Original Research Article

Effect of foliar application of plant growth promoting *Bacillus amyloliquefaciens* on feeding characteristic of *Spodoptera frugiperda* on maize leaves

Abstract

Maize is a third important cereal crop which is heavily affected by invasive pest *Spodoptera frugiperda*. Alternate biological mode of control is necessary instead of seeking inorganic chemical based control. Plant endophytes could be of great option for controlling plant pathogens and pest. To this context, the present study aimed to evaluate the potential of *Bacillus amyloliquefaciens* from maize (COH6) leaf apoplastic fluid. This bacterium was found to have plant growth promoting traits like indole acetic acid, siderophore, ammonia and hydrogen cyanide production. In addition, it was found to produce hydrolytic enzymes such as protease, pectinase, chitinase, and lipase which imply its bioprotective potential. Further foliar spray of *B. amyloliquefaciens* with cell concentration of 10^8 cfu ml^{-1} on 4 days old maize @ 5 ml per plant showed greater colonization percentage over other doses (1, 2, 3 & 4 ml $plant^{-1}$). The highest feeding deterrence was observed when *Spodoptera frugiperda* fed on leaves inoculated with 5 ml of *B. amyloliquefaciens*.

Key words *Bacillus*, fall armyworm, endophytes, plant protection and maize

Introduction

Maize is an important cereal crop used as food, feed and forage. It is also one of the components of various industrial products. Production of maize grain accounts for around 6% of all cereals production (Erenstein et al., 2022). The production and productivity of maize grain in the past few years reduced significantly due heavy infestation by the invasive pest *Spodoptera frugiperda* (Assefa et al., 2019). Although many chemical agents are available for control, it is necessary to develop eco-friendly management techniques.

Plant associated beneficial microbes not only improve plant nutrition. They also improve plant health by imparting resistance and/or resilience against abiotic and biotic stressors. Particularly, endophytes which reside inside the plant are essential for the growth and health of plants. In plants, apoplast is a place of interaction between external invaders and microorganisms (Wang et al., 2020). This particular niche is considered as major space for endophytic microorganisms with the ability to induce plants tolerance against various stressors (Du et al., 2016). Among various

endophytic bacterial genera, *Bacillus* spp are common and dominant endophytic bacteria which reside in most of the plant species (Deng et al., 2019). Metabolites of *Bacillus* sp were found to play important role in plant growth and defence elicitation against various environmental stressors (Shafi et al., 2017). In addition, *Bacillus* sp with the ability to produce plant growth hormones such as indole acetic acid (IAA), gibberellic acid (GA) improves plant growth and defence (Hashem et al., 2019). In yet another study (Shahid et al., 2021), it was revealed that plant endophytic *Bacillus* spp producing cell-wall-degrading enzymes such as chitinases, protease, cellulase, glucanase, and metabolites like lipopeptides and hydrogen cyanide is capable of providing defense against numerous pathogenic bacteria, fungi, nematodes, viruses and pests. *Bacillus* induced physiological changes such priming antioxidants and defence related metabolites against biotic and abiotic stressors in plants was also evidenced (Meena et al., 2017).

In this context, the present study was aimed to characterize apoplastic fluid isolate namely *Bacillus amyloliquefaciens* MZ895491 for its potential plant growth promoting and bioprotective traits against *S. frugiperda* infestation in maize under gnotobiotic conditions.

Material and methods

Maize seeds, bacterium and larvae used for the study

Maize seeds were obtained from the Department of Millets, Tamil Nadu Agricultural University, Coimbatore. The bacterium, *B. amyloliquefaciens* (MZ895491) was isolated from maize (COH6) leaf apoplastic fluid (unpublished data). *Spodoptera frugiperda* egg mass was obtained from National Bureau of Agricultural Insect Resources (NBAIR), Bangalore, India.

Plant growth promoting characteristic of *B. amyloliquefaciens*

Indole acetic acid (IAA)

10 ml of Luria-Bertani medium was inoculated with 1 ml *B. amyloliquefaciens* culture and incubated at room temperature for 7 days. After incubation, the broth was centrifuged at 12,000 g for 15 min. Then 1 ml supernatant was mixed with 2 ml of Salkowski reagent. Development of pink colour indicated positive test for IAA production (Gang et al., 2019).

