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ANTAGONIST EFFECT OF *BACILLUS* SPECIES AGAINST ALTERNARIA ROT OF TOMATO

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A B S T R A C T

The PGPR possesses antifungal activity against a broad range of pathogens. In this research study, the LPs of *Bacillus* strain B1 and B2 against *Alternaria alternata* were assessed. In vitro and *in* Planta assay on tomato fruit was made to check the post-harvest quality of fruits. The lipopeptides produced by both strains suppress the growth of the fungal pathogen *Alternaria alternata* and lessen the disease severity of the *Alternaria* rot on tomato fruits. The result revealed the quality of the *Bacillus*-treated fruits was also maintained and satisfactory. The lipopeptides of bacillus strain B1 and B2 represent a new innovative method to lessen the use of fungicides and chemical approaches to reduce the post-harvest loss caused by *Alternaria alternata*.

Keywords: Antifungal activity; *Alternaria alternate;* Planta assay

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INTRODUCTION

The tomato *Solanum lycopersicum* is regarded as the second most significant crop worldwide and in Mexico due to the large number of products derived from the fruit and the extensive surface devoted to its production (Ascencio-Álvarez et al., 2008). It is ingested in a variety of ways and is good for human health in large part because of the abundance of folic acid, lycopene, ascorbic acid, flavonoids, tocopherol, potassium, and phenolic chemicals it contains (Erba et al., 2013).

Most plant infections, particularly those of fungal origin, are the main cause of loss of between 10% and 40% of the yield both before and after harvest (Savary et al., 2019; Szalewski et al., 2018). Most of the pathogens including *Alternaria alternata* and *Penicillium digitatum* spp. induce huge degradation losses during the shipping and storage of tomatoes (Akhtar et al., 1994).

Synthetic fungicides are frequently employed to impede their formation and growth (Strange and Scott, 2005). When these several unfavorable effects, include a decline in soil biodiversity and pollution of the soil and food supply. Additionally, fungal diseases create defenses against fungicides, necessitating the usage of more of the currently available fungicides or the creation of brand-new fungicides (Crouzet et al., 2020; Strange and Scott, 2005).

Alternative techniques for eradicating plant pests have been the subject of numerous investigations, and biological control strategies are seen to hold promise. Biopesticides, particularly fungicides, bactericides, and other substances created by living organisms, are examples of biological control agents either the bacteria themselves or both (Sachdev and Cameotra, 2013). In contrast to traditional fungicides, which break down into poisons and persistent chemicals in food or the environment, bio fungicides are believed to be both effective and safe for future consumers (Kumar et al., 2021). As a result, there is a lot of interest in biocontrol agents for treating crops at the end of the vegetative season as well as for treating food and feed after harvest.

Biosurfactants can be used in agriculture to prevent and treat

plant illnesses as well as to encourage plant growth. The most well-studied groups of biosurfactants are classes of rhamnolipids and lipopeptides produced by bacteria of the *Pseudomonas* and *Bacillus* genera, respectively (Caulier et al., 2019; Ongena et al., 2005; Rudakova et al., 2021). Biosurfactants are substances with a variety of chemical structures and lengths. The diverse biosurfactants that are often produced by bacteria are complex mixtures whose individual structural variations affect the mixture's surface-active and bioactive properties (Geissler et al., 2017).

Several of them are based on *Bacillus* species, which are well-known for their abilities to fight against phytopathogens. The *Bacillus velezensis* species has been identified as a plant-associated bacterium in the *Bacillus amyloliquefaciens* operational group (*B. amyloliquefaciens*, *Bacillus siamensis*, and *Bacillus velezensis*), and they can directly or indirectly form beneficial connections with plants (Fan et al., 2017; Mishra et al., 2016). One of the most promising approaches for reducing the use of synthetic fungicides is biological control utilizing microbial antagonists, either on its own or as a component of an integrated control strategy (El Ghaouth et al., 2002).

The study aimed to assess the effectiveness of *Bacillus* species and investigate the effect of their LPs to control the *Alternaria alternata* in both *vitro* and in *Planta assay* experiments and investigate the LPs impact on the post-harvest quality of tomato fruits.

