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Production of *Bacillus velezensis* Strain GB1 as a Biocontrol Agent and Its Impact on *Bemisia tabaci* by Inducing Systemic Resistance in a Squash Plant

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Abstract: Pests represent a huge problem in crop production causing significant losses. Currently, biocontrol is utilized as an eco-friendly approach for controlling pests and reducing the shortage in crop production. In the current study, the production of a biocontrol agent, which was identified based on sequencing of the 16S rRNA gene as *Bacillus velezensis* strain GB1 with GenBank accession No. OM836750, was carried out in the stirred tank bioreactor using a batch fermentation process. For the first time, *B. velezensis* strain GB1 was tested as a biocontrol agent with soil drench application (10^9 cfu mL^{-1}) for management of *Bemisia tabaci* and induction of squash plant systemic resistance under greenhouse conditions. β-1,3-glucanase, chitinase, polyphenol oxidase, and peroxidase activity were measured in squash leaves at 24, 48, 72, 96, and 120 h. The influence of *B. velezensis* strain GB1 on population density, fertility, and hatchability of *B. tabaci* on squash plants was studied. The batch fermentation process of *B. velezensis* strain GB1 maximized the production of secondary metabolites and culture biomass, which reached a maximum value of 3.8 g L^{-1} at 10.5 h with a yield coefficient of 0.65 g cells/g glucose. Treatment with *B. velezensis* strain GB1 induced squash plants to boost their levels of β-1,3-glucanase, chitinase, polyphenol oxidase, and peroxidase enzymes. On the other hand, *B. velezensis* strain GB1 could significantly reduce the mean number of the attracted *B. tabaci* on squash plants. Additionally, whiteflies laid a lower mean number of 2.28 eggs/female/day on squash plants inoculated with *B. velezensis* strain GB1 compared to control. The percentage of *B. tabaci* egg hatchability declined by 5.7% in the *B. velezensis*-inoculated squash plants.

Keywords: *Bemisia tabaci*; *Bacillus velezensis*; biocontrol agent; fermentation process; pathogenesis-related (PR) proteins; induced systemic resistance

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1. Introduction

Bemisia tabaci (Gennadius) (Homoptera: Sternorrhyncha: Aleyrodidae) is a major pest to ornamental plants and horticultural crops, in both the field and greenhouses around the world. Over 600 plants have been identified as hosts for this common pest [1]. A heavy *B. tabaci* infestation can result in decreased plant vigor as well as a variety of physiological disorders [2,3]. Furthermore, the development of nymphs is commonly linked to the formation of sooty molds, which limit photosynthesis and cause defoliation and stunting [4]. Virus transmission can potentially cause significant damage as *B. tabaci* is a vector for over 100 plant viruses [5–7]. Tomato yellow leaf curl virus (TYLCV) and cucumber vein yellowing virus (CVYV) are two examples. These viruses are a severe problem for tomato and cucumber plants, causing yield loss of 50 to 100 percent [8,9].

Chemical pesticides are frequently employed to manage *B. tabaci* because of their instantaneous action, but this method has various downsides, including food safety concerns, insecticide resistance, ecological hazard, and non-target organism effects. To effectively control *B. tabaci*, biocontrol agents have been developed as a substitute for the traditional use of chemical pesticides in an integrated pest management (IPM) system [10].

Induction of plant resistance against the herbivore, which is a new biological strategy for dealing with plant stress conditions could be investigated as a possible approach for managing whitefly infestation [11]. Many investigations revealed that one of the plant defense mechanisms that defend against pathogens or insects attack is increasing the concentrations of the secondary metabolites or some of the host proteins, several of which are referred to as pathogenesis-related (PR) proteins. They currently comprise 17 families of stress proteins including β-1,3-glucanases, chitinases, and peroxidases [12,13]. This can change nutritional quality and palatability, raise toxicity, and change the host plant's anatomy, phenology, and physiology. These chemicals have attracted interest due to their possible causal role in resistance as seen by their high induction during induced local and systemic resistance by which it inhibits the growth and spread of such pathogens and insects.

Beneficial bacteria that live freely in the soil and rhizosphere are known as plant growth-promoting rhizobacteria (PGPR) [14,15]. It enhances plant growth and induces systemic resistance, making the plant more resistant to a wide spectrum of pathogen attacks in the future. This long-term systemic resistance induced by PGPR has been termed induced systemic resistance (ISR) [16,17], which is characterized by the formation of a primed state for defense in which defense-related responses are elicited more quickly in response to pathogen or insect attack [18]. ISR has been reported to be used by certain PGPR to protect plants from pathogens infections [19,20]. However, there have been a few types of research on the ISR employed by PGPR to combat insects [21–24].

Genus *Bacillus* is frequently used as a biocontrol agent [9]. It can improve plant growth and supply plant protection in a variety of crops such as cucumber [8,15,25]. *B. velezensis* is a widely distributed aerobic, endospore-forming, and Gram-positive species of *Bacillus*, which was named by Ruiz-García et al. [26]. It has been extensively researched and employed because of its direct or indirect growth promotion effect for many plants. *B. velezensis* has been observed to suppress the growth of a variety of pathogenic fungi, including *Fusarium oxysporum* [27], *Aspergillus flavus* [28], bacteria, and nematodes via the biosynthesis of secondary metabolites, such as β-1,3-1,4-glucanase, lipopeptide antibiotics (surfactin, iturin, and fengycin, for example), and iron carriers [29–31], which play significant roles in trigger systemic resistance in plants [32]. However, its activity against insects attack and underlying cellular and molecular defense mechanisms has not yet been widely elucidated [33].

The main objective of the current study is the production of *B. velezensis* as a biocontrol agent using a batch fermentation process in the stirred tank bioreactor, and evaluating for the first time the effects of squash (*Cucurbita pepo*) root colonization by *B. velezensis* on *B. tabaci* population density, females fecundity, and the egg hatchability under greenhouse conditions by elucidation of its underlying mechanism in terms of enhancing the expression of pathogenesis-related proteins such as enzymes involved in the build-up a defense strategy against whitefly, which could be used through the integrated pest management programs.

2. Materials and Methods

2.1. The Laboratory Culture of B. tabaci

Adults of *B. tabaci* (Gennadius) (Order: Homoptera, Suborder: Sternorrhyncha, Family: Aleyrodidae) were reared on healthy tomato plants *(Lycopersicon esculentum* Miller). The mother colony of whiteflies was established by Prof. El-Helaly and has been reared at the Department of Applied Entomology, Faculty of Agriculture, Alexandria University since

the 1960s. Recently, the mother colony of whiteflies was re-identified by Dr. Jon Martin, Insect/Plant Division, Department of Entomology, The Natural History Museum, UK.