Siderophore

A loopful of log phase culture was streaked on chrome azurol succinic (CAS) acid medium and incubated for 48 hr (Lenin et al., 2012). Yellow colour halo zone formation around the colonies indicated positive test of siderophore production

Ammonia

The bacterium was cultured in 10 ml peptone broth and incubated for 72 h at 30°C. After incubation, the culture was centrifuged at 10,000g for 10 min and the supernatant was collected. To the supernatant (1 ml), 0.5 ml of Nessler's reagent was added. Development of yellow colour indicated positive result of ammonia production (Kumari et al., 2018)

Hydrogen cyanide (HCN)

The bacterial culture was streaked on tryptic soy agar medium containing glycine (4.4 g/l). Alkaline picric acid soaked filter paper was placed in the lid of petriplate and sealed it with parafilm and incubated at room temperature for 4 days. A change in colour of the filter paper from yellow to brown indicated positive test of HCN production (Kesaulya et al., 2015).

Lipase

The bacterium was streaked on tributyrin agar medium and incubated for 2 days at room temperature. The positive lipase activity was observed from formation of clear zone around the colony (Veerapagu et al., 2013).

Protease

A loopful of *B. amyloliquefaciens* was streaked on skimmed milk agar medium. Clear zone around the colony indicated positive result (Olajuyigbe et al., 2005).

Pectinase

Log phase culture of *B. amyloliquefaciens* was streaked on pectinase screening medium and incubated for 2 days at room temperature. Clear zone around the colony indicated positive test for pectinase activity (Rania et al., 2016).

Chitinase

Log phase culture of *B. amyloliquefaciens* was streaked on colloidal chitin agar medium and incubated for 7 days at 30°C. Clear zone indicated positive test for chitinase activity (Wang et al., 2014).

Growth curve analysis of *B. amyloliquefaciens*

The growth pattern of *B. amyloliquefaciens* was assessed by measuring the optical density (OD) at 600 nm at 4h interval for 48 h (Sethuraman and Balasubramanian, 2010). Using the OD value growth curve was obtained. Generation time and specific growth rate was calculated as follow

$$G = t/n$$

$$N = \frac{(\log N1 - \log N0)}{\log 2}$$

$$R = \frac{1}{G}$$

'G' is the generation time, log N0 and log N1 are the number of cells at an early and late time point in exponential phase respectively,'t' is the time between 'N0' and 'N1' and 'R' is the specific growth rate.

Maize leaf colonizing potential of foliar treated *Bacillus amyloliquefaciens* and leaf feeding bioassay with *S. frugiperda*

Experimental design

Maize seeds (COH6) were surface sterilized with 1.6% sodium hypochlorite and placed on Hoagland's nutrient agar medium (gnotobiotic condition). *B. amyloliquefaciens* grown in nutrient broth (24h) was centrifuged at 12,000 g for 15 min and the bacterial concentration (10^8 cfu ml^{-1}) was adjusted with sterile distilled water. After 4 days of seed germination, the culture was sprayed over the foliar region using hand sprayer with different volume (1, 2, 3, 4 and 5 ml). Control plants were sprayed with sterile distilled water. Totally two sets were maintained. One set was used for whole plant bioassay and another set was used for re-isolation of *B. amyloliquefaciens*. Each set contained six treatments and three replications.

Re-isolation of *Bacillus amyloliquefaciens* from treated maize leaves

B. amyloliquefaciens colonization in maize was evaluated through re-isolation technique.After 48 h of foliar spray, the plants were uprooted and the leaves were surface sterilized with 70% alcohol for 1 min. After that immersed in 2.5% sodium hypochlorite and finally 30 sec in 90% ethanol; then thoroughly washed with sterile distilled water ten times (Nxumalo et al., 2020). After surface sterilization, the leaves were blotted on sterile filter paper. Surface sterilized leaves (1g) were ground with 5 ml of phosphate buffer (pH 7.2). After settlement, 1 ml of the leaf extract was serially diluted up to 10^8 and plated on nutrient agar medium. After 24 h of incubation, the colonies were counted.

Whole plant bioassay

After 48h of *B. amyloliquefaciens* spray, two numbers of second instar larvae of *S. frugiperda* (starved for 2h) were allowed to feed on maize leaf for 24 h. Then, the nutritional indices such as relative growth rate (RGR), relative consumptive rate (RCR), efficiency of conversion of ingested food (ECI) and feeding deterrence index(FDI) of *S. frugiperda* larvae were calculated following standard procedure (Waldbauer, 1968).

Plant biomass

After 24h of larval attack, the plant total biomass was calculated and denoted as gram per plant (on dry weight basis).

