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Research Article

ENDOPHYTIC COLONIZATION AND GROWTH PROMOTION OF CAULIFLOWER PLANT BY *Bacillus thuringiensis*

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Abstract- Bacterial endophytes are endosymbiotic microorganisms live inside plants for at least part of their life cycle without causing visible symptoms and involved in plant growth promotion. The present investigation the endophytic *B. thuringiensis* was isolated from the plant sample. Five isolates were confirmed on the basis of crystal formation and identified as *Bacillus thuringiensis*. The colonization capacity of the isolates was studied by recovering *B. thuringiensis* from the inoculated leaf and stem (2.9×10^7/g) and root (3.9×10^7/g) of the plants. The cauliflower seed germination and seedling development, seven days after planting showed significant increase over un-inoculated control. Further, the selected isolates were screened for the production of cell wall degrading enzymes and multiple plant growth promoting (PGP) traits *viz*. phosphate (P) and zinc (Zn) solubilization abilities. All five endophytic *B. thuringiensis* isolates were found positive for the production of cell wall degrading enzymes *viz*., cellulase, pectinase, protease, endoglucanase, and also having capacity to solubilize phosphate and zinc. The maximum phosphate and Zn solubilization was achieved by the standard strain HD1 followed by isolate CF4 followed by CF2. The ability of *Bacillus thuringiensis* isolates for IAA production, Gibberellic acid production and Siderophore production too was assessed. IAA and GA3 production were found to be maximum with CF4 (42.67 and 356.8 µg l^{-1} respectively), followed by CF2 (31.51 and 296 µg l^{-1}). Maximum siderophore production was observed in CF4 (374 mg l^{-1}) followed by CF2 (270 mg l^{-1}).

Keywords- *Bacillus thuringiensis, Cauliflower, PGPR*

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Introduction

Endophytes were defined as microorganisms such as bacteria and fungi that inhabited inside the plant during their life cycle without causing any apparent damage to the host plant [1]. The endophytic colonization provides a good environment that protects the microbes from adverse conditions. The presence of beneficial endophytes enhances the plant growth and development. Plant growth promoting ability of these endophytes could be directly established by assessing the production of plant growth hormones. The interaction between the plant and endophyte results either in production of plant hormones or in any activity that increases availability of nutrients like nitrogen and phosphorus [2].

Many species of bacteria belonging to the genera *viz., Agrobacterium, Azospirillum, Azotobacter, Arthrobacter, Bacillus, Bradyrhizobium, Burkholderia, Caulobacter, Flavobacterium, Herbaspirillum, Klebsiella, Mesorhizobium, Micrococcus, Pseudomonas, Rhizobium, Rhodococcus,* and *Serratia* enhance plant growth and are thus termed as plant growth promoting bacteria (PGPB) [3]. Common characteristics of endophytes include, the ability to synthesize plant hormones like indole-3-acetic acid, Gibberllic acid, siderophore production and to solubilize phosphate and zinc nutrients which enhances the hosts' tolerance to biotic and abiotic stresses [4].

For their plant growth promoting and disease control abilities, endophytes are used as bioinoculants in agriculture [5]. Studies have confirmed the presence of endophytic *B. thuringiensis* in cabbage, cotton, legumes and some medicinal plants [6]. In an endophytic relationship, *B. thuringiensis* would take up nutrients from the plant to survive, but would compensate by protecting them against biotic and abiotic stresses. Recent studies indicate that *Bacillus thuringiensis*, if developed as a PGPR, can promote seed germination and, root and shoot elongation apart from imparting insecticidal ability. Since *B. thuringiensis* is a Gram-positive, spore forming bacterium that produces parasporal protein crystal that exhibits insecticidal activity [7].

