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ORIGINAL ARTICLE **OPEN ACCESS**

In- vitro study of Plant Growth Promoting Traits (PGP) of *Cytobacillus firmus* strain VG5

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ABSTRACT

Increase in crops and yield productivity is becoming the utmost important for farmers all over the world to fulfil the demands of increasing population. But to achieve this goal, farmers excessively use chemical fertilizers and pesticides in fields which ultimately deteriorate soil fertility and soil quality, soil minerals and nutrients and also pollute ground water. Numerous microorganisms associated with rhizosphere play very important role in sustaining agriculture for long term. Application of such naturally occurring biological agents in fields can control pathogens as well as increase plant growth and crop production. These bacteria can also deal with the biotic and abiotic stress during the crop development. Soil isolate Cytobacillus firmus strain VG5 when tested in vitro for the PGPR features such as siderophores, ammonia, hydrogen cyanide (HCN), and IAA production, showed the ability to produce these PGPR traits. Cytobacillus firmus strain VG5 can be a boon to farmers for the increased growth and rise in production of crop yield. Hence this strain, due to its PGPR traits can be used as a biofertilizers/ biocontrol agent in future.

Keywords: *Bioinoculants, Biofertilizers, Cytobacillus sp., PGPR, Pesticide hazards.*
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INTRODUCTION

Many biotic and abiotic factors, extreme temperatures, drought, salinity, plants pathogens and pests severely affect the agricultural products and their productivity. To deal with the adverse conditions of biotic and abiotic factors, organic farming can be a positive ray of hope which not only can help in increasing crop production and yield but can also improve soil quality and fertility and also help in protecting and maintaining environmental conditions (1, 2). Numerous naturally occurring biological agents play very important role in sustaining agriculture for long term. Application of such rhizospheric microorganisms in fields can control pathogens as well as increase plant growth and crop production. Among such microorganisms, bacteria possess multiple plant growth promoting traits are called as Plant Growth Promoting Rhizobacteria (PGPR) commonly used as bio-inoculant. These are capable of enhancing and improving crop production and removing directly or indirectly biotic and abiotic stress. PGPR accelerate uptake of plants nutrients from the surrounding environment by producing many PGPR traits such as siderophores (to seize iron), phosphorus solubilization and/or by nitrogen fixation (3). Along with these PGPR also produce phytohormones such as indole acetic acid (IAA), the most widely studied plant hormone capable of enhancing root growth, also promote other beneficial plant-microbe symbiosis (4, 5). PGPR can reduce ethylene production by the secretion of 1-Aminocyclopropane-1-carboxylate (ACC) deaminase enzyme, also produce cellulose, protease, and antibiotics and cyanide and protect plant from bacteria, fungal and nematodes infection (6, 7).

Researchers' interest in isolating and investigating novel strains of plant growth promoting bacteria (PGPB) has grown **over the recent** years. **Several** PGPB identified globally during the past few years has significantly increased. Numerous bacteria related to *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Burkholderia*, *Bacillus*, *Azospirillum*, and *Serratia* species have already been identified from diverse soils and reported aiding in the growth of various plants (8, 9). *Pseudomonas*, *Glomus*, *Bacillus*, and other strains have already been commercialized. The putative imidacloprid degrading bacteria (*cytobacillus firmus* VG5) was isolated from imidacloprid contaminated soils. Although this bacteria had already been reported earlier for PGPR qualities, this work focuses on the *in- vitro* evaluation of soil bacterial isolate, *Cytobacillus firmus* strain VG5 for its hydrogen cyanide (HCN), phosphate solubilization, ammonia, IAA production, and siderophores production capacity.

MATERIAL AND METHODS

***In vitro* assessment of plant growth promoting traits of *Cytobacillus firmus* VG5: Qualitative assay:**

The chemicals, media and reagents used for bioassay were purchased from HiMedia.

Study on Biostimulant activity: In this the ability production of phytohormones indole acetic acid by the isolates was studied.

Indole Acetic Acid (IAA) Production (10): **Indole Acetic Acid** production ability of bacteria was determined qualitatively by Salkowski reagent method with some modifications. 1 ml of each bacterial culture was grown in 100 ml of 1.5% (w/v) tryptone broth with or without imidacloprid incubated at 30^0 C under shaking conditions for 7 days. Centrifugation of each inoculated broth was done after 7 days at 3000 rpm for **half an hour**. About 4 ml of Salkowasky's reagent (0.2g $FeCl_3$ in 50 ml distilled water and 30 ml Conc.H_2SO_4) was added to each 2 ml of supernatant with two drops of orthophosphoric acid and incubated in dark. **Negative control was prepared by mixing the Salkowski reagent to un-inoculated tryptophan br**oth. **Appearance** of pink color in inoculated broth confirms the production of IAA.

