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RESEARCH ARTICLE

First report isolation, molecular identification and biological control of *Pantoea* leaf blight on *Phaseolus vulgaris* caused by *Pantoea eucrina* using *Trichoderma* species in Egypt.

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Abstract

This study aimed to isolate and identify the bacteria causing severe naturally occurring blight on leaves of common beans sprayed in several places under the governorate of Qena, both physiologically and molecularly. Four isolates were obtained from infected bean plants; however, the isolates' levels of pathogenicity varied. Isolate P-1 was the most virulent one against bean plants under greenhouse conditions. Molecularly, Isolate P-1 showed 99.55% similarity with the type strain o*f Pantoea eucrina* (Accession N0. NR116246T). *Trichoderma harzianum* and *T. viride in vitro and in vivo* were evaluated for the protection of bean plants against *Pantoea* leaf blight. Both biocontrol agents had an inhibitory effect on pathogen growth in in vitro experiments. In a pot experiment conducted in a greenhouse, the soil treated with *Trichoderma harzianum* and *T. viride* showed significant reductions in the number of bacterial pathogens and diseases. To our knowledge, this is the first report of isolation of the bacterium *Pantoea eucrina* from bean in Egypt and examined *Trichoderma harzianum* and *T. viride in* vitro and *in* vivo for controlling Pantoea leaf blight pathogen. It could be concluded that *Pantoea eucrina*, a new emergency plant pathogen, was originally isolated from *Phaseolus vulgaris* in Egypt for the first time and caused severe bean leaf blight in Upper Egypt. *In vitro* and *in vivo* applications of *Trichoderma harzianum* and *T. viride* have the potential to suppress bacterial pathogens, and disease incidence rates, and decrease pathogen populations in the leaves of treated bean plants.

Keywords: *Pantoea eucrina;* molecular identity; leaf blight; *Phaseolus vulgaris.*

1. Introduction

One of the most significant vegetable crops grown worldwide is the green bean (*Phaseolus vulgaris* L.), which is grown on over 25.000 hectares of land in Egypt and yields 3.00000 tonnes and 12.0000 kg71 ha of productivity (FAO 2009). According to Abo-Elyousr (2006) and El-Mougy *et al.* (2007), bean plants are susceptible to bacterial, fungal, and viral diseases in Egypt. Poor yields are primarily caused by bacterial and fungal infections (Ferreira *et al.*, 2006). Roughly 36% of global production potential is lost to pests and illnesses (Agrios, 2005).

Pantoea Species are known to cause leaf blight diseases in many crops like onion, cotton, eucalyptus, rice, maize, and sorghum (Morales-Valenzuela *et al.*, 2007, Swart, 2010; Doni *et al.*, 2019; Tufail *et al.*, 2020; Sepúlveda-Chavera *et al.*, 2023). Despite the fact that diseases belonging to this genus are becoming more and more important globally, identification procedures remain relatively simple. The majority of identification methods rely on phenotypic traits and basic genotypic approaches

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such as 16S rRNA sequencing (Janda and Abbott, 2007).

A sustainable and environmentally friendly alternative to the unfavorable use of chemical fungicides is plant-growth-promoting fungi (PGPFs). Among various methods of controlling plant diseases, using antagonistic fungi, like Trichoderma, is seen to be a desirable substitute for suppressing phytopathogens and enhancing plant development (Abdelmoteleb *et al.*, 2023). According to Mendoza-Mendoza *et al.* (2018), Trichoderma species have been shown to promote both systemic and local resistance against a range of bacterial, viral, and fungal infections. Through the activation of defense enzymes including peroxidases, hydroperoxide lyases, and phenylalanine ammonia lyases, as well as signaling cascades based on jasmonate/ethylene (JA/ET), Trichoderma colonizes plant roots and promotes induced systemic resistance (ISR) (Mayo *et al.*, 2016). Additionally, *Trichoderma* spp. triggers the expression of pathogenesis-associated (PR) proteins such as chitinase and β-1,3-endoglucanases through a salicylic acid (SA)-based systemic acquired resistance (SAR) response. According to Pimentel *et al.* (2020), the induced resistance can last for a long period and is often non-specific, offering protection against several diseases. There are no reports using *Trichoderma* sp. against Pantoea leaf blight on *Phaseolus vulgaris*, even though several reports of using *Trichoderma* fungi against various plant disease pathogens exist. During the years 2021 severe damage of common beans occurred in the natural field with leaf blight symptoms under Qena governorate -Upper Egypt. Our research aimed to isolate, purify, and identify the pathogen of the bacterial causal agent physiologically and molecularly. Examine the *in* vitro and potted capacities of *Trichoderma harzianum* and *T. viride* fungi to reduce the pathogen population.