The efficient *B. thuringiensis* colonization in cabbage seedling roots may be the main route of interaction with plants [8]. Endophytic strains colonize various parts of the plant, including the roots, leaves, stems, flowers and seeds. [9] reported that IAA-producing *B. thuringiensis* strain C25 isolated from the cabbage (*Brassica oleracea*) significantly enhanced the growth of *Lactuca sativa* under greenhouse condition. Similarly, co-inoculation of *Bradyrhizobium japonicum*-SB1 with *B. thuringiensis* strain KR1 promoted the growth of soybean and increased shoot weight, nodule number, root weight, root volume and total biomass, compared to rhizobial inoculation and control [10]. Several plant growth-promoting rhizobacteria that act as biofertilizers, contain viable microorganisms mainly found on the surface of plants or in the soil, rhizosphere and plant interior [11]. It was reported that *B. thuringiensis* and *B. subtilis* strains isolated from the wheat rhizosphere showed high PGPR activity, including phosphate solubilization act as a biostimulent [12]. The efficiency of endophytic bacteria in protecting plants from a series of abiotic stresses like drought, temperature and salinity [13].

B. thuringiensis produce spores which is suitable agents to enhance plant growth even in extreme environments having high temperatures, high salt concentrations, high heavy metal concentration and presence of organic toxicants. This feature is advantageous compared to non-sporulating PGPB.

Materials and Methods

Seed material

Seeds of hybrid cauliflower Astro Plus were used in this study, to evaluate the colonization of *B. thuringiensis* strains in cauliflower seedlings grown under controlled conditions.

Strains and culture condition

New endophytic *B. thuringiensis* isolates were isolated from plant samples

collected from different sites in Tamil Nadu. *B. thuringiensis* subsp. kurstaki HD1 was used as reference strain. The isolates were grown in Luria-Bertani medium (LB) at 30°C, on a rotary shaker (200 rpm). T3 medium was used for enhancing parasporal crystals formation of *B. thuringiensis*. The pure cultures were maintained at 4°C. For extended storage, stock cultures were stored in 25% (v/v) glycerol at -20°C

Pot culture study

The cauliflower seeds (1g) were surface sterilized with ethanol 70% for 5 minutes and subsequently in sodium hypochlorite 2 % in 70% ethanol for 30 minutes. Then the seeds were washed three times in sterile distilled water and blot dried using sterile filter paper. After disinfecting, seeds were soaked in cell suspensions of four different isolates of *B. thuringiensis* 109 CFU/ml) for 1 hour. The seeds were then transferred to filter paper for drying. Once dry, the seeds were sown in Protrays containing sterile potting mixture (1: 4 vermicompost : coir pith) and were incubated in a growth chamber at (25±2)˚C for germination, in the dark. After seven days, the seedlings were transferred to pots containing sterile soil. After two weeks, the rhizosphere soils were inoculated with 3 ml of suspension containing spores and crystals (1.26 × 108 spores), twice a week during subsequent 3 weeks period. Un-inoculated plants were kept as control. Pots were incubated at 24°C with 16h exposure to light. Four replications were maintained. The colonization was observed the over the period of plant growth.

Recovery of *B. thuringiensis* strain from treated cauliflower plants

Colonization was studied by re-isolation of *B. thuringiensis* from the inoculated plants. The samples (leaf, stem and root) from 7 to 30 days old healthy plants were checked. The plant samples were rinsed thoroughly, then surface sterilized with 70% ethanol for 30 seconds and 0.1% mercuric chloride for 2 min and washed with sterilized water three times to remove surface bacteria [14]. The sterility of the leaf, stem and root was reconfirmed by incubating the final surface wash water on a Luria-Bertani (LB) agar plate. Then the plant samples from each treatment were macerated separately in sterile centrifuge tubes containing 1 ml LB broth (amended with 0.25 mM CH_3COONa), vortexed for 2 min and incubated at 28°C for 24 h, following which the tubes were subjected to a heat treatment at 80°C for 20 min in a shaker cum water bath . After the heat treatment, an aliquot of 0.1 ml from each tube was spread plated on Luria Bertani agar and incubated for a further 48 h at 28°C. The heat stable bacterial colonies were enumerated and examined under phase contrast microscope for the presence of spores and crystals. Bacterial colonies that produce crystals during sporulation were designated as *B. thuringiensis*. The reisolated *B. thuringiensis* colonies were purified by repeated streaking on Luria-Bertani agar plates.

