

## ORIGINAL ARTICLE

# The feeding behavior of *Diaphorina citri* monitored by using an electrical penetration graph (DC-EPG) on citrus plants treated with *Bacillus cereus* and *Bacillus velezensis*

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## Abstract

*Diaphorina citri*, an important pest and insect vector that can transmit the pathogenic bacteria *Candidatus Liberibacter asiaticus*, causing Huanglongbing disease, is one of many challenges in citrus agriculture. Integrated pest management by utilizing microorganisms is a wise and efficient alternative without damaging the environment. Utilization of Plant Growth Promoting Rhizobacteria (PGPR), such as *Bacillus cereus* and *B. velezensis*, is a potential strategy for the biological control of plant diseases or insect vectors. By inducing systemic resistance in plants, PGPR can enhance plant defense against diseases and insect pests while activating molecular and physiological changes in plants. This research aimed to determine the effect of *B. cereus* and *B. velezensis* on the plant growth and feeding behavior of *D. citri*. The height and volume of the plant canopy were observed periodically for 6 months, while the feeding behavior of *D. citri* was monitored using the Electrical Penetration Graph (DC-EPG). The results showed increased height and volume of the citrus plant canopy treated with *B. cereus*, indicating that *B. cereus* could act as a PGPR. The application of *B. cereus* and *B. velezensis* to citrus seedlings affected the feeding behavior of *D. citri*. *D. citri* showed difficulty in penetrating the phloem tissue of citrus plants.

**Keywords:** *Bacillus cereus*, *Bacillus velezensis*, citrus, *Diaphorina citri*, electrical penetration graph

## Introduction

Huanglongbing (HLB) disease, caused by the pathogenic bacteria *Candidatus liberibacter asiaticus* (Clas), is one of the most destructive and economically important citrus diseases that can lead to reduced yields, fruit drop, reduced fruit quality, and death of citrus plants (Johnston *et al.* 2019). The spread of this disease depends on the presence of inoculum and vectors in the field. This bacterium is transmitted continuously via the psyllid vector *Diaphorina citri* or through grafting. A long incubation period occurs before symptoms appear after vector inoculation in citrus phloem by an insect Vector. The formation and accumulation of callose in phloem inhibit the transport of nutrients and cause

typical symptoms, such as asymmetrical spots on the leaves, loss of productivity, and death of infected trees after several years (Gabriel *et al.* 2020). *Diaphorina citri*, as an insect vector for HLB and can transmit the disease at the imago and nymph stages. At the imago stage, symptoms appear more quickly because *D. citri* can quickly move from one leaf shoot to another. The imago and nymphs of *D. citri* suck young tissue fluids, causing damage and even death to the plant (Widyaningsih *et al.* 2019).

Developing host resistance is one aspect of pest management that helps reduce the need for chemical pesticides to control insect vectors and the spread of disease (Hong-xing *et al.* 2017). Control of the insect vector *D. citri* by utilizing microorganisms is a good alternative and provides efficient control with little

or no negative impact on the environment (Bidima *et al.* 2022). Plant Growth Promoting Rhizobacteria (PGPR) is a potential strategy for successful biological control. PGPR associated with the roots of higher plants increases the host's adaptation to biotic and abiotic stress to increase growth and tolerance to stress and pest attacks (Navitasari *et al.* 2020; Arsène *et al.* 2023). PGPR plays a role, among others, by producing phytohormones such as ethylene, jasmonic acid (JA), and salicylic acid (SA) as signal molecules to coordinate immune responses (Pieterse *et al.* 2012; Amalia *et al.* 2023). In addition, volatile compounds and lipopeptides produced by PGPR influence host plants to activate Induced Systemic Resistance in defense responses and metabolic changes in plants (Henry *et al.* 2011; Rahma *et al.* 2019).

