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Evaluating biocontrol traits of *Bacillus Subtilis CAB3* for growth promotion of *Arachis hypogaea L.*

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ABSTRACT
Biocontrol is an effective and sustainable alternative to conventional pesticide for plant disease management. This study mainly designates about the biocontrol potential of rhizobacteria against phytopathogenic fungi such as Fusarium solani, Fusarium oxysporum and Aspergillus spp which causes plant diseases like root wilt and aspergillosis. Indigenous bacterial strains were isolated from central Gujarat region and primary and secondary Screening was carried out using crowded plate technique and dual culture assay respectively. 9 strains were selected based on antagonistic activity for several biocontrol traits including Hydrogen Cyanide (HCN) production, Ammonia (NH3) production, Zinc (Zn) solubilization, glucanase and cellulase the growth promoting trait was carried out with Phosphate (P) solubilization assay. Result showed that 4 strains were positive for HCN production, and 5 strains were positive for NH3 production. Qualitative analysis of Zn solubilization showed 16.9 mm zinc solubilizing index (ZSI) by CAB3 and quantitative analysis showed 2.87µg/ml Zn solubilization. From the above study 3 superlative isolates CAB3 , AB1 and AB2 were selected for pot study of on Arachis hypogaea L. The pot experiment clearly demonstrates disease management by producing jasmonic acid (JA) and salicylic acid (SA) along with the growth promoting and antagonistic traits by desired strains. Amongst the isolates the best potential isolates were further carried out for their identification process by morphological, cultural, biochemical, and molecular assays. Results of 16s rRNA sequencing revealed the most potential isolate as Bacillus subtilis CAB3.
Keywords:*Biocontrol, Phytopathogenic fungi, PGPR, Induced systemic resistance, Fusarium spp.*

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INTRODUCTION

Biological control is an environment friendly alternative to chemical pesticides and it is an alternative method of protecting the plant from pathogens. Biocontrol agents were isolated by screening of rhizospheric soil. It is an effective & sustainable alternative to conventional pesticide for plant diseases management. Bacterial isolates were checked for their antagonistic activity against a range of phytopathogenic fungi [1][2]. Biocontrol traits like production of HCN, ammonia and catalase was checked. The isolate strains were capable of producing a number of hydrolytic enzymes as well as antimicrobial metabolites. The aim of this study was to directly compare antagonistic strain isolated from rhizospheric soil. Screening of sample was done by crowded plate technique and dual culture assay. Another important feature of the bacteria is the direct growth promotion phenomenon, the bacteria known as plant growth promoting rhizobacteria live in a close vicinity of the plants and play a key role in the transformation of many organic and inorganic compounds making them available for plant growth such as nitrogen Phosphorus, Potassium, Iron, and Zinc [3], [4]. *Fusarium* is a fungal genus comprising numerous species that cause a range of plant diseases. These diseases can affect a wide variety of crops, including wheat, maize, cotton, and tomato. *Fusarium* fungi can cause root rot, stem rot, leaf rot, wilt, and seedling blight, leading to reduced plant growth and yield loss. *Fusarium oxysporum* is one of the most destructive species, causing vascular wilt diseases in over 100 plant species worldwide [5]. *Fusarium* infection can also lead to the production of mycotoxins, which are harmful to human and animal health [6]. The control of *Fusarium* diseases in plants is challenging, and chemical pesticides have limited effectiveness. Therefore, alternative approaches, such as the use of biocontrol agents, are being explored for the management of *Fusarium* diseases [7, 8].

Plants have various mechanisms to defend against biotic stresses caused by pathogens and parasites, ranging from viruses to insects. The timing of the defence response is critical in determining whether the plant can cope or succumb to the challenge of necrotizing pathogens and parasites. If defence mechanisms are triggered prior to infection, the disease can be reduced. Induced resistance is a state in which a plant's defensive capacity is enhanced after being appropriately stimulated. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance[9], [10], where the plant's defences are preconditioned by prior infection or treatment, resulting in resistance against subsequent pathogen or parasite challenges. Certain strains of plant growth-promoting rhizobacteria (PGPR) can suppress diseases by inducing systemic resistance in the plant against both root and foliar pathogens, as well as by antagonizing soil-borne pathogens [11].This study aimed to identify potential rhizobacterial strains exhibiting plant growth promotion as well as biocontrol potential agent a broad spectrum of phytopathogenic fungi. The future objective is to develop dual purpose inoculum exhibiting biofertilizer and sustainable agriculture. Zinc is an essential micronutrient which is required by the plants in adequate concentration for growth and development. Zinc solubilizers solubilizes Zn through several mechanisms which include excretion of metabolites such as organic acid, production of chelating agents[12]. The method used was based on observing clear zones or hollows around colonies growing on Bunt and Rovira media amended with selected Zn compound. PGPR are widely studied because of their potential for plant production under three characteristics, Firstly PGPR acting as biofertilizer, they provide nitrogen via nitrogen fixation, phosphorus via phosphate solubilization and zinc via zinc solubilization reaction which can subsequently be used by plants as essential nutrients [13],[14], secondly phyto-stimulation can directly promote the growth of plant, by production of plant hormones [15]. Finally, third and most important biological control agents can protect plant via root systems from phytopathogenic organisms and induce the natural plant immunity via "induced systemic resistance" phenomenon. The application of PGPR in agricultural system as inoculants is being very attractive since it would substantially reduce the use of chemical fertilisers, pesticides, and fungicides [10].