Evaluation of development of cabbage seedlings

The *B. thuringiensis* treated cauliflower seedlings were evaluated for percentage of germination and other biometrics like root length, shoot length, biomass production and dry weight of the plants were assessed. Observations are recorded after 3rd day and 27th day after seed treatment and root inoculation respectively [15].

Screening for cellulase, pectinase and protease production

All the isolates were checked for the enzyme production. However, for cellulose isolates were grown on basal salt medium supplemented with 1% carboxymethylcellulose (CMC) [16]. The formation of clear zones around the colonies was observed and measured in order to select the highest cellulase producer [17]. For pectinase production the yeast extract peptone (YEP) agar medium was used. After incubation, iodine-potassium iodide solution was used to observe the presence of clear zones around the isolates [18]. Production of protease and endogluconase was determined by the presence of clear zones around the colonies grown on skimmed milk agar and CMC medium [19].

Estimation of plant growth promoting traits

Production of indole acetic acid

The *B. thuringiensis* isolates were allowed to grow in the LB medium [20] supplemented with and without tryptophan at 28±2°C for 48-96 h. After the incubation period, the broth was centrifuged and the supernatant was mixed with 2 ml of Salkowski's reagent (2 % of 0.5 M $FeCl_3$ in 35 % perchloric acid) and incubated in dark. After 30 min of incubation, the OD was measured at 530 nm using spectrophotometer. A standard curve was prepared using pure IAA in the range of 0-250 µg/ml by plotting at 530 nm. Estimation of IAA production was calculated according to the procedure described by [21].

Production of Gibberllic acid (GA3)

The GA3 produced by the *B. thuringiensis* isolates was quantified by following the protocol described by [22]. The cultures were grown in nutrient broth for 7 days at room temperature. After the incubation period the cell free supernatants were collected by centrifugation (10,000 rpm/10 min) in the presence of phosphate buffer and acidified to pH 2.0 with 5N HCl. The acidified supernatants were extracted with equal volumes of ethyl acetate using separating funnels. After mixing 5 ml aliquots of the solvent extracts, with 2 ml of zinc acetate solution (21.9 g of zinc acetate in 80 ml DW with 1 ml of acetic acid glacial and the final volume was made upto 100 ml), 2 ml of potassium ferrocyanide solution (10.6 g of potassium ferrocyanide in 100 ml of DW) and 5 ml of 30 % HCl, the mixtures were incubated at 20° C for 75 min. Appropriate blank was prepared using 5 % HCl and the changes in absorbance were measured using spectrophotometer (M/s, Cary 50 Bio- Varian, Australia) at 254nm.

Siderophore production assay

The modified method for estimating siderophore production was carried out using micro-titer plates. The cultures were inoculated in the basal medium and incubated at 28° C for 48 to 72 h so as to obtain a cell concentration of 108CFU ml^{-1} broth. Centrifuged supernatant 100 µl, from each bacterial culture was added in separate wells of the micro plate followed by addition of 100 µl of CAS reagent. After incubation, the optical density of each sample was recorded at 630 nm [23].

Siderophore production (PSo) = (Ar - As) * 100/Ar

Where As- absorbance of the sample (CAS solution and cell free supernatant of the sample) and Ar- Absorbance of reference (CAS solution and uninoculated broth)

Phosphate solubilization

The *B. thuringiensis* isolates were screened for the phosphate solubilization employing modified Pikovskaya agar medium, containing 0.3% insoluble calcium triphosphate (HiMedia, India). The test cultures were inoculated into the plates and incubated at 28◦C. The presence of clear zones around the colonies after 7 days of incubation qualitatively confirmed the phosphate solubilizing potential of the isolates [24].