2.2. Isolation and Molecular Identification of the Bacterial Isolate

The bacterial isolate used in this study as a biocontrol agent was isolated from the rhizosphere of eggplant roots (Alexandria—Egypt) by Dr. Abo-Zaid, G.A., Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), City of Scientific Research and Technological Applications (SRTA-City), Egypt.

Total DNA was extracted from an antagonistic bacterial isolate isolated in the current investigation according to Istock et al. [34]. Universal primers, Start (forward) 5′AGAGTTTGATCMTGGCTCAG 3′ and End (reverse) 5′TACGGYACCTTGTTACGACTT 3′ were used to amplify the whole length of the 16S rRNA gene [35]. The amplified 16S rRNA gene of the antagonistic bacterial isolate was purified and sequenced based on the enzymatic chain terminator approach by the use of a Big Dye terminator sequencing kit. The nucleotide sequences were then compared to other 16S rRNA gene sequences in the GenBank database (http://www.ncbi.nlm.nih.gov) (accessed on 2 March 2022). The phylogenetic tree was built using the Neighbor-Joining method in MEGA software version 5 (SRTA-City), with a total of 2000 bootstrap replications.

2.3. Production of the Bacterial Isolate

2.3.1. Preparation of Stirred Tank Bioreactor

Batch fermentation was carried out in a 10 L bench-top bioreactor (Cleaver, Saratoga, NY, USA) equipped with three six-bladed disc-turbine impellers and four baffles and controlled by a digital control unit with a working volume of 4 L. The process was automated using a control unit with a 10.4-inch color touch-screen interface and the ability to store up to 59,994 distinct programs for various situations. The temperature and pH levels were established at 30 °C and 7, respectively. Automatic feeding of 2 mol L^{-1} NaOH and 2 mol L^{-1} HCl kept the pH in check. The air was compressed and adjusted to 1 VVM (air volume per broth volume per minute) after passing through a sterile filter. The dissolved oxygen level was kept over 20% by changing the agitation speed among 200 and 600 rpm. The dissolved oxygen level and pH values were determined online using METTLER TOLEDO electrodes.

2.3.2. Batch Fermentation Process

The bacterial isolate was pre-cultured by inoculating a single colony of the isolate into a 500 mL Erlenmeyer flask containing 100 mL of Number 3 production medium [36]. The bacterial isolate was grown overnight at 30 °C and shacked at 200 rpm. The batch fermentation process of the bacterial isolate was initiated in the bioreactor using Number 3 production medium and an optical density ($O.D_{550}$) of 0.5. Several samples of culture were taken throughout the fermentation period, and optical density at 550 nm was used as an indicator of cell number.

2.3.3. Biomass Estimation

Dry cell weight was determined using a 10 mL sample of culture broth, which was centrifuged at 894× *g* for 10 min, and the pellet was resuspended, washed, and centrifuged again as before. After that, drying the pellets was performed overnight in a dry-air oven at 80 °C [37].

2.3.4. Glucose Estimation

An enzymatic colorimetric kit (Diamond Diagnostics, Egypt) was used for measuring glucose concentration, which is based on glucose oxidase activity and peroxidase activity. The final product is a red-violet quinoneimine dye, which is used as an indicator of glucose concentration.

2.4. Induction of Pathogenesis-Related (PR) Proteins in Squash Plants

2.4.1. Experimental Design

Seeds of *C. pepo* were soaked in water for 2 days to synchronize germination and then they were sown individually in pots (15 cm diameter) with one kilogram of mixed soil that contains clay, sand, and peat moss (1:1:1). When they reached the developmental stage of four expanded leaves, they were arranged into four groups. Every 15 plants represented a treatment. The first group represented control plants, and the second group was drenched with 10 mL of bacterial culture broth on the rhizosphere in each pot (10^9 cfu mL^{-1}). Squash plants of the other two groups were covered by glass lantern, and the third group was exposed to 50 adults of unknown age or sex *B. tabaci* alone, whereas the fourth group was exposed to bacterial culture broth along with *B. tabaci* at the same rate as the second and third groups, respectively. Treatments were performed for 24, 48, 72, 96, and 120 h. All groups were kept in an insect-proof greenhouse at 25 ± 5 °C, 65 ± 5 RH, and under natural light conditions.

2.4.2. Sample Extraction for Determination of Enzymes Activity

One gram of leaf samples was collected from all plants of the four treatments groups and crushed with a pre-cooled mortar and pestle in 10 mL of 0.05 M sodium acetate buffer (pH 5.0) in the presence of 0.3 g polyvinyl pyrrolidone (PVP, also, commonly called polyvidone or povidone). Finally, samples were centrifuged at 16,000× *g* for 15 min at 4 °C. The activity of *β*-1,3-glucanase, chitinase, polyphenol oxidase, and peroxidase were determined using the supernatants as cruds enzymes.

2.4.3. Assay of β-1,3-Glucanase Activity

The beta-1,3-glucanase activity was colorimetrically assayed by Saikia et al. [38]. The reaction mixture consisted of 62.5 µL of 0.04% laminarin and 62.5 µL of enzyme extract. The temperature and incubation time of the reaction was 40 °C for 10 min, respectively. The reaction was stopped by adding 375 µL of dinitrosalicylic acid and heating for 5 min in a boiling water bath, and Eppendorf tubes were shaken using a vortex and their absorbance was measured at 500 nm. The enzyme activity was expressed as U g^{-1} fresh weight (quantity of enzyme that liberates one µM glucose per minute under experimental conditions).

2.4.4. Assay of Chitinase Activity

One mL of the enzyme extract was added to one mL of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) and mixed by shaking in a test tube then kept in a water bath at 37 °C for 75 min with shaking. The reaction was then stopped by adding one mL of dinitrosalicylic acid [39] and heated for 5 min then cooled and centrifuged at 3000× *g* for 5 min to get rid of chitin before measuring O.D. at 540 nm. Chitinase activity was defined as Ug^{-1} fresh weight (µM N-Acetylglucosamine liberated per minute under experimental conditions).

2.4.5. Assay of Polyphenol Oxidase Activity

Polyphenol oxidase activity was determined according to Mayer et al. [40]. Two hundred µL of the enzyme extract was added to 1.5 mL of 0.1 M phosphate buffer (pH 7). To start the reaction, 200 µL of 0.01 M catechol in phosphate buffer (pH 7) was added and the activity was expressed as a change in absorbance at 495 nm min^{-1}.

2.4.6. Assay of Peroxidase Activity

The reaction mixture consisted of 0.5 mL of enzyme extract and 0.5 mL of 1% H_2O_2. 1.5 mL of 0.05 mL pyrogallol was added to every sample separately and incubated at room temperature. The enzyme activity was expressed as the change in absorbance at 420 nm at 1 min intervals [41].