MATERIALS AND METHOD

Sample collection and isolation

Phosphate solubilizing bacteria:

A composite sample of 10 g rhizospheric soil (root adhering soil) was carried to the laboratory followed by serial dilution and isolation of rhizobacteria was completed within 48 h. loosely adhering soil was removed from the roots by washing it with sterile distilled water, serial dilution was done and 0.1 ml from 10^{-6} dilution was taken and spread on modified Pikovskaya's (PVK) agar containing: 10 g glucose; 5 g Ca3(PO4)2; 0.2 g KCl; 0.5 g (NH4)2SO4; 0.2 g NaCl; 0.1 g MgSO4.7H2O; 0.002 g MnSO4.H2O; 0.002 g FeSO4 .H2O. The pH was adjusted to 7.0 before autoclaving. Bacterial colonies surrounded by a halo zone (indicating removal of phosphate) on Pikovskaya's agar were assumed to be phosphate-solubilizers. Pure cultures were obtained by restreaking in triplicates in the fresh medium. Over-all 9 isolates initially obtained in this manner were maintained on nutrient agar slants at 30°c as stock cultures.

The bacterial isolates obtained above were re-tested by plate assay using PVK medium. The halo zone around the colony was probable confirmation of phosphate solubilization and was measured after 7 days of incubation at 30° C. Tricalcium phosphate was used as a source of phosphate in the medium [16].

Isolation of pathogenic organism

The pathogenic organism was isolated from the infected plant of *fusarium spp* as a pure culture on potato dextrose agar (PDA) medium. The plant specimens were washed with normal tap water, the rotten parts cut into small pieces (4-5 mm), sterilized with 0.1% sodium hypochlorite (NaOCl) for two minutes. rinsed three time in sterile water and dried between sterilized filter paper. The sterilized plant pieces were transferred to sterilized PDA agar plates later it was purified by the hyphal tip method and transferred to potato dextrose agar (PDA) slants and pure cultures of the pathogens were maintained for further studies [17], [18].

Screening of potential biocontrol isolates:

Screening for the antagonistic activity was done by using the dual plate assay method. The nutrient agar and potato dextrose agar medium were mixed in a flask and autoclaved. The plates were poured with that medium. Newly grown pathogenic fungi i.e., *Fusarium spp* were placed at the centre of the plates and the bacterial isolates were streaked at the edge of plates to observe the antifungal activity against isolated bacteria. Plates were incubated for 5-6 days at 28±2°C. The antifungal activity was assessed by measuring the growth inhibition zone against the test fungi [19].

Biocontrol traits of selected isolates

Ammonia production:

Ammonia production by bacterial isolates was tested in sterile peptone water. A loopful of bacterial isolates were inoculated in 10 ml of peptone water and incubated for 48-72 hour at 28°C. After incubation Nessler's reagent was added in each tube. Colour change from brown to yellow was a positive test for ammonia production [20].

HCN and Catalase production:

All the isolates were screened to produce hydrogen cyanide by adapting the method of lorck (1943). Briefly, nutrient broth was amended with 4.4g glycine/l and bacteria were streaked on modified agar plate. A whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at top of the plate. Plates were sealed with parafilm and incubated at 28±2 for 4 days. Development of orange to red colour indicate HCN production [21].

The test bacteria were grown on nutrient agar at 30°C for 24–48 h. A loopful of each culture was mixed with 50 µl of 3% (v/v) H2O2 on a glass slide and incubated at room temperature (26 ± 2°C) for 1 min to observe the evolution of oxygen which was recorded as a positive catalase reaction [22].

Zinc Solubilization:

Plate and Broth Assay:

Zinc solubilizing ability of bacterial isolates was estimated using Bunt and Rovira medium. Zinc Oxide (ZnO) 0.1% was added as an insoluble source of Zinc. The bacterial isolates were spot inoculated on the agar plates. The plates were incubated at 28°C for 3-5 days. After incubation halo zones were observed around the bacterial colony which showed zinc solubilization. The diameter of the colony and halo zone was checked, and Zinc Solubilizing Index was calculated. Three selected bacterial isolates were inoculated in Bunt and Rovira medium in which ZnO was added as a source of zinc. The broth was divided in two flasks having 25 ml broth and 0.1% of insoluble ZnO and sterilized for 30 minutes in autoclave. After sterilization 0.1% inoculum is added and incubated [23].