*Bacillus* is one of the PGPR groups reported by many studies that has the ability to increase plant growth and induce resistance, so it has the potential to be used to control insect pests. Harun-Or-Rashid *et al.* (2018) reported that *B. velezensis* could increase the resistance of rice plants to brown planthopper pests. Systemic resistance to brown planthoppers is induced by *B. velezensis* via pathways dependent on SA and jasmonic acid (JA). Elsharkawy *et al.* (2022) also reported that the development and death rate of *Rynochophorus ferrugineus*, an insect that destroys palm trees, can be suppressed by *B. cereus* and *B. thuringiensis*. Another *Bacillus* species, the endophytic bacteria *B. subtilis* isolated from citrus plants, was also reported to be able to reduce the population of Clas and reduce the intensity of HLB disease to <3% (Munir *et al.* 2022). Likewise, Zhao *et al.* (2016) reported that the larvae of the insect *Helicoverpa armigera* showed different feeding behavior responses, in which the area consumed by the larvae on cotton leaves treated with *B. thuringiensis* was smaller than that on cotton leaves without *B. thuringiensis* treatment. In addition, the number of foraging places on cotton plants without *B. thuringiensis* was higher than that on cotton plants treated with *B. thuringiensis*. The larvae of *H. armigera* showed consistent feeding behavior on cotton plants without *B. thuringiensis* treatment. Research by de Oliveira Dorta *et al.* (2018) also found that translocation of *B. thuringiensis* strains from citrus roots to shoots could control nymphs and cause the highest mortality rate of *D. citri*.

Plant defense mechanisms can be observed through the feeding behavior response of piercing-sucking insects, including *D. citri*. The feeding behavior of *D. citri* can be monitored using the Electrical Penetration Graph (DC-EPG). The basic principle of EPG recording is connecting plants and insects as an electronic circuit, so when the stylet pierces plant tissue, the voltage differences resulting from different stylet activities and positions in plant tissues will be recorded

and visualized as various waveforms. The waves will fluctuate along with the activity of insect stylets in other plant tissues (Gaffar *et al.* 2011; Soffan and Aldawood 2015). The waveforms are observed in real-time on a computer monitor via EPG software. EPG waveforms are usually associated with stylet position and activities in the epidermis, parenchyma, xylem, and phloem (Tjallingii 1978). This research aimed to determine the effect of applying *B. cereus* RC76 and *B. velezensis* B-27 on plant growth enhancement and feeding behavior of *D. citri* using DC-EPG on citrus plants.

## Materials and Methods

### Bacterial culture

The *B. cereus* RC76 and *B. velezensis* B-27 used were from the collection of the Plant Pathology Laboratory, Department of Plant Protection, Faculty of Agriculture, Universitas Gadjah Mada. Bacteria were cultured on Yeast Peptone Agar (YPA) media (20 gr agar, 10 gr bacto peptone, 5 gr yeast) and incubated at 25°C for 24–48 hours. The 48-hour-old bacteria were then suspended in distilled water to obtain a bacterial density of  $10^8$  cfu · ml<sup>-1</sup> for seed treatment (Ilmiah *et al.* 2021).

### Insect and plant

Rearing of *D. citri* was carried out in the greenhouse of the Faculty of Agriculture UGM, with a temperature of 26–30°C. *D. citri* was reared on a potted plant of *Murraya paniculata* inside screened cages. Young adult females (5–10 days old) were used in the EPG study after an adaptation period on healthy citrus seedlings (*Citrus nobilis*). Seedlings, 20 cm tall, were used for the EPG recordings; these seedlings were grown in pots containing soil and manure (1 : 1) inside a greenhouse.

### Application of *Bacillus cereus* RC76 and *Bacillus velezensis* B-27 on citrus seedlings

This research was arranged in a completely randomized design (CRD). A total of 15 citrus seedlings (*C. nobilis*) were used with five replications each for the application of *B. cereus* RC76, *B. velezensis* B-27, and the control. The seedlings were immersed in the *B. cereus* RC76 and *B. velezensis* B-27 suspension for 1 hour. Subsequently, the seedlings were planted in 25 × 30 cm polybags filled with a 1 : 1 soil and manure combination. Following planting, a suspension of *B. cereus* RC76 and *B. velezensis* B-27 was drenched to the seedlings' root area once a week. Agronomic observations of plants, including plant height and canopy volume, were done every 6 months.

Canopy volume measurements were carried out according to Rodrigues *et al.* (2020):

$$V = \frac{2}{3}\pi \left(\frac{D}{2}\right)^2 H,$$

where:

$V$  – volume of canopy ( $\text{m}^3$ ),

$D$  – diameter of canopy (m),

$H$  – plant height (m).