2.5. The Effect of the Bacterial Isolate on B. tabaci Population Density

To study the effect of the bacterial isolate on the attraction of *B. tabaci* adults, plants of the same germination, cultivation, pot diameter, soil weight, and developmental stage as in the enzyme assay experimental design were studied. Plants were divided into two groups in a greenhouse under the same conditions with each group consisting of five plants. The first group was the control that was drenched only by water, and the second group was drenched with 10 mL of bacterial culture broth (10^9 cfu mL^{-1}) on the rhizosphere in each pot. Whitefly adults were collected from the mother colony and released in the center of the greenhouse in the free-choice test. For each treatment, the numbers of attracted whiteflies/cm^2/plant were daily recorded and calculated as a mean of daily record. To avoid whitefly movement, the counting was done gently and in the early morning. The experiment was replicated for five successive days. The obtained data were calculated as a mean of five replicas.

2.6. The Effect of Inoculation of Squash Plants by the Bacterial Isolate on Egg-Laying and Hatchability of B. tabaci

To study the effect of bacterial isolate on the attraction of egg-laying of whitefly females and the egg hatchability, five plants inoculated with bacterial culture broth (10^9 cfu mL^{-1}) and non-inoculated plants (control) were covered by a glass lantern and infested with the natural sex ratio (2 males:3 females) of *B. tabaci* for four days to allow the oviposition. After removing the adults by shaking the plants, the laid eggs/plant were recorded and calculated as a mean of five replicas. Plants were transferred to an insect-proof greenhouse. To keep the plants isolated free from newly *B. tabaci* adults' infestation, each plant was fully covered by a glass lantern until egg emergence, and then the percentage of hatchability was recorded and calculated as a mean of five replicas.

2.7. Statistical Analysis

Randomized complete blocked design (RCBD) was used for the analysis of the obtained results (a two-way ANOVA) using SAS software [42]. The significant differences between treatments were determined according to the least significant differences (LSD) at a $p \leq 0.05$ level of probability.

3. Results

3.1. Molecular Identification of the Bacterial Isolate

The bacterial isolate used in the current study was identified based on sequencing of 16S rRNA. A search in the database to identify the bacterial isolate was performed in BLAST search at the National Center for Biotechnology Information site (http://www.ncbi.nlm.nih.gov) (accessed on 2 March 2022). The research revealed that the sequence of the investigated bacterial isolate was almost similar to several *B. velezensis* strains with a homology percentage of 99%. The PGPR isolate was identified as *B. velezensis* strain GB1 with the accession number, OM836750. A phylogenetic tree was built using the nucleotide sequence of the 16S rRNA gene of *B. velezensis* strain GB1 obtained in the current investigation and nucleotide sequences of the 16S rRNA of other Bacillus species obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) (accessed on 2 March 2022). The phylogenetic tree revealed that two major clusters exist. Cluster 1 included *Escherichia coli*, whereas *B. velezensis* strain GB1obtained in this study and all *Bacillus* spp. strains provided from GenBank were clustered in Cluster 2, which was divided into two groups. The first group included *B. amyloliquifaciens*, *B. subtilis*, and *B. licheniformis* provided from GenBank, whereas the second group contained *B. velezensis* strain GB1 isolated in this investigation, and all *B. velezensis* strains collected from GenBank have a high percentage of identity that reached 95% (Figure 1).

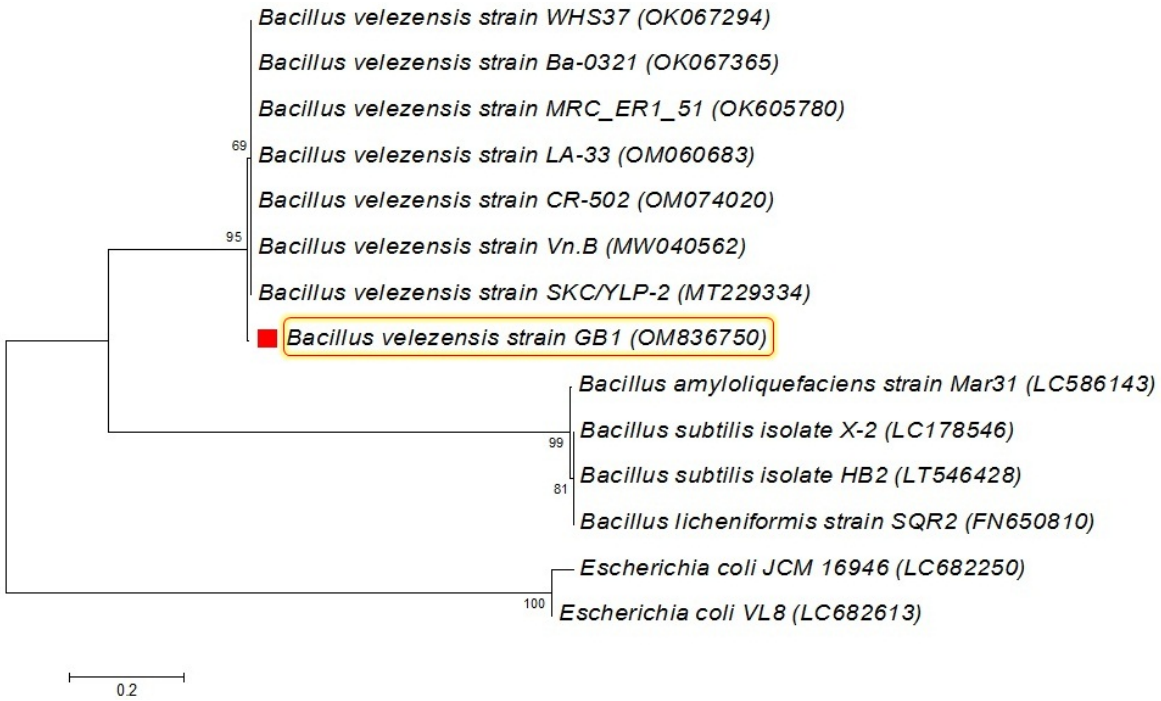

Figure 1. Phylogenetic tree of *Bacillus velezensis* strain GB1 obtained in the current study and validly described members of the genus *Bacillus* based on the nucleotide sequences of the 16S rRNA gene. The phylogenetic tree was constructed with the Neighbor-Joining method using MEGA version 5.