MATERIALS AND METHODS

Bacterial and fungal cultures

The bacterial and fungal cultures were available at Plant Pathology Department, Islamia University Bahawalpur. The bacterial culture was revived in the Luria Bertani broth (Lb) medium for this experiment. The recipe of LB medium contained (10 g of peptone, 5 g of yeast extract, 10 g of sodium chloride, and 20 g of agar per 1000 ml of distilled water) and the culture was incubated at 37 °C at 160 rpm.

The fungal culture was grown on Potato dextrose agar (PDA) medium, an artificial growth medium. A single spore culture technique was used to create a pure culture of an isolated fungus, and it was then transferred to a fresh Petri plate with PDA medium at 26 °C for two weeks (Kilani-Feki et al., 2016). With the aid of a hemocytometer made from an old fungal culture, 10^4 or 10^6 cfu spores/ml of fungal conidial suspension were created in sterilized water.

Pathogenicity test

The fully-ripped tomato was purchased by the local fruit market in Bahawalpur, Pakistan. The final selection was based on color, size homogeneity, and the absence of physical defects. Fruits were first washed with tap water before treatment, then disinfected with a 75 % alcohol solution, rinsed with sterile distilled water, and dried before creating a puncture wound. Using a sterile needle, each sample was uniformly punctured (3 mm) in the fruit's equator and 10 mm deep (Khedher et al., 2015).

Antagonism assay with *Bacillus* strains

A dual culture assay was used to measure the antibiosis activity of bacteria according to the method that was explained by Boulter et al. (2002). The fungal culture (5mm block) was placed at one corner of the PDA plates from the 6-day-old culture was used. The bacterial strains were pipette away from the block of fungal culture at the other corner of the PDA plates. Then the plates were incubated at 26 ºC in the incubator and checked the growth of the fungal culture was until it reached the edge of the PDA plates to check the efficiency of the bacterial strains. Eight *Bacillus* strains (Table 1) were evaluated and three replicates of each treatment were used in this experiment. For control, only fungal culture was inoculated in the PDA plates and instead of bacterial strain sterilized water was used.

Table 1. *Bacillus* strains used in the study.

Sr. No.	Species	Strains
1	*Bacillus atrophaeus*	B1
2	*Bacillus subtilis*	B2
3	*Bacillus altitudinus*	B3
4	*Bacillus velezensis*	B4
5	*Bacillus thuringiensis*	B5
6	*Bacillus altitudinus*	B6
7	*Bacillus amyloliquefaciens*	B7
8	*Bacillus velezensis*	B8

Extraction of LPs

For the LPs extraction Surveyor HPLC on line with a LCQ DECA XP Plus Ion Trap mass spectrometer with an ESI source was used. Separations were carried out using a C18-

Security Guard cartridge, 4 × 3.0 mm, and an analytical Luna 5 m C18(2), 150 4.6 mm. The injection had a 10 µL volume. The lipopeptides were eluted using the following linear gradient: A: B (50: 50) for 3 minutes, A: B (0: 100) for 18 minutes, and 100% B over 5 minutes at a flow rate of 0.8 ml/min. The mobile phase components were: A = water, 1% formic acid; B = acetonitrile. Full LC/MS scan. From m/z 100 to 2000, positive and negative modes were carried out; alternatively, LC/ESI-MS/MS modalities were applied to the chosen precursor ions in accordance with the parameters established during the infusion analysis.

***In vitro* Antagonism assay with *Bacillus* strains LPs**

LPs of the two best performing strains were evaluated at *in vitro* antagonism assay by well diffusion method to check the efficacy of *Bacillus* strains LPs against *Alternaria alternata*. The fungal culture (5mm block) was placed at one corner of the PDA plates from the 7-day-old culture was used. The bacterial strains were pipette away from the block of fungal culture at the other corner of the PDA plates. Then the plates were incubated at 26 ºC in the incubator and checked the growth of the fungal culture was until it reached the edge of the PDA plates to check the efficiency of the bacterial strains. Ten *Bacillus* strains were evaluated and three replicates of each treatment were used in this experiment. For control, only fungal culture was inoculated in the PDA plates and instead of bacterial strain sterilized water was used.

