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Research Article

Induced Resistance Mechanism of Twisted Disease Suppression of Shallot by *Bacillus* spp.

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ABSTRACT

Plant Growth Promoting Rhizobacteria has been known for its ability to induce plant resistance on shallot against twisted disease. Its ability as a bioprotectant agent is estimated to be comparable to the efficacy of Trichoderma which is currently widely used as a biological control agent.. This study aims to determine the content of jasmonic acid, salicylic acid, peroxidase, and disease suppression in shallot by application of *Bacillus velezensis* B-27, *Bacillus cereus* RC76, and application with combination of both rhizobacteria. The application was carried out with tuber dipping for 30 minutes in each treatment with a bacterial density of 10^8 CFU mL^{-1}. Application using Trichoderma was used as the comparison treatment, and the control plot was not given any treatment. Pathogen inoculation was carried out simultaneously as planting using *Fusarium acutatum* with a spore density of 10^6 CFU mL^{-1}. The jasmonic and salicylic acids content was measured using the *High-Performance Liquid Chromatography* method, and the peroxidase content was determined using the spectrophotometric method. Disease suppression was measured at 10-day intervals. The results showed that treatment with *Bacillus cereus* RC76 increased jasmonic and salicylic acid levels, while application with *Bacillus velezensis* B-27 showed the highest level of peroxidase. Treatments with *Bacillus* spp. were able to suppress twisted disease by 72.2% to 100%. This study demonstrated that application *Bacillus* spp. suppressed twisted disease on shallot and quantitatively increased the content of jasmonic and salicylic acid as induced resistance mechanism against pathogens.

Keywords: *Bacillus* spp.; induce resistance; shallot; twisted

INTRODUCTION

Twisted disease is one of the limiting factors in shallot production. Twisted disease can be caused by several species of Fusarium, such as *F. solani*, *F. oxysporum*, and *F. acutatum*. These pathogens species have different roles in the onset of the symptoms. The attack of *F. solani* and *F. acutatum* caused wilting symptoms in plants, *F. solani*, *F. oxysporum* and *F. acutatum* caused tuber rotting, while the typical symptoms of twisted (leaf twisting and wilting) were caused by *F. solani* and *F. acutatum* (Lestiyani *et al*., 2021). The biological control agent widely used by growers and proven to suppress disease in shallots is

Trichoderma spp. (Jumadi *et al*., 2021). In addition, other microbes that are widely studied and developed as biological agent is *Plant Growth Promoting Rhizobacteria* (PGPR). Its ability as a bioprotectant, biostimulant, and biofertilizer (Tuhuteru *et al*., 2018) makes the utilization of PGPR has great potential in improving plant productivity (Joko *et al*., 2012).

One of the mechanisms of disease suppression by PGPR is the induction of plant resistance. PGPR can induce plant resistance by mediating the formation of *Induced Systemic Resistance* (ISR) involving jasmonic acid and ethylene production. Several Bacillus species reported to generate ISR and significantly reduce the incidence and intensity of several diseases in various host plants are *B. amyloliquifaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilis*, *B. mycoides*, and *B. sphaericus*. Although PGPR generally induces plant resistance through the ISR mechanism, some pathogenic rhizobacteria can stimulate the Systemic Acquired Resistance (SAR) pathway involving salicylic acid (SA) and Pathogenesis-related protein (PR protein) (Choudhary *et al*., 2007). A study by Taufik *et al*. (2010) showed that the application of PGPR *Pseudomonas fluorescens* and *Bacillus* sp. increased the content of salicylic acid and peroxidase in chili to suppress the *Cucumber Mosaic Virus* infection. The combination of ISR and SAR mechanisms can improve plant protection against pathogen infection, compared to ISR or SAR mechanisms alone (Choudhary *et al*., 2007).

Research by Rahma *et al*. (2020) showed that tuber dipping and plant spraying using *B. velezensis* B-27 could reduce the intensity of purple blotches and twisted disease on shallot. Dwimartina *et al*. (2017) also reported that *B. cereus* has antagonistic ability against the pathogen *R. syzygii* subsp. *syzygii*. This study evaluated the application of PGPR using bacteria from the genus Bacillus, namely *Bacillus velezensis* B-27 and *Bacillus cereus* RC76, by tuber dipping method on shallot. The application of PGPR is expected to suppress the incidence and intensity of twisted disease in shallot and increase the activity of enzymes and hormones involved in the induction of plant resistance (Choudhary *et al*., 2007).