Assay of β-1, 3- glucanase

The activity of **β** -1, 3-glucanase was determined by measuring the release of reducing sugars by using laminarin or starch as substrate and glucose as standard. The reaction mixture consisted of 500 µl of culture supernatant, 500 µl of 1 M citrate buffer (pH 5.0) and 500 µl of 4% laminarin. The reaction was carried out at 37 °C for 30 min. The reaction was stopped by adding 2 ml of dinitro salicylic acid and heating for 5 min on a boiling water bath, vortexes and its absorbance measured at 500 nm [24].

Assay of cellulase

The activity of cellulase was determined by measuring the release of reducing sugars using carboxymethylcellulose as substrate and glucose as standard. The reaction mixture consisted of 500 µl of culture supernatant, 500 µl of 1 M citrate buffer (pH 5.0) and 500 µl of 1% carboxymethylcellulose. The reaction was carried out at 37 °C for 30 min. The reaction was stopped by adding 2 ml of dinitro salicylic acid and heating the reaction mixtures for 5 min on a boiling water bath, vertexing and measuring absorbance at 500 nm [25].

Jasmonic acid and Salicylic acid detection:

Jasmonic acid was detected by Plant jasmonic acid (JA) ELISA Kit by sun long, This ELISA kit uses Sandwich-ELISA as the method. The Micro Elisa strip plate provided in this kit has been pre-coated with an antibody specific to jasmonic acid (JA). Standards or samples are added to the appropriate Micro Elisa strip plate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for jasmonic acid (JA) is added to each Micro Elisa strip plate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain jasmonic acid (JA)and HRP conjugated jasmonic acid (JA)antibody will appear blue in colour and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm [26].

Salicylic acid detected by the strains were cultured in the standard succinate medium (SSM) at 28 °C for 48 hours on a shaker incubator at 180 rpm to ascertain salicylic acid production of the isolates. Centrifugation at 10,000 rpm for 5 minutes was used to collect the cells, which were then re-suspended in 1 ml of 0.1 M phosphate buffer. An identical volume of chloroform was used to extract SA after acidifying 4 ml of the cell free culture filtrate with 1N HCL to pH 2.0. The pooled chloroform phases received five millilitres of 2M ferric chloride and four millilitres of water. In a UV-visible spectrophotometer, the absorbance of the purple iron-SA complex that had developed in the aqueous phase was measured at 527 nm against a blank [27].

Pot Study:

The pot experiment was carried out in order to check the potential of the bacteria as biopesticide and biofertilizer agents. *Arachis hypogaea L.* was grown in four different pots. After the plants were grown the three most potential isolates *Bacillus subtilis CAB3*, AB1, AB2 were added. 10 ml of bacterial cultures of the isolates were added in three different pots. The fourth pot was named as control in which no bacterial

culture was added. This experiment was carried out to check the potential of the bacterial isolates in plant growth promotion.

Disease induction

Groundnut root rot and stem rot disease was artificially introduced by challenge inoculation of plants with *fusarium spp* on the 21st day of plant growth. Conidial suspension of *fusarium spp* was prepared in sterilized distilled water by harvesting acervuline from freshly sporulating cultures by scraping the surface of PDA slants in sterile condition. Serial dilutions of the spore suspension were prepared, and inoculum density was regulated to 5×100 spores /ml using a haemocytometer and the resultant fungal suspension was used to introduce root rot and stem rot disease in the groundnut plants according to the slightly modified method described by Oh et al. (1999) [28]. The control plants were inoculated with sterilized dilution blank. The roots and stems of all the plants were imperilled to examination of the development of rot symptoms. Disease incidence was calculated according to the formula:

Percentage of disease incidence in roots = No. of roots infected ÷ Total no. of leaves x 100

Percentage of disease incidence in stems = No. of stems infected ÷ Total no. of fruits x 100.

Bacterial Identification:

Cultural, Morphological and Biochemical Characterization:

The most potential bacterial isolate was grown on nutrient agar plate and its colony characters were observed then gram Staining was performed for the most potential bacterial isolate observed under microscope and different biochemical tests were carried out to identify the bacteria.

Phylogenetic analysis of 16srDNA sequencing:

DNA was extracted by following the protocol of wizard Genomic DNA purification kit. By using the universal primer 16s rRNA was amplified. The PCR cycle was run for 30 cycles of 20 seconds. Nucleotide sequences of 16s rRNA were aligned by Mega 11.0 and phylogenetic tree was created.

