Exploring the Potential of Chromium Reducing Bacillus sp. and their Plant Growth Promoting Activities

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Abstract A large number of different microorganisms are commonly found in the soil including bacteria, fungi, actinomycetes, protozoa and algae of these bacteria are by far the most common type of soil microorganism possibly because they can grow rapidly and have the ability to utilize a wide range of substances as either carbon or nitrogen sources. Use of naturally occurring, free living bacterial species, which can protect and promote plant growth by colonizing and multiplying along the surface of the root and/or root cortex. In our present investigation was to study the plant growth promoting (PGP) activities and chromium reducing Bacillus sp. from rice fields of in and around Erode district. From 25 soil samples 63 different Bacillus sp. were isolated. Among the 63, eight Bacillus sp. (BA1 to BA 8) possess effective PGP activities. In eight different Bacillus isolates particularly (BA1, BA3, BA4 and BA6) exhibited maximum plant growth promoting and chromium reducing activities. In addition to these traits, plant growth promoting bacterial isolates must be rhizospheric competent, able to survive and colonize in the rhizospheric soil.

Keywords Bacillus, Chromium, Plant Growth Promoting Activities, Rhizospheric

1. Introduction

Agriculture is increasingly dependent on the use of chemical fertilizers, growth regulators and pesticides to increase yield. This dependency is associated with problems, such as environmental pollution, health hazards, interruption of natural ecological nutrient cycling and destruction of biological communities that otherwise support crop production. The use of bioresource to replace chemical pesticides, growth regulators and fertilizers is growing. In this context, plant growth promoting rhizobacteria (PGPR) are often novel and potential tools to provide substantial benefits to agriculture[1,2].

A large number of different microorganisms are commonly found in the soil including bacteria, fungi, actinomycetes, protozoa and algae[3] of these bacteria are by far the most common type of soil microorganism possibly because they can grow rapidly and have the ability to utilize a wide range of substances as either carbon or nitrogen sources. Use of naturally occurring, free living bacterial species, which can protect and promote plant growth by colonizing and multiplying along the surface of the root and/or root cortex of the inoculated plants is said to be one such safe and suitable alternative[4]. The organisms that establish positive interactions with plant roots and show observable benefits on the plant growth are collectively called as plant growth promoting rhizobacteria.

The rhizosphere of plants is a zone of intense microbial activity, and some bacteria from this zone, termed rhizobacteria exhibit active root colonization in the presence of the existing native micro flora. Rhizobacteria that exert beneficial an effect on plant development is also are referred to as PGPR[1], because their application is often associated with increased rates of plant growth. It is widely accepted that rhizosphere and rhizoplane microorganisms can influence plant growth and development. Some years later, the term plant growth promoting bacteria (PGPB) was proposed to designate rhizobacteria that enhances plant growth by other ways[5], plant growth promoting bacterial species including Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligens, Arthobacter, Bacillus and Serratia[6,7].

PGPR accounts for about 2-5% of the total the rhizobacteria involved in plant growth promotion[8]. Such PGPR use one or more direct or indirect mechanisms to improve the growth and health of plants. These mechanisms can be active simultaneously or independently at different stages of plant growth. Among these, P-solubilization, biological nitrogen fixation, improvement of other plant nutrients uptake, and phytohormone production like, Indole-3-acetic acid are some of the regulators that profoundly influence plant growth[9]. Moreover, biological control of plant pathogens and deleterious microbes, through the production of antibiotics, lytic enzymes, hydrogen cyanide, and siderosphores or through competition for nutrient and space can improve significantly
plant health and promote growth as evidenced by increases emergence, vigor, and yield[8].