MATERIALS AND METHODS

This research was performed using a completely randomized design (CRD) in the Greenhouse of Faculty of Agriculture, Universitas Gadjah Mada, from November 2021 until January 2022. Jasmonic and salicylic acid content was analyzed at the Laboratory of Agrochemical Residue, The Agricultural Environment Research Institute (*Balai Penelitian Lingkungan Pertanian* [BALINGTAN]), Bogor. Analysis of peroxidase level was performed in the Laboratory of Plant Pathology, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada. The shallot variety used in this research was Bima Brebes. The

planting medium was sterilized soil and manure mixed with a 2:1 ratio. The treatments for this research is as follows:

A = application of *Bacillus velezensis* B-27

B = application of *Bacillus cereus* RC76

C = application of bacterial consortia (*Bacillus velezensis* B-27 + *Bacillus cereus* RC76)

D = application of *Trichoderma asperellum*

E = control

PGPR Application (*Bacillus* spp.)

The PGPR isolates used were *B. velezensis* B-27 and *Bacillus cereus* RC76. Bacterial isolates were grown on Yeast Peptone Agar (YPA) media and incubated for 48 hours. The colonies were then suspended with sterile water to a density of 10^8 CFU ml^{-1} measured with a spectrophotometer wavelength of 600 nm. The tuber dipped for 30 minutes in 120 mL of bacterial suspension for each treatment, then air-dried before planting (Tuhuteru *et al*., 2019; Rahma *et al*., 2020).

Shallot Planting

Shallot planting was carried out in the greenhouse of the Faculty of Agriculture, Universitas Gadjah Mada. The planting was done in the afternoon using the treated tuber that had been air-dried. Planting media was put in polybags with a size of 40×40 cm. The planting media was then moistened, and five tubers were planted in each polybag.

Pathogen Inoculation

The pathogen used for inoculation was *Fusarium acutatum*, as it has the highest virulence among other twisted-causing pathogens (Lestiyani *et al*., 2021). *F. acutatum* isolates were grown on Potato Dextrose Agar (PDA) medium for seven days, then the spores were harvested and suspended to obtain a spore density of 10^6 CFU/ml. Pathogen inoculation was carried out by pouring 10 mL of pathogen suspension for each plant simultaneously as shallot planting (Wijoyo *et al*., 2020).

Trichoderma Application

The isolate used in this treatment was *T. asperellum* obtained from the Biological Control Laboratory, Pakem, Yogyakarta. *T. asperellum* suspension was prepared by suspending conidia and conidiophores growing on corn media in sterile water and mixed until homogeneous to obtain a spore density of 10^6 CFU mL^{-1}. Conidia density was calculated

using the haemacytometer. *T. asperellum* was applied by pouring 50 mL of spore suspension for each polybag at the time of planting and repeated 20 and 40 days after planting (DAP).

Determination of Twisted Disease Incidence

Twisted disease incidence was observed at 10 DAP with 10-day intervals. Observations were made by counting the number of diseased plants per polybag and then determined using the following formula (Korlina & Baswarsiati, 1995):

$$Disease\ incidence = \frac{Total\ number\ of\ infected\ plant}{Total\ number\ of\ plant\ per\ polybags}\ x\ 100\%$$

Determination of Twisted Disease Intensity and Area Under Disease Progress Curve (AUDPC)

Twisted disease intensity was observed at 10 DAP with 10-day intervals, adopting the method of Nugroho *et al*. (2015) with the following formula:

$$Disease\ intensity = \sum \frac{(nxv)}{NxZ}\ x\ 100\%$$

Where n = the number of infected plants showing a certain score; v = severity score (0 = no symptoms, 1 = partially yellowed leaves, 2 = yellowed leaves began to dry, 3 = the leaves dried and wilted, 4 = The tuber began to rot, 5 = The plant died); N = the highest score value; Z = total number of plants observed.

