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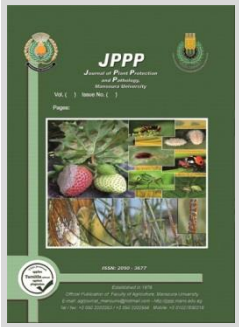
Evaluation of Cytotoxicity Effects of the Biocontrol Bacterium *Bacillus velezensis* BE1

Esraa M. Aboelez [1]; M. A. E. Selim[2]; Safaa A. Yousef[3]; Safaa Hamza[1]; Y. M. Shabana [1,4] and E. A. Elsherbiny[1*]

[1]Plant Pathology Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt
[2]Agricultural Microbiology Department, Faculty of Agriculture, Mansoura University, Mansoura 35516, Egypt
[3]Mycology Research and Plant Disease Survey Department, Plant Pathology Research Institute, Agricultural Research Center, Giza 12619, Egypt
[4]National Agricultural and Food Research Council, Academy of Scientific Research and Technology, Cairo, Egypt



ABSTRACT

Bacillus velezensis controls plant pathogens and reduces dependence on synthetic pesticides within sustainable agriculture practices. In the current study, the cell suspension of *B. velezensis* BE1 did not reduce the viability of Vero cells, kidney cells from an African monkey, HFB4 human skin cell line, or WI-38 cells, which are diploid human fibroblasts generated from the lung cell of a female fetus, at concentrations ranging from 19×10^{11} to 0.59×10^{11}. Additionally, the study indicated that cell suspensions of *B. velezensis* BE1 exhibited no cytotoxicity at any concentration tested on the three cell lines. Cytotoxicity levels ranged between 0 and 1.45 % for Vero cells, 0 and 0.91 % for WI-38 cells, and 0.40 to 6.27 % for HFB4 cells. These findings confirm the biosafety of the endophytic bacterium *B. velezensis* BE1 and could be used for the control of plant pathogens in both pre- and postharvest diseases.

Keywords: Cytotoxic effects, Endophytic bacteria, Biological control, Biosafety

INTRODUCTION

Annually, a considerable amount of crops is destroyed in the pre and post-harvest phases due to invasions by various pathogens including fungi, oomycetes, bacteria, viruses, and nematodes. These phytopathogens consistently assault crops, resulting in direct and indirect global financial losses estimated to be around 40 billion dollars (Jamiołkowska 2020; Pandit *et al.* 2022). Biological control stands out among non-chemical control methods as the most suitable for organic agriculture (Calvo-Garrido *et al.* 2014; Habashy *et al.* 2016). It's environmentally benign, sustainable, cost-effective, and precise (Bardin *et al.* 2015; Elsherbiny *et al.* 2017). This method entails diminishing plant pathogen populations via the activity of living organisms and their byproducts through antagonistic interactions or by enhancing plant resistance (Conrath *et al.* 2015; Yousef *et al.* 2024). Biological control agents protect crops from diseases by employing mechanisms that either stimulate the plants' resistance to pathogen infections or compete for nutrients and space (Di Francesco *et al.* 2017; Elsherbiny *et al.* 2024). Additionally, these agents may directly interact with pathogens through hyperparasitism, antibiosis, or the generation of bioactive secondary metabolites (Arseneault and Filion 2017; Ayaz *et al.* 2023).

In the kingdom of bacteria, different genera such as *Bacillus*, *Pseudomonas*, and *Agrobacterium* are crucial for biological control due to their association with soil and plants (Pignatelli *et al.* 2009). The genus *Bacillus* is a Gram-positive bacteria (Ruckert *et al.* 2011). To date, 142 species of *Bacillus* have been documented, with the number continually rising (Mian *et al.* 2024). These bacteria thrive in various ecological niches, such as air, soil, water, plants, and the rhizosphere (Mora *et al.* 2015). *Bacillus* species are capable of producing different types of bioactive substances with unique antagonistic properties (Li *et al.* 2014; Dimkić *et al.* 2017).

In our previous results (Aboelez *et al.* 2024), we identified the BE1 strain of *B. velezensis* through 16S rRNA analysis, which exhibited an enormous inhibition rate against *Botrytis cinerea* in a dual culture assay. Consequently, this study focuses on evaluating the viability and cytotoxicity of this bacterial strain on three cell lines: Vero cells, WI-38 cells, and HFB4 cells.

MATERIALS AND METHODS

1. Isolation and molecular identification of *Bacillus velezensis* BE1

Bacillus velezensis strain BE1, isolated from healthy tomato plant leaves (*Solanum lycopersicum*), was comprehensively characterized through the 16S rRNA gene sequences. Accession Number PP538030.1 is for the nucleotide sequence of this strain in the GenBank. Our previous study by Aboelez *et al.* (2024) details the findings.

