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ANTAGONISTIC POTENTIAL OF *BACILLUS* STRAIN AGAINST THE POST-HARVEST BLUE MOLD OF LEMON

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A B S T R A C T

The present study focuses on the reduction of significant postharvest blue mold disease of lemon (*Citrus limon* L) fruit through the use of *Bacillus* strains in vivo and in vitro conditions. Blue mold which is caused by *Penicillium italicum* is the most dangerous postharvest disease of lemon fruit and causes significant losses in citrus fruits. In this study, antagonistic compounds like Cyclic lipopeptides (CLPs) produced by *Bacillus* strains were used to control *P. italicum.* CLPs are considered best the antagonistic compound of *Bacillus* Strains that primarily play an important role in the reduction of plant diseases and have three important families such as iturin, fengycin, and surfactin. This study was carried out by using seven strains of the genus *Bacillus* that were used in vitro assay. Among these seven strains, two strains *Bacillus licheniformis* and *Bacillus thuringiensis* showed wonderful inhibition zone in vitro assay carried out in Petri plates. Further CLPs were extracted from the both best strains and used in vivo assay on lemon fruit and showed less disease incidence and lesion diameter of disease. Furthermore, lemon fruits treated with CLPs showed increasing defense mechanisms of enzymes P AL, PPO, and PO. This study indicates the strong antifungal effect of *Bacillus* strains and their metabolites CLPs against the blue mold of lemon.

Keywords: *Bacillus* strains; Lipopeptides; Biocontrol; *P. italicum;* Lemon; PAL; PPO; POD

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INTRODUCTION

Citrus is one of the world's most commercially significant horticulture crops. Citrus is highly nutritious and has a wide range of types, the much more popular of which is the Lemons (*Citrus limon* L.) fruit, which is a tropical fruit with a significant market value in the world - wide fruit market (Luo et al., 2013; Zeng et al., 2010).

Citrus fruit is particularly vulnerable to microbiological pathogen attack between harvesting and consumption because of its greater moisture content and nutritional quality (Tripathi and Dubey, 2004). Several postharvest diseases can affect citrus fruits and result in severe losses throughout the postharvest period. However, the most widespread and dangerous citrus fruit diseases are blue and green molds caused by *Penicillium italicum and Penicillium digitatum* respectively (Caccioni et al., 1998; Palou et al., 2002; Zheng et al., 2005). They are wound-specific pathogens that can contaminate fruits inside the field, in the transfer station, or during storage.

The primary means of controlling citrus postharvest diseases, such as green and blue molds, has been chemical fungicides (Eckert, 1989). However, due to potential risks to human health and the environment, there is rising concern on a global scale about the typically haphazard use of chemical fungicides on crops (Norman, 1988). The continued usage of synthetic pesticides has also led to the introduction of pathogenic resistance strains. As a more efficient alternative to using chemicals to control plant diseases, biological control is becoming a more popular method of controlling plant diseases. However, alternatives to synthetic chemicals, especially biocontrol agents, are frequently ineffective compared to a large number of the

industrial fungicides presently that is being used (Conway et al., 1999; El-Ghaouth et al., 2000). Regarding the multiple biological approaches, microbiological antagonism, particularly *Bacillus* strains and yeasts is preferred and exhibits great promise to control fruit diseases in agriculture (Abraham et al., 2010; Osman et al., 2011; Sharma et al., 2009a). Species of the genus *Bacillus* have been used in controlling citrus postharvest diseases for a long time, such as *B. subtilis* was used to control green mold of citrus(Wilson and Wisniewski, 1989). The development of various antimicrobials to inhibit the growth of plant pathogens has commonly been linked to the mechanism of action of biological controlling agents (Ongena and Jacques, 2008; Stein, 2005).

Bacillus species are being analyzed for a wide range of possible usage because of their diversity in producing biologically active compounds. Such as several members of bacillus genes generate several antagonistic cyclic lipopeptides (CLPs), and most common family members of CLPs are iturin, fengycin, and surfactin. These CLPs may primarily play an important role in decreasing plant diseases during treatments with Bacillus species, based on certain researchers. One of the biggest risks to crops and plant productivity is mainly Plant-pathogenic fungi. Therefore, the use of *Bacillus* species to prevent fungal infections could be a significant approach to agricultural technology.