2. Materials and Methods

2.1. Sample collection

Symptomatic common bean-infected plants were collected from natural fields and photographed.

2.1.1. Isolation of the bacterium

Bacteria were isolated from samples of diseased plants taken from every commercial field looked over. The infected leaves, stems, and pods were washed for ten minutes under running water, surface-sterilized for three minutes in 3.5% sodium hypochlorite, and then twice rinsed for one minute each in sterile water. A droplet of sterile water was used to macerate with infected pieces before they were scattered over a nutritional agar medium (NA). After 3-5 days of incubation at 28ºC, the development of colonies was observed on the plates. In order to obtain pure cultures, the single colony approach was used. A total of five individual colonies were chosen for morphological and genetic analysis.

2.1.2. Pathogenicity test under greenhouse condition

After being cultivated in nutritional broth for 12 hours, the bacterial isolates were harvested using a centrifuge and resuspended to 10^6 cfu/ml in sterile water. The susceptible bean variety (Balady) plants' leaves two weeks old were sprayed with bacterial suspension with 0.02/ml tween in a greenhouse with 26°C and >85% relative humidity. Lesions due to bacterial blight were noted after 15 days. Control plants were sprayed with sterilized distilled water. three replicates were used for each treatment.

2.1.3. Disease index

Disease index was determined 15 and 30 days after inoculation according to Louws *et al.* (2001). A five-grade diseases index was used to rate plants as follows: 1 has no symptoms; 2 has a few necrotic spots; 3 has a lot of necrotic spots; 4 has the majority of the leaf area impacted; and 5 has died. The study was conducted twice using three replicates.

2.1.4. Identification of the pathogenic bacteria

Based on their morphological, cultural, and physiological characteristics, the isolated bacterial culture that was shown to be pathogenic and induce symptoms of blight in bean plants was identified in accordance with Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984).

2.1.5. Molecular identification of bacterial isolate

According to Zumbro *et al.* (2015), the bacterial isolate was cultivated in sterile test tubes with 10 ml of nutritional broth medium. After 48 hours of incubation at 28ºC, the cultures were transferred to the Molecular Biology Research Unit at Assiut University to be extracted of their DNA using a Patho-gene-spin DNA/RNA extraction kit, which was donated by the Korean company Intron Biotechnology Company. The DNA of the sample was sent to SolGent Company, Daejeon South Korea for polymerase chain reaction (PCR) and gene sequencing. PCR was performed using two universal primers where 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'- GGTTACCTTGTTACGACTT-3'). Using a size nucleotide marker (100 base pairs), the purified PCR products (amplicons) were verified using electrophoreses on 1% agarose gel. After adding dideoxynucleotides (dd NTPs) to the reaction mixture, the amplicons were sequenced. Using 27F and 1492R primers, bacterial amplicons were sequenced in both the sense and antisense directions (White *et al.*, 1990). The National Centre of Biotechnology Information (NCBI) website's Basic Local Alignment Search Tool (BLAST) was used to further analyze the sequences. MegAlign (DNA Star) software version 5.05 was used to perform phylogenetic analysis on the sequences.

2.2. In vitro antagonistic

2.2.1. Sources of Trichoderma isolates

The microbiology lab generously contributed one isolate of the fungus *T. viride* and *T. harzianum* that were used in the present study. South Valley University, Faculty of Agriculture.

2.2.2. Evaluation of antifungal assay

Dual culture assay: five millimeter-diameter *T. viride* and *T. harziaum* discs were challenged *in* vitro to inhibit the growth of the isolated bacteria. Picked fungus was taken out of the periphery of 4-day-old PDA cultures and put on one side of Petri dishes filled with nutrient agar medium. On the other side of the plate were two isolated bacterial streaks. The pathogen bacteria alone were used to inoculate the NA medium (control plate). After incubating the plates for 72 hours, the inhibition percentage was measured. The study was conducted twice using three replicates.