2. Preparation of concentrated cell suspensions

The BE1 strain was initially cultured on Nutrient Agar (NA) medium and incubated at 28 °C for 48 h. The bacterial biomass from the NA medium was then used to prepare a suspension in the Nutrient Broth (NB) medium. This suspension inoculated a fresh NB medium, with the initial concentration at 1×10^6 CFU mL^{-1} (at 28 °C 48 h at 180 rpm).

* **Corresponding author.**
E-mail address: sherbiny@mans.edu.eg
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Then, the broth was centrifuged at 10000 × *g* for 15 min, separating the cells and supernatant. The supernatant underwent a second centrifugation and was filtered using a filter (0.22 µm). The cells were washed with sterile distilled water two times, with centrifugation and disposal of the wash liquid after each. Finally, a bacterial cell suspension in sterile distilled water was prepared at various concentrations for bioassays.

3. Cytotoxicity assay

Three cell lines were utilized: Vero cells (ATCC: CCL-81), originating from the kidney epithelial cells of an African green monkey; a standard cell line, the human skin melanocyte cell line (HFB4); and WI-38 cells (ATCC: CCL-75), which are diploid human cells made up of fibroblasts from the lung tissue of a female fetus at three months gestation.

The viability and cytotoxicity assays were performed using an MTT reduction method (3–4,5-dimethyl-thiazol-2-yl-2,5-diphenyltetrazolium bromide), adhering to the protocol recommended by Mosmann in 1983. The cells under examination were put in tissue culture plates (96-well) with a concentration of 1×10^5 cells per 100 µL. They were incubated with varying concentrations of cell suspensions from *B. velezensis* BE1 (24 h at 37 °C) under humidified conditions to form a complete monolayer. The cell monolayer underwent two washes and then incubated in RPMI medium supplement with 2 % serum (48 h). Each dilution was tested with 0.1 mL in separate wells, and three wells were designated as controls containing only RPMI. Then, 20 µL of PBS, BIO BASIC CANADA INC (MTT solution) was added after removing the culture media. This was followed by thorough mixing at 150 rpm (5 min).

All samples were put for 4 h in a 5 % CO_2 environment at 37 °C to facilitate the metabolization of MTT. Subsequently, the resultant formazan crystals were dissolved in 200 µL of DMSO (10 %) after discarding the media. All samples were agitated at 150 rpm for 30 min in darkness to ensure complete dissolution of the formazan into the solvent. The OD was then calculated at 570 nm. Alterations in the morphology of the tested cells were observed using a phase-contrast microscope. The viability of tested cells was determined using the formula provided by Pournejati *et al.* (2021), Haq *et al.* (2022), and Ibrahim *et al.* (2022):

Viability of tested cells (%) =

[OD at 570 nm of treatment / OD at 570 nm of control] × 100.

4. Statistical analysis

The data were analyzed by SAS (version 9.1, USA) and involved ANOVA. The significance differences at $P < 0.05$ were determined by Tukey's test (Elsherbiny *et al.* 2023).

RESULTS AND DISCUSSION

1. Cell viability

Data were collected from cell viability assessments using the MTT method. The results indicated no significant differences ($P < 0.05$) across all concentrations, ranging from 0.59×10^{11} to 19×10^{11} of cell suspension for *B. velezensis* BE1, in the viability of Vero and WI-38 cells. Vero cell viability was between 98.54 to 100 % (Table 1), and WI-38 cell viability ranged from 99.08 to 100 % (Table 2). However, a significant decrease in viability ($P < 0.05$) was observed in HFB4 cells treated with the highest concentration of 19×10^{11}, with viability at 93.72 % (Table 3).

Table 1. Cytotoxicity levels of *Bacillus velezensis* BE1 on Vero cells.

Concentration (CFU mL^{-1})	Viability (%)	Cytotoxicity (%)
19×10^{11}	98.54 a	1.45 a
9.5×10^{11}	99.69 a	0.30 a
4.75×10^{11}	100 a	0 b
2.37×10^{11}	99.34 a	0.65 a
1.18×10^{11}	99.84 a	0.15 a
0.59×10^{11}	100 a	0 b

No significant difference with the same letters in each column at $P < 0.05$ (Tukey's test).

Table 2. Cytotoxicity levels of *Bacillus velezensis* BE1 on WI-38 cells.

