormone indole acetic acid, hydrogen cyanide, ammonia and protease as well as phosphate solubilization. Thirty nine *Bacillus* strains were isolated from 25 soil samples of different regions of Kathmandu and Chitwan districts of Nepal. These isolates were tested for plant growth promoting traits *in vitro*. Among the total isolates, about 48.7% were indole acetic acid producers, 38.4% of the isolates showed the ability to solubilize the phosphate, 71.8% were able to produce ammonia and all the isolates had the ability to produce hydrogen cyanide and protease. The isolated strains showed positive results to maximum PGPR traits and exhibited a potential to be used as alternatives to chemical fertilizers and pesticides and could be used as low-cost bio-based technology to promote plant growth in the agricultural sector.

Keywords: PGPR, Biocontrol Agents, Plant Growth Promotion, *Bacillus*, Biofertilizers

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Introduction

The application of chemical fertilizers has long been used in conventional agriculture. While chemical fertilizers have aided farmers in increasing crop production there are also several harmful effects of chemical fertilizers which may include water pollution, chemical burn to crops, increased air pollution, acidification of the soil and many other direct and indirect effects to the human health ecosystem itself [1]. So, substantial research efforts are now focused on finding new alternatives to supplement the use of chemicals in agriculture. The use of beneficial rhizobacteria to increase the productivity and growth of plants could be one of the substitutes to agrochemicals.

The strains of bacteria available in the rhizosphere that stimulate plant growth are termed as Plant Growth Promoting Rhizobacteria (PGPR) [2]. The mechanism by which PGPR promotes plant growth can be direct or indirect [3]. PGPR can promote plant growth directly by facilitating resource acquisition i.e., fixation of atmospheric nitrogen, solubilization and mineralization of soil phosphorus, sequestering iron producing phytohormone and modulating phytohormones level by producing phytohormones like cytokinin, gibberellins, ethylene, indole acetic acid etc. Biocontrol bacteria produce antibiotics, siderophores and lytic enzymes including chitinases, cellulases, proteases that cause deleterious effects to phytopathogens and indirectly promote plant growth. Competition between pathogens and plant growth promoting bacteria can also check the disease incidence and severity [3].

Bacillus species are abundant in the rhizosphere, so they can be one of the major aspects of bio-based products to supplant agrochemicals. *Bacillus* spp are Gram positive common rhizobacteria and widely considered as a major aspect of plant growth promoting rhizobacteria [4]. The ability to replicate rapidly and resistant to adverse environmental conditions provide a unique feature to *Bacillus* species [5]. Their ability to produce hard, resistant endospores and antibiotics that limit wide ranges of phytopathogens make *Bacillus* spp an attractive option for biocontrol agents [6].

Various researches done worldwide identified *Bacillus* spp as PGPR [2,4-6]. But studies regarding PGPR Bacillus spp in Nepal are limited [7,8]. This study was thus aimed to isolate *Bacillus* spp from soil samples of different areas of Kathmandu and Chitwan of Nepal and screen the isolates for some direct and indirect plant growth

promoting traits. This includes the test for the production of indole acetic acid (IAA), solubilization of phosphate and production of hydrogen cyanide (HCN), ammonia and protease.

Methodology

Sample collection and processing

Soil samples (10 g each) were collected from rhizospheric soils of different regions namely Khairahani and Fasera in the Chitwan District and Hanumate Khola, Jagati in the Bhaktapur district of Nepal. The soil samples were collected from a depth of 5-10 cm in sterile plastic bags and carried to the laboratory, Nepalese Farming Industry, Kathmandu for further processing and analysis. The study was conducted from January to March 2019.

One gram of soil sample was dispensed into 99 mL of sterile distilled water and homogenized. One mL of homogenized soil sample was transferred into 9 mL sterile distilled water and serial dilution was carried out up to 10^{-8} dilution. Serially diluted bacterial cultures (100 µL) were spread on nutrient agar media and incubated at 37°C for 24 h and examined for the appearance of colonies. Identification of the isolated colonies was done on the basis of colony characteristics, Gram reaction, spore staining and catalase test [5]. Screening of isolates was done for plant growth promoting properties – IAA production, phosphate solubilization activity, HCN production, ammonia production and protease enzyme production.