Heavy metal contamination has been on the rise in proportion to the pace of worldwide industrialization, leading to significant health problems and toxic effects on plant and microbial biodiversity[10]. Chromium compounds are being used in a wide variety of commercial processes like tanneries, metal cleaning and processing and alloy formation. Despite various treatments, procedures in use, unregulated disposal of the chromium containing effluent has led to the contamination sites by extracting metals from the soil and concentrating them in the above ground biomass leading to impaired metabolic activities and reduced plant growth[11]. Hence, the alternate way to reduce the toxicity of heavy metals in the plant could be using the rhizospheric microbes to enhance phyto remediation efficiency[12]. Microbes and plants have attracted attention because of the biotechnological potential site or the possible transfer of accumulated metals to higher plants and the diversion of heavy metals towards microbial metabolism and growth[13]. Several species of the genus Bacillus sp. are found predominantly in the rhizosphere of various crops for their ability to control plant diseases. The resistant endospore of Bacillus sp. provides toleration to heat and cold as well as to pH extremes, pesticides, fertilizers and heavy metals. In addition, the application of some Bacillus sp. has shown increased grain yield and plant biomass accumulation[14].

The inoculants bacteria used for these purpose mainly to the genus Bacillus. Bacillus sp. is spore-forming gram positive, rod shaped bacteria. They are highly tolerant of adverse ecological condition. Bacillus sp. comprises one of the most common soil bacteria groups and they are frequently isolated from the rhizospheres of plants. Because of their spore-forming ability, plant growth promoting Bacillus strains are readily adaptable to commercial formulation and field application[15]. Therefore, it is necessary to develop efficient strains in field conditions, one possible approach is to explore in field conditions, one possible approach is to explore soil microbial diversity for PGPR having combination of plant growth promoting activities in terrestrial ecosystem and well adapted to particular soil environment.

So, keeping in view the above constraints, the present investigation was designed to screen heavy metal and plant growth promoting activities of Bacillus sp. from rice rhizospheres.

2. Materials and Method

2.1. Collection of Sample

Twenty five different rhizospheric soil samples were collected from rice field grown in Erode district of Tamil Nadu. The sample was collected in 1 cm depth and it was packed in a sterile polythene bag and labeled properly for further processing[16].

2.2. Isolation of Bacillus Isolates

The isolation of Bacillus sp. from soil samples, 1g of soil sample was serially diluted in sterile distilled water, 0.1 ml of soil suspension from 10⁻⁷ to 10⁻⁷ was spreaded on the nutrient agar plate. The plates were incubated for 24hrs at 37°C ± 2°C[17].

2.3. Identification of Bacillus sp.

The bacterial isolates were identified by using cultural, morphological and biochemical characteristics features described in Bergey’s manual of determinative bacteriology[18] and stored at 4°C on slants and maintained through sub-culturing. The isolates were characterized by Gram staining, motility test, carbohydrate fermentation, oxidase test, catalase test, H₂S production and starch hydrolysis as per the standard methods[19].

2.4. In vitro Screening of Multiple Plant Growth Promoting Activities of Bacillus sp.

2.4.1. Detection of Indole Acetic Acid (IAA) Production

IAA production was detected by method as described by Brick et al.[20].Bacterial cultures were grown in nutrient broth amended with tryptophan (20, 40, 60, 80 and 100 µg l⁻¹) for 72 hrs at 28°C and then fully grown cultures were centrifuged at 3000 rpm for 30 minutes. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4ml of the salwaksi reagent (50ml, 35% perchloric acid, 1ml of 0.5M FeCl₃ solution). Development of pink colour indicates IAA production. Optical density was taken at 530 nm with the help of spectrophotometer.

2.4.2. Hydrogen Cyanide (HCN) Production

Bacillus isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck[21]. Nutrient broth was amended with 4.4 glycine l⁻¹ and bacteria were streaked on modified agar plate. Whatman filter paper No.1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of the plate. Plates were sealed with para-film and incubated at 28°C for 4 days development of orange to red colour indicated the production of hydrogen cyanide.