***In Planta* assay with *Bacillus* strains LPs**

Select healthy tomato fruit and surface sterilized for 2 minutes with 1 percent NaClO and wash fruits with distilled water. Injure these fruits with a sterilized needle and make wounds. Inoculate these wounds with 10 µL of *Bacillus* strain LPs and then inoculate wounds with 10mL conidial suspensions of the pathogen by micropipette keep fruits in sterilized autoclave able square plastic boxes and incubated at 26 ºC for 7 days. After 7 days of treatment observe the fruit and measured the incidence and severity of the infection. The formula for the evaluation of disease incidence and severity is as follows; For disease incidence;

$$\text{Disease incidence (DI)} = \frac{\text{Total No. of infected fruits}}{\text{Total No. of fruits examined}} \times 100$$

For disease severity;

$$\text{Disease severity (DS)} = \frac{\text{Diameter of infected area}}{\text{Total diameter of fruit}} \times 100$$

Post-harvest quality test

Fruit firmness

A testing Machine Instron with a 6.00 mm plunger tip, was used to measure the force (N) needed to puncture a hole in the tomato fruit, and a reading was noted at the end of compression. As in Zapata et al. (2008), the machine was adjusted for maximal compression at a speed of 20 mm/min.

Weight loss and pH

According to the conventional AOAC (1984) procedure, weights of tomato samples (6 fruit per replication) before the treatment and after 7 days of treatment were taken. The difference between the original and final fruit weight after 7 days was accounted as the total weight loss. The pH of all the samples of tomato was recorded by using a pH meter.

Total soluble solids

Using a Digital Refractometer, the soluble solids content (SSC) of the tomatoes from each treatment was calculated from the fruit juice. Before taking readings, the machine was standardized using pure water.

Titratable acidity and Ascorbic acid content

The amount of 0.1N NaOH was measured in order to calculate the titratable acidity (TA) according to Ranggana's method, 1977. After Rangana (1979) the amount of ascorbic acid was calculated using the dye 2,6-dichlorophenol-indophenol titration (DCPIP) method.

Statistical analysis

The CRD design was applied and 6 replications were used. The data were analyzed through ANOVA using software Statistics 8.1 V while the Tukey test was employed to compare different treatments. Prism software was used for the graphical representation.

RESULTS AND DISCUSSION

Antagonism assay with *Bacillus* strains

A total of 8 *Bacillus* strains were employed in this experiment by the Well diffusion method to check the efficacy of the strains against *Alternaria alternata*. The *Bacillus* strain B5 failed against *Alternaria alternata* and gave no inhibition zone. Two Bacillus strains B1 and B2 gave maximum inhibition zones as compared to the control Figure 1.

***In vitro* assay with Bacillus strains LPs**

In this experiment LPs of the two best Bacillus strains B1 and B2 were evaluated by well diffusion method. Both B1 and B2 have good inhibitory potential against *Alternaria alternata* in *in vitro* experiments with inhibition zones 1.8 cm and 1.5 cm respectively as compared to the control (Figure 2).

***Planta assay* with LPs**

The LPs were tested in the Planta assay to investigate the effect of LPs on fruit against *Alternaria alternata* rot of tomato. Both B1 and B2 have good inhibitory potential against *Alternaria alternata* at *in Planta assay* experiment but the lowest incidence and severity of disease was shown by *Bacillus* strain B1 as compared to the control (Figure 3, 4).

Fruit firmness

The result indicates the firmness of tomato fruit was good and satisfactory in the *Bacillus*-treated fruits when compared to the results with healthy and infected control. The *Bacillus* strains B1 treated fruits have a high value of fruit firmness.

