

INVESTIGATION OF THE EFFECT OF *Bacillus subtilis* AND *Bacillus thuringiensis* AS BIO-AGENTS ON ANTHRACNOSE (*Colletotrichum* spp.) DISEASE IN KALANCHOE

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Abstract

Kalanchoe is a perennial succulent plant belonging to the *Crassulaceae* family and is highly susceptible to various pathogens, particularly fungal and bacterial diseases. Among these, anthracnose is one of the most prevalent disease factors affecting *Kalanchoe*. This study aimed to evaluate the efficacy of a bioformulation containing four plant growth-promoting rhizobacteria (PGPR) strains—*Bacillus subtilis* (61.29e and 3.3a) and *Bacillus thuringiensis* (2B3-1 and 2B2-2)—in controlling anthracnose disease. PGPR solutions were prepared at two concentrations (3×10^8 and 3×10^3 CFU/mL), and six treatment groups were established by foliar spraying on the plants: (1) application of diluted bacterial solution (3×10^3 CFU/mL) four days after anthracnose inoculation, (2) full-dose bacterial solution (3×10^8 CFU/mL) applied four days after inoculation, (3) repeated full-dose application at four-day intervals post-inoculation, (4) culture medium application four days after inoculation, (5) bacterial application followed by anthracnose inoculation after four days, and (6) control group with anthracnose inoculation only. The results demonstrated that the application of PGPR prior to disease inoculation was the most effective strategy in reducing disease symptoms. This was followed by the application of the full-dose bacterial solution post-inoculation. In contrast, application of the diluted solution after infection showed higher disease incidence. Nevertheless, all PGPR treatments, whether applied before or after infection, significantly outperformed the control and culture medium treatments in suppressing anthracnose. These findings highlight the potential of *Bacillus* spp. based PGPR formulations as promising biological control agents for anthracnose management in ornamental plants, contributing to the development of sustainable and environmentally friendly plant disease management strategies.

Keywords: Bio-Agents, Ornamental plant, PGPR

1. INTRODUCTION

Kalanchoe is a perennial succulent plant belonging to the *Crassulaceae* family. The *Kalanchoe* genus naturally spreads in regions with tropical climates, mostly in Madagascar, East and South Africa, South America, India, Arabian Peninsula and Southeast Asia (Descoings, 2006). The genus *Kalanchoe* includes about 140 species, and the species grows in nature as semi-shrubs, shrubs and rarely small trees (Descoings, 2006; Currey and Erwin 2011; Kahraman et al., 2021). Among the *Kalanchoe* genus, which has started to attract more attention recently, the most commercially grown species is *Kalanchoe blossfeldiana* Poelln. (Kahraman et al., 2021). *Kalanchoe*, which is in the group of succulent plants, is susceptible to many diseases and pests, especially fungal and bacterial diseases. Certain environmental conditions, especially high humidity and temperature, play an

important role in the development and sporulation of these pathogens (Cannon et al. 2012). Anthracnose is one of the most common disease factors in kalanchoe. One of the species that causes anthracnose is *Colletotrichum* spp., and the genus *Colletotrichum* spp. has been reported as one of the ten most important plant pathogens in the world based on economic importance (Dean et al. 2012; Guarnaccia et al. 2021).

Fungicides indeed play a crucial role in combating fungal diseases and are widely used in agriculture. They are effective in controlling fungal pathogens and preventing the spread of diseases in crops and plants. However, their use comes with certain limitations and drawbacks such as limited spectrum of activity, resistance to disease, environmental negative impact, residue concerns (Wu et al., 2015).

Indeed, the limitations of conventional approaches to combat soil-borne diseases have led farmers to seek alternative solutions that are more environmentally friendly and sustainable; one such alternative method is the biocontrol of diseases (Safaei-Asadabadi et al., 2021; Bilgili and Bilgili, 2023). Biocontrol involves the use of beneficial microorganisms, such as bacteria, fungi, and other microbes, to suppress or control plant diseases. These beneficial microorganisms can act as antagonists to the soil-borne pathogens, either by directly inhibiting their growth or by stimulating the plant's natural defense mechanisms. The goal of biocontrol is to enhance plant tolerance to diseases and reduce the impact of pathogens on crop health (Silva and Canellas, 2022; Devi et al., 2022).

