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Soybean Bacterial Endophytes *Bacillus subtilis* (EB-1) and *Bacillus amyloliquefaciens* (EB-2) against Anthracnose survival in Leaf and Soil

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ABSTRACT: Bacterial endophytes employ various direct and indirect mechanisms for plant growth promotion under biotic stress upon colonization. To successfully establish endophytes as plant growth promoters, colonization and population density-dependent process, quorum sensing is essential. Therefore, the present study aimed at assessing the efficacy of foliar and basal application of two soybean plant growth-promoting bacterial endophytes having biocontrol activity against soybean anthracnose, namely *Bacillus subtilis* strain 5 (EB-1) and *Bacillus amyloliquefaciens* strain 14 (EB-2) in the soybean seedling. These two endophytes priliminarily screened for antibiotic resistance using a disc diffusion method to make the selective culture medium suitable for bacterial re-isolation from plant tissues. Bacterial endophytes were applied as foliar and basal inoculation on the soybean variety. Then, the bacterial population within the leaf tissues and rhizosphere soil was enumerated in the nutrient agar plate amended with antibiotics. Our study showed that the EB-1 and EB-2 showed significantly lower survival in the leaf tissues and the rhizospheric soils. However, the bacterium EB-1 and EB-2 survival rates were significantly higher in the leaf and soil tissue, respectively, compared with each other. Further, we identified the maximum survival period of these bacterial endophytes was 30 days in leaf tissue. Therefore, the present findings showed that endophytic bacterial suspension has to be applied at 30-day intervals. And we identified the maximum survival period of these bacterial endophytes in leaf tissue and soil and explained their role in providing biological control against diseases.

Keywords: *Bacillus subtilis* (EB-1), *Bacillus amyloliquefaciens* (EB-2), colonization, antibiotic susceptibility, Anthracnose, and survival.

INTRODUCTION

Soybean is a proven driver of the rural economy and has acted as an oil and protein supplement source in India since its introduction in the late sixties (Natraj *et al.*, 2019). It in great demand in the livestock industry, which generates economic wealth due to lucrative prices in the world market Rajput *et al.* (2021a) stated that at several phases of its growth cycle, from seed germination to full maturity, soybeans are vulnerable to a variety of stress factors originating from both living organisms and environmental variables. Approximately 3.33% of the world's human calorie intake is attributed to soybeans (FAOSTAT, 2018). Soybeans are susceptible to a variety of stresses deriving from living organisms and environmental elements at different phases of their growth, ranging from seed germination to full maturity. Its production is mainly hindered the increased frequency of biotic stress due to climate change. The key biotic stress includes diseases like anthracnose, *Rhizoctonia* root rot, charcoal rot and yellow mosaic virus (Rajput *et al.*, 2023). *C. truncatum* is prevalent in almost all locations across the world, where soybean is grown. Soybean is susceptible to this pathogen at all its growth stages. The disease becomes more prevalent by warm temperatures (30-35°C) and rain mist, which provides moisture for 12 hours or more and causes 16-25% of yield loss in India (Nataraj *et al.*, 2019; Nataraj *et al.*, 2023). Recently, researchers observed that each percentage increase in soybean anthracnose disease severity caused nearly 1 t/ha yield loss by Rajput *et al.* (2022). Recently, Kumar *et al.*, (2023) suggested milkweed can act as alternative and primarily source for spread of soybean anthracnose that make more difficult to manage the disease.

Modern agriculture needs sustainable production to meet food and nutritional security with minimum ecological disturbance by cheaper, low-cost input. Microbial entities associated with soybean plants constitute phytomicrobiomes with immense potential to combat abiotic and biotic stresses and boost crop production. As per Du *et al.* (2017), specific endophytic bacteria assume a crucial role in plant defense by integrating into the plant system and