RESULTS AND DISCUSSION

Phosphate solubilisation

Plants required Phosphorus (P) as second most essential source of nutrient after nitrogen Because P plays a vital role in every phase of plant growth and development, nevertheless in soil, P is mostly present in insoluble form, So PGPR are having a capacity to solubilize P and increases the availability for crops [29], [30]. It is well recognised that the rhizosphere's advantageous plant-microbe interactions play a significant role in determining plant health and soil fertility. Plant growth-promoting rhizobacteria (PGPR) are a wide variety of soil bacteria that, when grown together, promote the growth of their host. Such rhizosphere microorganisms gain from using the nutrients generated by plant roots as metabolites for their growth and support plant growth through a variety of mechanisms, including the generation of hormones that stimulate growth and the control of plant diseases. Twenty bacterial isolates were screened initially on PVK agar plates and further phosphate solubilization checked in PVK broth-based assay (figure.1); five of these (CAB1, AB1, AB2, AB3, and AB4) were able to solubilize P in the plate-based assay, as shown by the development of a clear halo around the colony in PVK agar medium. These five were carried forward for secondary screening.

Secondary screening by dual culture assay:

Dual plate assay was performed on mixed medium PDA and nutrient agar plates. It was observed that there was a development of inhibition zone in the direction of actively growing bacteria as the fungi was not able to grow further. As a result of the cellulase glucanase present in fungal cell walls, which bacteria can breakdown, mycelial growth can be inhibited or invaded. It was clearly visible that certain bacterial isolates inhibited the mycelial growth which shows their antagonistic activity against *Fusarium spp*. Antifungal activity was shown by *Bacillus subtilis CAB3* was highest compared to other isolates AB1,AB2 and AB3 [20].

Ammonia production:

Ammonia is a key rhizosphere fungistatic factor in agriculture soils. It can inhibit the conidial germination of the nematophagous fungus *A. oligospora*. As we know, a small portion of ammonia can ionize as NH4+ and OH– in water reported by Liu T et al., 2021 [31]. Nitrogen is necessary for the plant growth and development. The bacterial isolates will be able to fix atmospheric nitrogen and covert it to ammonia to make it available for the plants. Ammonia production by the bacterial isolates help impact plant growth indirectly out of the 20 bacterial isolates It was observed that Bacillus subtilis CAB3, AB1, AB2, AB3 and AB4 isolates produced ammonia in peptone water gave positive test which was confirmed by the colour change from brown to yellow. [20].

HCN and catalase production:

Ability for hydrogen cyanide production was observed for bacterial isolates *Bacillus subtilis CAB3* and AB1. Microbial cyanides seem to play a role in suppression of many plant diseases. Additionally, it is well known

that the antifungal properties of both catalase and HCN are closely connected. Plants' ability to produce HCN has been proven to have both positive and negative effects (Cattelan et al. 1999) [32]. According to Flaishman et al. (1996), excessive HCN synthesis may be crucial in the management of fungal infections in groundnut [33]. Catalase is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS), catalase enzyme breakdown the hydrogen peroxide and converts into water and oxygen molecules. Isolates CAB3, AB1, AB2 gave positive results, AB3 and AB7 gave negative results (Table. 1) [34].

Zinc Solubilization:

Plate assay and Broth assay :

Nearly half of the cultivated soils in the world are deficient in available zinc (Zn) [35]. Low availability of Zn in alkaline soil is a chronic stress in cereal production in Turkey, India, Pakistan, and China [36], Zinc is a prosthetic group in many proteins and is involved in the activation of all the six groups of enzymes, namely, oxidoreductases, transferases, hydrolases, lysases isomerases and ligases [37]. Zinc deficiency leads to inhibition of protein synthesis in plants, which is marked by a decline in RNA [38]. The capacity of the bacterial isolates to solubilize insoluble zinc compound was confirmed in Bunt and Rovira Agar medium. There was a formation of clear halo zone around the bacterial colonies which confirmed zinc solubilization by the bacterial isolates. The zinc solubilizing index (ZSI) was found 16.9, 13.9, 10.34 by *Bacillus subtilis CAB3*, AB1 and AB2 respectively. *Bacillus subtilis CAB3* gave the highest Zinc Solubilizing Index on the plate assay as well as in broth assay amongst the three bacterial isolates, *B.subtilis CAB3* showed the highest amount of zinc solubilization i.e., 2.87 µg/ml. The other two isolates i.e., AB1 and AB2 showed 2.46 µg/ml and 2.11µg/ml of zinc solubilization respectively (figure.2) [39].