Concentration (CFU mL^{-1})	Viability (%)	Cytotoxicity (%)
19×10^{11}	99.41 a	0.58 a
9.5×10^{11}	99.19 a	0.80 a
4.75×10^{11}	99.08 a	0.91 a
2.37×10^{11}	99.78 a	0.21 a
1.18×10^{11}	99.89 a	0.10 a
0.59×10^{11}	100 a	0 b

No significant difference with the same letters in each column at $P < 0.05$ (Tukey's test).

Table 3. Cytotoxicity levels of *Bacillus velezensis* BE1 on HFB4 cells.

Concentration (CFU mL^{-1})	Viability (%)	Cytotoxicity (%)
19×10^{11}	93.72 b	6.27 a
9.5×10^{11}	98.37 a	1.62 b
4.75×10^{11}	98.83 a	1.16 b
2.37×10^{11}	99.12 a	0.87 b
1.18×10^{11}	99.47 a	0.52 b
0.59×10^{11}	99.59 a	0.40 b

No significant difference with the same letters in each column at $P < 0.05$ (Tukey's test).

2. Cytotoxicity assessment

The cytotoxicity of *B. velezensis* BE1 cell suspension at various concentrations was evaluated using Vero, WI-38, and HFB4 cells. The results showed no cytotoxicity for cell suspensions of *B. velezensis* BE1 at all concentrations (Fig. 1, 2, and 3). As detailed in Tables 1 and 2, the bacterial suspension exhibited minimal toxicity, ranging from 0 to 1.45 % for Vero cells, and 0 to 0.91 % for WI-38 cells. The highest bacterial cell suspension concentration, 19×10^{11}, resulted in 6.27 % cytotoxicity for HFB4 cells (Table 3), demonstrating the biosafety of the endophytic bacterium *B. velezensis* BE1.

The cytotoxic impacts on mammalian cell lines have been observed with bacteriocins from *Bacillus* strains. Vaucher *et al*. (2010) indicated that the IC50 for *B. licheniformis* P40 bacteriocin on Vero cells was 0.30 µg mL^{-1}. Abdhul *et al*. (2015) found that *B. coagulans* BDU3 bacteriocin exhibited low cytotoxicity to HEK 293 (human embryonic kidney cells). Additionally, a bacteriocin from *B. velezensis* BUU004 shows potential as a safe food preservative, as suggested by Butkhot *et al*. (2019).

B. velezensis BUU004 could be awarded the QPS status following the criteria recommended by EFSA (2014). It is thus justifiable to emphasize that *B. velezensis* BUU004 has fundamental biosafety characteristics, poses no harmful risks to human health, and is safe for use in humans.

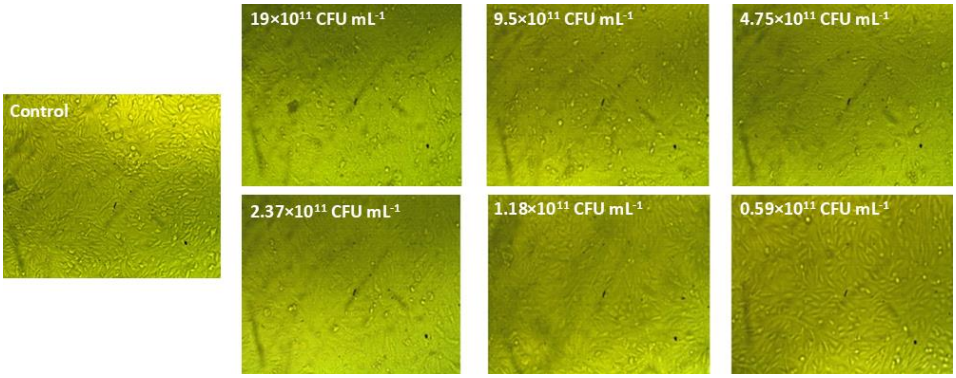

Fig. 1. Effect of *Bacillus velezensis* BE1 on Vero cells at different concentrations .