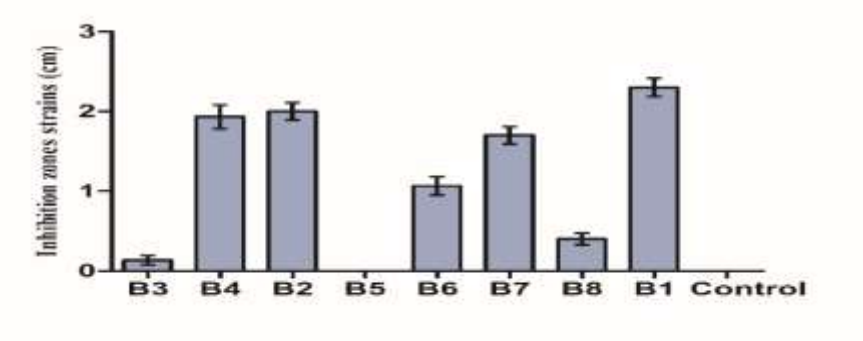

Figure 1. Antagonism effect of *Bacillus strains* against *Alternaria alternata* colony growth.

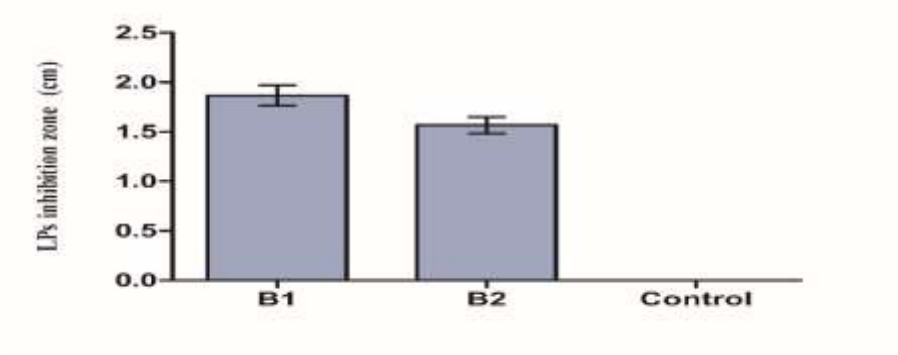

Figure 2. Antagonism effect of *Bacillus* LPs against *Alternaria alternata* colony growth.

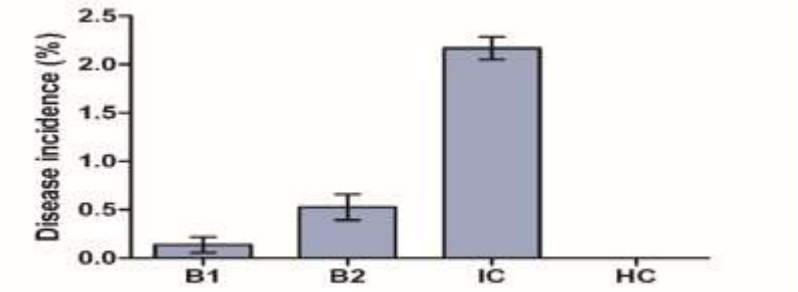

Figure 3. Antagonism effect of *Bacillus* LPs on the disease incidence of *Alternaria alternata*. HC = Healthy Control, IC = Infected Control.

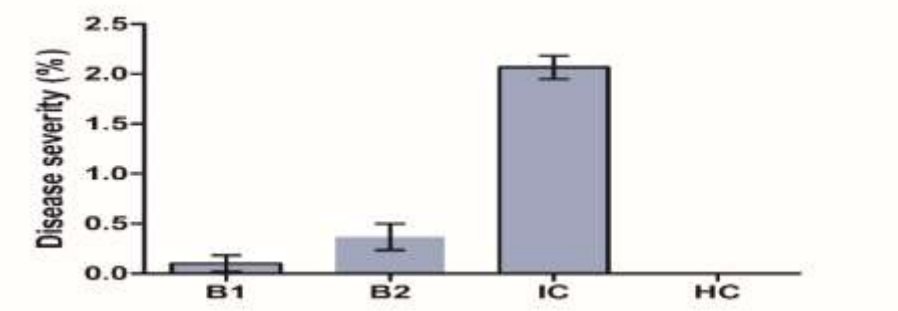

Figure 4. Antagonism effect of *Bacillus* LPs on the disease incidence of *Alternaria alternata*. HC = Healthy Control, IC = Infected Control

Titratable acidity, Ascorbic acid content, and total soluble solids

The titratable acidity and ascorbic acid content were satisfied and good in the *Bacillus*-treated tomato fruit as compared to the healthy and infected control. The highest ascorbic acid content and titratable acidity were observed in the Bacillus strain B1. The effect of LPs-treated fruits indicated the highest total soluble solid content as compared to the infected control and positively correlated with the healthy control (Figure 5).