IAA production

IAA production was qualitatively estimated [9]. All the isolates were incubated in nutrient broth containing 5µg/mL L-tryptophan for 48 h at 28°C. Following incubation, culture was centrifuged at 5,000 rpm for 20 min and 1 mL of supernatant was mixed with 2 mL of Salkowski reagent and kept in a dark room for 20 min. Appearance of pink color indicated the IAA production.

Phosphate solubilization activity

Screening for phosphate solubilization ability of isolate was done using Pikovskaya's agar medium [10]. The isolates were spot inoculated on Pikovskaya's agar plates and incubated at 28°C for 48 h. Clear zones around the colonies indicated the positive test. The diameter of the halo zone was measured.

HCN production

Qualitative estimation of HCN was done by using the methods described by Lorck (as cited by [11]). Each isolate was streaked on nutrient agar plate supplemented with 4% glycine. A Whatmann filter paper soaked in a solution of 2% Na_2CO_3 in 0.5% picric acid was placed between base and lid of petriplate and incubated at 28 °C in inverted position for 48 h and observed for color change from yellow to brown.

Ammonia production

All the bacterial isolates were tested for the production of ammonia [12]. For ammonia production strain was inoculated into 5 mL peptone medium and incubated for 48 h at 28 °C. After the bacterial growth, Nessler's reagent (0.5 mL) was added to the tube in 2:1 ratio. Development of brown to yellow color indicated positive test for ammonia production.

Protease test

For protease production test, isolated *Bacillus* spp were spot inoculated on skim milk agar plate and kept for incubation for 24h at 28 °C. Appearance of halo zone around the colonies was considered as positive for protease production [10]. The diameter of the halo zone was measured.

Results

Thirty nine isolates of *Bacillus* were obtained from 25 soil samples. The isolates were Gram positive rods (**Figure 1**), endospore forming, and catalase enzyme producers. The colonies on nutrient agar were rough, creamy white, dry and folded, opaque and irregular edged.

Figure 1. Gram stained *Bacillus* species under oil immersion objective

Plant growth promoting properties

The plant growth promoting properties of the isolated were evaluated based on the ability to produce IAA, solubilization of phosphate, ability to produce HCN, ammonia and protease.

IAA production

Out of 39 isolates of *Bacillus*, 19 isolates (48.7%) showed the ability to produce IAA. Development of pink color after 20 min of addition of 2 drops of Salkowski reagent

Table 1. Properties of 39 isolates tested for different plant growth promoting traits.

Bacillus Strain (BS)	IAA Production	P-Solubilization Width of halo zone (mm)	HCN Production	Ammonia Production	Protease Production Width of halo zone (mm)
BS00	-	-	+	+	1
BS01	++	-	+	++	2
BS02	-	-	+	+	3
BS03	+	-	+	-	1
BS04	+	-	+	+	2.5
BS05	+++	1.5	+++	-	1
BS08	+	1	++	-	1.5
BS09	+	1	+	+++	1
BS10	+	1.5	+++	+	1
BS12	-	-	+	+++	1
BS13	++	1	+++	++	2.5
BS14	-	-	++	+	1.5
BS15	+++	2	+	-	3
BS16	+	1.5	+	++	2.5
BS17	-	1	+	++	3
BS18	-	-	+	-	1
BS19	-	-	++	+	3
BS20	-	1	+	+++	1
BS21	+	-	++	-	1
BS22	-	-	+	++	2
BS23	-	-	+	+	1
BS24	-	3	+	-	1.5
BS25	+++	-	+++	+	3
BS26	++	-	++	+	2
BS27	-	1.5	+	-	2.5
BS28	-	-	+	++	2
BS29	++	3	+++	+++	2
BS30	+++	-	+++	-	1
BS31	-	-	+++	++	2
BS32	-	-	+	+++	1
BS33	-	-	+++	+	1
BS34	+	1.5	+	-	1.5
BS35	-	1	+	+	1.5
BS36	-	-	+	++	1
BS37	++	0.5	+	+	1.5
BS38	-	-	+++	-	1.5
BS39	++	-	++	+	1
BS40	++	-	+	+	1
BS41	-	-	+++	+++	1