Glucanase and Cellulase production:

The primary ß1,3-glucanases that hydrolyse laminarin are endo- and exo-ß-glucanase, which release oligosaccharides and monosaccharides, respectively. Under circumstances, Trichoderma also produces ß-1,6-glucanases, which hydrolyse the ß-1,6-glucans, a minor structural polymer of the cell wall. In fungus, ß-1,3-glucanases serve a variety of purposes, including differentiation and development as well as mobilising ß-glucans when energy sources are depleted. Like chitinases, it seems like glucose prevents the production of ß-1,3-glucanases [40]. Direct evidence for the involvement of glucanases in mycoparasitism has been demonstrated by Lorito et al.,1994 [41]. Bacterial cellulases can help to inhibit the growth of fungi by breaking down the cell walls of fungal cells. This can prevent the fungi from being able to penetrate the roots of plants and cause disease. Additionally, the breakdown of cellulose by bacterial cellulases can release nutrients that are essential for plant growth, making the soil more fertile. When cellulose is broken down, it releases nutrients like nitrogen, phosphorus, and potassium, which are important for plant growth. The release of these nutrients also helps to improve the soil structure and texture, making it easier for plants to take root and grow [42]–[44]. AB4 and AB6 are the only isolates in this group with negative cellulase enzyme activity, while all other isolates had positive findings. All isolates, apart from AB3, AB6, and AB7, generate the enzyme glucanase, for providing antifungal activity. Those isolates developed cellulase and glucanase enzymes by clearing zones in the growth medium.

Jasmonic acid and salicylic acid production

Jasmonic acid and salicylic acid are both important signalling molecules that play a key role in the induction of systemic resistance in plants. Salicylic acid is known to play a major role in the induction of systemic acquired resistance (SAR) [45], which is a long-lasting and broad-spectrum resistance response that can protect plants against a wide range of pathogens. SAR is triggered when a plant is exposed to a pathogen or pathogen-derived elicitor, which leads to the accumulation of salicylic acid in the plant tissues. Salicylic acid then activates a series of defence responses, including the expression of pathogenesis-related (PR) genes and the production of reactive oxygen species (ROS), that can help to protect the plant against subsequent pathogen attacks [46], [47]. Jasmonic acid, on the other hand, is involved in the induction of induced systemic resistance (ISR), which is a defence response that can be induced by certain beneficial microorganisms. ISR is characterized by the upregulation of defence-related genes, such as those involved in the biosynthesis of jasmonic acid itself, and the production of defence-related metabolites [10], such as phytoalexins and volatile organic compounds (VOCs) [48]. ISR can also lead to changes in plant morphology and physiology, such as increased root growth and altered photosynthetic rates, that can help to enhance the plant's resistance to pathogens. Isolates CAB3, AB1, AB2, and AB3 are produce JA and SA similarly which was indicated that JA and SA dependent responses are incorporated with each other. Production of JA and SA is directly proportional to the induction of ISR and SAR responses [49], [50].

Pot Study and induction of disease:

The seeds of groundnut plants were treated with carboxymethyl cellulose (CMC) and bacterial inoculum as part of a pot study. The plants were exposed to *Fusarium* fungi as a pathogen after 21 days. The comparison

study's findings between the isolation, fungicide, and control (figure.3) groups revealed that while the illness affected 100% of the control plants, neither the fungicide-treated pots nor the isolate-inoculated pots displayed any signs of root infection by the pathogen. On the stem, 77.77% of the control plants had disease, whereas there was none in the pots treated with fungicide and isolate. Salicylic acid (SA) and jasmonic acid (JA) were examined to determine their existence after the experiment. The findings demonstrated that although the control plants lacked both JA and SA production, the isolate CAB3 produced both. These results imply that the isolate CAB3 treatment may cause groundnut plants to produce JA and SA, which would confer systemic resistance against *Fusarium* fungi. It is possible to do additional study to validate the results, such as molecular analyses of the PR and NPR genes to confirm these findings and investigate the potential mechanisms underlying the observed effects.

Bacterial Identification:

According to (Table. 5) CAB3's biochemical tests for citrate, gelatinase, urease, oxidase, and nitrate reduction were all negative. Catalase, lipase, phosphate MR-VP, and carbohydrate utilization were discovered to be positive for CAB3. The (Table. 4 & 6) also includes morphological and cultural features because of the large, off-white roads that are gram-positive bacilli.

Table 1- Biocontrol traits of isolated Bacteria.

Sr.No.	Bacterial Isolates	HCN Production	Ammonia Production	catalase	Cellulase	Glucanase	JA	SA
1	Control	-	-	-	-	-	-	-
2	CAB3	+	+	+	+	+	+	+
3	AB1	+	+	+	+	+	+	+
4	AB2	+	+	+	+	+	+	+
5	AB3	+	+	-	+	-	+	+
6	AB4	-	+	+	-	+	-	-
7	AB5	-	-	+	+	+	-	-
8	AB6	-	-	+	-	-	-	-
9	AB7	+	-	-	+	-	-	-
10	AB8	+	+	+	+	+	-	-

A phylogenetic tree for CAB3 would typically show its evolutionary relationships to other bacteria, based on genetic data. Blast run for our isolated bacterial species it matches 100% similarities with other *Bacillus subtilis* strains, it is highly indicative that our isolated species is indeed *B.subtilis*. This is because a 100% match between genetic sequences strongly suggests that the isolated species shares the same genetic identity as other *B.subtilis* strains. The branching pattern of the tree would indicate the degree of genetic relatedness between *B.subtilis* and other organisms. Organisms that share a common ancestor with *B.subtilis* more recently would be located on branches that are closer together, while those that are more distantly related would be located on branches that are farther apart.