The AUDPC value was determined using the formula from Campbell and Maddeen (1990) as follows:

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{Yi + Y_{i+1}}{2}\right)(t_{i+1} - ti)$$

Note:

AUDPC = area under disease progress curve

n = total number of observations

Y_{i+1} = assessment of disease intensity at the i^{th} observation + 1

Yi = assessment of disease intensity at the i^{th} observation

t_{i+1} = time at the i^{th} observation + 1

ti = time at the i^{th} observation

Analysis of Jasmonic and Salicylic Acid Content

The sample used is the leaves of the plant at 10 Day After Inoculation (DAI) (Taufik *et al.,* 2010). The leaves were selected as samples because the salicylic acid formation pathway of the isochorismate acid pathway is in plant chloroplasts (Vicente & Plasencia, 2011). Besides that, the high content of jasmonic acid in leaves is related to its role in the leaf deterioration process as well as its role in inducing Reactive Oxygen Species (ROS), which damages chloroplasts first (Ullah *et al*., 2019). The analysis was carried out using the method of Tenhaken and Rubel (1997). Extraction was done by grinding 1 g of plant sample in a mortar added with 3 ml of methanol and acetone mixture with a 1:1 ratio. The suspension was then placed into the Eppendorf tube. The supernatant was separated from the pellet. The pellet was then extracted again by adding 1 ml methanol and acetone mixture (1:1, v/v) and centrifuged at 5,000 rpm for 10 minutes. The supernatant was then mixed with the supernatant from the first extraction before being centrifuged again at 5,000 rpm for 10 minutes. The supernatant was then air-dried, and the dried residue was suspended by adding 30% methanol. The suspension was then centrifuged at 5,000 rpm for 10 minutes. The pellet was discarded, and the supernatant was submitted for qualitative and quantitative analysis using High-Performance Liquid Chromatography (HPLC) at Laboratory of Agrochemical Residue, The Agricultural Environment Research Institute (*Balai Penelitian Lingkungan Pertanian* [BALINGTAN]), Bogor.

The mobile phase used a solution of methanol-sodium acetate buffer 50 mM pH 4.5 (30:70 in 500 ml), homogenized for 10 minutes using a magnetic stirrer with a flow rate of 0.6 ml/minute. The sample and the mobile phase solution were filtered using a 0.45 µm RC cellulose acetate filter membrane. The stationary phase used colon C18, with Shimadzu C-R7A plus chromatopaque. The wavelength used was 280 nm with a VP-ODS Ultrasphere column and UV detector at 280 nm.

Quantitative analysis was performed to determine the levels of metabolites in the sample by converting the sample area to a standard area with a known concentration on the calibration curve. Calibration curves were obtained from area data of several standard compounds with known concentrations.

The difference between salicylic and jasmonic acid measurements is their standard chromatograms. Standard chromatograms of salicylic acid and jasmonic acid were prepared with a concentration of 0.063 ppm with the following calculation formula (Tenhaken & Rubel, 1997):

$$\text{Enzyme activity (mg Kg-1)} = \frac{Area\ Spl}{Area\ std} \times Kons.std \times \frac{FP}{BT}$$

Note :

Area spl = sample area on chromatogram reading

Area std = standart area

Kons std = standart concentration

FP = dilution factor

BT = total sample weight (mL)

Analysis of Peroxidase Level

The sample used is the leaves of the plant at 10 DAI (Taufik *et al.,* 2010). The leaves were selected as sample because the hight content of peroxides is related to its role in the ROS mechanism which damages the chloroplasts first (Vellosilo *et al*., 2010). Peroxidase level analysis was accomplished using the method by Suswati *et al*. (2015). Extraction was carried out by grinding 1 g of plant sample in a mortar added with a mixture of 0.5 M potassium phosphate buffer pH 7 and 0.1 g polyvinylpolypyrrolidone (1:1, 2.5 mL). The mixture was then filtered using two layers of gauze and centrifuged at 6000 rpm for 15 minutes at 4°C. The supernatant was then used for the analysis of enzyme activity. Determination of peroxidase enzyme activity was carried out by mixing 5 ml of pyrogallol solution (containing 0.631 g of pyrogallol and 0.05 M phosphate buffer pH 6 with a final volume of 100 ml) with 0.2 ml of enzyme extract into a tube. The absorption values were measured at a maximum wavelength of 420 nm using a Genesys 10S UV-VIS spectrophotometer. 0.5 ml of 1% H_2O_2 was then added to the buffer and enzyme solution. The solution was then incubated for 5 minutes for the control extract and 30 minutes for the sample extract for each treatment. The absorbance value was measured again, and the changes were recorded.