A significant category of proteins called pathogenesis-related (PR) proteins plays roles in the defensive mechanisms of plants against pathogen infections as well as other physiologic challenges (Ballester et al., 2006). These PR proteins are recognized as the significant marker enzymes that may directly restrict infection growth through the breakdown-down of chitin and 1,3-glucan which are two of the main components of the fungal cell wall(Bill et al., 2017). Peroxidase (POD), Phenylalanine lyase (PAL), and Polyphenol oxidase **(**PPO) enzymes are important PR proteins and have been linked to plant resistance against pathogens resistance and have been involved in the production and oxidizing of phenol chemicals (Bill et al., 2017). The two major enzymes in the phenylpropanoid biological process to stimulate manufacturing are Phenylalanine lyase (PAL) and Peroxidase (POD), whereas Polyphenol oxidase **(**PPO) plays a critical function in the release of oxygen plant metabolism and oxidizing phenol chemicals into harmful quinones to prevent pathogen growth.

MATERIALS AND METHODS

Fungal culture

P. italicum fungus was isolated from lemon fruit infected with the blue mold of lemon. A sterilized disease portion of the fruit (peel) was grown on potato dextrose agar medium (PDA) and incubated at 25±1°C for 24 hours. To obtain a pure culture of pathogen single-spore method was used and pure cultures were kept at -80 in 30% glycerol solutions for further investigation.

Bacterial culture

Seven *Bacillus* strains such as *B. cereus, B. subtilis, B. velezensis, B. atrophaeus, B. pumilus, B. thuringiensis, and B. licheniformis* for biocontrol have been taken from the bacterial culture bank of the Molecular Laboratory of Plant Pathology department of the Islamia University of Bahawalpur, Pakistan.

Lemon fruits

Healthy and equal-sized fruits cv. 'Meyer Lemon' was taken and sterilized with 1% NaOCL for 3 minutes, washed with distilled water, and dried to remove extra water on the fruits.

In vitro assay of *Bacillus* strains

To check in vitro activity of Seven *Bacillus* strains against *P. italicum* dual culture technique was used. From a 7-day-old pathogen culture, a 4mm culture block was used. It was positioned in the middle of a Petri plate with PDA and incubated at 25°C for two days. Filter paper discs that had been disinfected were positioned 3 cm away from the plate's edges. On both sides of the filter paper disks, bacterial culture of 10 µL was placed and then these plates were incubated at 25±1℃ for 7 days. Five replications of each treatment were used in the test, which was carried out three times under the same procedures.

Extraction of LPs from *Bacillus* strains

Lipopeptides from the best *Bacillus* Strains *Bacillus licheniformis* and *Bacillus thuringiensis* were extracted. Luria Bertani (LB) broth of 250 ml was prepared and the best *Bacillus* strains were inoculated. Then placed on a rotary shaker at 180 rpm at 30°C After 3 days, to obtain the cell-free supernatants (CFS) the bacterial culture was centrifuged at 10,000 rpm at 4°C for 15 minutes and CFS was collected in a sterilized glass beaker, incubated at 4°C for 12 h and again centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was discarded, and the pellet was collected and dried. To remove impurities, the pellet was dissolved in 5 mL HPLC-grade methanol (pH 7) and passed through a 0.22 µm syringe filter.

In vivo assay of LPS

In vivo assay, LPs of the best strains were inoculated at healthy, equal-sized, and surface sterilized lemon fruit. An artificial 5 mm hole was produced on the fruit using a sterile needle. The pathogen was then injected into each hole with a 10 µL spore solution (1×10^6 spore's mL^{-1}). Then in each

hole, 10 µL of LPs solution was injected. The fruits were incubated for seven days at room temperature in square plastic boxes that had been disinfected. Each treatment was replicated fifteen times and repeated three times under the same conditions for the experiment and carried out in a CRD layout. Measurements of lesion diameter were made of the lemon fruit and Disease incidence was measured by the following formula (Sukorini et al., 2013).

$$\text{Disease incidence (\%)} = \frac{\text{No. infected fruits}}{Tota\ No.of\ fruits} \times 100$$

The formula given by Masood et al. (2010) was used to calculate the disease severity (Lesion diameter). The fruit lesion diameter was measured using Vernier calipers.

$$\text{Disease severity} = \frac{\text{Lesion diameter}}{\text{Total area of the fruit}} \times 100$$

Assay of defense enzymes

After 7 days of treatment fruit peel (Sample) was taken and washed with distilled water. The Coseteng and Lee (1987) method was modified to produce fruit peel extraction. About 0.5g of sample with 5 ml buffer solution was placed in a postal motel. The sample was then centrifugation between 8000 and 13000 rpm for twenty minutes at 4^{o}C. The obtained supernatant was collected to determine enzyme activity.