Study on Biofertilization activity: Phosphate solubilization and nitrogen fixing ability of bacterial isolate *Cytobacillus firmus* strain VG5 was determined.

Ammonia Production (10):

In 10 ml of each sterile peptone water broth, a bacterial culture was introduced along with imidacloprid and grew for 3–4 days at 30^0 C. Similar sets were prepared for each isolate in absence of imidacloprid. In a result, **formation of** NH_3 was **validated** by observing the formation of dark yellow color with the addition of the Nesseler's reagent (10g % Mercuric Chloride, 7g % Potassium Iodide, 16g % Sodium Hydroxide mixed in water at pH 13.0). The uninoculated peptone water mixed with Nesseler's reagent was taken as a negative control.

ii) Phosphate Solubilization (11):

The capacity of the bacterial isolate *Cytobacillus firmus* strain VG5 to dissolve inorganic phosphate was assessed by spot inoculation of bacterial culture on Pikovskaya's agar medium containing tri calcium phosphate as insoluble phosphate source. The presence of clear halo zone around the colonies following one week of incubation at R.T. indicated phosphate solubilization (12).

Study on Biocontrol activity: Ability of isolate *Cytobacillus firmus* strain VG5 to produce HCN was determined which can act against plant pathogens.

i) Hydrogen Cyanide Production (10): The isolates *Cytobacillus firmus* strain VG5 was also tested for its biocontrol properties. **Glycine (4.4 %) was added to a tube containing King's B broth**. The broth was inoculated with individual bacterial culture. The set was prepared in presence and absence of imidacloprid. Whatman filter paper no.1 was **immersed** for 15 min in 2ml of 2% KOH, which was mixed in 1 ml picric acid and 5g of Na_2CO_3 prepared in 200 ml distilled water. The soaked filter paper was laid on the top of the test tube. The test tubes were incubated at 30 °C for 4 days. A similar set was prepared for negative control without inoculation of bacterial isolate. Development of orange to red color on Whatman filter paper no.1 showcased prominent hydrogen cyanide production.

ii) Siderophores Production (10): Siderophores are the molecules produced by microorganisms that have an extremely high affinity for ferric iron. They are produced in an iron limiting **environment**. Therefore, King's B minimal medium was used as an iron-restricted medium. To minimize the amount of contaminating iron, all media and components were prepared with milli-Q water. **The glassware used for** media storage and for growth of the culture was treated with **concentrated HNO_3 followed by ri**nsing with milli-Q water.

Bacterial culture was grown for 24 to 48 hrs in King's B medium with and without imidacloprid at 30^0 C. The supernatant was collected after centrifugation of completely grown cultures at 3000 rpm for 30 min. Siderophores production in the supernatant was detected by following qualitative methods in absence of Chrome Azurol S (CAS) blue agar medium.

Arnow's Assay (For Catechol type siderophores):

In 3ml of supernatant 0.3 ml of 5N HCl was mixed. This was spiked with 1.5 ml of Arnow's reagent (10g $NaNO_2$, 10g Sodium Molybdate, 100 ml Distilled Water in 5N HCl) and 0.3 ml of 10N NaOH (13). Development of pink color showcased the positive test.

Tetrazolium Salt Assay (For Hydroxamate type siderophores):

In a 0.1 ml of cell supernatant, pinch of tetrazolium salt and few drops of 2N NaOH were added (14). Development of deep red color is indicative of positive test.

Vogel's Chemical Test (For Carboxylate type siderophores):

In 5 ml water, 3 drops of 2N NaOH and 1 drop phenolphthalein was added together. After the development of light pink color, 2 drops of supernatant was added (14**)**. Disappearance of pink color indicated positive test.