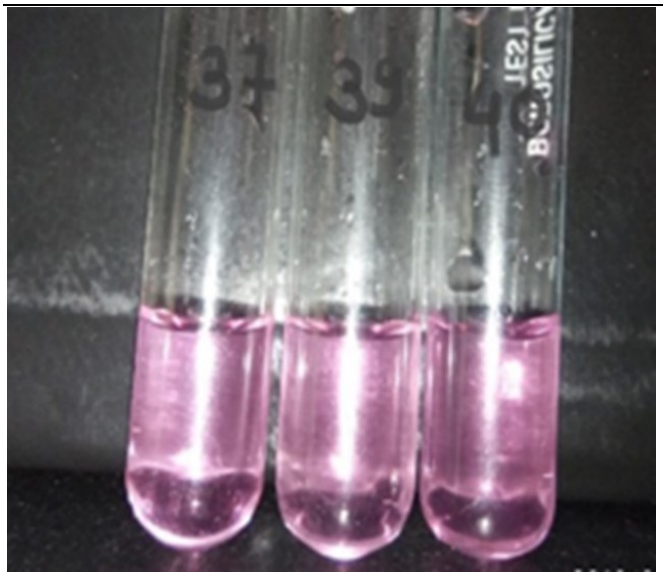

Figure 2. Appearance of pink color after the addition of Salkowski reagent in IAA test

in 2mL of the cell free supernatant indicated the positive test for IAA (**Figure 2**). The IAA producing strains were further classified into three groups as +++ (strong), ++ (moderate) and + (weak) based on the intensity of color visible. The strains showing deep pink, pink and light pink were placed in the group +++, ++ and + respectively. Four isolates showed deep pink color after 20 min of addition of Salkowski reagent into the cell free supernatant liquid whereas 7 isolates showed pink color and 8 showed light pink color (**Table 1**).

Phosphate solubilization

The isolates showing a halo zone around the colonies after 48 h of incubation following spot inoculation in Pikovskaya's agar plate were taken as positive tests for Phosphate solubilization (**Figure 3**). Out of 39 isolates, 15 (38.4%) isolates exhibited the halo zone. The width of the halo zone was also measured (**Table 1**). The width of the

halo zone was as high as 3mm (BS24 and BS29) to most of the *Bacillus* spp showing the clear zone of 1mm diameter.

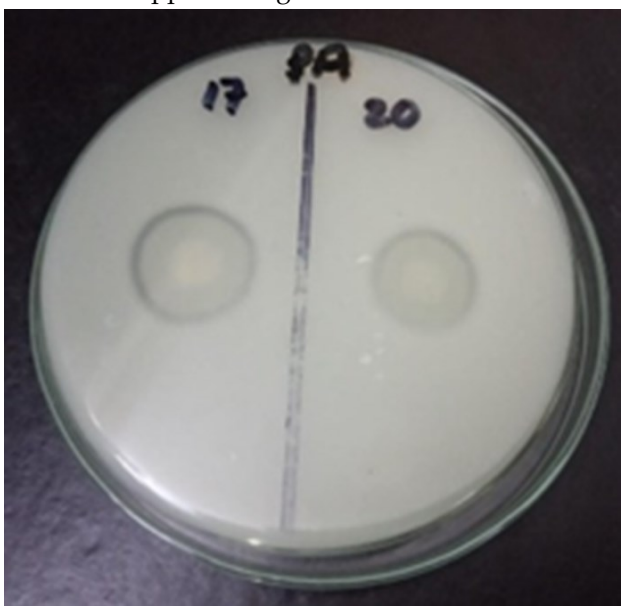

Figure 3. Appearance of halo zone around colony in phosphate solubilization test

HCN production

All 39 isolates of *Bacillus* produced HCN as evidenced by the change in color of the Whatmann filter paper from yellow to brown. In the presence of glycine, the brown color of filter paper was observed giving a clear indication of HCN production by *Bacillus* strains (**Figure 4**). However, different strains produced the different intensity of brown color as light brown, orange brown to reddish brown. Based on the distinction in the color of filter paper the strains are grouped into +++ (strong), ++ (moderate) and + (weak) for those producing reddish brown, orange brown and light brown respectively (**Table 1**). Ten strains changed the color of filter paper to reddish brown, 6 changed the color to orange brown while 23 strains changed the color to only light brown.