After the before and after incubation absorbance values were obtained, the enzyme activity was determined by the following formula (Yang *et al.,* 2019):

$$\text{Unit ml}^{-1}\text{ enzyme} = \frac{(rA420nm/20SecSample - rA420nm/20SecBlank)(3)(df)}{(12)(0.2)}$$

Note:

3 = total volume (mL)

df = dilution factor

12 = coefficient of 1 mg/mL purpurogalin at 420 nm.

0.2 = volume (mL) used

One (1) unit defines the change of 1 mg of pyrogallol to 1 mg of purpurogalin in 20 seconds at 20ºC, pH 6.

RESULTS AND DISCUSSION

Effect of Application with *Bacillus* spp. in Disease Suppression

A common symptom of twisted disease is leaf wilting from tip to base, along with leaf twisting (Abdel-Kader *et al*., 2019). Wiyatiningsih *et al*. (2009) documented that the fastest incubation period for twisted disease was 15 days after planting (DAP) for shallot grown in the rainy season, and the longest incubation period was 50 DAP for shallots planted in the dry season. The twisted disease incidence and intensity observation was done at 10-day intervals, starting on the tenth day after pathogen inoculation. The results of the disease incidence observation (Table 1) showed a significant difference in the incidence of disease between the controls and all other treatments. Twisted disease incidence in plants treated with *B. velezensis* B-27 (4%), *B. cereus* RC76 (0%), as well as with combination (16%), showed a lower incidence level compared to the control plot (36%). The disease incidence in plants treated with *Bacillus* spp. is not significantly different from the plot treated with *T. asperellum* (12%). The study results found that the incidence in control plants was still relatively low, this might be related to the virulence of the pathogen used. Wijoyo *et al.* (2020) showed that the incidence of moler disease in control plants with *F. acutatum* inoculation was only about 28.33% to 34.03%.

The disease intensity (in Table 1) showed that the control plot had the highest disease intensity compared to the other four treatments. There was no significant difference between the plots treated with *B. velezensis* B-27, *B. cereus* RC76, bacterial consortia, and *T. asperellum*. The disease suppression in the *B. velezensis* B-27 treated plot reached 89%, in the *B. cereus* RC76 treated plot reached 100%, and in the bacterial consortia treated plot reached 72.2% compared to the control.

The area under the disease progress curve (AUDPC) was determined using disease intensity development data from time to time (Milati *et al.,* 2021) to estimate plant resistance to disease. Figure 1 showed that the control treatment had the highest AUDPC value (798), while the lowest AUDPC value was in the *B. cereus* RC76 treatment (0), followed by *B. velezensis* B-27 treatment (70), bacterial consortia treatment (175), and *T. asperellum* treatment (322). A low AUDPC value indicates a higher suppression of twisted disease in

shallots, whereas a higher AUDPC value indicates a lower suppression of the disease (Hersanti *et al*., 2019).

Disease suppression in *Bacillus* spp. treated plots are in accordance with Chang *et al*.'s (2003) study, which documented that *B. cereus* could inhibit the growth of plant pathogenic fungi such as *Fusarium oxysporum*, *F. solani*, and *Phytium ultimum*. *Bacillus cereus* produced secondary metabolites, namely chitinase, which inhibited the hyphae growth of *F. oxysporum* and *P. ultimum*. A study by Resti *et al*. (2017) also showed that *B. cereus* suppressed the growth and development of *F. oxysporum*, *Colletotrichum capsici*, and *C. gloeosporioides* in shallots through several mechanisms such as the production of salicylic acid, which has an important role in the SAR mechanism, lipase enzymes, proteases, and phosphate solvents. Protease and lipase enzymes are involved in the hydrolysis of pathogen cell walls (Wu *et al*., 2017). Resti *et al*. (2017) reported that *B. cereus* suppressed the growth of *F. oxysporum* by 14.54% in *in-vitro* tests.