Weight Loss and pH

The minimum weight loss was observed in the Bacillus-treated tomato fruits as compared to the infected control and weight loss was positively correlated with the healthy control. The value of pH indicates that the infected control has low pH as compared to the treated tomato fruits (Figure 5). In the agriculture industry, the usage of PGPR is expanding and could provide a useful alternative strategy for synthetic fertilizers and chemicals. Microorganisms that stimulate plant growth are effective microbial competitors that can stimulate plant growth by creating phytohormones, metabolites, or used as biocontrol agents against phytopathogens (Chandrasekaran and Chun, 2016; Grimstad et al., 2012; Vessey, 2003; Zehnder et al., 2001). Tomato fruits are a good source of antioxidant chemicals that can lessen harmful oxidation events in the body, hence reducing the number of diseases like cancer and cardiovascular and neurological disorders that are linked to free radical oxidation (Choi et al., 2014; Klee and Giovannoni, 2011; Saini et al., 2017). Up to 30 % of the cultivated tomato crop may be wasted due to postharvest diseases caused by the pathogen *Alternaria alternata* brings a huge economic loss to the agriculture industry and have a broad range of host and infect tomato fruit at any stage of fruit ripening (Bashir et al., 2020).

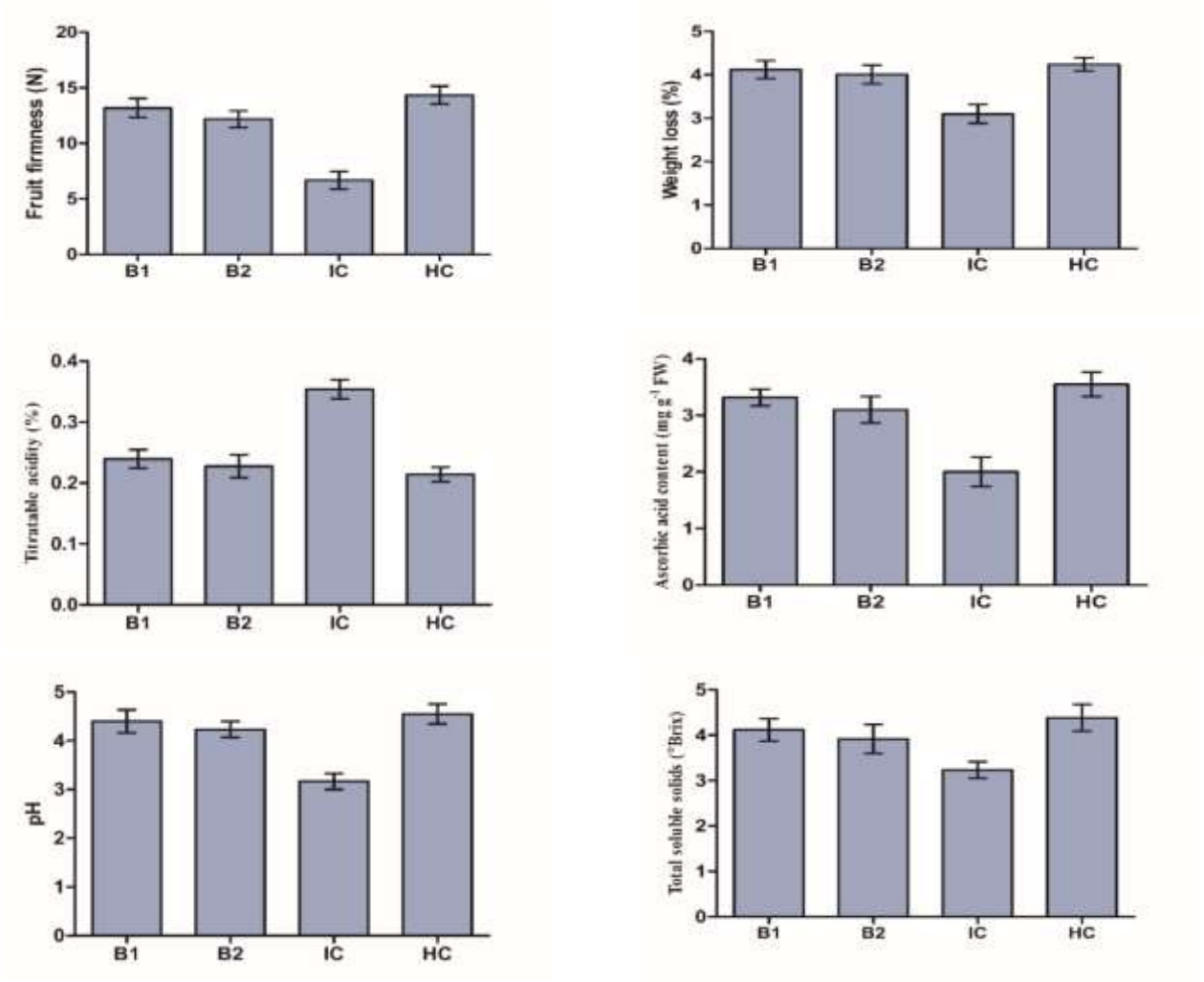

Figure 5. The effect of *Bacillus* strains LPs on the post-harvest quality of tomato fruits.

The *Bacillus* strains proved effective as a biocontrol agent to promote plant growth and eco-friendly strategy to reduce the post-harvest loss caused by microbes. Their beneficial antibacterial, antiviral, and anticancer qualities are what have people interested in them. Two strains B1 and B2 proved effective in antagonist assay and have high inhibitory potential against *Alternaria alternata*. A report of Engindeniz and Cosar (2013), correlate to my findings, that revealed that the combined release of diffusible and volatile organic compounds to the culture medium may be the cause of the maximum inhibition seen in the direct antagonism test.

The LPs of *Bacillus* strain B1 and B2 were evaluated *in vitro* and *In Planta* assay experiments and gave good inhibition control against *Alternaria alternata*. A previous study supports my work that revealed the bioactive lipopeptides known as surfactins have outstanding surface activity (Kameda et al., 1972).

