AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology

Spotlight Selection | Host-Microbial Interactions | Full-Length Text

Calcium modulation of bacterial wilt disease on potato

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ABSTRACT *Ralstonia solanacearum* species complex (RSSC) is a phytopathogenic bacterial group that causes bacterial wilt in several crops, being potato (*Solanum tuberosum*) one of the most important hosts. The relationship between the potato plant ionome (mineral and trace elements composition) and the resistance levels to this pathogen has not been addressed until now. Mineral content of xylem sap, roots, stems and leaves of potato genotypes with different levels of resistance to bacterial wilt was assessed in this work, revealing a positive correlation between divalent calcium (Ca) cation concentrations and genotype resistance. The aim of this study was to investigate the effect of Ca on bacterial wilt resistance, and on the growth and virulence of RSSC. Ca supplementation significantly decreased the growth rate of *Ralstonia pseudosolanacearum* GMI1000 in minimal medium and affected several virulence traits such as biofilm formation and twitching motility. We also incorporate for the first time the use of microfluidic chambers to follow the pathogen growth and biofilm formation in conditions mimicking the plant vascular system. By using this approach, a reduction in biofilm formation was observed when both, rich and minimal media, were supplemented with Ca. Assessment of the effect of Ca amendments on bacterial wilt progress in potato genotypes revealed a significant delay in disease progress, or a complete absence of wilting symptoms in the case of partially resistant genotypes. This work contributes to the understanding of Ca effect on virulence of this important pathogen and provides new strategies for an integrated control of bacterial wilt on potato.

IMPORTANCE *Ralstonia solanacearum* species complex (RSSC) includes a diverse group of bacterial strains that cause bacterial wilt. This disease is difficult to control due to pathogen aggressiveness, persistence, wide range of hosts, and wide geographic distribution in tropical, subtropical, and temperate regions. RSSC causes considerable losses depending on the pathogen strain, host, soil type, environmental conditions, and cultural practices. In potato, losses of $19 billion per year have been estimated for this pathogen worldwide. In this study, we report for the first time the mineral composition found in xylem sap and plant tissues of potato germplasm with different levels of resistance to bacterial wilt. This study underscores the crucial role of calcium (Ca) concentration in the xylem sap and stem in relation to the resistance of different genotypes. Our in vitro experiments provide evidence of Ca's inhibitory effect on the growth, biofilm formation, and twitching movement of the model RSSC strain *R. pseudosolanacearum* GMI1000. This study introduces a novel element, the Ca concentration, which should be included into the integrated disease control management strategies for bacterial wilt in potatoes.

KEYWORDS calcium, plant ionome, bacterial wilt, disease resistance

Ralstonia solanacearum species complex (RSSC) is the causal agent of bacterial wilt and one of the most important gram-negative plant pathogenic bacteria. This pathogen affects more than 250 plant species, including important crops such as tobacco, tomato, potato, peanut, and banana in tropical, subtropical, and temperate

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The authors declare no conflict of interest.

See the funding table on p. 16.

Received 19 February 2024
Accepted 22 March 2024
Published 1 May 2024

regions (1–4). The disease affects more than 1.5 million hectares of potato crops worldwide having a significant economic impact estimated in $ 1 billion per annum (5, 6). The bacterium infects the roots of host plants, rapidly colonizes the vascular system, and releases large amounts of exopolysaccharide (EPS) that block water flow within xylem vessels, causing wilting symptoms and subsequent plant death (7). RSSC can persist, spread, and survive in different natural habitats including soil, water, numerous weed hosts, and debris, with latently infected seed potatoes being mainly responsible for international spread (3). Genetic and phenotypic diversities of this xylem-invader make a sustainable disease control difficult to achieve (8).

RSSC colonizes vascular vessels formed by a network of metabolically dead xylem vessels that transport sap from roots to leaves. Xylem sap flow disperses planktonic cells of RSSC upward. In addition, RSSC has two forms of active motility, swimming motility mediated by flagella and twitching motility mediated by type IV pili. Twitching motility allows bacteria to move basipetally in the plant xylem vessels against sap flow. Type IV pili, lectins, and adhesins participate in bacterial attachment, which is also influenced by physicochemical properties of host surface, pH, and temperature. Attached cells can grow in aggregates forming biofilms that fill vessels and may obstruct xylem sap flow (9, 10).