Zn solubilization

Tris-minimal medium supplemented separately with zinc oxide (ZnO) and zinc phosphate Zn3 (PO4)2 at a concentration equivalent to 0.1% Zn was used to study zinc solubilization. In which the *B. thuringiensis* isolates were grown in the Zn medium for seven days at 28°C, after the period of incubation, observed the formation of clear halo zone around bacterial colonies [25].

Result and Discussion

Colonization study- Recovery of *B. thuringiensis* from treated cauliflower plant

Bacillus thuringiensis is mainly recovered from soil, where it is extensively found [26]. However, some groups of *B. thuringiensis* are present on the leaves of different plant species. *B. thuringiensis*is a plant mutualistic bacterium offers protection to plants from phytophagous insect larvae and in return gets nutrients, and environment protection. Some *B. thuringiensis* strains were found to colonize the plant roots (maximum average colonization observed was 3.679 x 105 CFU root g^{-1} [27]. In this present investigation, the ability of the spores of the *B. thuringiensis* strains to penetrate and colonize the cauliflower plant was studied. Colonization was confirmed by recovery of *B. thuringiensis* from different plant parts of cauliflower after heat treatment.

Table-1 *Cell wall degrading enzymes and functional traits of Bacillus thuringiensis*

Bt Isolates	Cell wall degrading enzymes				Functional traits (cm)	
	Celluase	Pectinase	Protease	Endoglucanase	P solubilization	Zn Solubilization
HD1	2.1(+++)	2.0(+++)	1.95(++)	2.1(+++)	1.4	0.9
CF2	1.5(++)	0.9	1.7	0.8	1.0	0.6
CF4	1.8(++)	1.6(++)	0.5 (++)	1.7(++)	0.8	1.2
EP1	1.2(++)	0.8	0.5	0.9	0.4	0.5
EP3	0.8(+)	1.2(++)	0.6	0.4	0.4	0.3

Further, phase contrast microscopic examination for parasporal crystals confirmed the presence of *B. thuringiensis*. This indicated that the *B. thuringiensis* strains were able to translocate from the rhizosphere to the upper parts of the cauliflower plant. In present investigation colony forming units (CFUs) of the isolate in different parts of were determined to quantify the endophytic colonization within plants. The studies on population dynamics and colonization efficiency of *B. thuringiensis* indicated higher in roots in contrast to leaf and stem samples. Average CFU counts on 28th day post inoculation, were 2.9 × 107 leaves g^{-1}, 1.5 × 107 stems g^{-1} and 3.9 × 107 roots g^{-1}. Recovery of the bacterium from the leaves was inconsistent in all treatments. In another study, significantly higher CFUs were observed in shoots rather than roots [28]. [29] identified that *Bacillus thuringiensis* was found in 51% of the leaf samples of maize and beans, and in 33% of the soil samples collected from near these plants. The results suggest that the *B. thuringiensis* can move up from the soils to roots to stems and leaves indicating its endophytic nature. [30] reported that plants grown in sterile soils inoculated with *B. thuringiensis* strain 146-15702 (pHT315) recorded highest recovery 14 days after sowing.

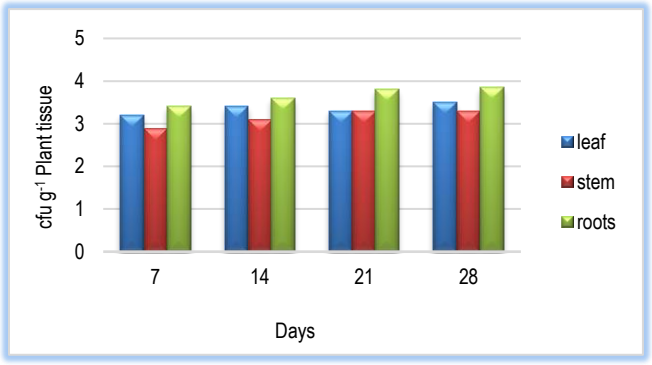

Fig-1 Colonization *B. thuringiensis* in leaf, stem and roots of the treated cauliflower plants