2.2.3. Inhibition percentage

By calculating the radial growth of the bacteria cultured on control and altered plates, the inhibition percentage was determined using the following formula (Harlapur *et al.*, 2007): P% % = 100 × (C – T) / C

where T is the average radial growth in plates altered with *T. viride* or *T. harzianum,* C is the average radial growth in control plates, and P% is the proportion of pathogen growth suppression.

2.3. Pot experiment

2.3.1. Plant material

Greenhouse-grown bean variety ''Balady'' was used for all experiments. Under greenhouse conditions at 25 C°, seeds were grown in 15 cm pots in a soil mixture that included sand and slow-release fertilizer (1% NPK 12:4:6). Watering was done as needed.

2.3.2. Inoculum production and seed inoculation with Trichoderma species

T. harzianum and *T. viride* were grown on PDA in Petri plates for 7 d at room temperature. After adding sterile distilled water (SDW) to each plate, a sterile spatula was used to scrape the colony surface, and cheesecloth was used to filter the resulting conidia suspension. The conidia suspension was adjusted to 10^8 conidia/mL. Tween-20 (0.01% v/v) was added as a wetting agent. *Phaseolus vulgaris* cv. Balady bean seeds were soaked in the conidial suspension for five minutes, then planted in soil that had been

improved with 10^8 conidia/g for a week earlier and kept in a greenhouse. In order to achieve this, the conidia suspension was made as previously mentioned and thoroughly combined with the soil (clay, sand, and compost) in a 1:1:1 v/v ratio. *P. eucrina* inoculum and disease symptom grading was carried out in accordance with the previously mentioned pathogenicity test protocol.

2.3.3. Effect of T. harzianum and T. viride on pathogen multiplication in vivo

Bacterial colonies were recovered from bean plant tissues as follows: 10 to 15 days after *P. eucrina* inoculation, bacterial colonies were harvested from the tissues of treated or untreated bean plants. using. The 5-mm diameter leaf discs were removed aseptically using a crook borer, homogenized in 1 ml of sterile 0.06% NaCl solution, and serially diluted. Onto (NA) medium agar plates, aliquots of several dilutions (0.1 ml) were plated. Following a 48-hour incubation period at 28°C, developing colonies on every dilution plate exhibiting bacterial growth were counted.

2.4. Experimental design and data analysis

There were four replicates of each treatment in the fully randomized experimental setup. Duncan's multiple range tests (Duncan's test P>0.05) were used to differentiate the treatment means obtained after the experiment was performed at least twice (Gomez and Gomez, 1984).

3. Results and discussion

3.1. Isolation of the casual pathogen and pathogenicity tests

On the plants leaves, stems and pods natural signs were evident. The plant seems burned, and the dead leaves are still attached to the water-soaked, huge, brown, angular patches of dead tissue that are encircled by a very thin zone of yellow tissue. Spots on pods are often round, black to reddish-brown in colour. Reddish-brown, water-soaked stems with a yellow border were seen to be girdled, resulting in places on the stems that were wilting. (Figures.1α 2). Four pure bacterial isolates were obtained from naturally diseased bean leaves. Pathogenicity of isolated bacteria was tested on bean plants.

Figure 1. Natural symptoms of bacterial leaf blight occurred on common bean.

Figure 2. Natural symptoms on leaves, stems and pods infected by *Pantoea eucrina* on common bean.

Data illustrated in Figure. (3) show that all four isolates were pathogenic to bean plants and varied in their pathogenicity. Isolates No. 1 caused the highest disease index followed by isolates No. 2, and 3, while isolate No. 4 caused the lowest one. Blight symptoms within two weeks of inoculation were distinct as dark brown angular lesions on plant leaves. (Figure .4). Control plants inoculated with sterile water had no symptoms. Our results are in agreement with those reported by (Swart, 2010; Tufail *et al.*, 2020). To our knowledge, this is the first report of *Pantoea eucrina* causing Pantoea leaf blight of common bean in Egypt.