RESULT AND DISCUSSION

Plant Growth Promoting (PGP) Activities of *Cytobacillus firmus* strain VG5:

i) Indole Acetic Acid Production: Development of pink colour was observed in *Cytobacillus firmus* VG5 in presence & absence of pesticide (Fig. 3.1). The presence of imidacloprid in the media was not found to affect the PGP activity of the isolate. IAA is one of the most important phytohormones producing auxin, which improves nutrient uptake and root development in plants and may be extremely crucial for controlling plant growth. Amino acid tryptophan is the main precursor molecule for the synthesis of IAA in bacteria and is commonly found in root exudates. (15) reported that PGPR like *Enterobacter, Bradyrhizobium*, *Pseudomonas*, *Rhizobium*, *Klebsiella* and *Agrobacterium* produced IAA via indole-3-pyruvic acid and indole-3-acetic aldehyde. The synthesis of IAA is a key mechanism of promoting **crop** growth as it helps root development and nutrients uptake (8). It has also been proposed that IAA coordinates nitrogen demand and improves crop yields. IAA not only enhances seed and tuber germination, increases the rate of xylem and root development, helps in lateral and adventitious root formation and also affects photosynthesis, plant cell division and differentiation but also resistance to stressful conditions (16). Our strain can produce IAA, which makes it potentially valuable for enhancing crop development.

ii) Phosphate solubilization: Clear zone of phosphate solubilization was observed in *Cytobacillus firmus* VG5 when grown on Pikovskaya's agar plate with and without imidacloprid (Fig. 3.2). This indicated that the strain was capable of solubilizing mineral phosphorus even in the presence of imidacloprid. Microbial phosphate solubilization was carried through a variety of solubilization reactions such as decrease in soil pH by release of organic acids via acidification, dissolution of phosphate by anion exchange reactions or chelation of Fe and Al associated with phosphate. This solubilization reaction makes phosphorus available in the soil for plant uptake, which in turn improves plant growth and yield (17). Bacteria belonged to the genera *Microbacterium Pseudomonas*, *Bacillus*, *Enterobacter*, *Serratia, Flavobacterium*, *Arthrobacter*, *Beijerinckia*, *Burkholderia*, *Rhizobium*, *Rhodococcus,* and *Erwinia* have been isolated from rhizosphere soil as phosphate solubilizing PGPR and gaining much attention of many agriculturists (18). In this aspect, our bacterial strain *Cytobacillus firmus* VG5 also played a very significant role in solubilization of mineral phosphorus and making it readily available to plants to improve their growth and crop productivity without any environmental pollution and ill effects to human health.

iii) Ammonia production: When isolate *Cytobacillus firmus* VG5 was grown in sterile peptone water, showed yellow to brown color development and was recorded positive for the production of NH_3 (Fig.3.3). The production of ammonia indirectly increases the plant growth. Joseph et al, 2007 found 95% ammonia production by *Bacillus* followed by 94.2% by *Pseudomonas* and 74.2% and 45% by *Rhizobium* and *azotobacter* respectively. *Bacillus cereus* and *Aneurinibacillus aneurinilyticus* and *Paenibacillus* sp. showed 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity which could convert ACC to α-ketobutyrate and ammonia under adverse environmental conditions of heat and salinity stress and helped in enhanced plant growth and development (19, 20).

iv) Hydrogen cyanide: Production of hydrogen cyanide, a vital component for plant growth promotion was found positive in *Cytobacillus firmus* VG5 strain. Development of orange to red color on Whatman filter paper no.1 in the media containing tubes showcased prominent hydrogen cyanide production (Fig.3.4). Microbial cyanides act as a bio-control and play very important role in plant disease control. The results of our investigation were in agreement with several previous studies. A positive correlation between overriding of root rot after the production of HCN by bacterial isolate was reported (21). Glycine was reported among one of the factors which influence the rate of HCN production. Glycine was found in root exudates and was reported to be the primary precursor of microbial cyanide synthesis (22). *P. fluorescent* strain CHAO produced HCN which stimulates root hair formation and suppresses root rot caused by *Thielaviopsis basicola* in tobacco plant (23). In many earlier published literatures *Bacillus firmus* was reported as an efficient bio-control agent against many pathogens and pests. *Bacillus firmus* I-1582 strain not only reported to colonize tomato roots more extensively than cucumber but also found to induce systemic resistance in tomato against *Meloidogyne* (24). *B. firmus* I-1582 and *Bacillus amyloliquefacien*s QST713 bacterial strains not only colonize roots and promote plant growth but also shield plants from infections or pests (25).