thriving within the host tissue without causing any harmful effect. The consensus is that bacterial endophytes and plants have a mutually beneficial relationship, wherein plants provide nutrition to endophytes in return for protection against plant diseases (Qin *et al.,* 2017). Plants can defend themselves against pathogens through antagonistic interactions or by triggering mechanisms like induced systemic resistance and the biochemical analysis revealed the production of cell wall-degrading enzymes, including chitinase, cellulase, amylase, protease, and β 1-3 glucanase, which play a crucial role in degrading the cell wall of the pathogen by Chauhan *et al*. (2022). Endophytes induce their host plants to develop a wide range of defense mechanisms, triggering both primary and secondary defense systems (Jaiswal *et al.,* 2023). Upon inoculation, the endophytic bacteria enter the plants actively and passively and are transferred horizontally and vertically. Passive entry occurs through natural openings like stomata, near lateral roots, and damaged tissues. Similarly, active entry occurs through the dissolution of the cell wall through the cell wall degrading enzymes like cellulases and pectinases (Khandel *et al.,* 2017). Vertical transmission occurs through seeds and pollen, whereas horizontal transmission occurs through rhizosphere and spermosphere transmission, entry through aerial parts like leaves, stem, flower, and fruits (Frank *et al.,* 2017). Pan *et al.* (2023) suggested that endophytic bacteria potentially enhance plant resistance to pathogens by niche competition, inducing host resistance, and activating the immune system. Bacterial endophytes are producers of antimicrobial substances. For example, the endophyte *Enterobacter* sp. strain 638 generates antibiotics 2-phenylethanol (Taghavi *et al*., 2010). In addition to their role in establishing the rhizosphere or rhizoplane, numerous bacteria from this region can infiltrate plants, occupying internal tissues, and have been shown to stimulate plant growth. Therefore, the present investigation aimed to understand the rhizospheric and leaf endosphere colonization and survival of the soybean bacterial endophytes having inhibitory activity against soybean anthracnose *Colletotrichum truncatum* in the leaf tissues and the soil upon foliar and basal application near the stem for successful establishment in the given niches.

MATERIALS AND METHODS

A. Soybean bacterial endophyte and inoculum preparation

Soybean bacterial endophytes *Bacillus subtilis* strain 5 (EB-1) and *Bacillus amyloliquefaciens* strain 14 (EB-2) have shown biocontrol activities against soybean anthracnose were obtained from the Division of Crop Protection, ICAR-Indian Institute of Soybean Research, Indore (Madhya Pradesh) were used for the endocolonization study. Previously, these two endophytes were characterised for plant growth-promoting traits, biochemically, and molecular identification through the 16s rRNA gene (Rajput *et al.,* 2021b). A loopful of bacterial culture was inoculated into the 100 ml nutrient broth (Composition: Yeast extract 3 g, NaCl- 5g, Peptone- 5 g, Distilled water- 1L) into a 250 ml flask and kept in a shaker at 28 °C and 180 rpm for 12 hr.

B. Antibiotic susceptibility test of the bacterial endophytes

The antibiotic susceptibility of two bacterial isolates was assessed using the disc diffusion method, by EI-Banna *et al.* (2021). These isolates were spread onto nutrient agar plates using a sterile L-shaped spreader following an overnight growth bacterial culture in nutrient broth. Standard antibiotic disc (Hi media) including Streptomycin (10 µg/disc), Cefazolin (10 µg/disc), Amoxyclav (30 µg/disc), Penicillin (10 µg/disc), Gentamicin (10 µg/disc), Cefuroxime (10 µg/disc), Rifampicin (10 µg/disc), Kanamycin (10 µg/disc), Deoxycycline (10 µg/disc), Tetracycline (10 µg/disc), Nalidixic acid (10 µg/disc), and Co-trimoxazole (10 µg/disc), were then positioned on the nutrient agar plates, followed by an incubation at 28 °C for 48 h. Subsequently, the antibiotic susceptibility pattern was assessed by measuring the inhibition zone. The organisms were categorised as either sensitive or resistant based on the recorded diameter of the zone of inhibition compared to nearby centimetres and following the guidelines in the DIFCO Manual 10th edition (1984).

C. Preparation of antibiotics nutrient agar

The nutrient agar (NA) medium was prepared by adding 18 gm of agar-agar into the nutrient broth medium composition and was autoclaved. After autoclaving and cooling the media, antibiotic amoxyclav 150 µl for EB-1 was prepared. Similarly, for EB-2, the combination of streptomycin 50 µl, cefazolin 50 µl, amoxyclav 150 µl, cefuroxime 50 µl, deoxycycline 50 µl, and co-trimoxazol 50 µl were added in nutrient agar before pouring into the petriplates.

D. Bacterial endophyte survival in soil and plant tissue

The soybean variety used in the present study was JS 95-60. In the first experiment, we applied a foliar spray of the endophytic bacterial suspension of a concentration of OD_{600}= 1.00 (5 × 10^8 cells/mL) into the soybean seedlings after 15 days of sowing. It was achieved by diluting the bacterial suspension with sterile distilled water. The bacterial suspension was then evenly sprayed onto the leaves at 5ml/plant in the early morning. In the second experiment, we inoculated 2 ml/ plant of bacterial suspension at the base of the stem after 15 d. For the first experiment, plant leaves were collected from plants inoculated with potential bacterial endophytes at various time intervals (3 d, 5 d, 7 d, 10 d, 15 d, and 21 d after inoculation).

The endophytes reisolated from the second trifoliate leaf by surface sterilising it into 70 % ethanol and 1% sodium hypochlorite, followed by washing it five times with sterile distilled water. Then, plant tissue was crushed into the sterilised mortar and pestle, and approximately 1 ml of crushed samples was serially diluted, from 10^{-1} to 10^{-4} dilutions. Further, 100 µl solution from the serially diluted samples was spread on a Nutrient agar medium amended with the antibiotics.