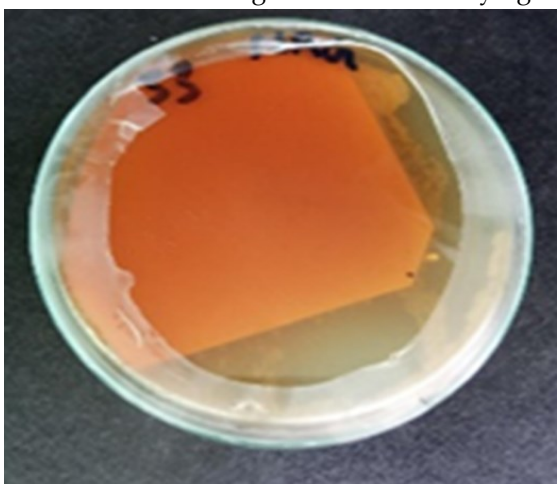

Figure 4. Change is color of filter paper to reddish brown following incubation in HCN production test

Ammonia production

For ammonia production, the development of brown to yellow color after the addition of 0.5 mL of Nessler's reagent was observed as a positive test (**Figure 5**). Among 39 isolates, 28 isolates developed the color of the medium to brown or yellow following the addition of Nessler's reagent. However, different strains produced different intensity of color as yellow, light brown and deep brown. Based on the distinction in the color of media the strains are grouped into +++ (strong), ++ (moderate) and + (weak) for those producing deep brown, light brown and yellow respectively (**Table 1**). Six strains changed the color of media to deep brown, 8 changed the color to light brown while 14 strains changed the color to yellow.

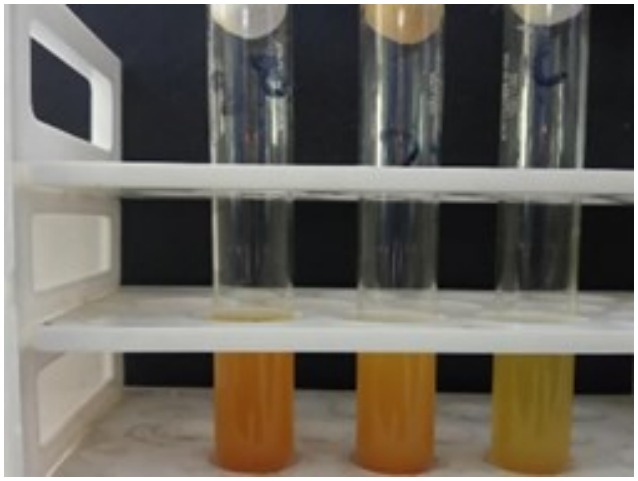

Figure 5. Change in color of peptone water in ammonia test

Figure 6. Appearance of halo zone in protease test

Protease production

The isolates showing the halo zone around the colonies after 28h of incubation following spot inoculation on skim milk agar were taken as positive tests for protease production (**Figure 6**). From the 39 isolates taken for experiment, all the isolates produced the halo zone. The

width of the halo zone was also measured (**Table 1**). The width of the halo zone was as high as 3 mm (5 isolates) to most of the *Bacillus* spp showing the clear zone of 1mm diameter.

Discussion

Certain strains of *Bacillus* spp have gained worldwide attention in recent years due to their abilities in promoting plant growth. Therefore, in this study, isolates of *Bacillus* spp. obtained from soil of different regions of Kathmandu valley and Chitwan district were primarily tested for the plant growth promoting traits *in vitro*.

The capability to increase plant growth parameters is highly related to the IAA level, which was produced by *Bacillus* spp isolates. IAA, the major auxin in plants, plays a major role in both the shoot and root development [13]. Among the 39 isolates, 19 isolates gave pink color on incubating the supernatant liquid with Salkowski's reagent. Salkowski's reagent when reacted with IAA, tris-(indole-3-acetato) iron (III) complex is formed which displays pink coloration due to the formation of IAA complex and reduction of Fe^{3+} [14]. In a similar study, 7 of 12 test strains of *Bacillus* produced IAA [15]. Similar observation of IAA production by *Bacillus* strains has been reported in a study [16]. In their study, 76.3% (n=90) were able to produce IAA. Similar method applied for IAA production in *Pseudomonas* exhibited all the isolates were able to produce IAA [10]. The availability of different level of precursors affects the ability of bacteria to produce IAA.