Estimation Cell wall degrading enzymes

Endophytic bacteria originate from seeds, vegetative planting materials, rhizosphere soil and phylloplane [31]. For initiating colonization, the endophytic bacteria need to first disrupt the plant cell wall that can facilitate the entry and further spread. Some bacterial endophytes are capable of producing plant cell wall-degrading enzymes that are active against cellulose, xylulose, pectin and endoglucon [32]. In the present study all five isolates of endophytic *B. thuringiensis* were tested for production of cellulose, pectinase, protease and endogluconase [Table-1]. However, All the five isolates produced cellulose, whereas only 4 isolates were able to produce pectinase. Highest cellulase and pectinase production were found in HD1 followed CF4 (1.8 and 1.6cm). Similarly [33] reported that among 78 bacteria isolated from the rice rhizosphere, 33 isolates produced cellulose. Most of the cellulase producers belonged to the genus *Bacillus*. [34] testified that 40 isolates of *B. thuringiensis* were found to produce pectinase and also [35] state that out of 23 alkaliphilic bacteria, 12 isolates were able to produce protease on skim milk agar medium. In our studies *B. thuringiensis* having the ability to solubilize the casein and CMC. Among the isolates (HD1, CF2, CF4, EP1 and EP3) isolate CF4 produced maximum protease and endogluconase.

Phosphorus solubilization

Phosphorous is particularly essential nutrient for growth and development of plants. It plays an important role in plants such as cell division, photosynthesis and development of root system and utilization of different carbohydrates.

All five *B. thuringiensis* isolates showed positive phosphate solubilization. The maximum phosphate solubilization was recorded by the isolate HD1-(1.4cm). The minimum phosphate solubilization was found in EP1-(0.4cm) isolates. The present study was comparable to [36] specified among the isolate 10 were showed positive phosphate solubilization. [37] also documented that the isolate PSB1 showed highest phosphate solubilization in (66.32 mg/L) followed by PSB8 (61.78 mg/L). The bacteria involved in P solubilization can enhance plant growth by increasing the availability of other trace elements such as iron and zinc [38].

Zn solubilization

Zinc (Zn) is an important micronutrient playing major role in various cellular functions in plant. Endophytic bacteria have the ability to convert insoluble form in to soluble form of Zn and improve the Zn availability to plants. The Zn solubilization potential of endophytic bacteria attributed to the production of certain chelators or secretory enzymes that convert insoluble Zn to the biologically available form. In plate assay method all the endophytic *B. thuringiensis* subjected to measure Zn solubilization efficiency on Tris-minimal medium amended separately with zinc oxide and zinc phosphate as inorganic source of zinc. All five endophytic *B. thuringiensis* solubilized zinc phosphate incorporated in Tris-minimal medium producing a clear halo zone around the colony [[Table-1]. Maximum diameter of halo zone was observed in CF4 (1.2cm) which higher than the reference strain. Our results are supported by the findings of [39] who reported that the zinc-solubilizing *Bacillus* strains recovered from soybean rhizophere soil significantly increased Zn content in soybean seeds. [40] isolated zinc solubilizing bacteria from the rhizosphere of rice and reported that rice seedlings inoculated with species from *Pseudomonas, Ralstonia, Burkholderia* and *Klebsiella* showed significant plant growth compared to uninoculated seedlings.

Phytohormones production enhance the plant growth

Phytohormones have important functions in plant growth and development as regulators and signals molecules produced by the microbes. They help in fixing atmospheric nitrogen, solubilization of minerals such as phosphorus and potassium, production of siderophore that solublize and sequester iron, and production of plant growth regulators [41]. In the present investigation five selected endophytic *B. thuringiensis* isolates were evaluated for qualitative production in vitro based on the phytohormones (IAA, GA3) and siderophore production in chemically defined medium.