### Electrical penetration graph (EPG) recordings

The feeding behavior analysis of *D. citri* was conducted using EPG, which was a technique for monitoring the feeding behavior, especially to observe in detail the position and activity of the *D. citri* stylet during feeding. The EPG monitoring was performed using one citrus plant with 7-day-old leaves infested with three *D. citri*, which were then EPG recorded for 12 hours. EPG monitoring was carried out for 15 replications to compare the feeding behavior of *D. citri* on citrus plants given *B. cereus* RC76, *B. velezensis* B-27, and without *Bacillus* application.

Insect electrodes for *D. citri* were made by immobilizing the *D. citri* individuals and connecting them to gold wire by silver glue, then to the EPG amplifier input through a copper rod (Luo *et al.* 2015). The plant electrode was constructed by inserting the copper rods into the planting medium, and the other side was connected to the EPG input. The real-time EPG of *D. citri* feeding monitoring was made possible through STYLET 3.0 software.

During *D. citri* feeding on the citrus, several waveforms were generated through the EPG system consisting of non-probing (Np), C, D, E1, E2, and G waveforms. The Np waveform had a straight-line shape, indicating no penetration of the probing stylet into citrus plant tissue. Waveform C, which had a complex shape, was a sign that the stylet was located between cells in the epidermis and parenchyma. Waveform D was in the phloem tissue and occurred between waveform C and E1. Waveform E1 represented the initial activity of the phloem phase. Waveform E1 showed the stylet making initial penetration into the phloem and salivation activity. Meanwhile, waveform E2 showed the stylet activity entering the phloem tissue and taking up fluid in the phloem (Bonani *et al.* 2009).

### Data analysis

Data was analyzed using the ANOVA test with a confidence level of 95% and the Kruskal-Wallis test using R-studio.

## Results

### Effects of *Bacillus cereus* RC76 and *Bacillus velezensis* B-27 on citrus seedlings

The application of *B. cereus* RC76 and *B. velezensis* B-27 to citrus seedlings affected growth enhancement during 6 months of observation, including plant height and canopy volume (Fig. 1). The citrus seedlings drenched with *B. cereus* RC76 were taller and had a larger canopy (Fig. 1B). Citrus seedlings treated with *B. velezensis* B-27 (Fig. 1C) also appeared to have better performance than the control treatment (Fig. 1A).



**Fig. 1.** Citrus seedlings treated with water (control treatment) – A; *Bacillus cereus* RC76 – B and *Bacillus velezensis* B-27 – C. The growth performances of each plant were evaluated 6 months after transplanting

The growth of citrus plants observed 6 months after transplanting showed that *B. cereus* RC76 treatment increased plant height and canopy volume. The plant height and canopy volume of citrus plants treated with *B. cereus* RC76 were statistically different from the control treatment. However, although citrus plants treated with *B. velezensis* B-27 looked better, they did not show statistical differences compared to the control treatment. Citrus plants treated with *B. cereus* RC76, *B. velezensis* B-27, and the control treatment had a height of 76.50 cm, 65.78 cm, and 65.78 cm with a canopy volume of 44  $\text{cm}^3$ , 30  $\text{cm}^3$ , and 27  $\text{cm}^3$ , respectively (Table 1).

**Table 1.** Plant height and canopy volume of citrus seedlings treated with *Bacillus cereus* RC76 and *Bacillus velezensis* B-27 for six months

Treatments	Parameter	
	plant height [cm]	canopy volume [cm <sup>3</sup> ]
<i>Bacillus cereus</i> RC76	76.50 a	44 a
<i>Bacillus velezensis</i> B-27	65.78 ab	30 b
Control	60.50 b	27 b

Means followed by the same letters are not significantly different

### Feeding behavior of *Diaphorina citri* monitored by DC-EPG

The EPG variables observed in this study included the number of and total duration of waveforms. The number of waveforms showed the frequency of stylet activity, while the total duration specified how long the stylet was in a particular tissue. In this study,