Statistical analysis

Statistical analyses were carried out using Microsoft Excel (version 2010) and SPSS (version 16.0). All the analyses were done with minimum of three replications. The Duncan's multiple range test (DMRT) was carried out at $P \leq 0.05$ for bioassay and biomass production analysis.

Results and Discussion

Bacillus spp is one of the common beneficial bacteria inhabiting many plants and improves plant growth and health (Hashem et al., 2019). Particularly, *B. amyloliquefaciens* gained greater interest among scientific community due to its potentiality to elicit plant defence against numerous phytopathogens (Ji et al., 2013) and herbivores (Li et al., 2015). The present study was aimed to evaluate the effect of *B. amyloliquefaciens* of maize leaf apoplastic fluid against *S. frugiperda* infestation in maize.

Plant growth promoting characteristics of *B. amyloliquefaciens*

The culture was qualitatively assessed for its ability to produce indole acetic acid (IAA), siderophore, ammonia, hydrogen cyanide (HCN) and hydrolytic enzymes such as lipase, protease and chitinase. Indole acetic acid is one of the important plant growth promoting phytohormones (Duca et al., 2014). Siderophore is an iron chelating compound which plays important role in plant growth through enhanced iron availability. At the same time affect the growth of plant pathogens by depriving them the iron (Villarreal-Delgadoet al., 2018). Hydrogen cyanide (HCN) is an important secondary metabolite which is toxic to biotic stressors (Brzezinska et al., 2020). In the present study, the apoplastic fluid bacterium *B. amyloliquefaciens* showed positive results for IAA, siderophore, ammonia and HCN production. The ability to produce hydrolytic enzymes such as

lipases, proteases, pectinases, and chitinases indicates the biocontrol property of microorganisms (Jadhav et al., 2017). Lipases hydrolyse waxes, lipoproteins and fat of the insects (Gandotra et al., 2018). Proteases affect insect cuticles, midgut and hemocoel (Sugio et al., 2015). Chitinases break the cuticle of insect cell wall (Veliz et al., 2017) and pectinases have a role in pest control by affecting the insect gut (Shelomi et al., 2016). In the current study, *B. amyloliquefaciens* culture was shown to produce all the above mentioned hydrolytic enzymes (table 1).

Table 1 Qualitative analysis of plant growth promoting and bioprotective characteristics of maize apoplastic fluid associated *B. Amyloliquefaciens*

Treatment	IAA	Siderophore	Ammonia	HCN	Lipase	Protease	Pectinase	Chitinase
BA	+	++	+++	++	++	+++	++	+++

Note: BA- *Bacillus amyloliquefaciens*, IAA- indole acetic acid, HCN- hydrogen cyanide, + - less; ++ - moderate; +++ - High

Growth curve of *B. amyloliquefaciens*

Growth curve of *B. amyloliquefaciens* grown in LB broth is shown in figure 1. The results revealed absence of lag phase and very lengthy log phase of 20 h. Similarly, the stationary stationary phase was observed between 20 and 40 hr. The generation time and the specific growth rate of the culture were 5.2 ± 0.03 h and 0.142 ± 0.01 h^{-1} respectively