v) Siderophore production: Production of siderophores was said to be advantageous for enhancing plant development and preventing disease. As different types of siderophores are present in nature, qualitative tests like Vogel's chemical test, Tetrazolium salt test and Arnow's assay are used in common to detect them (Fig. 3.5, 3.6, 3.7). The isolates *Cytobacillus firmus* VG5 was tested for their iron chelating properties and it was found that *Cytobacillus firmus* VG5 was not able to produce catechol type siderophore tested by Arnow's assay (Fig. 3.6). An instantaneous development of deep red colour was observed in the test samples of both the bacterial isolates after the addition of NaOH and tetrazolium salt

showed the presence of hydroxamate type siderophore (Fig. 3.7). Tetrazolium salt test is based on the reduction of tetrazolium salts by hydroxamic acid by hydrolyzing hydroxamate groups under strong alkaline conditions (26). Whereas, when tested for the production of carboxylate type of siderophore by Vogel's chemical test, the bacterial isolate *Cytobacillus firmus* strain VG5 was found negative (Fig. 3.5). This strain was able to produce hydroxamate type of siderophore and was found negative for carboxylate type of siderophore. Ferric or Fe^{3+} ion though present as one of the predominant micronutrient in nature but is poorly soluble and hence not readily available to plants or microorganisms. Many previous reports showed isolation of PGPR siderophore producing microorganisms. It was mentioned in the previous report that microorganisms have developed specific mechanisms for assimilating iron by producing siderophores. Microorganisms produce a low molecular weight chemical called a siderophore that chelates iron and transports it in cells (27). Similar to our strain, many other strains also reported to produce siderophore in earlier literature. These siderophore producing PGPR confined Fe^{3+} around the root area. Siderophores then bind with Fe^{3+} to form siderophore - Fe^{3+} complex. This complex subsequently binds with iron- limitation- dependent receptors on the bacterial cell surface and releases Fe^{3+} which activates in cytoplasm as ferrous ion (28). The strain used in the current study was also capable of chelating ferric ion by producing siderophores. Similarly, bacteria producing siderophores, restrict iron availability to pathogens and indirectly can stimulate plant growth by inhibiting the spread of plant diseases (29). Recently identified *V. paradoxus* TF20 and *B. cereus* P8 isolates showing higher growth promoting and biocontrol activity than other microorganisms , whereas other 4 identified bacterial isolates were showing comparatively more PGP activity (potassium solubilization, nitrogen fixing activity, and phosphorus solubilization) in the soil of West Sikkim (30). Our results are also in agreement with (31) where isolates showed high PGPR activity with increased level of seed germination, seedling vigor, IAA production and biocontrol activity to protect plants from Phytophthora crown rot caused by *Phytophthora capsici*.

Fig. 3.1 IAA production: Positive results for IAA production by *Cytobacillus firmus* strain VG5

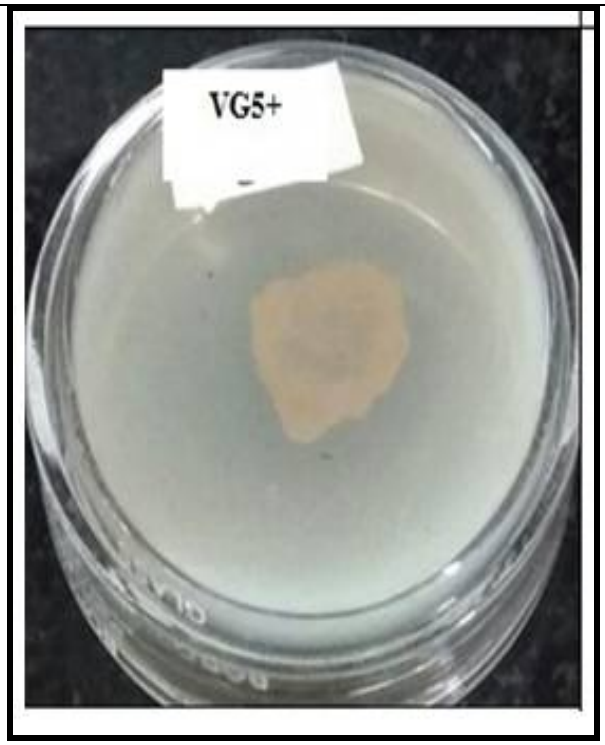

Fig 3.2 Phosphate solubilisation by *Cytobacillus firmus* strain VG5: Clear halo zone of solubilisation around colonies on Pikovskaya's agar plate