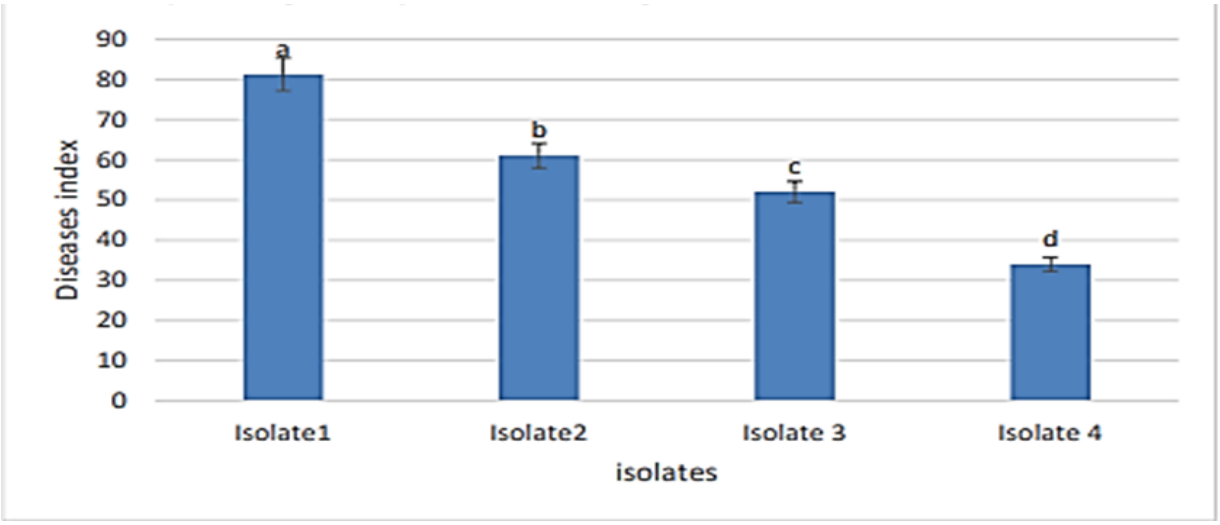

Figure 3. Pathogenicity tests of four isolate of *Pantoea eucrina* on bean plants. Bars indicate the standard deviation. The values in the column followed by the same letter are not significantly different according to Duncan's at P<0.05.