Rahma *et al*. (2020) explained that tuber dipping treatment using *B. velezensis* B-27 could reduce the growth and development of twisted disease; this report is in accordance with the results of this study (Table 1). The reduction was due to compounds produced by *B. velezensis* B-27. *B. amyloliquefaciens* subsp. *Plantarum* FZB42, identified by L.T. Wang *et al*. (2008), has similarities with *B. velezensis*, producing antifungal compounds such as surfactin, fengisin, and bacilomycin. Basilomycin has the highest inhibition of *Fusarium graminearum* growth based on the in-vitro test. The inhibition mechanism by bacilomycin occurs through swelling of the fungal hyphae tips and conidia. This mechanism reduced fungal germination by up to 5.44% (Rahma *et al.,* 2020). Basilomycin D suppresses the growth of fungal pathogens by disrupting the plasma membrane of hyphae and conidia, causing cytoplasmic rupture and plasmolysis. In addition, another inhibitory mechanism of bacilomycin D reported was the induction of ROS accumulation in the hyphae and conidia of *F. graminearum*. Basilomycin D induced the expression of glutathione reductase and thioredoxin genes that play a role in ROS synthesis in *F. graminearum* (C. Wang *et al*., 2020).

Another study by Khan *et al*. (2020) showed that the inhibition percentage of *F. oxysporum* by *B. velezensis* LIe-9 reached 68.56% in the in-vitro test. The same study also identified bioactive compounds that act as antimicrobials such as diketopiperacin, cyclopeptide, latrunculin A, triamtrene, rubiadin, and others that may contribute to the antifungal activity of *B. velezensis* Lie-9. Chen *et al*. (2020) reported that *B. velezensis* CLA178 could suppress the incidence of crown gall disease in *Rosa multiflora* through the

ISR mechanism.

Jasmonic Acid and Salicylic Acid Content

The results of this study (Figure 2) showed that the highest jasmonic acid content was found in *B. cereus* RC76 treated plot, followed by *T. asperellum* and *B. velezensis* B-27 treated plots. The lowest jasmonic acid content was obtained in the control treatment, which was not significantly different from the bacterial consortia treatment.

Analysis of salicylic acid content (Figure 3) showed that plants treated with *T. asperellum* had the highest salicylic acid content, followed by *B. cereus* RC76 treatment. The lowest value of salicylic acid content was obtained in the control treatment and was not significantly different from the treatment using *B. velezensis* B-27 and the consortia of the two bacteria.

There was an increase in jasmonic acid and salicylic acid content in the *B. cereus* RC76 treated plot compared to the control. This result is in accordance with the previous study by Niu *et al*. (2011), which reported that *B. cereus* could activate plant resistance that depends on the jasmonic acid and salicylic acid pathways in *Arabidopsis thaliana*. Niu *et al*. (2012) explained that *B. cereus* AR156 could induce the expression of genes related to resistance through both the salicylic acid and jasmonic acid pathways. In that study, it was reported that the salicylic acid-dependent pathway was stimulated first, which was indicated by an increase in PR1 expression, then followed by the induction of the jasmonic acid-dependent pathway. The initiation mechanism of ISR induced by PGPR is not fully understood; however, several elicitors have been identified, such as flagellins, lipopolysaccharides (LPS), volatile organic compounds (VOCs), and siderophores. In plants, PGPR generally activates ISR via the jasmonic acid or ethylene pathway, but it is possible that in some cases, the ISR mechanism via the salicylic acid pathway is also increased (Romera *et al*., 2019).

The high jasmonic acid content in treatment using *B. velezensis* B-27 was in accordance with the study by Chen *et al*. (2020), which recorded that *B. velezensis* CLA178 was able to induce the resistance of *Rosa multiflora* against crown gall disease through the jasmonic acid and or ethylene signaling pathway. In some cases, it is known that there is a crosstalk mechanism between resistance signals that depend on salicylic acid, jasmonic acid, or ethylene (Koornneef & Pieterse, 2008 as cited in Niu *et al*., 2011). This crosstalk activity acts as a plant efficiency control mechanism to suppress or stimulate one pathway, depending on the type of resistance the plant requires (Kunkel & Brooks, 2002 as cited in Niu *et al*.,

2012). Several studies have shown that the salicylic acid pathway and the jasmonic acid pathway are antagonistic to each other, which suppress one or stimulate the activity of one another (Koornneef & Pieterse, 2008 as cited in Niu *et al*., 2011). The signaling pathways in plant resistance may differ depending on the plant species and the microbe involved (Romera *et al*., 2019).