2.4.3. Phosphate Solubilization

Bacterial isolates were evaluated from the ability to solubilize inorganic phosphate. Pikovskaya’s agar medium (HiMedia, Mumbai) containing calcium phosphate as the inorganic form of phosphate was used in this assay. A loopful of bacterial culture were placed on the plates and kept for incubation at 28°C for 7 days. The presence of clear zone around the bacterial colonies indicates the solubilization of phosphate.

2.4.4. Heavy Metal Tolerance

The selected bacterial isolates were tested for their resistance to heavy metals by agar dilution method. Freshly prepared Nutrient agar plates amended with soluble heavy
metal chromium (Cr) at various concentrations ranging from 50 µg to 100 µg were inoculated with bacterial cultures. Heavy metal tolerance was determined by the appearance of bacterial growth after incubating the plates at 28°C for 72 hrs[22].

2.4.5. Screening of Antifungal Activity

The bacterial isolates tested for their antifungal activity by agar well diffusion method. Test fungi Fusarium sp., Penicillium sp. and Cercospora sp. were grown on potato dextrose agar (PDA) slants. The spores were scraped and suspended in 10 ml of sterile PDA broth. Diluted spore suspension (10² CFU l⁻¹) of the fungi was spread on PDA plates 8 mm diameter wells were punched into the agar medium with sterile cork borer and filled with different volume (50, 75, 100 µl) of bacterial culture. The plates were incubated for 5 days at 28±2°C. The antifungal activity was evaluated by measuring the zone of inhibition[23].

2.5. Statistical Analysis

The results are reported as mean ± SD. The statistical variation in IAA production by Azotobacter isolates at different tryptophan concentration and antifungal activity against the test fungi were analyzed using an analysis of variance (ANOVA), following a mean separation according to the Tukey multiple range test. In all statistical analysis, P <0.05 was considered significant[24,25].

3. Results

Rhizosphere soil along with roots and leaf samples from tea plants grown in different sites situated at three different altitudes in Erode district of Tamil Nadu, were collected and used for the isolation of Bacillus sp. using specific media. The attempts yielded 63 Bacillus isolates, as far as the sites at three different filed were concerned, samples collected at Erode district yielded 16 Bacillus isolates. Among the 63 Bacillus sp., 8 species of Bacillus sp. were exhibited efficient plant growth promoting activities by screening methods.

The isolates were identified based on morphological and biochemical characteristics and were tested for their beneficial traits like ability to solubilize of insoluble inorganic phosphate, production of plant growth promoting substances and biocontrol potential. Efficient isolates selected based on the above characters were examined for their in vitro screening methods. The results obtained on these aspects are presented as follows.

3.1. Isolation and Identification of Bacillus sp.

On the basis of cultural, morphological and biochemical characteristics a total of 63 Bacillus isolates were identified from 25 rhizospheric soil samples as described in Bergey’s Manual of Determinative Bacteriology[18]. Among the 63 isolates, 8 (BA1 – BA 8) were selected for further studies based on the efficiency of multiple plant growth promoting activities exhibited in preliminary studies (data not shown).

General features of the test isolates are illustrated in Table 1. The isolates observed as transparent, watery, mucoid, slimy colonies in Ashby’s mannitol agar medium; gram negative rods; positive for catalase, indole, MR, VP, citrate, nitrate reduction; ferment glucose, lactose, mannitol, and sucrose producing both acid and gas; ability to hydrolyze the starch; and prototrophic for biotin.

Table 1. Morphological and cultural characteristics of Bacillus isolates from the rhizospheric soil of rice fields

| Morphological and cultural characterization | Bacillus species |
|-------------------------------------------|-----------------|
| Number of isolates | Eight |
| Colony morphology on Ashby’s mannitol agar | Cream, spreading, opaque |
| Pigmentation | Transparent, milky |
| Gram reaction, cell shape | Gram negative rods |
| Growth on nitrogen free medium | Positive |
| Biotin prototrophy | Positive (90 %) |
| Motility | Motile |
| Indole | Positive (100 %) |
| Methyl red | Positive (80 %) |
| Vogesproskauer | Positive (70 %) |
| Citrate | Positive (100 %) |
| Catalase | Positive (100 %) |
| Glucose | Acid and gas production (100 %) |
| Lactose | Acid and gas production (80 %) |
| Mannitol | Acid and gas production (40 %) |
| Sucrose | Acid and gas production (90 %) |
| Nitrate reduction | Positive (100 %) |
| Starch hydrolysis | Positive (100 %) |