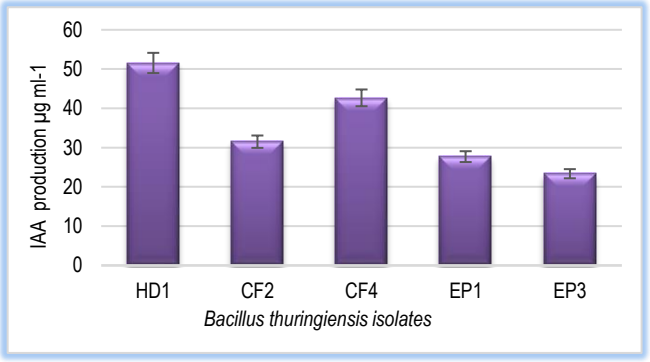

Fig-2 Estimation of IAA production of *Bacillus thuringiensis*

Indole-3-acetic acid (IAA) production

A member of the auxin family of phytohormones IAA influences many cellular functions in plants and therefore is an important signal molecule for the regulation of plant growth and development.

Table-2 *Endophytic Bacillus thuringiensis on development cauliflower seeding Treatment*

Treatment	Length of aerial part (cm)	Length of Roots (cm)	Fresh Biomass (g/Plant)	Dry Biomass (g/Plant)	Numbers of Leaves / Plants
HD1	7.40±1.51	7.52±2.04	0.28±0.06	0.027±0.03	2.9±0.31
CF2	8.08±1.94	9.88±1.41	0.50±0.06	0.019±0.05	3.0±0.03
CF4	8.96±1.52	9.67±5.14	0.45±0.07	0.023±0.04	3.0±0.47
EP1	8.75±0.99	8.70±1.27	0.31±0.04	0.020±0.04	2.7±0.48
EP3	8.27±1.04	9.07±3.37	0.31±0.11	0.021±0.05	3.1±0.56

Endophytic *B. thuringiensis* isolates (HD1, CF2, CF4, EP1, EP3) were tended to produce high amount of IAA in the range of (23 - 51) µg ml^{-1} at 7th day of incubation. In the presence of tryptophan, significantly high amount of IAA production was observed in HD1 (51.55 µgml^{-1}) followed by CF4 (42.67 µgml^{-1}) [Fig-2]. [42] reported all the seven *Bacillus* isolates were exhibited IAA production in which one *Bacillus* sp BPR7 produce maximum IAA production (17 µgml^{-1}).

Gibberllic acid (GA3) production

The Gibberellic acid produced by the isolate CF4 was 356.80 µg ml^{-1} of GA3. Least amount was showed by isolate EP3 (281.1 µg ml^{-1}) The standard strain showed the maximum amount GA3 production [Fig-3]. This result was in accordance with [43]. [44] also reported that the gibberellic acid was produced by Pseudomonas fluorescens and *Bacillus subtilis*.