3.2. Production of B. velezensis Strain GB1

Batch fermentation of *B. velezensis* strain GB1 was started in a stirred tank bioreactor with an optical density ($O.D_{550nm}$) of 0.5. Biomass and glucose concentration as a carbon source of batch fermentation of *B. velezensis* strain GB1 was plotted against time, and the maximum biomass recorded was 3.8 g L^{-1} at 10.5 h (Figure 2A). Glucose concentration decreased rapidly, reaching 4.22 g L^{-1} at 5 h; however, it reached 0 g L^{-1} at 11 h. Cell mass increased exponentially over time with a constant specific growth rate (μ) of 0.09 h^{-1} (supplementary materials). One of the important factors estimated in the exponential phase of the bacterial cell growth is the yield coefficient $Y_{X/S}$, which represents the amount of obtained biomass against the amount of consumed carbon source (glucose). In this batch fermentation process, the yield coefficient recorded 0.65 g cells/g glucose (Figure 2B). Dissolved oxygen is an important factor affecting bacterial cell growth in the bioreactor, which can be controlled by agitation speed. Batch fermentation of *B. velezensis* strain GB1was started with a low agitation speed (200 rpm) and a higher value of dissolved oxygen of 99.5%, which was reduced gradually. The dissolved oxygen percentage decreased gradually during the first two hours of the batch fermentation process to reach 21.4% at 2.15 h. The decline of dissolved oxygen percentage is an indicator of growing the bacterial culture (Figure 3). Dissolved oxygen percentage was reserved at above 20% to guarantee adequate oxygen delivery. So, agitation speed was raised gradually step by step from 200 to 600 rpm related to the growth of bacterial cells and glucose consumption from the culture broth.

3.3. Induction of Pathogenesis-Related (PR) Proteins in Squash Plants

3.3.1. β-1,3-Glucanase Activity

As shown in Table 1, squash plants treated with *B. velezensis* strain GB1 alone and *B. velezensis* + *B. tabaci* showed an increase in their levels of *β*-1,3-glucanase enzyme from the first day. After that, the *β*-1,3-glucanase level in both treatments started significantly rising to reach the maximum value of 40.34 U g^{-1} fresh weight on the fourth day (2.98 folds over control) and 42.21 U g^{-1} fresh weight on the fifth day (2.87 folds over control), respec-

tively. Although that *B. tabaci*-infested squash plants showed a significant enhancement in the activity of *β*-1,3-glucanase from the second day of infestation compared with control and fluctuated high and low until the fifth day, it remained significantly less than what was recorded by exposure to *B. velezensis* alone or with *B. tabaci* and *B. velezensis.*

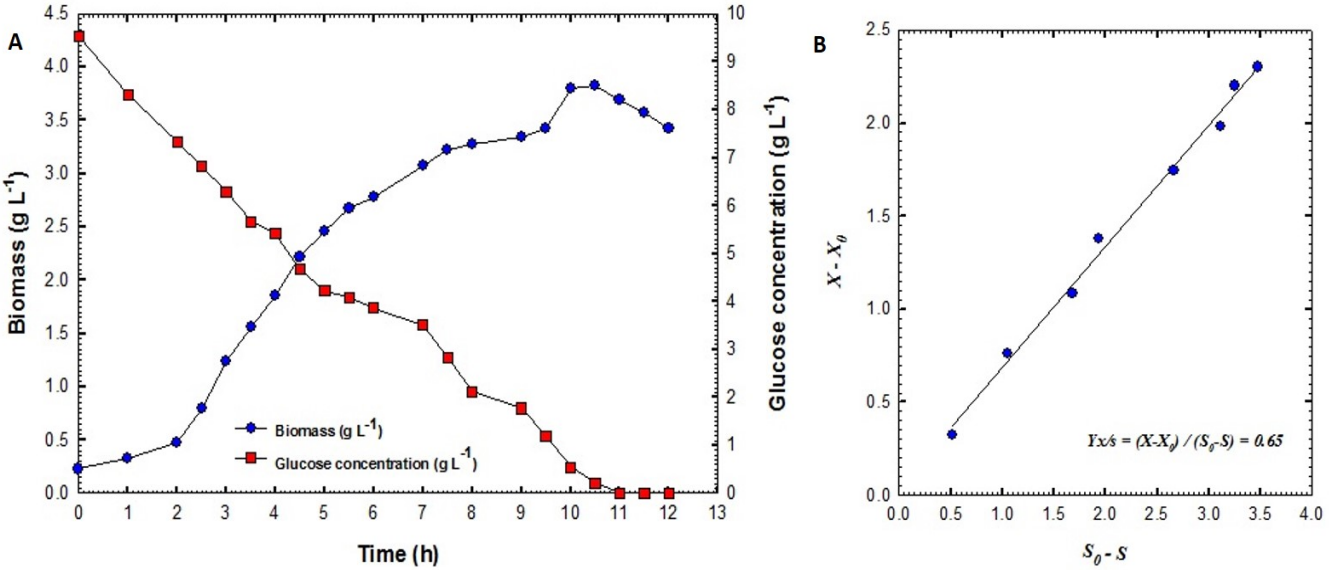

Figure 2. (**A**) Biomass and glucose concentration of fermentation broth as a function of time for batch fermentation of *Bacillus velezensis* strain GB1, and (**B**) yield coefficient for growth of *Bacillus velezensis* strain GB1on glucose. X_0 represents the cell mass in the broth at initial time t_0 (g L^{-1}); X, the cell mass concentration in the broth at time t (g L^{-1}); S_0, glucose concentration in the broth at initial time t_0 (g L^{-1}); and S, glucose concentration in the broth at time t (g L^{-1}).

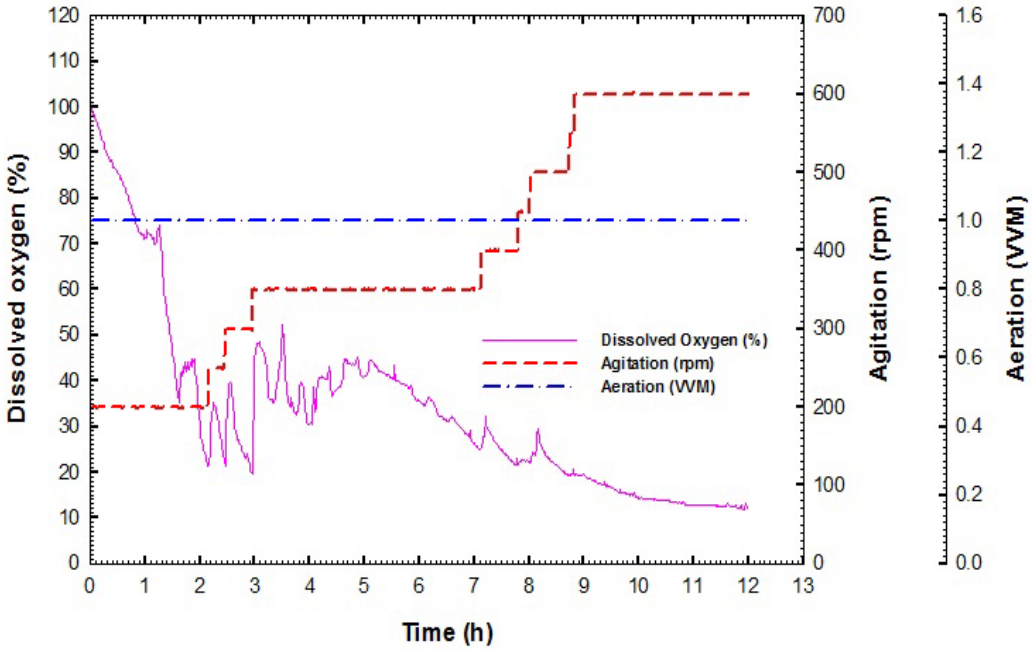

Figure 3. Dissolved oxygen, agitation, and aeration as a function of time during batch fermentation of *Bacillus velezensis* strain GB1.