Despite phosphorus being abundant in soil it is insoluble and cannot contribute to the plant growth [3]. So, the solubilization and mineralization of insoluble phosphate in soil by rhizobacteria makes an important property of plant growth promoting bacteria. Rodriguez and Fraga studied that *Bacillus* and other phosphate solubilizing bacteria (PSB) like *Pseudomonas* and *Rhizobium* were capable of converting insoluble phosphate available in the soil into soluble form [17] . Phosphate solubilizing ability of isolated strains was tested using Pikovskaya's agar medium. Fifteen out of the total isolates produced halo zone on Pikovskaya's agar medium after incubation following the spot inoculation on the plates as a result of phosphate solubilization. The halo zone around the colony was due to the polysaccharides, organic acids or phosphatase produced by the phosphate solubilizing *Bacillus* strains (18). The bigger diameter of halo zones may be due to their greater ability to solubilize phosphate. In a similar study of phosphate solubilization by *Bacillus* strains out of 12 isolates that promoted soybean seedling significantly, 11 isolates showed phosphate solubilization (16). Another study documented only 11.5 % of *Bacillus* spp. isolated from the samples obtained from various sites such as vineyard soil, fig orchard soil, forest soil, sewage soil, coastal area soil, compost of mushroom and paddy field were able to solubilize phosphate (19). The study indicates that the phosphate solubilization capacity of *Bacillus* differs according to their site of existence.

Production of HCN by rhizobacteria is believed to promote plant growth by indirect mechanism. Hydrogen cyanide is supposed to act synergistically with bacterially encoded antibiotics [3]. Rijavec and Lapanje in their study concluded that HCN increases the availability of phosphate for rhizobacteria and plant hosts, especially in oligotrophic alpine environments and thus indirectly contributing to plant growth [20]. Picric acid present in the filter paper reacts with free cyanide produced by the bacteria to produce colored iso purpuric acid, thus the change in color of filter paper is visible. The color developed is directly proportional to free cyanide.

Plants can only utilize the reduced forms of the nitrogen; hence, nitrogen first must be fixed and converted to a combined form (either ammonia/nitrate) and then trapped by the plants [21]. Twenty eight isolates (71.8%) were able to produce ammonia. These rates of ammonia production are lower than 95% and 80% demonstrated by other researchers [11, 22].

The enzyme protease, produced by most of the microorganisms, causes the hydrolysis of the peptide bonds that link amino acids in the polypeptide chain [23]. The production of protease by beneficial rhizobacteria helps to lyse a portion of pathogenic fungi and act as a biocontrol agent [24]. Screening of protease producing ability of *Bacillus* isolates was carried using skim milk agar medium. The casein present in the Skim milk agar medium is hydrolyzed by the proteolytic bacteria, *Bacillus* which is indicated by the formation of clear zones around the colonies [25]. All the isolates tested for protease enzyme production produced the halo zone around the colonies on skim milk agar medium following the incubation displaying the ability of *Bacillus* to produce protease enzymes. The greater the ability to produce protease, the bigger is the diameter of halo zones.

Six isolates BS09, BS10, BS13, BS16, BS29 and BS37 showed positive results for PGPR traits assessed in the study. Among these, BS29 showed maximum IAA production, phosphate solubilization, HCN production, ammonia production and proteolytic activity.

Conclusion

It is evident from the present studies that the *Bacillus* species tested were able to demonstrate multiple plant growth promoting traits. The results concluded that *Bacillus* strains have a huge potential to be used as alternatives to chemical fertilizers and pesticides for the promotion in growth and production of plants. The study on *Bacillus* strains can be further detailed so that these strains can commercially be developed as low-cost bio-based products to promote plant growth in the agricultural sector.

Author's contribution

EP is the principal investigator, carried out laboratory works, and also prepared the manuscript. A Sanjyal and CRR are co-investigators, and carried out laboratory works. SP is a laboratory supervisor and helped to carry out laboratory works. AS is the corresponding author, an academic supervisor of the research and prepared the manuscript.

Competing Interests

The authors declare no competing or financial interests.

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Ethical Approval and Consent

Not applicable.

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