Table 2 - antagonism of microbial antagonists *Bacillus subtilis CAB3* against root rot in ground nut.

Sr no	Treatments	Total number of roots **	Number of infected roots **	% of disease incidence in roots
1	Control	09	09	100
2	Fungicide (Thiophanate methyl 0.2 %)	09	00	00
3	*Bacillus subtilis CAB3*	09	00	00

*All values are means from two repeated experiments with three replications each with 3 plants (3 plants/pot)
** Values are per treatment i.e., for all the plants in all replications

Table 3 - Antagonism of microbial antagonists *Bacillus subtilis CAB3* against stem rot in ground nut.

Sr no	Treatments	Total number of stems **	Number of infected stems **	% of disease incidence in stem
1	Control	09	07	77.77
2	Fungicide (Thiophanate methyl 0.2 %)	09	00	00
3	*Bacillus subtilis CAB3*	09	00	00

*All values are means from two repeated experiments with three replications each with 3 plants (3 plants/pot)
** Values are per treatment i.e., for all the plants in all replications

Table 4 - Cultural Characteristics of CAB3	
Size	Medium
Shape	Roughly circular
Margin	Uneven
Elevation	Slightly Raised
Surface	Smooth
Opacity	Opaque
Pigmentation	Off-white

Table 5 – Biochemical tests of CAB3 isolate.		
Sr.No.	**Biochemical Test for CAB3**	**Result**
1	Carbohydrate Utilization	+ve
2	Urea Utilization	-ve
3	Oxidase	-ve
4	Citrate Utilization	-ve
5	Catalase	+ve
6	Indole production	+ve
7	Voges-Proskauer	+ve
8	Methyl Red	+ve
9	Phosphatase	+ve
10	Nitrate Reduction	-ve
11	Casein hydrolysis	+ve
12	Lipase	+ve

Table 6- Morphological Characteristics of CAB3	
Size	Big
Shape	Rod
Arrangement	Chain/Pair
Gram Reaction	Gram Positive