Table 1. The time-course of β-1,3-glucanase activity in squash plants exposed to *Bemisia tabaci* and *Bacillus velezensis*, and both agents compared with control.

Treatments	β-1,3-Glucanase Activity (U g^{-1} Fresh Weight)				
	24 h	48 h	72 h	96 h	120 h
Control	* 15.11 ± 0.99 ^{jk,**}	14.83 ± 0.76 ^{k}	16.34 ± 1.68 ^{j}	13.52 ± 1.42 ^{k}	14.70 ± 0.82 ^{k}
B. tabaci	15.31 ± 0.81 ^{jk}	23.69 ± 0.51^{g}	20.29 ± 0.43 ^{h}	18.81 ± 0.75 ^{i}	25.22 ± 0.51 ^{f}
B. velezensis	24.73 ± 0.67 ^{f}	33.66 ± 0.63 ^{d}	35.79 ± 0.71 ^{c}	40.34 ± 0.55 ^{b}	40.20 ± 0.51 ^{b}
B. velezensis + *B. tabaci*	30.76 ± 0.87 ^{e}	29.71 ± 1.4 ^{e}	35.86 ± 0.31 ^{c}	40.17 ± 0.19 ^{b}	42.21 ± 0.47 ^{a}

* Means in each column followed by the same letter do not differ significantly ($p \leq 0.05$); ** significant letters.

3.3.2. Chitinase Activity

As illustrated in Table 2, the accumulation of chitinase started to increase with time one day after exposure to the tested agents with the absence of a considerable change in the case of *B. tabaci*. Treating squash plants with *B. velezensis* strain GB1 induced a significant accumulation of chitinase to reach the maximum level of 0.0497 U g^{-1} fresh weight on the fifth day (9 fold more than control), which is the most significant level of activity among all treatments. Also, the presence of bacteria in combination with the insect resulted in the enhancement of the chitinase level from the first day, which then gradually increased with time to reach the maximum increase of 0.0486 U g^{-1} fresh weight on the fourth day (8.5 fold more than control). The chitinase accumulation in the squash leaves during the first three days of exposure to *B. tabaci* was almost equal and without any significant difference to its level in the control, which was then slightly elevated on the fourth and fifth days (1.2 and 1.4 fold increase compared to control, respectively).

Table 2. The time-course of chitinase activity in squash plants exposed to *Bemisia tabaci* and *Bacillus velezensis*, and both agents compared with control.

Treatments	Chitinase Activity (U g^{-1} Fresh Weight)				
	24 h	48 h	72 h	96 h	120 h
Control	* 0.0054 ± 0.00015 ^{j,**}	0.0055 ± 0.00025 ^{j}	0.0053 ± 0.0002 ^{j}	0.0057 ± 0.00011 ^{j}	0.0055 ± 0.0001 ^{j}
B. tabaci	0.0057 ± 0.00005 ^{j}	0.0060 ± 0.00011 ^{j}	0.0066 ± 0.0001 ^{ij}	0.0070 ± 0.0001 ^{i}	0.0078 ± 0.00025 ^{h}
B. velezensis	0.0142 ± 0.0003 ^{g}	0.0379 ± 0.00052 ^{e}	0.0426 ± 0.0010 ^{d}	0.0474 ± 0.0007 ^{c}	0.0497 ± 0.0006 ^{a}
B. velezensis + *B. tabaci*	0.01843 ± 0.0006 ^{f}	0.0378 ± 0.0004 ^{e}	0.0383 ± 0.0005 ^{e}	0.0486 ± 0.00005 ^{b}	0.0382 ± 0.0005 ^{e}

* Means in each column followed by the same letter do not differ significantly ($p \leq 0.05$); ** significant letters.

3.3.3. Polyphenol Oxidase Activity

As shown in Table 3, inoculation with *B. velezensis* strain GB1 recorded the most significant treatment in increasing the level of polyphenol oxidase in squash plants where it reached the highest level of 28.82 U g^{-1} fresh weight on the second day (4.6 fold more than control). Subsequently, the enzyme activity retreated gradually with time. The treatment with *B. velezensis* + *B. tabaci* and infestation with *B. tabaci* alone recorded the maximum levels of polyphenol oxidase enzyme of 24.15 and 19.20 U g^{-1} fresh weight on the third day of the treatments, respectively, then gradually retracted at 96 and 120 h post-treatment.

Table 3. The time-course of polyphenol oxidase activity in squash plants exposed to *Bemisia tabaci* and *Bacillus velezensis*, and both agents compared with control.

Treatments	Polyphenol Oxidase Activity (U g^{-1} Fresh Weight)				
	24 h	48 h	72 h	96 h	120 h
Control	* 4.33 ± 0.02 ^{n,**}	6.25 ± 0.09 ^{l}	3.80 ± 0.23 ^{o}	4.71 ± 0.16 ^{n}	5.43 ± 0.4 ^{m}
B. tabaci	9.73 ± 0.55 ^{k}	14.17 ± 0.045 ^{j}	19.20 ± 0.20 ^{h}	18.62 ± 0.36 ^{i}	14.15 ± 0.09 ^{j}
B. velezensis	25.48 ± 0.22 ^{c}	28.82 ± 0.3 ^{a}	27.82 ± 0.43 ^{b}	25.87 ± 0.08 ^{c}	23.82 ± 0.29 ^{d}
B. velezensis + *B. tabaci*	22.16 ± 0.50 ^{f}	23.25 ± 0.8 ^{e}	24.15 ± 0.19 ^{d}	19.12 ± 0.14 ^{h}	20.34 ± 0.17 ^{g}

* Means in each column followed by the same letter do not differ significantly ($p \leq 0.05$); ** significant letters.

3.3.4. Peroxidase Activity

As displayed in Table 4, peroxidase activity was affected by different treatments. It was observed that the maximum rate of activity was significantly exhibited on the fifth day of inoculation with *B. velezensis* strain GB1 to reach 1883.67 U g^{-1} fresh weight, which is equivalent to 1.87 fold greater than the control plant at the same time. The combined effect of *B. velezensis* strain GB1 and the whitefly on the peroxidase activity was evident from the first day of exposure. The peroxidase activity gradually increased with time to reach the maximum value on the fourth day to reach 1801.33 U g^{-1} fresh weight (1.82 fold more than control). Feeding of *B. tabaci* induced the accumulation of peroxidase from the beginning and then progressively increased with time to reach the ultimate of 1375.67 U g^{-1} fresh weight (1.4 folds more than control) on the fourth day.