By using an exogenous catechol substrate, PPO activity was measured. 200 µL of sample extract was mixed with 2.5 mL of 0.2 M potassium phosphate buffer, 500 µL of cold acetone, and 500 µL of 50 mM catechol to make up the reaction mixture. A spectrophotometer was used to detect absorbance at 420 nm.

POD assay was determined by using a guaiacol substrate. Reagent solutions were consisting of 5 mM H2O2 in 0.2 M potassium phosphate buffer with 6.8 pH and 5 mM guaiacol.200 µL of peeling extract was mixed with 800 µL of the reaction medium to complete the reaction. By placing 100 L of each sample onto an Elisa reader plate and analyzing the absorbance at 470 nm using a spectrophotometer, the peroxidase activity in each sample was identified (Siegel and Galston, 1967).

Ballester et al. (2006) adopted the procedure to determine the PAL activity. PAL activities were measured by using acetone powders.5g of fruit peel was grinded with 50 µL of the supernatant filtered by using Bencher filter. The PAL was measured by mixing 0.05 g of acetate powdered and 100 ml of sodium borat1.5 ml and 20mM mercaptoethanol at PH 8. The purification of the resulting supernatant was achieved by the process of drying out the proteins with 60% ammonia. PAL activity was determined by analyzing the absorbance for cinnamic acid at 290nm. The activity of PAL was measured and showed as nmoles g-1 h-1 of cinnamic acid.

RESULTS AND DISCUSSION

In vitro antifungal effect of *Bacillus* strains

By dual culture technique seven *Bacillus* Strains were used to test the antifungal effect of *Bacillus* strains in vitro against *P. italicum.* Among seven strains two strains *Bacillus licheniformis* and *Bacillus thuringiensis* showed the highest inhibition zone (Figure 1). *Bacillus licheniftheormis* showed the highest inhibition zone followed by *Bacillus thuringiensis.* All the other strains showed lowest inhibition zone.

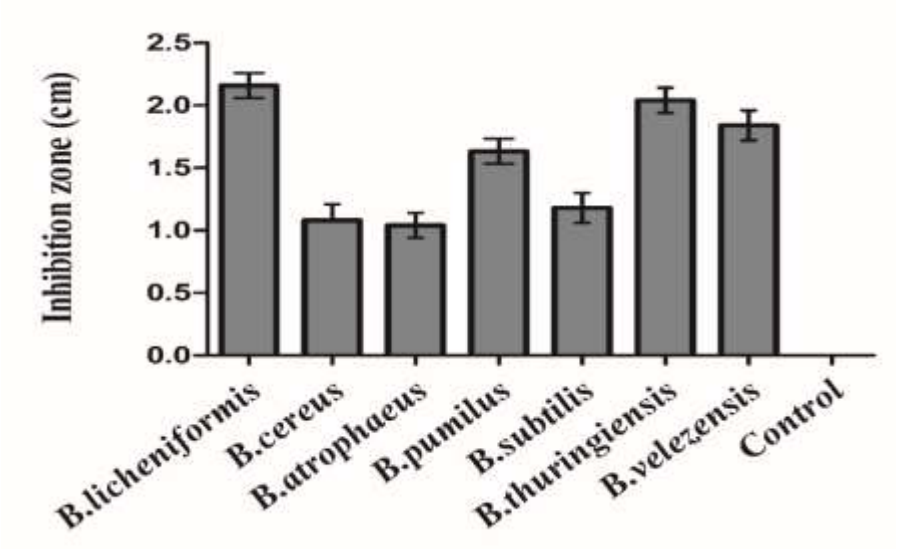

Figure 1. In vitro antifungal effect of *Bacillus* strains against *P. italicum.*
Each bar is an average of 15 replicates from three experiments, analyzed by Tukey's HSD test in a CRD design.