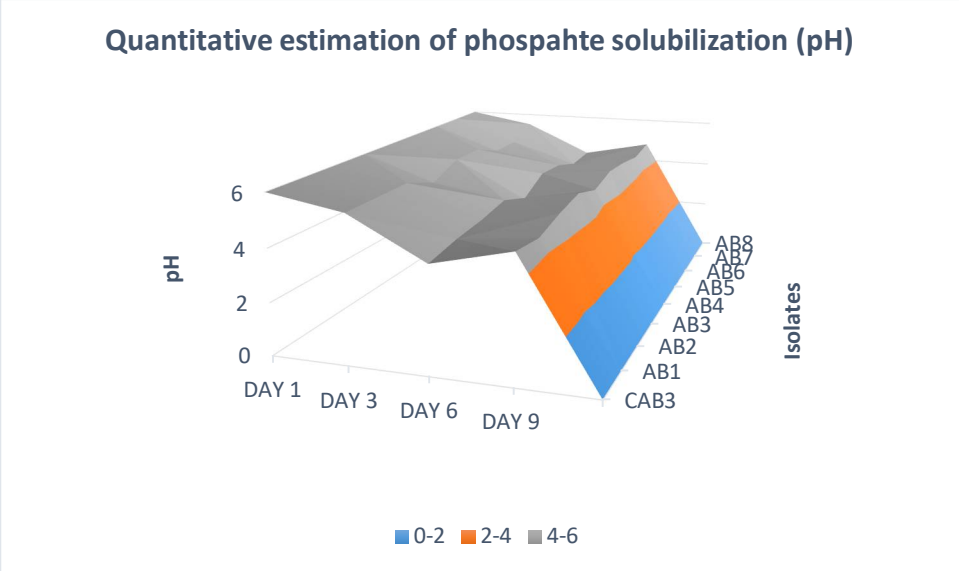
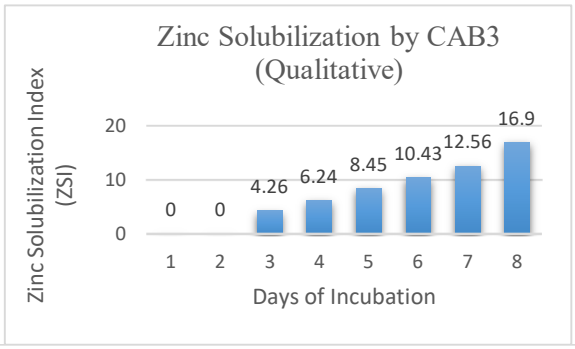
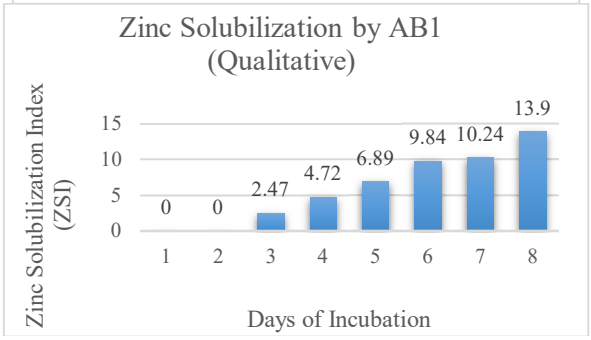
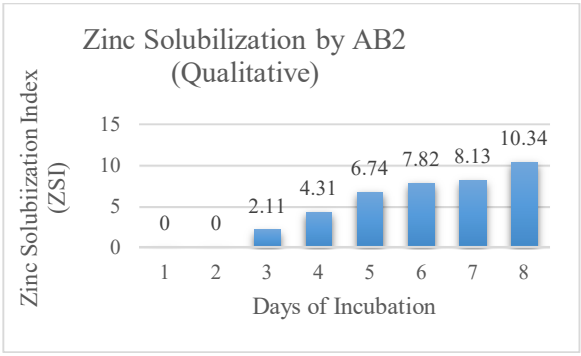
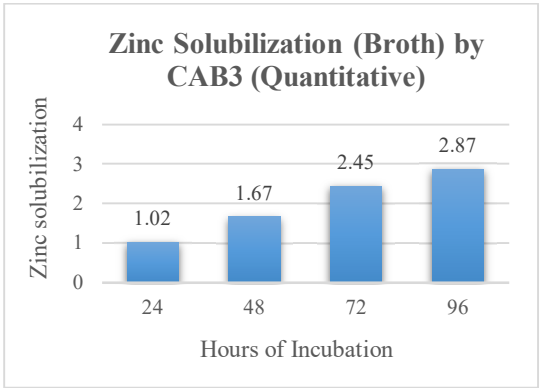


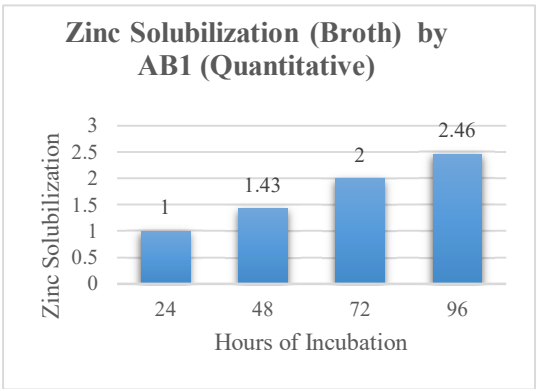
Figure 1- Quantitative estimation of phosphate solubilization (pH).


Zinc Solubilization by CAB3 (Qualitative)
Zinc Solubilization Index (ZSI)
20
10
0
0
0
4.26
6.24
8.45
10.43
12.56
16.9
1
2
3
4
5
6
7
8
Days of Incubation


Zinc Solubilization by AB1 (Qualitative)
Zinc Solubilization Index (ZSI)
15
10
5
0
0
0
2.47
4.72
6.89
9.84
10.24
13.9
1
2
3
4
5
6
7
8
Days of Incubation


Zinc Solubilization by AB2 (Qualitative)
Zinc Solubiization Index (ZSI)
15
10
5
0
0
0
2.11
4.31
6.74
7.82
8.13
10.34
1
2
3
4
5
6
7
8
Days of Incubation


Zinc Solubilization (Broth) by CAB3 (Quantitative)
Zinc solubilization
4
3
2
1
0
1.02
1.67
2.45
2.87
24
48
72
96
Hours of Incubation



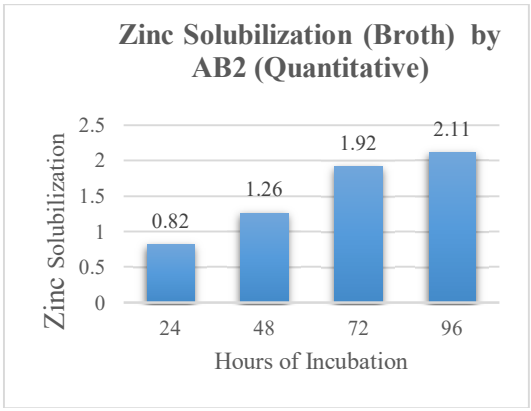


Figure 2- Zinc solubilization assay.