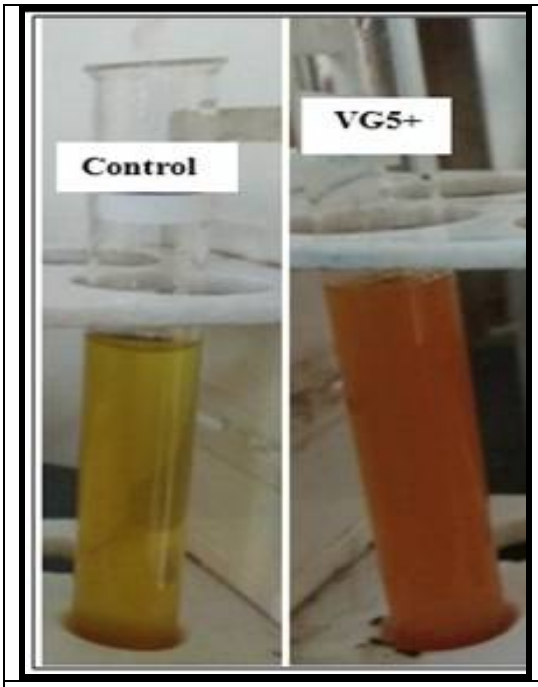

Fig. 3.3 Ammonia Production by *Cytobacillus firmus* strain VG5
Positive results: Development of intense yellow color after addition of Nesseler's reagent in the inoculated broth

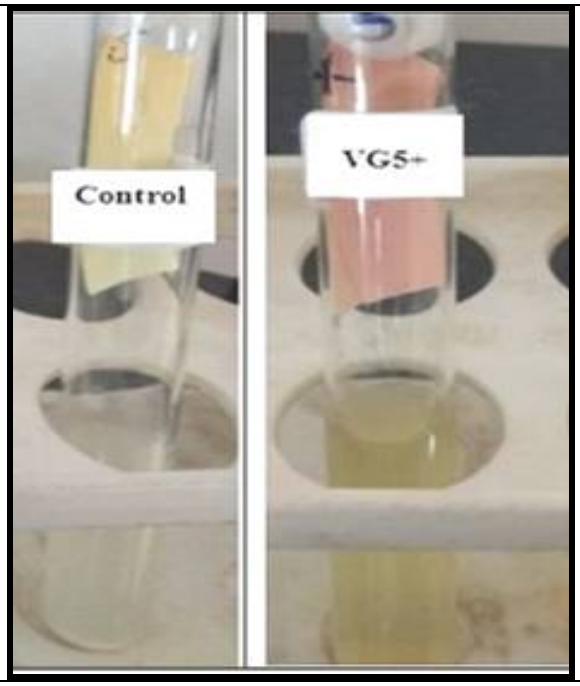

Fig. 3.4 HCN production by *Cytobacillus firmus* strain VG5
Positive results: Development of orange to red color on Whatman filter paper no.1 in the media containing tubes

Fig. 3.5 Siderophore production by *Cytobacillus firmus* strain VG5: (A)Vogel's chemical test : Negative for the carboxylate type of siderophore production

Fig. 3.6 Siderophore production by *Cytobacillus firmus* strain VG5: Negative Arnow's test for Catechol type siderophore**:**

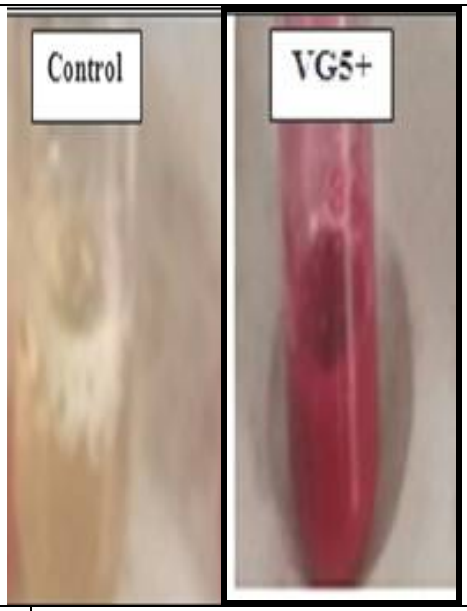

Fig. 3.7 Siderophore production by *Cytobacillus firmus* strain VG5: Tetrazolium salt assay Positive for hydroxamate type siderophore:

CONCLUSIONS

Cytobacillus firmus strain VG5 used in the current study is emerged out to be the efficient PGPR as this strain was found capable of producing phytohormones IAA, ammonia, HCN and siderophore along with

phosphate solubilisation under *in vitro* conditions. This indicates that *Cytobacillus firmus* VG5 can be used as bio-inoculants or bio-fertilizers and as well as biocontrol agents as a substitute of chemical fertilizers for improving crop growth and productivity without any environmental pollution or health hazards. Therefore it is pivotal to test the potential of these plant growth promoters in pot or *in situ* for further formulation as bio-inoculant for farmers' use.

CONFLICT OF INTEREST

The authors claim no conflicts of interest because none financial support was received from any government, non-government agency or organization to conduct this research work.

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