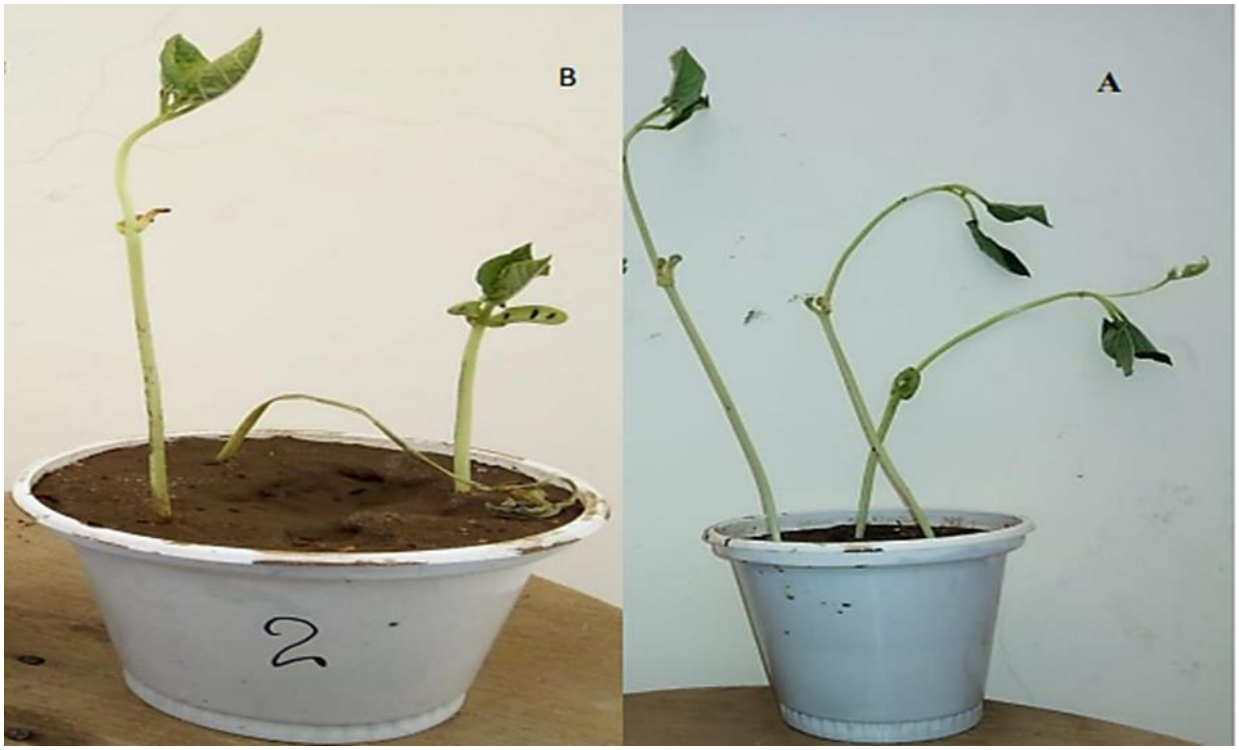

Figure 4. Pathogenicity test under greenhouse condition(a). control plants, (b). sprayed plants with *P. eucrina* isolate No.1

3.2. Identification of pathogens

The pathogenic bacteria that were isolated were identified based on their morphological, cultural physiological, and pathological features, as well as those documented by Krieg and Holt (1984). All examined isolates had yellow pigmentation, formed mucoid colonies, were gram-negative, and generated sticky slime when tested with the potassium hydroxide (KOH) test. The isolate's molecular profile exhibited 99.55% similarity to that of the *Pantoea eucrina* type strain. (Accession N0. NR116246T) figure (5). Within the Enterobacteriaceae family, the genus Pantoea comprises a diverse group of rod-shaped, yellow-pigmented Gram-negative bacteria that have been identified as plant pathogens that cause wilting, galls, soft rot, and necrosis in a variety of crops of susceptible plants (Muraschi, Friend and Bolles, 1965; Ewing and Fife, 1972; Brady *et al.*, 2008; Volksch *et al.*, 2009; Nadarasah and Stavrinides, 2014; Tufail *et al.*, 2020). Tatumella, Erwinia, and Pantoea are closely related (Brady *et al.*, 2010a, b). The bacterial species Pantoea and Xanthomonas are becoming more significant as newly discovered bacterial diseases (Swart, 2010).

3.3. Effect of on common blight causal organisms in vitro

Results in figure (6) showed that, *T. harzianum* and *T.viride* were able to suppress bacteria and prevent the pathogen's proliferation *in* vitro. The inhibition percentage of *T. harzianum* was 53%, while that of *T. viride* was 66%. The application of biocontrol in the management of plant diseases has various known modes of action (Ran and others, 2005). *Trichoderma* sp. is an antagonistic fungus that directly competes with diseases for resources and available space. either direct parasitism in fungi or the production of metabolites and antibiotics (Ezziyyani *et al.*, 2004a).