Enzymatic Assay of Peroxidase

The enzymatic assay of peroxidase (Figure 4) showed a similar result of peroxidase levels between the control plot, *B. velezensis* treated plot, and *T. asperellum* treated. treatment with *B. cereus* RC76 and bacterial consortia resulted in a lower peroxidase level than the control plot. This result is similar to a previous study by Chen *et al*. (2020), which recorded that the treatment using *B. velezensis* CLA178 did not give a significantly different effect of peroxidase content with the control treatment.

Peroxidase is an enzyme group oxidoreductase that can catalyze the oxidation reaction or reduction (Al-Baarri, 2016). Peroxidase is involved in plant resistance response to pathogens and is included in PR proteins. Peroxide also has a role in the biosynthesis of lignin which serves as a mechanism of one form of physical resistance in plants by inhibiting pathogenic infections (Sukma *et al*., 2012). The lower peroxidase enzyme content in the treatment using *B. cereus* RC76 could occur because the compounds or signaling pathways that play a role in plant resistance depend on the species and types of microbes involved, as reported by Romera *et al*. (2019).

CONCLUSION

Application using *Bacillus* spp. suppressed moler disease on shallot. *Bacillus cereus* RC76 applied on shallot increased the content of jasmonic and saliyclic acid, while application with *Bacillus velezensis* B-27 increased the content of jasmonic acid. The increased of jasmonic and salicylic acid content is one of induced resistance mechanism against pathogen on shallot.

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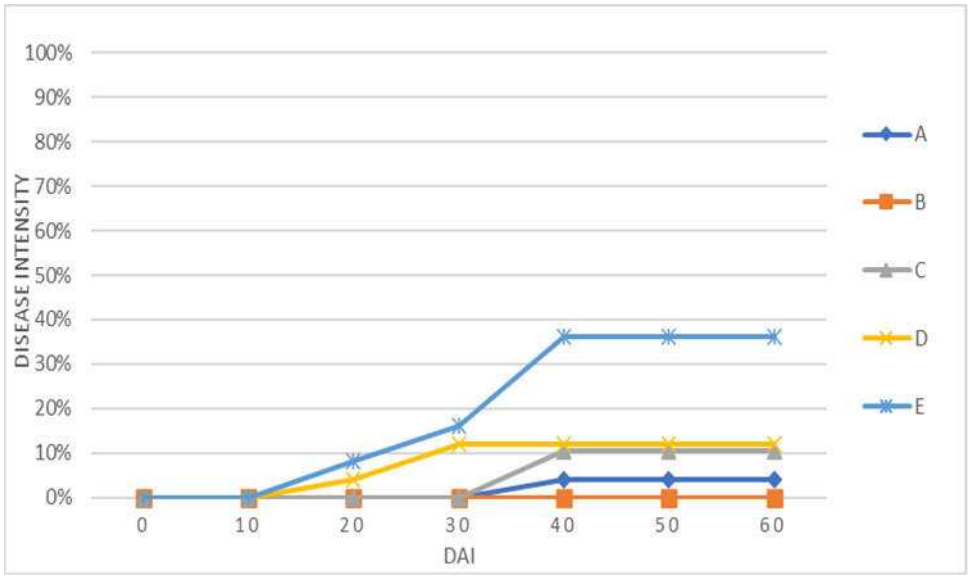
APPENDIX

Table 1. Effect of treatments with *Bacillus* spp. in development of twisted disease on shallot.

Treatments (%)	Disesase Incidence (%)	Dicease Severity (%)	Disease Suppression (%)
A	4 a	4 a	89
B	0 a	0 a	100
C	16 a	10 a	72,2
D	0 a	0 a	100
E	36 b	36 b	0

Note: A = application of *B. velezensis* B-27, B = application of *B. cereus* RC76, C = application of *Bacillus* spp., D = *T. asperellum* application, E = control. The data has been transformed. The number followed by the same letter indicates there is no difference according to DMRT 95%.