All plates were plated in triplicate and incubated at 28°C for 48 h. Similarly, in the second experiment, we checked the presence of the bacterium in the bacterial-inoculated soil by taking 1 gm of rhizospheric soil and serially diluting up to 10^{-4}. Then 100 microliters were spread into the nutrient agar plates amended with antibiotics.

E. Statistical analysis

The data was statistically analysed using IBM SPSS version 28.00, and graphs were prepared using GraphPad Prism software (San Diego, CA, USA).

RESULTS

A. Bacterial endophytes antibiotic susceptibility test

The antibiotic susceptibility test showed that EB-1 was resistant to Amoxyclav and was sensitive against all the tested antibiotics. Similarly, EB-2 showed resistance against Streptomycin, Cefazolin, Amoxyclav, Cefuroxime, and Co-trimoxazol (Table 1).

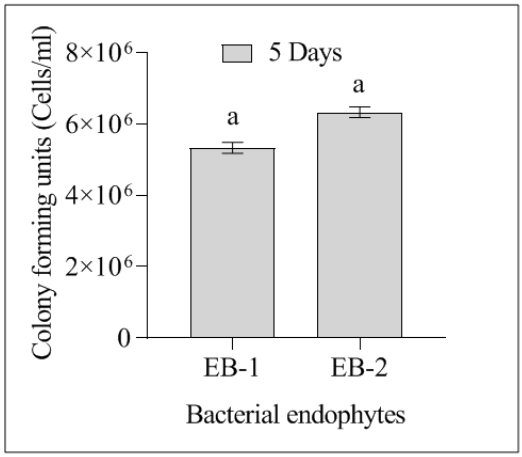
Table 1: Antibiotic susceptibility test of the soybean bacterial endophytes.

Isolate no.	EB-1	EB-2
Streptomycin	Susceptible	Resistance
Cefazolin	Susceptible	Resistance
Amoxyclav	Resistance	Resistance
Penicillin	Susceptible	Susceptible
Gentamycin	Susceptible	Susceptible
Cefuroxime	Susceptible	Resistance
Rifampicin	Susceptible	Susceptible
Kanamycin	Susceptible	Susceptible
Deoxycycline	Susceptible	Resistance
Tetracyclin	Susceptible	Susceptible
Nalidixic acid	Susceptible	Susceptible
Co-trimoxazole	Susceptible	Resistance

B. Bacterial endophyte survival in leaf tissue and soil

Our study observed that the EB-1 isolate exhibited a biological cyclic change in bacterial populations within leaf tissues. Specifically, there was an increase in bacterial population after five days, followed by a subsequent decrease after seven days. EB-2 isolate showed a continuous decrease in the bacterial population after five days in leaf tissues. The initial bacterial population inoculated as spray and basal inoculation was approximately 5×10^8 cells/mL. After five days, the bacterial count for EB-1 and EB-2 was 5.3×10^6 cells/ ml and 6.3×10^6 cells/ ml, respectively (Fig. 1). However, the bacterial count was below < 25 (Too less To Count) on 7 DAI, 10 DAI, 21 DAI, and 30 DAI in the leaf tissues. Similarly, we could not count the bacterial cells in the rhizospheric sample as the bacterial colony was below < 25 after five days of inoculation.

Similarly, the bacterial survival of the EB-2 isolate was significantly (p<0.001) higher than EB-1 in the leaf endosphere. Likewise, irrespective of the bacterial isolates, the bacterial colony survival significantly decreased as the days progressed. Furthermore, the two-way interaction between bacterial isolates and days was significantly (p<0.001) higher (Fig. 2[A]) and (Table 2a and 2b). Similarly, for soil samples observed at 5 d only, EB-1 showed a significantly higher bacterial survival than EB-2 (Fig. 2 [B]).



Colony (%) in leaf tissue

Fig. 1. Endophytic bacterial population colony forming units (cells/mL) in leaf tissues.