A previous report revealed that the direct application of numerous lipopeptides families found in an organic extract as a potential bio pesticide may also help by preventing the growth of biofilms caused by other pathogens in the fruits and managing superinfections caused by bacteria (Feliziani and Romanazzi, 2016). Recent research revealed that these chemicals interactions can have synergistic, additive, or antagonistic effects on this mixture (Mihalache et al., 2018; Sabaté et al., 2017).

In this study, the antagonism assay with *Bacillus* strains revealed the highest inhibition zones as compared to the assay with LPs and in *Planta* assay experiments. Two strains B1 and B2 gave the highest inhibition zones that's why their LPs were evaluated in the well diffusion method and then also employed in *Planta* assay to check the efficacy of LPs on fruits and analyze the post-harvest quality of fruits. And our result indicated the bacillus LPs treated fruits showed good satisfactory conditions of fruit when compared to healthy and infected controls.

Since the nutritional state of plants is improved by improving the availability of nutrients in the rhizosphere, PGPR may increase crop output and fruit size by facilitating plant nutrition (Richardson, 2001).

CONCLUSION

In this investigation, we evaluated the efficacy of *Bacillus* strains to suppress the growth of *Alternaria alternata* in vitro experiments, and then we choose the best two performing strains and evaluate their LPs in vitro as well as *In Planta assay* experiments and analyze the efficacy of *Bacillus* LPs on the post-harvest quality of tomato fruits. The *Bacillus atrophaeus* B1 and *Bacillus subtilis* B2 have proven best and we conclude that the tomatoes treated with the *Bacillus* LPs have a high source of ascorbic acid, titratable acidity, and total soluble solid contents as compared to the infected control. The quality of the fruit was also good and weight loss was minimum in the treated tomato fruits.

CONFLICTS OF INTEREST

The authors declared no conflict of interest. The funders had no part in the design, collection analyses and interpretation and writing of short communication.

AUTHOR'S CONTRIBUTION

All authors contributed and supported towards writing of this manuscript.

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