A



B

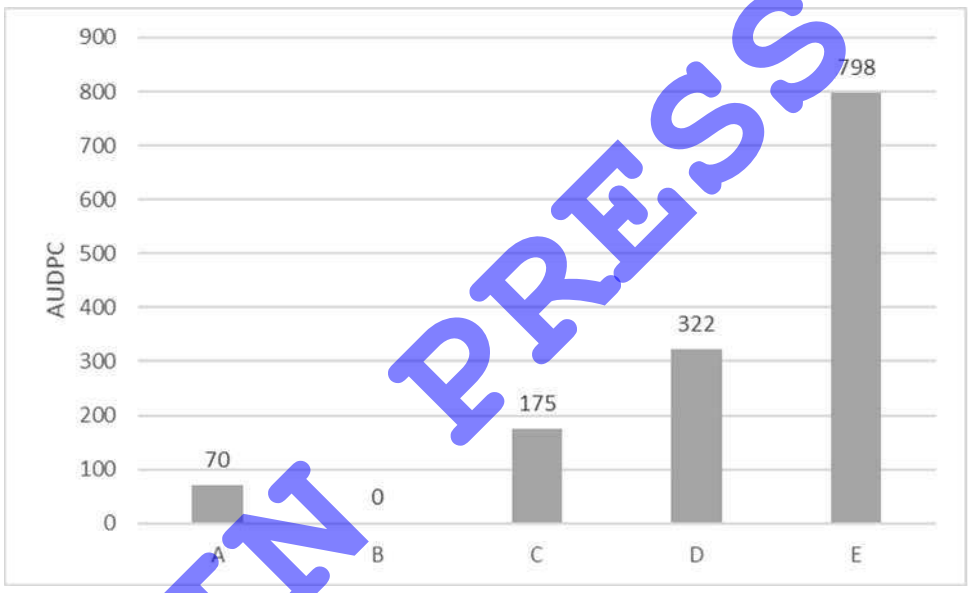


Figure 1. Area under the disease progress curve (AUDPC) calculated of twisted disease in the greenhouse for 60 days after the inoculation of *Fusarium acutatum* (A = application of *B. velezensis* B-27, B = application of *B. cereus* RC76, C = application of *Bacillus* spp., D = *T. asperellum* application, E = control). A-Disease progress curve expressed in percentage of disease intensity, B-AUDPC calculated from the disease intensity.

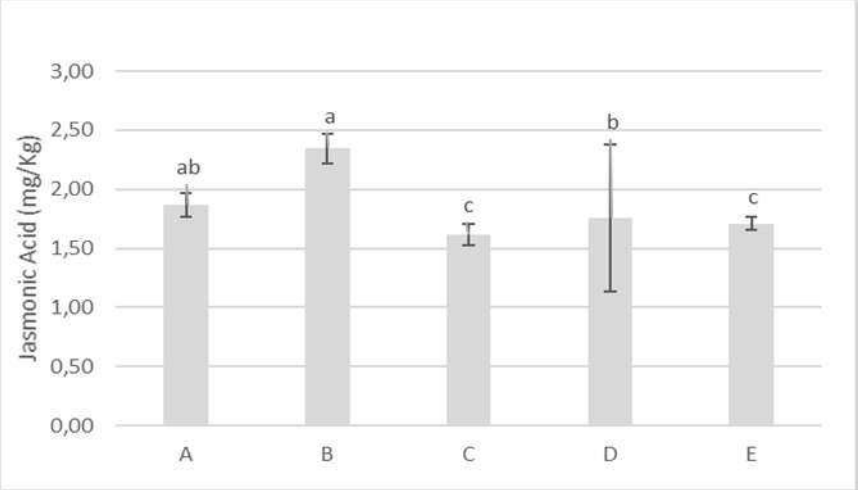


Figure 2. Effect of tuber dipping treatments with *Bacillus* spp. in content of jasmonic acid (JA) [A = application of *B. velezensis* B-27, B = application of *B. cereus* RC76, C = application of *Bacillus* spp., D = *T. asperellum* application, E = control]; the same letters above the bars indicates there is no difference according to DMRT 95%