Table 2. Multiple plant growth promoting activities of Bacillus isolates from the rhizospheric soil of rice fields

| Bacillus isolates | Indole acetic acid production | Ammonia production | Hydrogen cyanide production | Phosphate solubilization |
|------------------|------------------------------|--------------------|-----------------------------|-------------------------|
| BA1              | +++                          | +++                | +++                         | +++                     |
| BA2              | +                            | +++                | +                           | -                       |
| BA3              | +++                          | +                  | +                           | +++                     |
| BA4              | +++                          | +++                | +++                         | +++                     |
| BA5              | +                            | +                  | +                           | -                       |
| BA6              | +++                          | +                  | +++                         | +                       |
| BA7              | -                            | +                  | +++                         | +                       |
| BA8              | +                            | -                  | -                           | +                       |

BA – Bacillus isolates from rice tomato rhizospheric soil, + low colour intensity / zone formation; ++ medium colour intensity / zone formation; +++ high colour intensity / zone formation; - no colour change / no zone formation

3.2. Multiple Plant Growth Promoting Traits of Test Isolates

Screening results of multiple PGP traits of 8 Bacillus sp. are depicted in Table 2. Out of eight isolates, four Bacillus isolates (BA1, BA3, BA4 and BA6) were showing multiple PGP activities in relation to indole acetic acid (IAA), ammonia, hydrogen cyanide and phosphate solubilization (Table 2). BA2, BA5, BA7 and BA 8 isolate neither producing
ammonia nor solubilizing the phosphates. Among the eight only five Bacillus isolates were tolerant to mercury and zinc. Higher growth was noticed in BA1, BA4 and BA6 in chromium and mercury up to the concentration of 200 µg l⁻¹ (Table 3).

### Table 3. Heavy metal tolerance among Bacillus isolates from Rhizospheric soil of rice fields grown on nutrient agar

| Bacillus isolates | Chromium concentration (µg l⁻¹) |
|------------------|---------------------------------|
|                  | 50  | 100  | 150  | 200  |
| BA1              | +++ | +++  | +++  | +++  |
| BA2              | +   | +    | +    | +    |
| BA3              | +++ | ++   | +    | +    |
| BA4              | +++ | +++  | ++   | ++   |
| BA5              | +   | +    | +    | +    |
| BA6              | +++ | +++  | +++  | ++   |
| BA7              | +   | +    | +    | +    |
| BA8              | ++  | +    | +    | +    |

BA – Bacillus isolates from rice rhizospheric soil; ++ low growth; +++ medium growth; +++ high growth; - no growth

#### 3.2.1. Quantitative assay of IAA Production

A total of eight isolates of Bacillus sp. were tested for the quantitative estimation of IAA in the presence of different concentrations of tryptophan. With no addition of tryptophan, production of IAA was not observed. With the addition of tryptophan from 20 to 100 µg l⁻¹ the production of IAA was increased significantly from a minimum of 0.56 – 9.50 µg l⁻¹. The maximum 9.50 µg l⁻¹ IAA production was observed in BA6 at 100 µg l⁻¹ of tryptophan concentration. The production of IAA was highest in the isolates of BA6 followed by BA1, BA2 and BA8. In BA3 and BA7, IAA production was detected only at higher concentration of tryptophan (Table 4).