Table 4. The time-course of peroxidase activity in squash plants exposed to *Bemisia tabaci* and *Bacillus velezensis*, and both agents compared with control.

Treatments	Peroxidase Activity (U g^{-1} Fresh Weight)				
	24 h	48 h	72 h	96 h	120 h
Control	* 932.67 ± 7.63 ^{m,**}	1004.66 ± 7.37 ^{k}	987.00 ± 14.52 ^{l}	985.66 ± 10.07 ^{l}	1005.33 ± 9.07 ^{k}
B. tabaci	1187.33 ± 9.71 ^{j}	1235.00 ± 4.00 ^{i}	1351.33 ± 22.01 ^{h}	1375.67 ± 5.03 ^{gh}	1363.67 ± 5.13 ^{h}
B. velezensis	1535.33 ± 15.63 ^{f}	1542.67 ± 11.59 ^{f}	1785.00 ± 15.1 ^{cd}	1838.66 ± 6.51 ^{b}	1883.67 ± 12.58 ^{a}
B. velezensis + *B. tabaci*	1387.67 ± 7.02 ^{g}	1390.33 ± 8.02 ^{g}	1782.33 ± 13.01 ^{d}	1801.33 ± 9.50 ^{c}	1764.00 ± 14.11 ^{c}

* Means in each column followed by the same letter do not differ significantly ($p \leq 0.05$); ** significant letters.

3.4. The Effect of the Inoculation of Squash Plants with B. velezensis Strain GB1 on B. tabaci Population Density

As shown in Figure 4A, the mean number of *B. tabaci* adults/cm^2 in the control did not show any significant difference throughout the experiment period from the first day (3.63 adults/cm^2) to the end of the fifth day (3.77 adults/cm^2). On the other hand, the mean number of *B. tabaci* in the case of *B. velezensis*-inoculated squash plants decreased significantly from the first day compared to that recorded on any day of the control. The mean number of *B. tabaci* recorded 2.56 adults/cm^2 on the first day then it decreased over time with a significant decrease to reach the lowest significant level of 0.95 adult/cm^2 on the fifth day. Figure 4B shows the rate of the reduction of *B. tabaci* attraction to *B. velezensis*-inoculated squash plants with time. The reduction percentage of attraction on the first day as a consequence of inoculation with strain GB1was 29.5%, which increased gradually to reach 74.03% on the fifth day. From the obtained results, it seems clear that the inoculation of squash plants with *B. velezensis* strain GB1 plays an important defensive role against whiteflies. It was observed that the inoculation of squash plants with strain GB1 reduced the mean number of whitefly adults/cm^2 by about 49.3% throughout the experiment period.

3.5. The Effect of the Inoculation of Squash Plants with B. velezensis Strain GB1 on Egg-Laying and Hatchability of B. tabaci

The obtained results from statistical analysis referred to a significant negative correlation between the inoculation of squash plants with strain GB1 and the oviposition rate. As shown in Figure 5A, the number of laid eggs per female per day was decreased by the inoculation of squash plants with strain GB1. Whiteflies fed on squash plants inoculated with *B. velezensis*, and laid 2.28 eggs/female/day, which was significantly lower by about 43.98% than the mean number of laid eggs by whiteflies fed on non-inoculated plants (4.07 eggs/female/day). The percentages of the egg hatchability take the same trend of oviposition. As shown in Figure 5B, the percentage of the egg hatchability in normal plants was 91.35%, which was significantly higher than its value of 86.11% in the inoculated plants, which was considered a 5.7% decline in hatchability percentage.

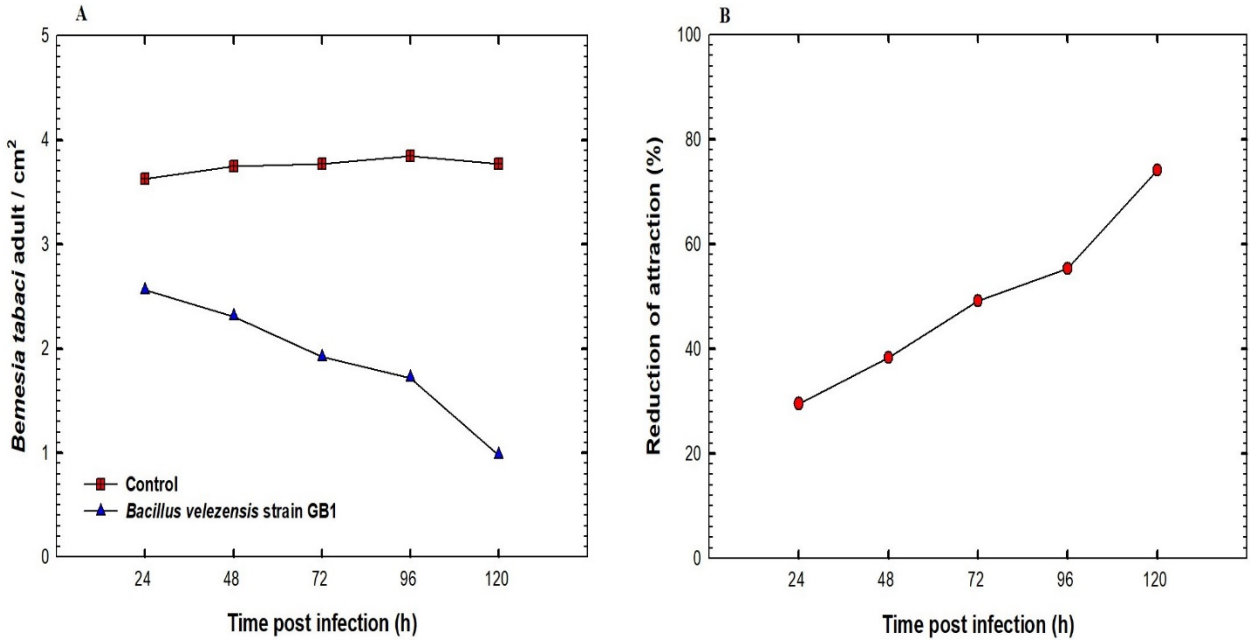

Figure 4. The effect of squash plants' inoculation with *Bacillus velezensis* Stain GB1 on (**A**) the population density and (**B**) the attraction reduction percentage of *Bemisia tabaci*.