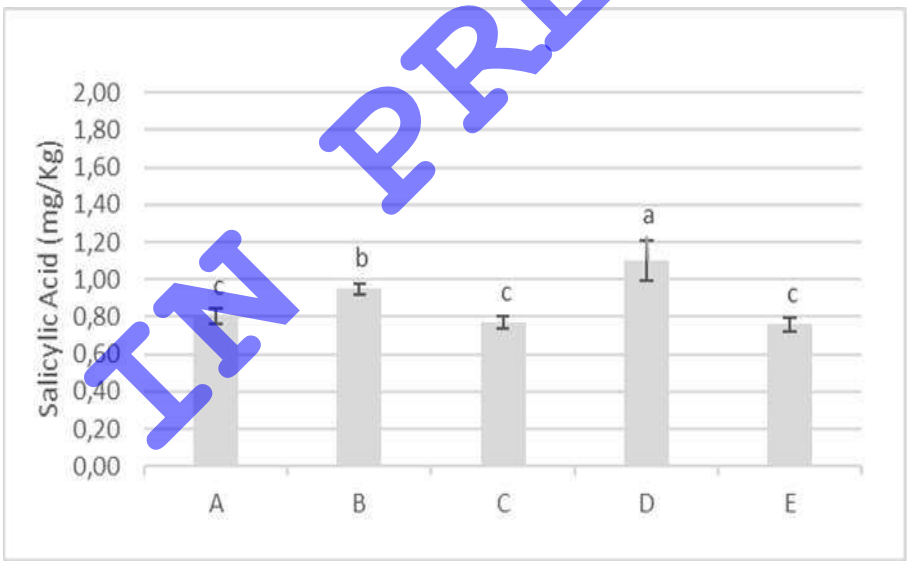


Figure 3. Effect of tuber dipping treatments with *Bacillus* spp. in content of salicylic acid (SA) [A = application of *B. velezensis* B-27, B = application of *B. cereus* RC76, C = application of *Bacillus* spp., D = *T. asperellum* application, E = control]; the same letters above the bars indicates there is no difference according to DMRT 95%

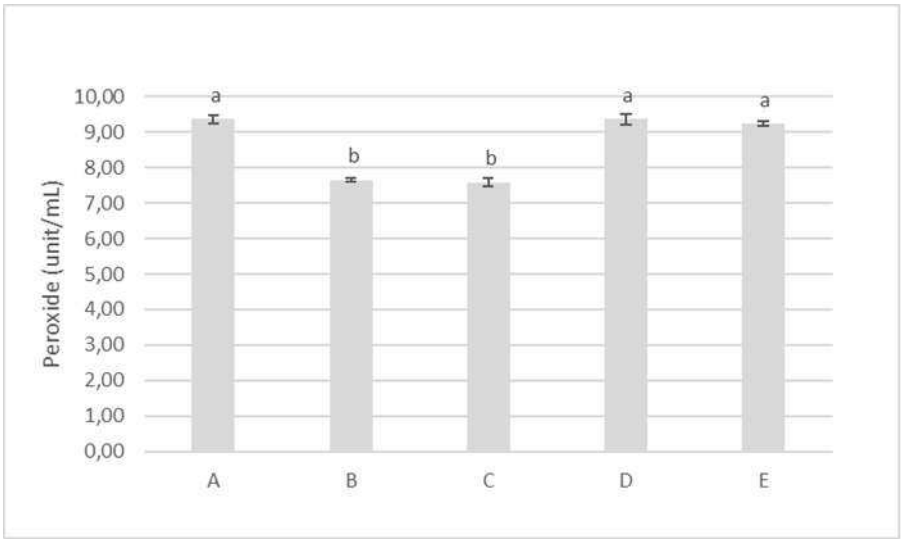


Figure 4. Effect of tuber dipping treatments with *Bacillus* spp. in content of peroxide (POD) [A = application of *B. velezensis* B-27, B = application of *B. cereus* RC76, C = application of *Bacillus* spp., D = *T. asperellum* application, E = control]; the same letters above the bars indicates there is no difference according to DMRT 95%