In vivo antagonist effect of CLPs

CLPs were extracted from *Bacillus licheniformis* and *Bacillus thuringiensis* and tested in vivo assay to check lesion diameter against *P. italicum.* Both strains showed less lesion diameter in vitro assay (Table 1).

Effect of CLPs on PAL, POD and PPO enzyme activity

Lemon fruits treated with CLPs extracted from *Bacillus licheniformis* and *Bacillus thuringtheiensis* showed increasing PPO, POD and PAL enzyme activity as compared to infected control (Figure 2).

Table 1. In vivo antagonist effect of *Bacillus* strains on lesion diameter and Disease incidence of blue mold of lemon.

Treatments	Lesion diameter(cm)	Disease incidence %
Bacillus licheniformis+PI	1.5	5.0
Bacillus thuringiensis+PI	1.6	6.0
P. italicum (PI)	3.7	100.0
Healthy control (HC)	0.0	0.0

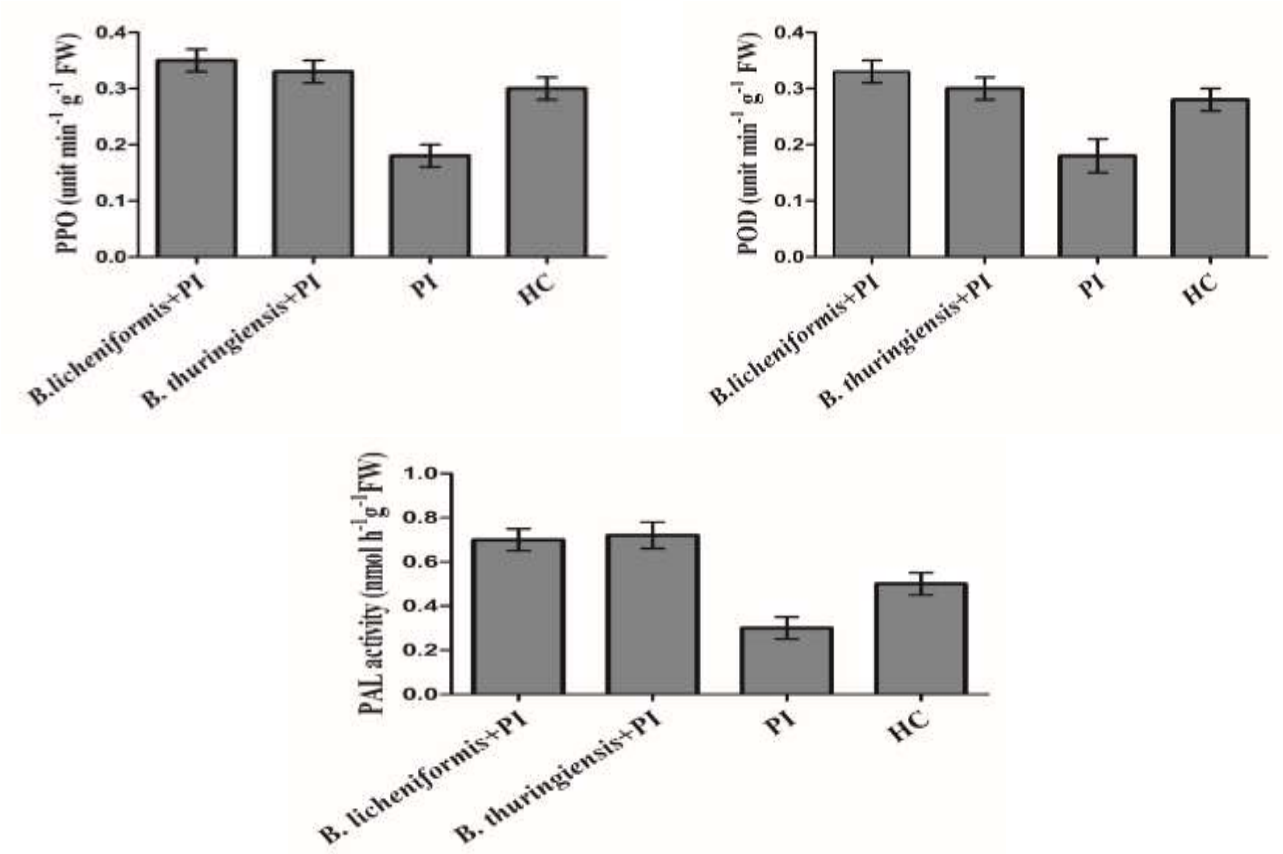

Figure 2. PPO, POD and PAL enzyme activity in lemon fruits treated with CLPs extracted from *Bacillus licheniformis* and *Bacillus thuringiensis* in vivo assay.