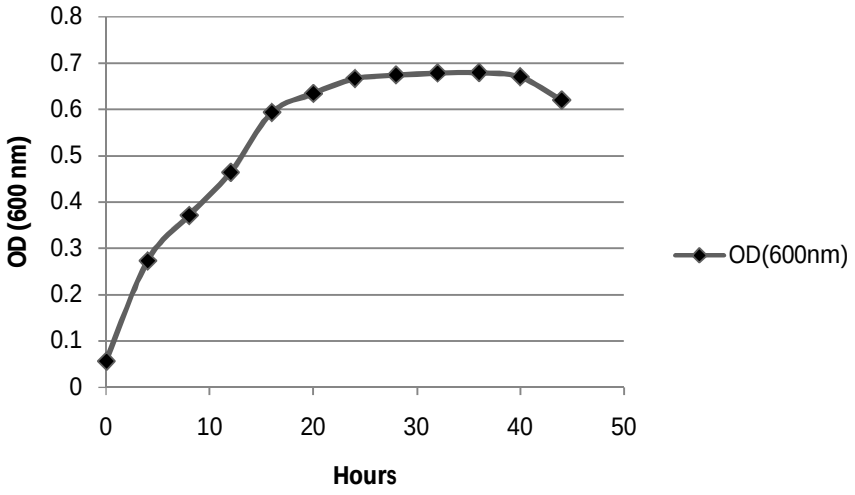

Figure 1. Growth curve of *B. amyloliquefaciens*

Re-isolation of *B. amyloliquefaciens* from maize leaf endophytic region

The apoplastic endophytic bacterium *B. amyloliquefaciens* was sprayed at different doses (1, 2, 3, 4, and 5 ml plant^{-1}) with a concentration of 3.2 x 10^8 cfu ml^{-1} on maize grown under gnotobiotic condition (table 2). Leaf endophytic colonization capacity of *B. amyloliquefacines* (BA) was analysed by re-isolation technique. The highest colonization (8.30×10^8cfu g^{-1}) was observed in T_6 (5 ml BA) followed by T_5 (11.93×10^7cfu g^{-1}). The lowest colonization was (1.90×10^4cfu g^{-1}) observed in T_2 (1 ml BA). Complete absence of the endophytes was noticed in un - inoculated control.

Table 2. Effect of various doses of *B. amyloliquefaciens* foliar spay on maize endophytic colonization under gnotobiotic condition

Treatments	**Bacterial count (cfu g^{-1} FL)**
T_1 (C)	ND
T_2 (1ml BA)	1.90×10^4 (±0.48)
T_3 (2mlBA)	3.13×10^6 (±0.52)
T_4 (3ml BA)	6.70×10^6 (±0.12)
T_5 (4mlBA)	11.93×10^7 (±0.37)
T_6 (5ml BA)	8.30×10^8 (±0.41)

Values are the mean ± standard deviation of experimental data intriplicate. FL- fresh leaf, BA- *Bacillus amyloliquefaciens*, ND - not detected.

Whole plant bioassay

B. amyloliquefaciens inoculation significantly altered the feeding characteristics of *S. frugiperda*. The relative growth rate (RGR) of larva was reduced with increase in the dose of *B. amyloliquefaciens* inoculation (table 3). The relative growth rate (0.53 ± 0.08mg g^{-1} day^{-1}) of *S. frugiperda* fed on maize inoculated with 1 ml BA (T_2) was on par with un-inoculated control T_1 (0.55 ± 0.03 mg g^{-1} day^{-1}). The RGR of *S. frugiperda* fed on plants of T_3 (2 ml BA) recorded 0.43 ± 0.01 mg g^{-1} day^{-1}) and T_4 (3 ml BA) recorded 0.43 ± 0.09 mg g^{-1} day^{-1}. The lowest growth rate of *S. frugiperda* (0.20 ± 0.02 mg g^{-1} day^{-1}) was observed in T_6 (5ml BA) which was on par with T_5 (0.27 ± 0.01 mg g^{-1} day^{-1}).

Other indices like the relative consumptive rate (RCR) of larva was lower in T_5 (5 ml BA) (20.0 ± 2.98 mg g^{-1} day^{-1}) which was on par with T_5 (4 ml BA) (21.67 ± 0.19 mg g^{-1} day^{-1}). Efficiency of conversion of ingested food was higher in T_1- C+SF (0.21%) and it was on par with T_2 - 1 ml BA. Of different doses of inoculation, T_3 (2 ml BA) and T_4 (3 ml BA) inoculation was one par with each other. The lowest conversion efficiency was observed in T_5 – 4 ml BA (0.12%) and T_6 5 ml BA

(0.10%). The feeding deterrence index was higher in T_6 - 5 ml BA (3.97%) followed by T_4 – 3 ml BA (3.79%) and T_5 – 4 ml BA (3.42%). The lowest feeding deterrence was observed in T_2 -1 ml BA (1.55%) and T_3 – 2 ml BA (1.54%).

Similarly, inoculation of endophytic *B. amyloliquefaciens* in hosta plant reduced the feeding of *S. frugiperda* larvae and increased the mortality rate by 30% (Li et al., 2015). Khedher et al., 2017 reported that surfactant produced from *B. amyloliquefaciens* AG1 reduced the *S. littoralis* infestation. *Myzus persicae* diet inoculated with cell suspension and cell free supernatant of *B. amyloliquefaciens* reported to cause 100% mortality rate (Guadalupe Lopez Isasmendi et al., 2019). *B. amyloliquefaciens* A1 inoculation was found to cause 84.29% mortality rate of citrus mealybug (Mohamedova et al., 2017).