### Table 4. Production of indole acetic acid at different tryptophan concentration by Bacillus isolates

| Bacillus isolates | IAA production (µg l⁻¹) |
|------------------|-------------------------|
|                  | 0   | 20  | 40  | 60  | 80  | 100 |
| BA1              | ND | 1.86 | 1.92 | 2.99 | 4.31 | 7.56 |
| BA2              | ND | 1.59 | 2.68 | 3.80 | 4.89 | 6.96 |
| BA3              | ND | ND   | 0.07 | 0.18 | 0.96 | 1.22 |
| BA4              | ND | 2.79 | 3.79 | 3.86 | 5.97 | 6.18 |
| BA5              | ND | 0.69 | 0.77 | 0.84 | 0.91 | 0.99 |
| BA6              | 0.56 | 2.79 | 5.86 | 7.96 | 8.23 | 9.50 |
| BA7              | ND | ND   | ND   | ND   | 0.50 | 0.61 |
| BA8              | ND | 1.76 | 2.87 | 4.98 | 5.31 | 6.40 |

ND – not detectable; BA – Bacillus isolates from rice rhizospheric soil

#### 3.2.2. Antiphytopathogenic Fungal Activity

Antiphytopathogenic fungal activity of the ten Bacillus isolates was checked against Pencillium sp., Cercospora sp. and F. oxysporum in SDA (Table 5). The antifungal activity of the Bacillus isolates tested varied with inhibition zones in diameter from 1.00 to 34.23 mm. Isolates BA1, BA3, BA4 and BA6 induced larger inhibition zones showing their high antifungal activity and exhibiting broad-spectrum activities against test fungi compared to the other Bacillus isolates. Neither BA2 nor BA5 showed antifungal activity against the three test fungi. No antifungal activity was observed in isolates BA7 and BA8 at lower concentrations 50 and 100 µl l⁻¹ against test fungi (Table 5). Antiphytopathogenic fungal activity of the ten Bacillus isolates was increased significantly with the concentration of the culture suspension against Pencillium sp., Cercospora sp. and F. oxysporum.

### Table 5. Antiphytopathogenic fungal activity of Bacillus isolates against Aspergillus flavus, Cercospora sp. and Fusarium oxysporum

| BA | Concentration of Bacillus suspension (µl l⁻¹) |
|----|---------------------------------------------|
|    | 50  | 100  | 150  | 200  |
|    | Pe  | Cs   | Fo   | Pe  | Cs   | Fo   | Pe  | Cs   | Fo   |
| BA1 | 15.61 | 18.42 | 16.62 | 17.33 | 20.00 | 19.50 | 21.33 | 25.25 | 23.50 | 26.65 | 32.62 | 29.00 |
| BA2 | 1.10 | ND   | ND   | 3.00 | 2.25 | 2.15 | 3.45 | 2.60 | 2.75 | 3.51 | 2.66 | 2.80 |
| BA3 | 12.25 | 15.10 | 13.10 | 16.45 | 22.68 | 19.00 | 23.45 | 28.45 | 25.00 | 29.62 | 34.23 | 31.00 |
| BA4 | 14.50 | 19.50 | 17.00 | 18.65 | 23.65 | 20.65 | 21.35 | 29.26 | 24.33 | 23.26 | 32.35 | 28.65 |
| BA5 | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   |
| BA6 | 12.32 | 17.65 | 15.52 | 14.55 | 21.00 | 18.83 | 18.17 | 24.33 | 20.67 | 24.17 | 30.35 | 28.75 |
| BA7 | ND   | ND   | ND   | 1.00 | ND   | ND   | 2.23 | 1.00 | 1.89 | 2.65 | 2.45 | 3.45 |
| BA8 | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   |

BA – Bacillus isolates from rice rhizospheric soil; ND – Not detected; Pe – Pencilliumsp; Cs – Cercospora sp.; Fo – Fusarium oxysporum
4. Discussion