PGPR are beneficial microorganisms that can enhance plant growth and health through various mechanisms, including disease suppression. *Bacillus* species have gained significant attention as effective Plant Growth-Promoting Bacteria (PGPR) for the control of soil-borne plant diseases (Devi et al., 2022; Wu et al., 2015). The abilities of *Bacillus* species as PGPR are attributed to their production of several bioactive compounds, such as siderophores, antibiotics, secondary metabolites, and hydrolytic enzymes (Bilgili and Bilgili, 2023). These substances play important roles in promoting plant growth and protecting plants from soil-borne pathogens. In addition to direct effects on pathogens, *Bacillus* species also play a role in enhancing the plant's immune response and defense mechanisms (Zauberman, 1991; Bilgili and Bilgili, 2023).

This study was conducted to investigate the success of a formulation containing *Bacillus* species four bacteria in preventing anthracnose in kalanchoe plants. Investigating the potential of *Bacillus* species PCPRs as a biological control agent for anthracnose in ornamental plants is an essential step in developing sustainable and eco-friendly disease management strategies for agriculture. This research will contribute to our understanding of biocontrol and offers practical solutions for farmers to combat plant diseases more effectively.

2. MATERIALS AND METHODS

Plant Materials

Commercially available Kalanchoe (*Kalanchoe blossfeldiana*) potted plants were used as plant material in the study. Kalanchoe plants were planted in pots containing a 1:1 mixture of peat and perlite. The greenhouse temperature was recorded as 22±24 °C on average for the experimental period. Routine irrigation of the plants was done manually.

Activation of Rhizobacteria

A formulation was formed as a mixture of four different isolates of *Bacillus subtilis* (61.29e and 3.3a) and *Bacillus thuringiensis* (2B3-1 and 2B2-2). Bacterial strains isolated and identified were used in a doctoral thesis conducted at Erciyes University, Institute of Science and Technology.

Rhizobacterial strains were taken from the stock culture at -20°C and allowed to grow in 250 ml Erlenmeyer flasks in autoclaved Nutrient Broth (NB) medium at $28-30^{\circ}\text{C}$ for 24 hours on a shaker at 150 rpm. Media used for the activation of bacteria; Nutrient Agar (NA) medium ($\text{pH } 6.8 \pm 0.2$): Prepared by suspending 20 g of solid medium in 1 liter of distilled water, sterilized by autoclaving at 121°C for 15-20 minutes. Nutrient Broth (NB) liquid medium ($\text{pH } 6.8 \pm 2$): It was prepared by dissolving 8 g of solid medium in 1 liter of distilled water and autoclaving at 121°C for 15-20 minutes.

Inoculation of the Anthracnose Disease Agent.

Bacterial solutions were prepared at 3×10^8 , and 3×10^3 concentrations. Six different treatments were used by spraying the solution on the leaves.

Treatment 1: Application diluted bacterial solution four days after anthracnose contamination (3×10^3)

Treatment 2: Application of two times diluted bacterial solution four days after anthracnose contamination ($2B/3 \times 10^8$)

Treatment 3: Application of full dose bacteria solution four days after anthracnose contamination ($A+B/3 \times 10^8$).

Treatment 4: Nutrient medium application four days after Anthracnose contamination ($A+\text{MEDIA}$).

Treatment 5: Application of a full dose of bacterial solution two days before anthracnose contamination. ($B+A/3 \times 10^8$)

Treatment 6: Control/anthracnose infected plants only (anthracnose spores reproduced on the susceptible kalanchoe plants were transmitted to the moistened leaves with a brush.) (C)

The anthracnose agent was contaminated in the greenhouse. Anthracnose isolates used in the study was obtained from pot Kalanchoe plants in Kayseri province.

Morphological Identification of Anthracnose

Isolation of the pathogen was carried out from plant leaves that were observed to be infected with the disease. Isolation of the pathogen from plant tissues was done according to Jamshidi and Salahi (2008). For this purpose, sections with a diameter of 5-6 mm were taken from the infected leaf part, covering the infected and healthy plant tissue. Sections taken were kept in 70% ethanol for 1 minute and passed through sterile distilled water 4 times. The obtained sections were divided into 2-3 parts and transferred to 1.5 ml Eppendorf tubes containing sterile distilled water. It was vortexed for 20 seconds and transferred to 2% water agar medium. After 48 hours of growth in WA medium, the germinated spores were transferred to potato dextrose agar medium containing 7 g of oat flour per liter. The isolates were incubated in the media for approximately one month since the growth of the fungus and the production of conidia were very slow.