Table 3 . Whole plant bioassay with *S. frugiperda* on maize leaves sprayed with *Bacillus amyloliquefaciens* (10^8cfu ml^{-1}) under gnotobiotic condition

Treatments	RCR (mg g^{-1} day^{-1})	RGR (mg g^{-1} day^{-1})	ECI (%)	FDI (%)
T_1 (C+SF)	26.59 (±0.93)^a	0.55 (±0.03)^a	0.21 (±0.03)^a	0.00
T_2 (1ml BA+SF)	26.66 (±1.32)^a	0.53 (±0.08)^a	0.20 (±0.04)^a	1.55 (±0.19)^c
T_3 (2 ml BA+SF)	26.50 (±2.41)^a	0.43 (±0.01)^b	0.16 (±0.02)^b	1.54 (±0.21)^c
T_4 (3 ml BA+SF)	26.21 (±1.12)^a	0.43 (±0.09)^b	0.16 (±0.08)^b	3.79 (±0.39)^a
T_5 (4 ml BA+SF)	21.67 (±0.19)^b	0.27 (±0.01)^c	0.12 (±0.01)^c	3.42 (±0.79)^b
T_6 (5 ml BA+SF)	20.00 (±2.98)^b	0.20 (±0.02)^c	0.10 (±0.01)^c	3.97 (±0.82)^a
P	0.046	0.06	0.055	0.07

Values are the mean ± standard error of experimental data in triplicates. Values with different letters are significantly different according to Duncan's test; $P \leq 0.05$. RGR- Relative growth rate; RCR- Relative consumptive rate; ECI- Efficiency of conversion of ingested food; FDI- Feeding deterrent index; C- Control; SF- *Spodopterafrugiperda*; BA-*Bacillus amyloliquefaciens*

Plant biomass

The plant biomass content of *B. amyloliquefaciens* inoculated (1ml to 2ml) maize after 24h of *S. frugiperda* attack was found to be on par with each other for doses of 1ml to 3ml $plant^{-1}$. However higher dry biomass value was observed in T_6 (5ml BA) and T_5 (4ml BA) which was on par with each other (figure 2).

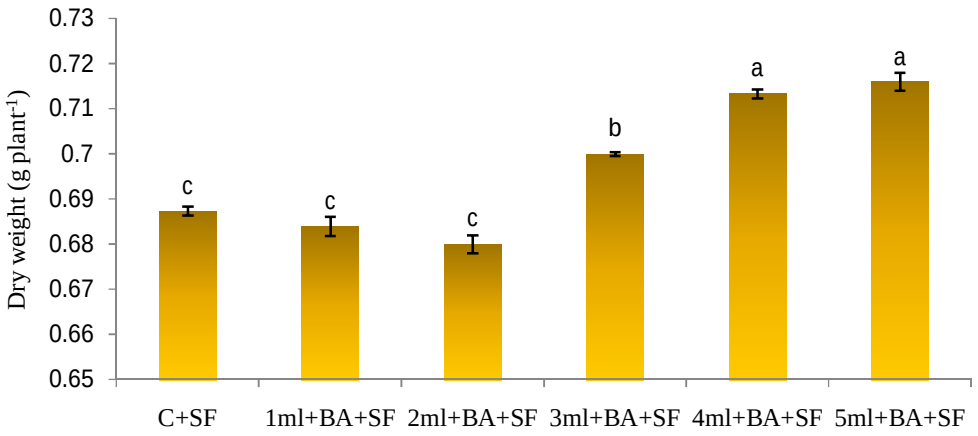

Figure 2 Biomass content of maize inoculated with different doses of *B. amyloliquefaciens* in the presence and absence of *S. frugiperda*

Conclusion

Changing climatic conditions increased the pest and disease attack. In this regard, it is imperative to uncover proper eco-friendly mitigation measures for sustainable agricultural production. The current study revealed the potentiality of apoplastic fluid *B. amyloliquefaciens* in reducing the feeding capacity *S. frugiperda* on maize leaves colonized with this endophyte. Foliar spray of *B. amyloliquefaciens* @ 5ml/plant significantly reduced the *S. frugiperda* growth. Thus, after confirming the effect of bacterial inoculants at field level, this can included as one of the components of integrated pest management system for sustainable agricultural production.

Data availability statement Raw sequence data of *Bacillus amyloliquefaciens* reported in this paper have been deposited in the NCBI GenBank under accession number MZ895491.

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