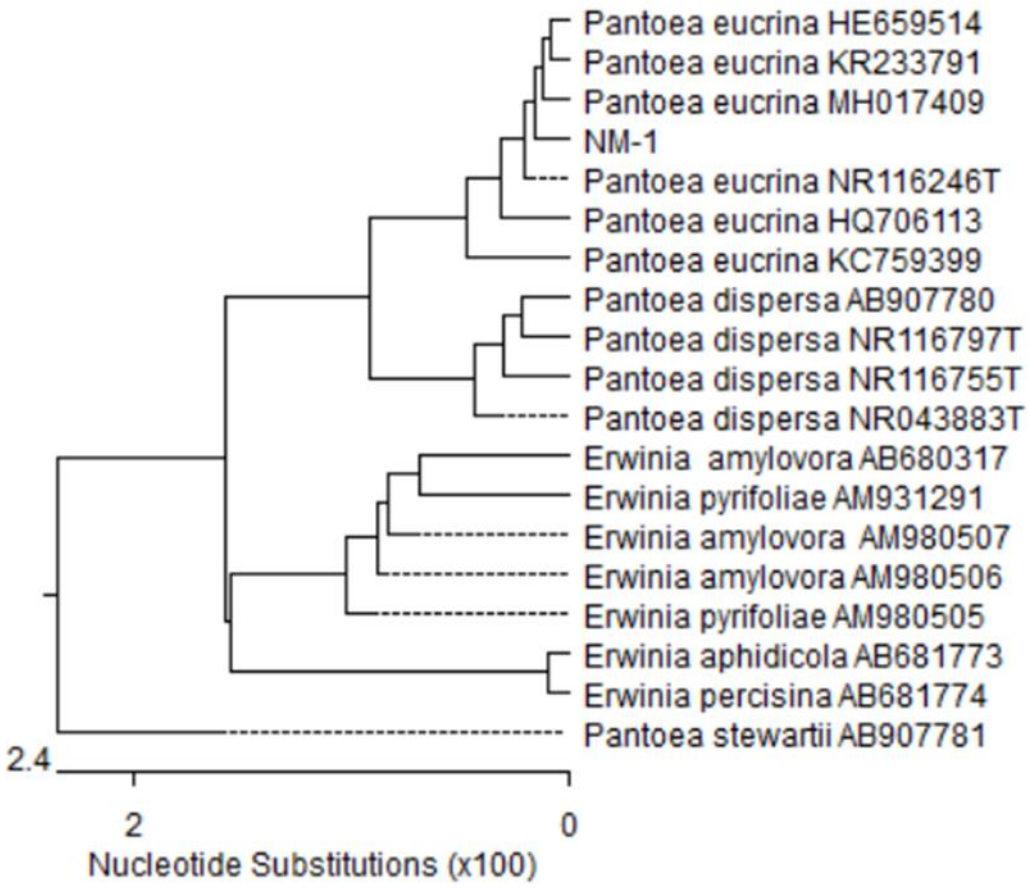

Figure 5. Phylogenetic tree based on 16S sequences of rDNA of the bacterial strain isolated in the present study (NM-1) aligned with closely related sequences accessed from the GenBank. The sample showed 99.55% similarity with the type strain of *Pantoea eucrina* (Accession N0. NR116246T)

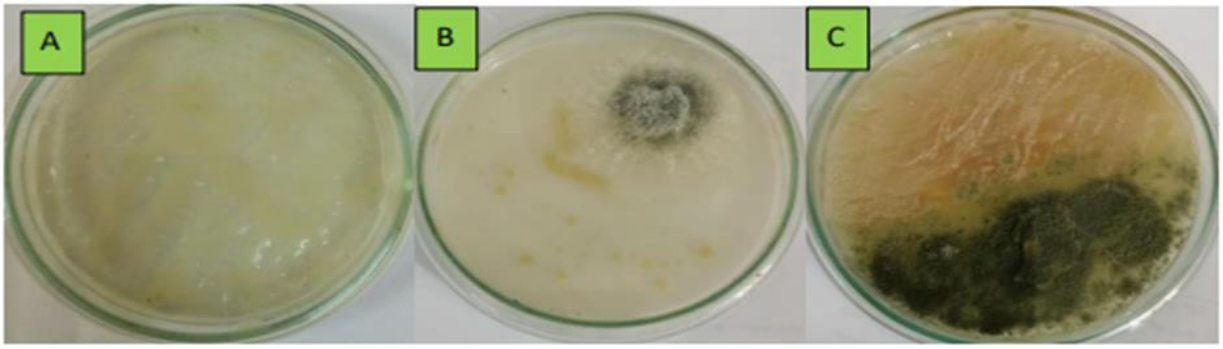

Figure 6. *In* vitro antagonistic of *T. harzianum* and *T. viride* against *Pantoea eucrina* on NA medium A, *Pantoea eucrina* control. B, *T. harzianum* + *Pantoea eucrina*. C, *T. viride* + *Pantoea eucrina.*