Of the developing colonies, Sogonov et al. (2008), morphological characterization studies were carried out. Morphological characterization of the isolates was made according to their macroscopic and microscopic properties. Microscopic identification was made according to the conidial characteristics using a binocular microscope after the isolates were grown in WA at 25°C . For macroscopic diagnosis, colony development and morphological characteristics of the isolates in the medium were examined, which were incubated in PDA at 25°C .

Determination Of Anthracnose Prevention Rate of Bacterial Solution Applications

The protection rates of the above-mentioned bacterial applications from anthracnose disease were determined in anthracnose infected Kalanchoe plants. The development of anthracnose disease in leaves was evaluated 30 days after contamination on a scale of 0-4 (Table 1., Delp and Milholland

1980). For all replications included in each application, disease progression was scored separately and averaged (Figure 1).

Table 1. Disease reaction scale to score disease phenotypes of Kalanchoe Anthracnose disease (Delp and Milholland 1980).

Disease reaction category	Diseased plant phenotype
0	Whole plant was healthy
1	$< \frac{1}{4}$ of the ratio of decay area and longitudinal section
2	$\frac{1}{4} - \frac{1}{2}$ of the ratio of decay area and longitudinal section
3	$(\frac{1}{2} - \frac{3}{4})$ of the ratio of decay area and longitudinal section
4	$> \frac{3}{4}$ of the ratio of decay area and longitudinal section and the whole plant died



Figure 1. Disease reaction scale to score disease phenotypes of Kalanchoe Anthracnose disease image

Protein isolation

Protein extraction was also performed by TCA/acetone method according to the classical method by Damerval et al. (1986) with minor modifications (Rampitsch, 2006)

For protein isolation, 1 g of sample powdered with liquid nitrogen will be homogenized in 4 mL of 10% TCA/acetone solution. The mixture will be taken into 2 mL Eppendorf tubes. The samples will be centrifuged at 12,000 rpm for 5 minutes at 4°C. The pellet will be suspended with 10% TCA/acetone+5 mM DTT solution (3 times). The pellet will be suspended with 80% acetone+5 mM DTT solution and kept at -20°C for 10 minutes. The samples will be centrifuged at 12,000 g at 4°C for 5 minutes. At the end of the process, the pellet will be left to dry at 25°C (Rampitsch, 2006).

Determination of Protein Concentration

"Bio-Rad Protein Assay" method was used for protein quantification. Bovine serum albumin (BSA) protein was used as a standard to generate the standard curve. Calculations were made by determining the absorbance values of determined BSA concentrations (0.125, 0.250, 0.500, 0.750, 1, 1.5 and 2 mg/mL) at 660 nm (Bradford, 1976).

SDS-PAGE Analysis (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis)

SDS-PAGE analysis will be performed to examine whether the proteins, which are the products of the relevant genes, are produced in abundance and in terms of size. The 80 µg of protein from each sample will be loaded onto polyacrylamide gel and analysis will be performed according to the method of Laemmli (1970). Concentrations of proteins isolated by appropriate methods will be determined before loading. For this, it was mixed with 4x loading buffer (0.5 M Tris.HCl 1.0 mL, Distilled Water 4.0 mL, %10 SDS 1.6 mL, Glycerol 0.80 mL, Beta-mercaptoethanol 0.4 mL, Bromophenol blue 0.2 mL) and incubated at 95-100 °C for 7 minutes. In this way, the proteins were denatured, and 30-50 Amps were run in SDS-PAGE. Also, the gel visualized with Silver Stain Plus and was determined using the method described by Temizkan and Arda (2004). The gels were fixed and stained with 0.1% Coomassie brilliant blue.

3. RESULTS AND DISCUSSIONS

Following anthracnose inoculation, kalanchoe plants were evaluated for disease development 30 days post-inoculation using the disease severity scale described in Table 1. Plants infected with the anthracnose pathogen were scored based on the extent of disease progression. Each treatment in the study was conducted in triplicate, and disease severity was individually assessed for each plant; the average disease severity score was then calculated. According to the findings, both the control plants inoculated with anthracnose and those treated with culture medium after inoculation exhibited a disease severity score of 5, indicating severe infection. In these groups, disease symptoms were prominently observed on both leaves and stems (Figure 2).