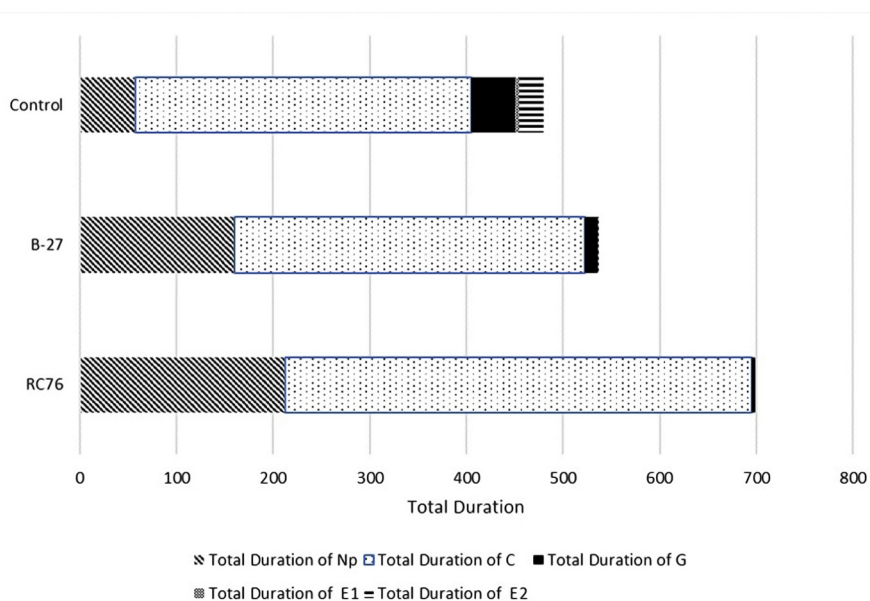
citrus seedlings treated with *B. cereus* RC76 and *B. velezensis* B-27 had a long total non-probing (Np) duration than the control treatment. Citrus seedlings with *B. cereus* RC76 treatment produced the lowest number of waveform C with the longest total waveform C duration, and *B. velezensis* B-27 treatment had the shortest total waveform C duration. Apart from that, the highest number of waveform D was found in citrus plants that were not treated. Treatment of *B. cereus* RC76 and *B. velezensis* B-27 showed a low number of waveform D. The number and total duration of waveform G were the shortest in citrus plants treated with *B. cereus* RC76 and the longest in the control treatment. The number of waveform E was the longest in the control treatment, whereas, in the treatments of *B. cereus* RC76 and *B. velezensis* B-27, there were no *D. citri* accessing the phloem during the 12 hours of observation. Statistically, the total duration of non-probing, waveform G, and waveform E in citrus plants treated with *B. cereus* RC76 was significantly different

**Table 2.** Feeding behavior of *Diaphorina citri* on citrus plants treated with *Bacillus cereus* RC76, *Bacillus velezensis* B-27, and control treatment observed for 12 hours on EPG's recording

No.	EPG variable	Treatments		
		<i>Bacillus cereus</i> C76	<i>Bacillus velezensis</i> B-27	control
Non-probing (Np) activities				
1	Number of non-probing	2.0 ± 1.0 b	2.0 ± 1.2 b	4.8 ± 2.4 a
2	Total duration of Np (min)	212.3 ± 149.2 a	160.1 ± 146.6 b	57.3 ± 42.9 b
Probe activities				
3	Number of probe	1.6 ± 0.9 a	2.0 ± 1.2 ab	4.8 ± 2.5 b
4	Total duration of probe (min)	483.1 ± 53.1 a	509.3 ± 286.6 a	562.6 ± 42.9 a
Waveform C				
5	Number of C	2.2 ± 0.8 b	2.6 ± 0.89 b	5.8 ± 2.8 a
6	Total duration of C (min)	483.1 ± 53.1 a	362.3 ± 288.7 a	347.7 ± 48.8 a
Waveform D				
7	Number of D	0.0 ± 0.0 a	0.4 ± 0.3 a	1.4 ± 1.3 b
8	Average D per probe	0.0 ± 0.0 a	0.1 ± 0.09 a	0.3 ± 0.3 a
Waveform G				
9	Number of G	0.2 ± 0.1 a	0.8 ± 0.4 a	1.2 ± 0.9 a
10	Total duration of G (min)	4.3 ± 4.2 a	13.6 ± 12.9 ab	45.6 ± 39.1 b
Waveform E1				
11	Number of E1	0.0 ± 0.0 a	0.0 ± 0.0 a	1.6 ± 1.4 b
12	Total duration of E1 (min)	0.0 ± 0.0 a	0.3 ± 0.2 a	4.1 ± 3.7 b
Waveform E2				
13	Number of E2	0.0 ± 0.0 a	0.4 ± 0.3 ab	1.2 ± 0.8 b
14	Total duration of E2 (min)	0.0 ± 0.0 a	0.1 ± 0.1 a	25.3 ± 24.5 b
15	Number of phloem phases	0.0 ± 0.0 a	0.0 ± 0.0 a	0.2 ± 0.1 b