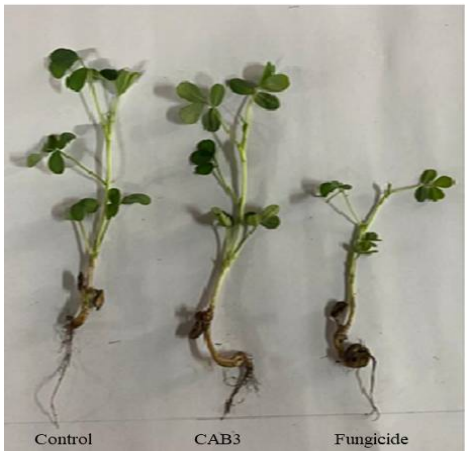


Figure 3 – Pot study of *Arachis hypogea L* with treatment of Control, CAB3 and fungicide (Thiophanate methyl 0.2 %).

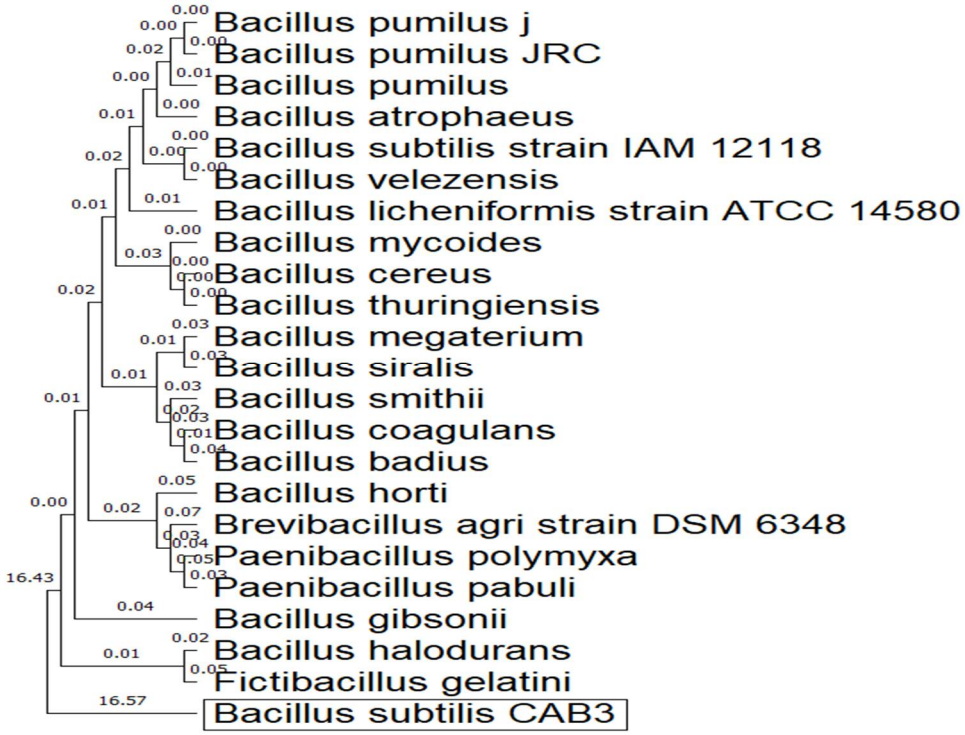


Figure 4- Phylogenetic analysis of CAB3 isolate.

CONCLUSION

This study serves as an excellent example of the value of screening rhizobacteria under in vitro circumstances for various PGPR features and their evaluation under regulated circumstances in a pot experiment. Due to its various PGPR features, the effective PGPR isolate *Bacillus subtilis CAB3* was chosen as a result, and it may be able to increase groundnut crop productivity and maintain soil fertility. In conclusion, our investigation showed that bacterial cellulase and glucanase producing isolate *Bacillus subtilis CAB3* inoculation of groundnut seeds and subsequent induction of systemic resistance via JA and SA production led to a significant reduction in protection against *Fusarium spp* infection. Additionally, the presence of JA and SA in the isolate-treated plants further supports the hypothesis that the induction of induced systemic resistance (ISR) through the production of these hormones can enhance plant defence mechanisms against pathogenic fungi. These findings have important implications for developing sustainable and eco-friendly disease management strategies in agriculture by harnessing the potential of microbial inoculants and plant hormones.

Ethics approval and consent to participate:
Not applicable

Availability of data and materials:
The manuscript compromises all the applicable data in the text, tables and in the figure.

Consent for publication:
AR and CKJ initiated the idea of research. AR and JR wrote the manuscript. AR, JR and CKJ reviewed and finalized the whole manuscript.

Conflict of interest:
All authors declare that there is not any conflict of interests for this publication.

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