In treatments where a low concentration of PGPR (3×10^3 cfu/mL) was applied after anthracnose infection, disease progression continued, and a disease severity score of 4 was recorded (Table 2). Conversely, in plants treated with a high concentration of PGPR (3×10^8 cfu/mL) after infection, the progression of the disease was notably slowed. Similarly, when PGPR at a concentration of 3×10^8 cfu/mL was applied twice at one-week intervals following infection, a significant reduction in disease severity was observed. The most effective result was obtained when PGPR at a concentration of 3×10^8 cfu/mL was applied prior to anthracnose inoculation. In this group, the plants exhibited a disease severity score of 1, indicating minimal symptoms.

Table 2. Disease reaction scale to score disease phenotypes of *Kalanchoe* Anthracnose

Treatment	Scale
Control	5
medium	5
A+ B/ 3×10^3	4
A+B/ 3×10^8	3
B/ 3×10^8 +A	1
A+ 2 times B/ 3×10^8	2

**Figure 2. Visual representation of anthracnose infection severity in the control group of *Kalanchoe* plants without PGPR treatment.**

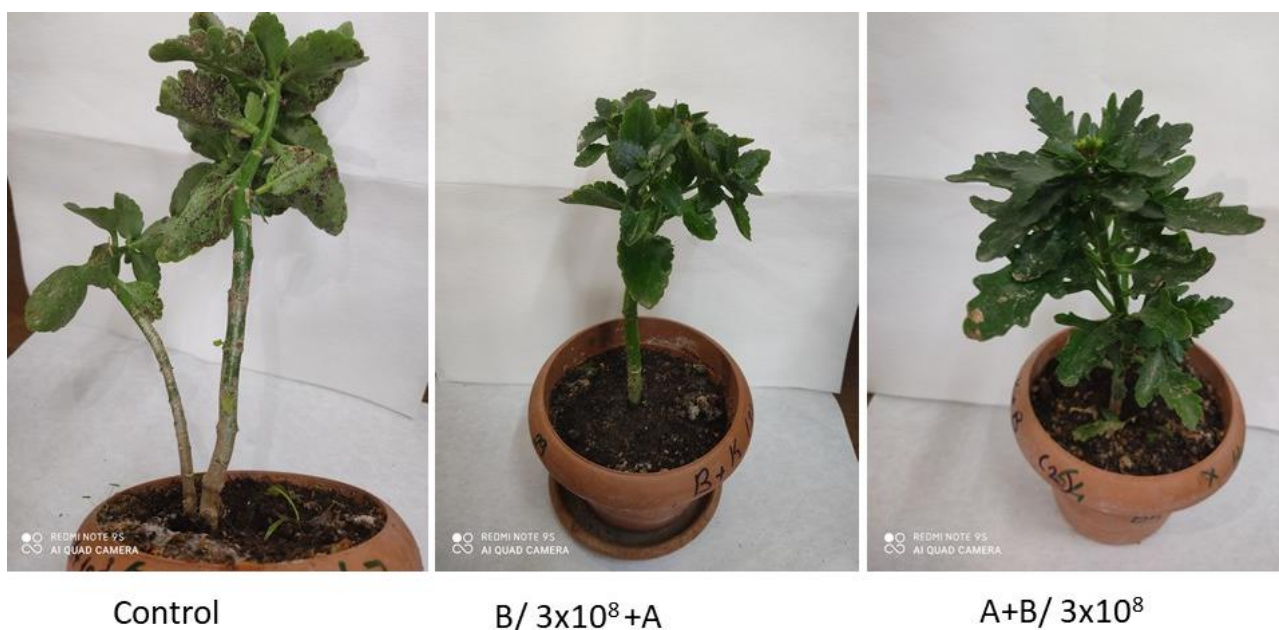


Figure 3. Visual representation of the effects of PGPR treatments on anthracnose disease in Kalanchoe. a) Control (inoculated with anthracnose without PGPR treatment), b) Anthracnose inoculation following PGPR treatment, c) PGPR treatment applied after anthracnose inoculation.

In addition, the bio-agent effects of PGPR treatments against anthracnose disease in Kalanchoe were also investigated in terms of protein profiles. When tissue samples from Kalanchoe leaves were analyzed, the protein profiles revealed electrophoretically heterogeneous patterns despite the presence of some common bands. Significant differences were observed in the protein banding patterns among treatments, particularly within the 20–150 kDa molecular weight range. In the B+A treatment group (bacterial solution application followed by anthracnose inoculation 4–5 days later), a greater number and intensity of protein bands were detected. Although the other three bacterial formulation treatments exhibited similar patterns to the B+A group, the number and intensity of the protein bands were comparatively lower (Figure 4).