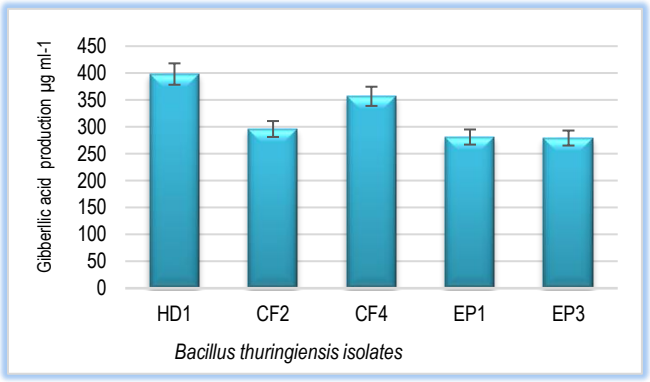

Fig-3 Estimation of GA3 production of *Bacillus thuringiensis*

Siderophore production

Siderophores are low-molecular weight secondary metabolites with iron-chelating potential. Which help in fulfilment of the iron requirement of plants by iron acquisition through chelation and facilitate plant bacterial association as well as colonization of roots, stem and leaf thus. Also, they make iron unavailable to pathogenic microorganisms. It was observed that all the *B. thuringiensis* strains taken in this study were positive for siderophore production. The isolate HD1 recorded maximum siderophore production (44.38µg ml^{-1}) and least siderophore production was by EP3 (16.04µg ml^{-1}) [Fig-4]. [45] demonstrated two endophytes *viz. Bacillus cereus* MQ23 and MQ23II isolated from *Sophora alopecuroides* root nodules were able to produce siderophores on CAS medium.

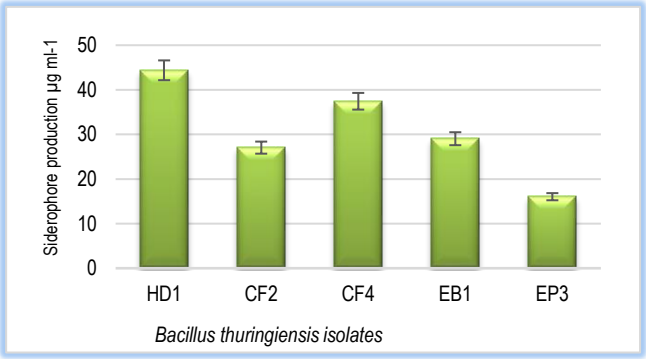

Fig-4 Estimation of siderophore production of *Bacillus thuringiensis*

Effect of *B. thuringiensis* strains on development of cabbage seedlings

Inoculation of *B. thuringiensis* directly promotes the growth of plants and can also indirectly induce growth by suppressing plant diseases. *B. thuringiensis* can colonize the roots of legumes, which leads to an increase of nodulation and growth of the plants [46]. In the present investigation the effect of inoculation of endophytic *B. thuringiensis* on the root and shoot length, and overall yield of the plant was studied. The seed germination and seedling development of cauliflower after seven days of planting resulted in the increase of seedling length. Thirty days after germination, the growth of the aerial parts was found improved by *Bacillus thuringiensis* strain (CF4) when compared to the controls. The root and shoot biomass were found increased during seedling stage, which were higher than control plants [Table-2]. Similarly, the results conform to that of [47]. [48] examined the tomato roots treated with *B. thuringiensis* culture filtrate increased the shoot length from 43.1% to 108% and root length from 13.7% to 65.6%.

Conclusion

Recent studies indicated that the *Bacillus thuringiensis* can successfully colonize cauliflower plants as endophyte and play an active role in plant growth. It is found that *B. thuringiensis* have the potential to enter through plant parts by using cell wall degrading enzymes. *B. thuringiensis* isolates are capable of releasing phytohormones which influence the growth of the inoculated plants. They also produce metabolites which solubilize the phosphate and zinc. The results obtained in this study with those of previous studies confirm *B. thuringiensis* as a polyvalent biocontrol, biofertilising and bio-stimulating bacterium whose plant growth promoting properties can very well be utilized in cabbage production.

Application of research: Endophytic *Bacillus thuringiensis* produce growth regulators IAA, Gibberllic acid, Siderophore which enhance plant growth and crop yield.

Research Category: Agricultural Microbiology

Abbreviations: PGRP: Plant growth promoting rhizobacteria,
CMC: Carboxymethylcellulose, IAA: Indole acetic acid, GA3: Gibberellic acid,
LB: Luria-Bertani, YEP: Yeast extract peptone

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University: Tamil Nadu Agricultural University, Coimbatore, 641003, India
Research project name or number: PhD Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Coimbatore

Cultivar / Variety / Breed name: Astra plus

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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