3.4. Effect of Trichoderma sp on Pantoea leaf blight causal organisms in vivo

The findings in figures 7 and 8 demonstrated that in comparison to control plants, treated soil and bean seeds treated with spore suspension of *T. harzianum* or *T. viride* before cultivating for 7 days considerably reduced the number of bacterial cells. The higher reduction in growth of

the pathogen within the host than the control treatments was after 15 days (Table 1). The ability of *T. harzianum* and *T.viride* to decrease the amount of bacteria present in host cells may be attributed to their indirect influence on lowering the amount of nutrients that the bacteria can use to thrive. Pathogen proliferation may be inhibited by a low concentration of nutrients in the intercellular space (Goodman *et al.,* 1986). *Trichoderma*. spp. has been known for its ability to act as biological control agents against plant pathogens (Askew and Laing 1993). Through mycoparasitism, antagonism, colonization of roots, and systemic action. *Trichoderma* spp. eliminate plant pathogen propagules in soil or on plant roots and affect foliar pathogens (Alfano *et al.,* 2007). This is the first report of a *Trichoderma* isolate causing systemic resistance against bean leaf blight. Our result explains that significant success in biocontrol is achieved under *in* vitro and *in* vivo conditions. Even though additional study is required to comprehend the antagonistic mechanisms. It is evident, that a microbial biocontrol agent is very effective, less expensive than chemicals and safe for both humans and animals.

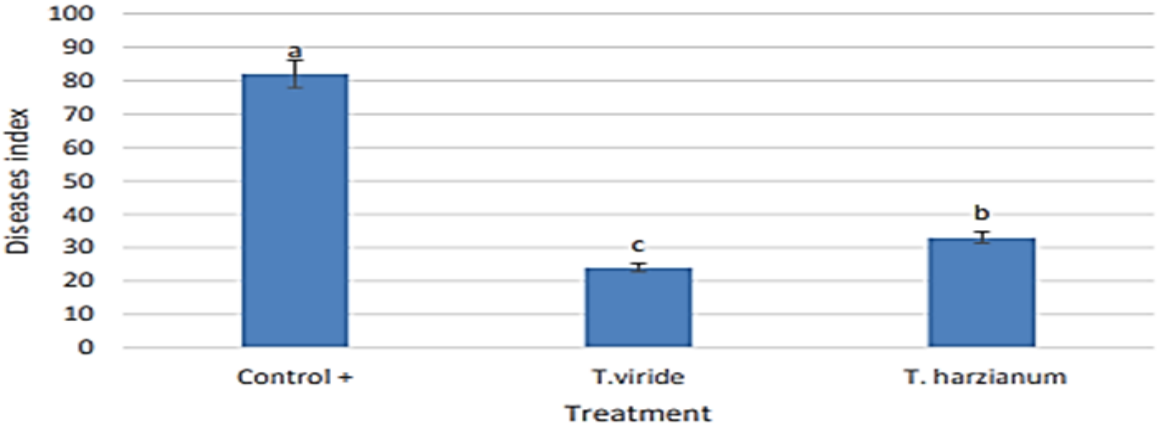

Figure 7. Disease index of common blight disease on bean after treatments with *T. harzianum* or *T.viride* under greenhouse conditions. Note: Different letters indicate significant differences among treatments within the same columns according to Duncan's at P<0.05.

Figure 8. showed the effect of *T. harzianum* and *T. viride in* vivo against Pantoea blight causal organisms on bean plants. A, bean plants inoculated with *Pantoea eucrina* only. B, bean plants inoculated with *Pantoea eucrina* + *T. harzianum*. C, bean plants inoculated with *Pantoea eucrina* + *T. viride*. D, control plants (non-inoculated).

Table 1. Population of *P. eucrina* in bean plants leaves after treatment with *Trichoderma*. sp for 10-15 days.

Dayes after application	Number of colonies	Number of colonies	Number of colonies
10	Control 9^a	*T. harzianum* 5.3^b	*T. viride* 4.4^c
15	*Control* 14^a	*T. harzianum* 4.2^b	*T. viride* 2.2^c

Note: Values in the column followed by different letters indicate significant differences among treatments according to Duncans at P<0.05.

4. Conclusion

Pantoea eucrina bacterium was isolated for the first time in Egypt as a bean leaf blight pathogen. *Trichoderma harzianum* and *T. viride in vitro and in vivo* could reduce the rate of the disease under greenhouse conditions and the number of the pathogen population on treated plants leaves against Pantoea leaf blight.

Declarations

- Ethics approval and consent to participate

Not Applicable

This manuscript is in accordance with the guide for authors available on the journal's website. Also, this work has not been published previously and is approved by all authors and host authorities

- Consent for publication

Not applicable

- Availability of data and material

Not applicable

- Competing interests

No potential conflict of interest was reported by the authors.

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All authors contributed equally in the manuscript

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