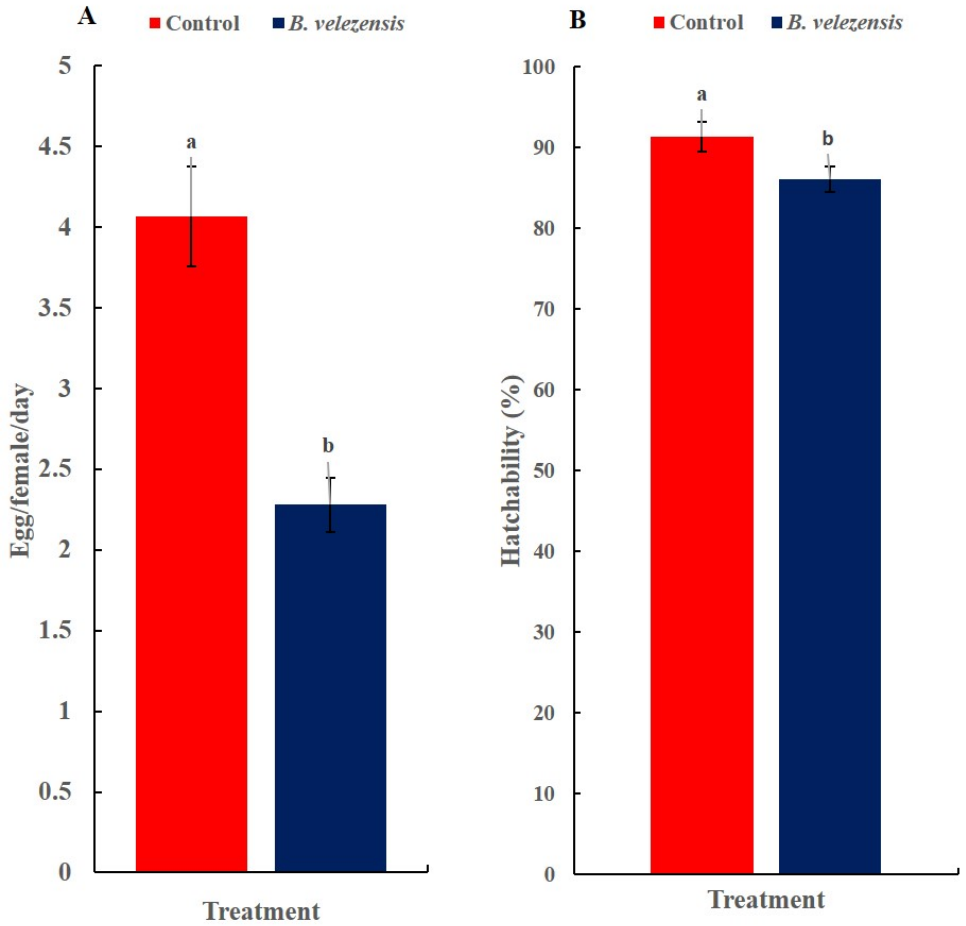

Figure 5. The effect of squash plants' inoculation with *Bacillus velezensis* on (**A**) the daily mean number and (**B**) hatchability of laid eggs by *Bemisia tabaci* females.

4. Discussion

Recently, integrated pest management approaches play an important role in reducing the role of chemical pesticides in the insect management approach, based on natural resources and behaviors such as host resistance and biological control [43]. Effectual control of pests with little or no natural environmental hazard is accomplished by biological control using microorganisms as a good alternative approach to chemical control [44]. Nelson [45] reported a prospective strategy for effective biological control that was performed using plant growth-promoting rhizobacteria (PGPR). So, the main target of the present study is maximizing the production of *B. velezensis* strain GB1 as a biocontrol agent using a batch fermentation process in the stirred tank bioreactor for biological control of whitefly *B. tabaci* on squash plants.

Cultivation in the stirred tank bioreactor compared to a shake flask provides the bacterial culture with the optimum conditions of temperature, pH, agitation, and aeration that are required for maximizing the growth of the bacterial cells and secreted secondary metabolites. Biomass of *B. velezensis* strain GB1 increased exponentially during the exponential phase with a constant specific growth rate of 0.09 h^{-1} to reach a high value of 3.8 g L^{-1} at 10.5 h with a production rate equal to 0.4 g L^{-1} h^{-1}. Matar et al. [35] used the batch fermentation process for scaling-up production of biomass of a biocontrol agent, *B. subtilis* isolate G-GANA7. The maximum biomass obtained by them reached 3.2 g L^{-1} at 11 h. The batch fermentation process of *B. velezensis* strain GB1 achieved a high yield of biomass, which recorded 0.65 g cell/g glucose. The same yield coefficient of biomass was achieved by Abdel-Gayed et al. [46] through a batch fermentation process of the biocontrol agent, *B. subtilis* isolate B4. In the exponential phase of our bacterial growth curve, the bacterial cells grow rapidly and consume a lot amount of oxygen and carbon source necessitated for growing with a consumption rate of glucose equivalent to 0.8 g L^{-1} h^{-1}. Our results revealed that the dissolved oxygen decreased gradually during the first two hours of the culture growth. Afterward, with the beginning of the exponential phase, the dissolved oxygen decreased rapidly so the agitation speed was raised to reserve it at above 20%.

Induced systemic resistance in plants against bacterial and fungal pathogens by *B. velezensis* has been established. Nevertheless, their activities against insect attack, and fundamental cellular and molecular defense mechanisms have not been illuminated up until now [47]. Molecular and physiological improvements in plants are activated by PGPR, mediating boosted plant defense against pathogens and insect pests by inducing systemic resistance (ISR) [47–50]. The present study revealed that the inoculation of squash plants by *B. velezensis* strain GB1 induced systemic resistance against *B. tabaci*. Rashid et al. [47] documented that *B. velezensis* YC7010 induced systemic resistance against the green peach aphid (GPA), *Myzus persicae*. Additionally, *B. velezensis* YC7010 was utilized as an inducer to systemic resistance of rice against brown planthopper (BPH; *Nilaparvata lugens* Stål), which is one of the most serious insect pests that reduces rice yield remarkably in many rice-growing areas [33]. In the current study, β-1,3-glucanase activity, chitinase activity, polyphenol oxidase activity, and peroxidase activity have been evaluated in squash plants treated with *B. tabaci* alone, *B. velezensis* strain GB1 alone, and *B. tabaci* + *B. velezensis* strain GB1 compared to control treatment. A positive correlation has been recorded between the inoculation of *B. velezensis* strain GB1, *B. tabaci* feeding, and the activity of all tested enzymes as follows:

In our study, PGPR *B. velezensis* strain GB1 has enhanced β-1,3-glucanase activity in squash plants against whitefly *B. tabaci* with a significant increase. Moreover, the present data indicate the presence of an effect of the whitefly feeding on the enzymatic activity of the squash plants as a natural and defensive reaction of the plant. These results are closed to the obtained results by Jimenez et al. Mayer et al. and Inbar et al. [51–53] showed that infestation of whiteflies resulted in the aggregation of pathogenesis-related proteins in tomato and other plants, which were thought to have a defensive role against insect pests. Gene-encoding pathogenesis-related proteins were induced locally in squash following silverleaf whitefly feeding [54]. Many studies confirmed that *B. tabaci* feeding boosted the

activity of pathogenesis-related proteins such as β-1,3-glucanase, chitinase, and peroxidase in cassava, tomato, black gram, and tobacco (*Vigna mungo*) compared to non-infested plants [55–59].