Means followed by the same letters are not significantly different

The Np waveform indicated no penetration of the probing stylet into citrus plant tissue. Waveform C was a sign that the stylet was located between cells in the epidermis and parenchyma. Waveform D represented the initial activity of the phloem phase. Waveform E1 showed the stylet making initial penetration into the phloem and salivation activity. Waveform E2 showed the stylet activity entering the phloem tissue and taking up fluid in the phloem. Means followed by the same letters are not significantly different



**Fig. 2.** Total duration of Np, C, G, E1, and E2 on citrus plants treated with *Bacillus cereus* RC76, *B. velezensis* B-27, and without treatment (control). The EPG test was performed using one citrus plant with 7-day-old leaves infested with three *Diaphorina citri*. EPG monitoring was carried out for 15 repetitions each to compare the feeding behavior of *Diaphorina citri*. Data was analyzed using the ANOVA test with a confidence level of 95% and the Kruskal-Wallis test using R-studio

than the control treatment. Citrus seedlings treated with *B. velezensis* B-27 showed a significant difference in the total duration of waveform E compared to the control treatment (Table 2).

The feeding behavior of *D. citri* on citrus seedlings treated with *B. cereus* RC76 appeared to have a longer total duration of Np, and C. The *B. velezensis* B-27 treated citrus had a longer total duration of Np waveform than the control, and the longest total duration of waveform G (Fig. 2).

## Discussion

*Bacillus* is a group of rhizosphere bacteria or PGPR that live naturally in the soil, in the rhizosphere of plants, or in plant tissue where the bacteria interact and form a mutualistic symbiosis with their host plants (Artanti *et al.* 2023). This research showed that the growth of citrus seedlings treated with *B. cereus* RC76 significantly increased plant height and canopy volume. This is in line with research by Dong *et al.* (2023), who reported that *B. cereus* could promote plant growth, help absorb nutrients, change bacterial diversity, and increase species abundance in the soil. The research results of Tsotetsi *et al.* (2022) also showed that *B. cereus* could increase cell development, trigger the formation of new roots, stimulate flowering, and increase plant hormone activity. *B. cereus* also increased plant growth directly

by producing cytokines and volatile compounds that influence plant hormonal networks. *B. cereus* has been reported to increase the growth of several types of plants, such as soybeans, corn, rice, and wheat. A study by Xu *et al.* (2014) showed that *B. cereus* strains can promote plant growth under abiotic stress, including drought and salinity. *B. cereus* also produces extracellular enzymes and antibiotic lipopeptides or induces systemic resistance, indirectly stimulating plant growth. Extracellular enzymes and antibiotic lipopeptides produced by *B. cereus* allow indirect plant growth stimulation (Kulkova *et al.* 2023).

The increase in canopy volume of citrus seedlings treated with *B. cereus* RC76 shows that the bacteria probably produce phytohormones and siderophores that encourage plant growth and development. An increase in the photosynthesis process in the leaves affects the photosynthesis released by the new canopy. Leaf development is characterized by the emergence of new leaves and an increase in the accumulation of leaves on the stems of citrus plants. This aligns with research by Ali *et al.* (2021), who stated that *B. cereus* increased leaf nitrogen, phosphorus, and potassium concentrations in plants inoculated with *B. cereus*. Based on the research by Hapsoh *et al.* (2022), *B. cereus* produces the IAA hormone, which can increase root formation and support rhizobium growth since more N is bound in the air, thereby stimulating vegetative growth. Bacterial activity in plant growth acts as a trigger for phytohormones, especially IAA, which can increase cell wall

permeability and help in the photosynthesis process. Park *et al.* (2017) stated that IAA-producing bacteria could help plants tolerate heat stress by activating antioxidant enzymes, gene regulation, osmoprotectant systems, and by increasing the accumulation of photosynthetic pigments. IAA-producing bacteria have been shown to increase the length and surface of plant roots and have good access to nutrients available in the soil.