Rhizospheric microorganisms have been reported to enhance plant growth by producing plant growth-promoting substances and by eliminating phytopathogens and nematodes (Mhatre et al., 2019). In a study conducted by Unlu (2007), two rhizobacterial isolates obtained from geranium cultivation areas were shown to inhibit leaf infections caused by *Xanthomonas axonopodis* pv. *pelargonii*, the causal agent of bacterial blight in geraniums, by 88–100%, while one PGPR isolate reduced stem infections by 63%. PGPRs have also been reported to act as potential agents for reducing damage caused by plant-parasitic nematodes (Tabatabaei and Saeedizadeh, 2017; Rashad et al., 2015).

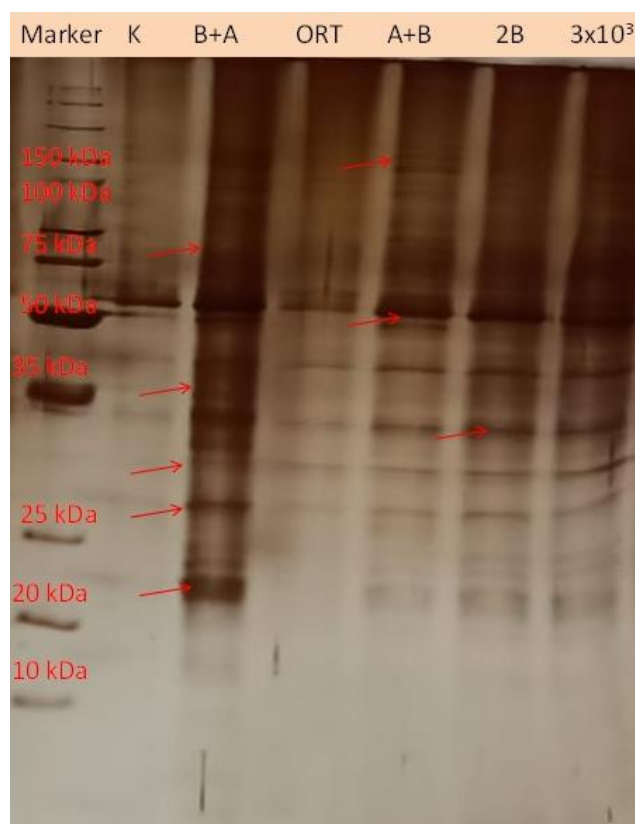


Figure 4. Gel image showing the effects of anthracnose inoculation and PGPR treatments on the protein profile of *Kalanchoe*.

4. CONCLUSIONS

Anthrachnose is a serious fungal disease affecting many ornamental plants, including kalanchoe. In this study, typical anthracnose symptoms were observed on the leaves and stems of kalanchoe, including small, circular to irregularly shaped lesions that were dark brown to black in color. All infected samples exhibited similar morphological characteristics consistent with anthracnose infection. The control plants showed intense disease symptoms, while plants treated with full-dose *Bacillus*-based PGPR formulations—both before and after disease onset—exhibited more healthy leaves with no visible symptoms. Disease severity increased as the bacterial concentration decreased, with more highest infection rates observed in plants treated with a threefold diluted bacterial solution.

Moreover, anthracnose infection caused a significant reduction in vegetative growth in control plants. In contrast, all PGPR-treated plants maintained better vegetative development, indicating the positive effect of bacterial formulations on plant growth under disease stress. These findings highlight the effectiveness of *Bacillus*-based PGPR formulations in suppressing anthracnose and promoting plant health, offering a promising, sustainable alternative to chemical control methods.

This research underscores the potential of rhizobacteria as biocontrol agents and provides a foundational understanding for their application in ornamental plant protection. While morphological observations provided clear insights into disease suppression, future studies

integrating molecular analyses will further validate and deepen our understanding of these biocontrol mechanisms. Overall, the use of *Bacillus* species as PGPR represents an environmentally friendly and sustainable approach to disease management. By enhancing plant growth, improving resistance to pathogens, and reducing the dependency on chemical fungicides, these beneficial microbes contribute significantly to the development of resilient and eco-conscious integrated pest management strategies. However, factors such as strain specificity, application timing, and environmental conditions should be carefully considered to optimize biocontrol efficacy in practical applications.

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