In the present study, *B. velezensis* strain GB1 alone induced chitinase activity to reach its maximum level on the fifth day followed by treatment with strain GB1 in the presence of *B. tabaci* infection. The mode of action of the chitinase enzyme is based on the degradation of chitin, a critical element of insect cells [60]. It has the potential to harm insects by destroying chitin-based structures such as the peritrophic membrane that provides a physical barrier to ingested pathogens and other substances that could be considered a danger to the insect [61]. The poplar chitinases were used as a method to control and inhibit the development of the Colorado potato beetle and pest population, which hinted at its involvement in the tomato plant defense [62].

In the current study, the application of *B. velezensis* strain GB1 has induced polyphenol oxidase activity as a defense enzyme in response to whitefly *B. tabaci*. *B. velezensis* XT1 increased polyphenol oxidase activity by 395%, indicating an enhancement of olive trees' resistance to *Verticillium dahlia* [63]. The inoculation of cotton roots with *B. velezensis* triggered induced systemic resistance (ISR) against *V. dahlia* and caused the activation of the antioxidant enzymes such as phenylalanine ammonia-lyase, polyphenol oxidase, peroxidase, and phenol contents [64]. Polyphenol oxidases exist in many plants [65]. They are metalloenzymes that contain a type-3 copper center and function as defense enzymes [66]. Polyphenol oxidase oxidizes phenolics to create highly reactive o-quinones that increase the anti-insect activity of phenolics. Quinones covalently link to proteins, reducing their permeability as nutritional resources [67,68]. Anti-nutritive action of polyphenol oxidase against insects is based on the formation of reactive oxygen species (e.g., superoxide radical and H_2O_2) that harm essential nutrients or fundamental elements for insects such as proteins, lipids, and nucleic acids [69–71]. The present study is in agreement with the studies on the Colorado potato beetle as a considerable positive correlation between polyphenol oxidase levels and larval mortality [72]. Furthermore, Bhonwong et al. [73] reported that overexpression of a polyphenol oxidase in tomato led to a reduction in the growth average and nutritional clue of cotton bollworm (*Helicoverpa armigera*) and beet armyworm (*Spodoptera exigua*), which also improved resistance against *Spodoptera litura* and *Malacosoma disstria* in tomato and poplar, respectively [74,75].

In our study, *B. velezensis* strain GB1 induced peroxidase accumulation in squash plants infected with whitefly *B. tabaci*. Peroxidase has been associated with a multitude of physiological processes, which could help plants stand against herbivore and pathogen attacks including polysaccharide crosslinking, oxidation of hydroxyl cinnamyl alcohol into free radical intermediates, phenol oxidation, hypersensitive response, crosslinking of extension monomers and lignification, production and polymerization of phenolics, negative effects on food digestibility, and protein availability to sucking pests [76,77]. Infestation with leaf miners resulted in an accumulation of peroxidase isoforms in groundnut plants [78]. Peroxidase-oxidized phenolics within the herbivore stomach and induced the production of semi-quinone radicals in the midguts of larvae, resulting in a lower growth rate of larvae that consumed poplars with induced peroxidase activity [79]. Dowd and Lagrimini [80] noticed that a high level of peroxidase activity in the infested plants with *Trialeurodes vaporariorum* whiteflies resulted in a reduction of the population density of whiteflies per plant. These results were closed to the obtained results in the present study. After treatment with *B. velezensis* SDTB022, the activities of defense enzymes in tobacco, such as polyphenol oxidase, peroxidase, and phenylalanine ammonia-lyase, significantly increased and, consequently, plant yield and disease defense responses in the field increased [81].

The present study showed that the inoculation of squash plants with *B. velezensis* GB1 significantly decreased the mean number of attracted *B. tabaci* and the percentage of the egg hatchability. The mixtures of the *B. velezensis* strain were responsible for emitting higher amounts of plant volatiles in cotton plants following *Heliothis virescens* larvae infestation [82].

The combination of *B. velezensis* strains that was referred to as Blend-8 and Blend-9 induced cotton resistance, and reduced the growth and development of *S. exigua* via the increased level of gossypol [24]. Systemic resistance in Arabidopsis seedlings against *M. persicae* was induced by inoculation with *B. velezensis*, which significantly reduced the settling, feeding, and reproduction of *M. persicae* on Arabidopsis leaves [47]. *Ostrinia nubilalis* laid significantly fewer eggs on maize plants treated with the different PGPR strains including *B. velezensis* compared to untreated plants, which can change maize plant volatiles with imperative ramifications for plant–insect interactions [83]. A mixture of lipopeptides (iturins, surfactins, and fengycins) was produced by *B. velezensis* B64a and *B. velezensis* B15, which were the best bioagent against *Aedes aegypti* [84]. *B. subtilis*-inoculated cotton plants showed raised levels of polyphenol oxidase, peroxidase, and chitinase, which had a substantial influence on reducing the aphid population under greenhouse conditions up to 14 days following treatment [85]. Hanafi et al. [86] found that inoculating tomato plants with the *B. subtilis* strain resulted in considerably reduced survival of *B. tabaci*'s nymphs and pupae, implying that inoculation with *B. subtilis* confers some type of resistance or avoidance behavior to plants, resulting in less *B. tabaci* propagation on the *B. subtilis*-inoculated plants. Valenzuela-Soto et al. [87] demonstrated that tomato plants treated with *B. subtilis* not only enhanced plant growth but also resulted in the establishment of an ISR through induction of several PR protein genes, resulting in the significant reduction in 4th instar nymphs, pupae, empty pupal cases (corresponding emerging adults), and emerged adults, with *B. tabaci* development favored over untreated controls.

5. Conclusions

The batch fermentation process of *B. velezensis* strain GB1 achieved in the stirred tank bioreactor was sufficient in maximizing the production of secondary metabolites and culture biomass, which reached a maximum value of 3.8 g L^{-1} with a yield coefficient of 0.65 g cells/g glucose. *B. velezensis* strain GB1 induced squash plants to enhance their levels of β-1,3-glucanase, chitinase, polyphenol oxidase, and peroxidase enzymes. Additionally, *B. velezensis* strain GB1 was effective in reducing the mean number of the attracted *B. tabaci* on squash plants with a significant decrease. The reduction percentage of *B. tabaci* attraction reached 74.03% on the fifth day. Squash plants inoculated with *B. velezensis* strain GB1 recorded a lower mean number of *B. tabaci* eggs/female/day compared to control with a decreasing percentage of 43.98%. The percentage of egg hatchability on squash plants inoculated with *B. velezensis* strain GB1 declined by 5.7%. Finally, *B. velezensis* strain GB1 could be considered one of the important biocontrol agents that plays a pivotal role in plant protection against whitefly *B. Tabaci* and a promising tool for integrated pest management (IPM) programs.

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