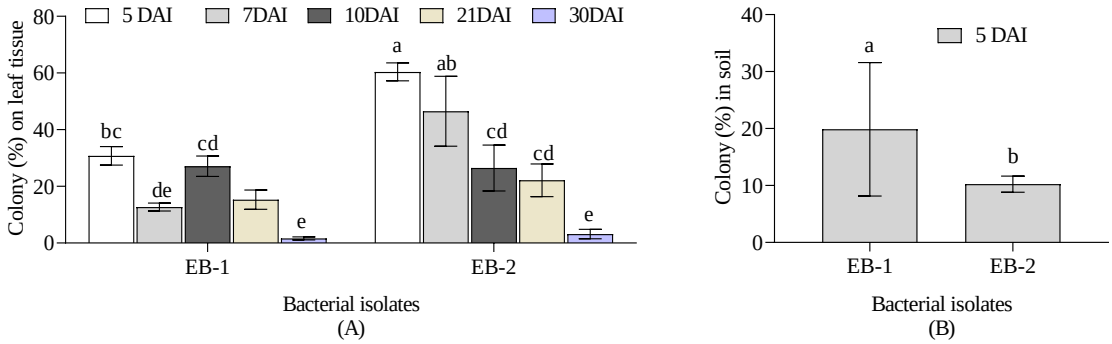
Table 2a: Two-way interaction ANOVA for the endophytic bacterial colony (%) upon re-isolation from leaf endosphere.

Isolate	Colony percentage	Days (df =4, 20)	Inoculation (df = 1, 20)	Days × Inoculation (df = 4, 20)
EB-1	17.50 ±11.08 [a]	P<.001	p<.001	p<.001
EB-2	31.73 ±21.47 [b]			

Table 2b: Two-way interaction ANOVA for bacterial colony (%) at different day intervals in leaf tissues.

Days	5 d	7d	10 d	21 d	30 d
Bacterial colonies (%)	45.57a	29.60b	26.78bc	18.71c	2.37d

Where, BE - Bacterial Endophytes, DAI - Days After Inoculation, LI - Leaf Inoculation, SI - Soil Inoculation.



Bacterial colony survival (%) on leaf tissue Bacterial colony survival (%) in soil

Fig. 2. Endophytic bacterial colony survival (%) of bacterial endophytes A) Leaf tissue (%), and B) soil (%).

DISCUSSION

The rhizosphere represents the hot spot for the signalling exchange between plant-microbe, microbe-microbe, or microbe-plant interactions. Plant roots release approximately 20-40 % photosynthetically fixed carbon compounds into the rhizosphere, which are compensated by chemoattracting beneficial microorganisms for various direct and indirect benefits for plant growth promotion under abiotic and biotic stresses. Heterotrophic rhizospheric microorganisms feed on the carbonaceous secretion of root exudates (Maheshwari *et al*., 2020). Similarly, microorganisms also release a wide array of microbial volatile organic compounds (MVOCs), phytohormones, and various secondary metabolites as microbial signals, which are involved in interactions with plants for plant growth promotion and suppress disease-causing soil-borne pathogens (Maheshwari *et al*., 2021). Both the inoculated endophytic bacterial population was significantly low compared to the initially inoculated population in leaf tissues and in the rhizospheric samples after five days. It indicates a possibility that, the plant excludes the soybean endophytes in the rhizosphere and the leaf endosphere.

In our study, the endophytic bacteria may have entered the leaf tissues using active or passive entry. However, driving factors for the phyllosphere colonization are host plant-based factors (genotypes, developmental stage, leaf age, leaf condition, canopy position, and plant immune system), seasonal variation (light, temperature, radiation, and precipitation), geographical location of plants, pollutants contamination (pesticides, herbicides, toxic metals and particulate matter) (Bashir *et al.*, 2022). Therefore, in the present investigation, the endophytic bacteria population decreased due to biotic factors upon inoculation. However, bacterial survival was observed in the leaf endosphere for up to 30 days. It showed that after 30 d, bacterial suspension has to be sprayed on the soybean.

Our results are in accordance with the previous report by Rai *et al.* (2006), who showed that bacterial endophytes isolated from field-grown maise, namely *Bacillus subtilis, Bacillus pumilus, Pseudomonas fluorescence and P. aeruginosa*, inoculated into maise grown in glasshouse, the bacterial population significantly decreased in the stem endosphere. Similar reports of inoculation of endophytic bacteria, namely *Pseudomonas aeruginosa* and *Burkholderia cepacia* in oil palm against suppression of Ganoderma showed a decrease in the bacterial population over time (Sapak *et al.*, 2008). The present findings involved the re-isolation using selective nutrient media. However, the present study findings had limitations of microscopic validation. Therefore, tracking the endophytic bacteria with green fluorescent protein-tagged upon plant inoculation and visualisation using the confocal scanning laser microscopy would reveal the bacterial colonization patterns.

CONCLUSIONS

We concluded that both the soybean bacterial endophytes applied through foliar and basal inoculation near the stem showed a significant decrease in the bacterial population. However, soybean bacterial endophytes could survive for up to 30 days in the leaf tissues. Therefore, we need to spray the bacterial suspension every 30 days to see the positive effect on plant growth and the inhibitory action against soybean anthracnose. Furthermore, other methods of bacterial delivery, such as seed treatments, should be tested for the survival and efficacy of the endophytes.

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