Each bar is an average of 30 replicates from three experiments, analyzed by Tukey's HSD test in a CRD design.

Blue mold is a most serious postharvest disease of lemon fruits and cause significant losses. The most widely method used for controlling fruit postharvest diseases are the use of chemical control through fungicides. But the application of fungicides that ultimately control the number of postharvest diseases has reduced because of public anxiety about chemical residues in fruits, the emergence of pathogens that are resistant to fungicides, the suspension of certification for some of the most effective fungicides, and updated environmental safety regulations (Droby et al., 2016). As a result, there is a need for novel techniques that offer effective control without harming human health or the environment.

Research on potential biological control techniques has been

encouraged by the demand for alternate control approaches, and as a result, biocontrol of different post-harvest disease of fruits through microorganism and certain bio fungicides have been approved. According to reports, this approach which utilizes antagonists is among the most interesting and potentially useful approaches to chemical treatment (Ab Rahman et al., 2018; Janisiewicz, 1998).

Biopesticides, specifically bio fungicides, bio bactericides, and other substances produced by microbes themselves, are considered biological control agents (Sachdev and Cameotra, 2013). In comparison to synthetic fungicides breakdown into poisons and permanent chemicals in food or the environment, bio fungicides are considered to be effective while also being safe for future consumers (Kumar et al., 2021). Therefore, there is a lot of interest in the use of microbial agents as biocontrol for controlling plant diseases after harvesting (Köhl et al., 2020).

Biological control, or the use of microbes or their metabolites to prevent plant disease, is environmentally friendly, usually risk-free, and might provide the crop with long-term (Fernando et al., 2005). *Bacillus* strains and other saprotrophic bacteria are effective biocontrol agents for plant diseases. Due to its inhibitory activities against several plant pathogens microorganisms, numerous *Bacillus amyloliquefaciens* strains have been extensively studied and employed in agriculture as plant growth boosters (Babalola, 2010; Cawoy et al., 2015; Choudhary and Johri, 2009; Pérez-García et al., 2011; Sharma et al., 2009a; Wu et al., 2015). Sharma et al. (2009b) reported that postharvest disease control may use a variety of mechanisms, such as antimicrobial substances, induced resistance, competing for nutrients and space, and parasitism.

In this study, we focused on screening the *Bacillus* strains for biological control of the postharvest blue mold of lemon. In vivo dual culture assay was used to screen the potential antagonist effect of *Bacillus* strains against *P. italicum.* The size of the inhibition zone was used to evaluate the antagonistic potential of *Bacillus* strains. Two *Bacillus* Strains *Bacillus licheniformis* and *Bacillus thuringiensis* showed highest inhibition zone.

Bacillus strains produce different type of metabolites that are responsible for reduction of pathogen infection. Mostly *Bacillus* strains produced Cyclic Lipopeptidesk 5z (CLPs) that control post-harvest diseases. CLPs produce fengcins, surfaction and turin. Previous study showed vbgi that Iturin A and bacillomycin D, which were present in the supernatant of *Bacillus amyloquefaciens* antifungal activity against *P. expansum* (Arrebola et al., 2010). Furthermore, we extract the Cyclic Lipopeptides (CLPs) from the *Bacillus licheniformis* and *Bacillus thuringiensis* and used in vivo assay. In vivo assay, CLPs has successfully reduced the incidence of blue mold of lemon.

Furthermore, we evulated the PAL, PPO and POD enzymes defense activity of lemon fruits treated with *Bacillus* strains. *Bacillus* strains increased the defense activity of PAL, PPO and POD enzymes. The results of the present study indicate that *Bacillus* strains and their metabolites such as Cyclic Lipopeptides (CLPs) has potential effect on the growth of *P. italicum* both in vivo and in vitro conditions and *Bacillus* strains has increase the defense activity of PAL, PPO and POD enzymes.

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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