Understanding the dynamics of root colonization by specific microbial components of the rhizoplane and rhizosphere is basic to the development of biological control of soil borne pathogens and the effective use of beneficial microorganisms to enhance plant growth[1]. Plant rhizosphere is known to be preferred ecological niche for various types of PGPR due to rich nutrient availability. The three main intrinsic characteristics of PGPR must be: (i) able to colonize the root, (ii) survive and multiply in microhabitats associated with the root surface, in competition with other microbiota, at least for the time needed to express their plant promotion/protective activities, and (iii) promote plant growth[26-29]. Further, exploration and evaluation of the isolates exhibiting multiple PGP traits on soil-plant system is needed to uncover their efficacy as effective PGPR[28,29]. In the present investigation multiple PGP activities were found in four Bacillus isolates (BA1, BA3, BA4 and BA6) out of eight isolates screened from rice growing rhizospheric soil.

Based on earlier reports[30-34] eighty percent of microorganisms (Azospirillum, Pseudomonas, Xanthomonas, and Rhizobiums as well as Alcaligenesfaecalis, Enterobacter cloacae, Acetobacterdiazotrophicus, A.chroococcumand Bradyrhizobiumjaponicum)isolated from the rhizosphere of various crops have the ability to produce IAA which help in stimulating plant growth. IAA production was detected in all the test isolates of Bacillus sp. The Bacillus isolates BA4, BA6, BA1 and BA3 produced higher amount of IAA, but in BA7 and BA8 the production was detected at higher concentration of tryptophan. Further, there was an increase in the level of IAA with the increasing concentration of tryptophan (10-100µg l-1) as evidenced by Ahmed et al.[29].

Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia and solubilization of phosphate. However, ammonia production and phosphate solubilizationobserved frequently in Bacillus sp. about 60-70% than the other isolates[35]. A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere soil[36-38,34]. Further, it was found that all the isolates of Bacillus obtained from rice soils are ammonia producers as well as solubilizing the phosphates, except BA8, BA2 and BA7, have good prospects to improve plant growth especially in soil with large amount of precipitated phosphate.

As per earlier reports[39-41]Azotobacter sp. protect several plants from root disease caused by soil borne fungi through HCN and siderophore production. Hydrogen cyanide production by Azotobacter isolates were about 60% for the inhibition of phytopathogens in the soil[34,29]. Most of the Bacillus sp. isolated (BA1, BA3, BA4, BA6, BA7 and BA8) from soils of vegetable plants are producing HCN as well as siderophore and act as potent antifungal agent. Synergistic interaction of these two with other metabolites may further function as stress factors including local and systemichost resistance[42] that led for the suppression of the root pathogens. Out of eight Bacillus isolates, four isolates (BA1, BA3, BA4 and BA6) were exhibiting high antifungal activity against Pencilliumsp. Cercospora sp. and F. oxysporum. The antifungal activity of the screened Bacillus isolates was concentration dependent.

Bacillus sp. had developed the mechanisms to cope with a variety of heavy metals for their survival in the rhizosphere of vegetable ecosystem[34] and can be used to reduce the toxicity of the metal or increase its bioavailability[10]. It was observed that few Bacillus rhizobacteria tolerate the Zn and Hg metal concentration to the level of 100-200µg l-1 and exhibiting a multiple PGPR activities like IAA, HCN, NH3 production and siderophores. This was evidenced in our study with Bacillus isolates BA1, BA3, BA4 and BA6. It was also apparent that most of the cultures of PGPR isolated from vegetable rhizosphere were tolerant to elevated levels of heavy metals that may decrease heavy metal toxicity and increase the PGPR activities for the plant growth[43].

5. Conclusions

The isolation of PGPR from different sources opens new doors to design strategies for improving the efficacy of biocontrol agents. Identification of key antimicrobials produced by superior agents can be exploited for streamlining strain discovery by targeting selection of new isolates that carry relevant biosynthetic genes. Determination of the role of edaphic parameters favourable for disease suppression, particularly those that stimulate antibiotic production and activity, can be exploited by targeting inoculants for soils that are more likely to support biocontrol. Biocontrol with plant growth promotion helps increasing the vegetative yield and thereby increasing crop yield.

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