Further analyses regarding the application of *B. cereus* RC76 and *B. velezensis* B-27 were carried out by testing the feeding behavior of *D. citri* using EPG. The feeding behavior of *D. citri* begins with the penetration of the stylet into plant tissue, which then finds the phloem to access nutrients. Stylet penetration of *D. citri* was inhibited in citrus seedlings which had the longest total non-probing duration and had been treated with *B. cereus* RC76 and *B. velezensis* B-27. In contrast, in the control treatment, stylet penetration of *D. citri* was not inhibited during the total non-probing duration. The non-probing number can be seen to determine the number of stylets that enter and exit the tissue. Lei *et al.* (1999) stated that events before contact with phloem are related to plant introduction, and events after contact with phloem are related to acceptance and consumption of phloem. The total duration of waveform C was the longest in citrus plants treated with *B. cereus* RC76, with the shortest waveform C indicating resistance from the *D. citri* stylets in the plant tissue to move. This study showed that it was more difficult for *D. citri* stylets to penetrate phloem tissue because *B. cereus* RC76 treatment caused the stylets to remain in the epidermis and parenchyma tissue.

Waveform G in the control treatment had the longest duration, which indicated that *D. citri* experienced a lack of water when accessing plant tissue. The purpose of stylet activity in the xylem tissue is to meet the water needs of *D. citri* because, according to Montlor and Tjallingi (1989), waveform G duration is thought to indicate whether or not *D. citri* is experiencing a water shortage. Apart from that, *D. citri* stylets in phloem tissue is related to the fulfillment of nutrition, where the fluid in phloem tissue is a nutrient for *D. citri*. In this study, the longest total duration of waveform E occurred in control citrus seedlings, indicating that *D. citri* could access phloem tissue. This aligns with research by Cen *et al.* (2012), who reported that *D. citri* spent most of its time in phloem tissue in healthy citrus plants.

The high non-probing activity and low total consumption duration in the phloem indicated that *D. citri* had difficulty accessing nutrients in citrus seedlings treated with *B. cereus* RC76 and *B. velezensis* B27 compared to the control treatment. However, the total duration of tissue penetration (C) in citrus seedlings treated with *B. cereus* RC76 was longer than without treatment. *B. cereus* RC76 treatment on citrus

seedlings changed the feeding behavior of *D. citri*, making it challenging to access plant tissue. This follows the study by Ma *et al.* (2022) that HLB, caused by phloem-restricted CLAs bacteria, stimulates systemic and chronic immune responses in phloem tissue, including callose deposition, production of reactive oxygen species (ROS) such as H<sub>2</sub>O<sub>2</sub>, and induction of immunity-related genes. The high incidence of cell death in leaves indicates CLAs infection in phloem tissue cells.

The feeding activity of *D. citri* is required to obtain nutrients that can affect its reproduction and growth. *D. citri* needs protein, carbohydrates, and fat to grow and develop. According to Cichoka *et al.* (2015), *D. citri* also has mutualistic symbiosis with the endosymbiotic bacteria CLAs, which can increase access to nutrients found in the phloem. The activity carried out by *D. citri* is caused by using young shoots as hosts, which *D. citri* prefers. *Bacillus* can control and maintain plant resistance, especially to the *D. citri* insect, a vector for citrus plant diseases (Fira *et al.* 2018).

## Conclusions

The citrus HLB pathogen, CLAs, was known as phloem-limited bacteria, which inhabited exclusively phloem tissue. This study revealed that citrus seedlings treated with *B. cereus* RC76 could increase plant growth, canopy volume, and number of shoots. The application of *B. cereus* RC76 also enhanced plant resistance to *D. citri* as seen by the high non-probing and pathway phases as well as the inability of *D. citri* to access phloem during 12 hours of recording using EPG. This also indicated that nutrient uptake by *D. citri* in phloem as well as CLAs acquisition was inhibited.

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