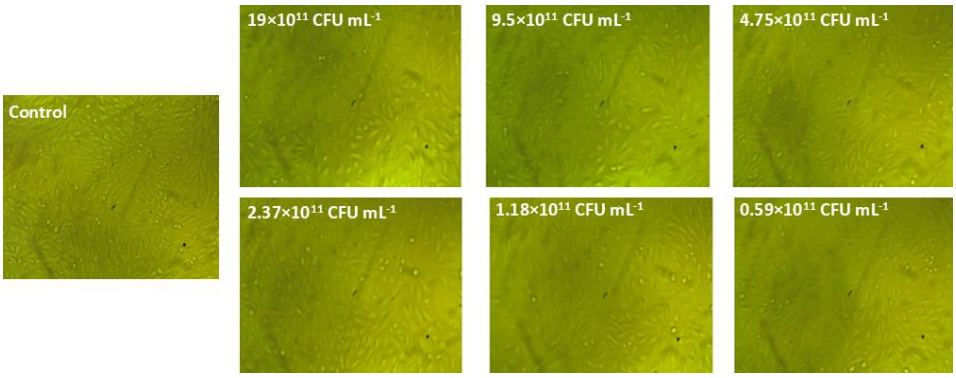

Fig. 2. Effect of *Bacillus velezensis* BE1 on WI-38 cells at different concentrations.

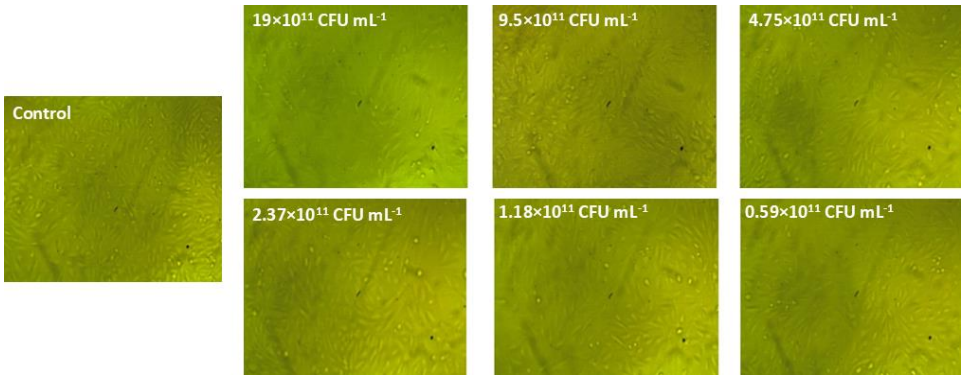

Fig. 3. Effect of *Bacillus velezensis* BE1 on HFB4 cells at different concentrations.

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تقييم تأثيرات السمية الخلوية لبكتيريا المكافحة الحيوية *Bacillus velezensis* BE1

إسراء محمد السيد عبد الوهاب[١]، محمد عبد الله العوضى سليم[٢]، صفاء أحمد يوسف[٣]، صفاء حمزة أحمد على[١] ، ياسر محمد نور الدين شبانة[١،٤]والشربينى عبد المنعم الشربينى[١]

[١] قسم أمراض النبات، كلية الزراعة، جامعة المنصورة، مصر
[٢] قسم الميكروبيولوجيا الزراعية، كلية الزراعة، جامعة المنصورة، مصر
[٣] معهد بحوث أمراض النباتات، مركز البحوث الزراعية، الجيزة، مصر
[٤] المجلس القومي للبحوث الزراعية والغذائية، أكاديمية البحث العلمي والتكنولوجيا، القاهرة، مصر

المخلص

يتم استخدام بكتيريا *Bacillus velezensis* في مكافحة مسببات الأمراض النباتية، مما يقلل من الاعتماد على المبيدات الحشرية الاصطناعية في ممارسات الزراعة المستدامة. في الدراسة الحالية، لم يقلل معلق الخلايا من *B. velezensis* BE1 من قابلية خلايا Vero (ATCC: CCL-81)، أو الخلايا الكلوية للقرد الأفريقي، أو سلالة خلايا الجلد البشري HFB4، أو خلايا WI-38 (ATCC: CCL-75)، وهي الخلايا الليفية البشرية ثنائية الصبغيات المشتقة من أنسجة الرئة لجنين أنثى، بتركيزات تتراوح من ١٩ × ١٠^١١ إلى ٠,٥٩ × ١٠^١١. بالإضافة إلى ذلك، أشارت الدراسة إلى أن معلق الخلايا من *B. velezensis* BE1 لم يُظهر أي سمية خلوية عند أي تركيز تم اختباره على سلالات الخلايا الثلاثة. تراوحت مستويات السمية الخلوية بين ٠ إلى ١,٤٥٪ لخلايا Vero، و٠ إلى ٠,٩١٪ لخلايا WI-38، و٠,٤٠ إلى ٦,٢٧٪ لخلايا HFB4. تؤكد هذه النتائج السلامة البيولوجية للبكتيريا الداخلية *B. velezensis* BE1.

الكلمات الدالة: السمية الخلوية - البكتيريا الداخلية - المكافحة البيولوجية - السلامة البيولوجية