Potato (*Solanum tuberosum* L.) is the third most consumed food crop worldwide and is essential part of the diet of around 1.3 billion people (3, 11, 12). The origin of commercial potato cultivars is limited to a restricted number of potato clones introduced from South America into Europe in the 16th century, leading to a narrow genetic base and a limited resistance to pathogens (13). Wild *Solanum* species and primitive forms of cultivated potato are considered an invaluable and diverse source of genetic variation for potato breeding for resistance to different pests and diseases (14). However, the resistance from these sources was variable depending on pathogen strain and environmental conditions, making potato breeding for bacterial wilt resistance a continuous challenge (15). Introgression of resistance through the potato breeding program in Uruguay makes use of the high genetic diversity available in the wild species *Solanum commersonii* Dun (14–18). Currently, the potato breeding program in Uruguay has advanced interspecific clones with high bacterial wilt resistance and low frequency of latent infections. These partially resistant clones showed a restricted multiplication of the pathogen in stems, and colonization patterns restricted to roots, and to a limited number of xylem vessels at the stem base. Furthermore, these clones showed higher lignin contents, callose deposition, and reactive oxygen species (ROS) production when plant defense responses were induced in comparison with susceptible potato (19).

Mineral micro- and macro-nutrients are essential for cellular functions, and their concentrations are tightly regulated because they can be toxic at high concentrations. Minerals are required by both pathogen and host. Pathogenic bacteria in both human and plant hosts have developed strategies to modify certain elements from the host for their own advantage during the infection process (20–24). Ionomics, defined as the study of mineral element concentrations, requires the application of high-throughput elemental analysis technologies (e.g., Inductively Coupled Plasma-Optical Emission Spectrometry, ICP-OES) to characterize the functional state of an organism under different conditions (25). The plant ionome is affected by various factors, including species, variety, organ, environment (26, 27), and by the presence of bacterial pathogens (22, 23, 28–30). Several studies highlighted ionomic studies as useful tools for selection of varieties with optimal nutritional mineral profiles, while minimizing harmful concentrations of toxic or radioactive elements (26, 31–34). In addition, the use of high throughput techniques for ionomic analysis in different plant species has enhanced our understanding of the pivotal role that mineral elements play during plant infection (21–23, 28, 35).

Calcium (Ca) is an important nutrient element that plays a major role in improving the structure and integrity of plant cell wall components. In addition, plants use Ca as a secondary messenger in the transmission of endogenous (developmental) and exogenous (environmental) signals (36, 37). Several studies have shown Ca association

with tolerance to adverse environmental conditions, promotion of plant growth and tuberization, and plant resistance to diseases involving tissue maceration (38–44). Studies done with the bacterial pathogen *Xylella fastidiosa* showed that Ca is accumulated during successful plant host infections (21, 22, 45), and it increases virulence traits of this pathogen such as biofilm and twitching movement (46, 47).

As per these findings, we hypothesized that the concentrations of mineral elements within potato plant tissues are indicative of the resistance to bacterial wilt in potato genotypes. Accordingly, the objectives of our research were: (i) to evaluate the correlation between resistance to bacterial wilt and ionome variations within xylem sap, stem, leaf, and roots of potato genotypes exhibiting diverse resistance levels to bacterial wilt; (ii) to elucidate the impact of identified minerals, particularly Ca, on both the growth and virulence of the model RSSC strain *Ralstonia pseudosolanacearum* GMI1000 under batch and flow conditions; (iii) to examine the effects of mineral amendments, with a focus on Ca, on enhancing bacterial wilt resistance in potatoes. Our findings revealed a significant increase in Ca concentrations within the xylem sap and stems of genotypes resistant to bacterial wilt compared to susceptible genotypes. Further, Ca demonstrated a notable inhibitory effect on growth, biofilm formation, and twitching motility of *R. pseudosolanacearum* GMI1000. Most importantly, Ca amendments applied via drenching to potato plants significantly improved their resistance against bacterial wilt, underscoring the potential of targeted mineral supplementation in disease management strategies.

MATERIALS AND METHODS

Bacterial strains and growth conditions

R. pseudosolanacearum strain GMI1000 (48) and the reporter strain GMI1000 Pps-GFP (49) were streaked from a glycerol stock at −80°C on triphenyl tetrazolium chloride (TZC) agar plates (50), and incubated at 28°C for 48–72 h. Liquid cultures were prepared in casamino acid-peptone-glucose rich broth (CPG) (0.1% casamino acid, 1% peptone, and 0.5% glucose, pH 7.0) or minimal medium supplemented with 0.2% glucose (MM) (51, 52). Ca effect was evaluated with $CaCl_2$ supplementation as specified for each experiment. Gentamicin selection (5 and 75 µg/mL in liquid and solid cultures, respectively) was used for the reporter strain. Optical density was measured spectrophotometrically at 600 nm to adjust bacterial suspensions for inoculation (OD_{600} of 0.1 corresponds to 10^8 cfu/mL).

Plant materials and growth conditions

Plant propagation and growth conditions were the same as described in previous studies (19). Briefly, plants were micro-propagated *in vitro* from a node in Murashige and Skoog (MS) medium with sucrose 30 g/L and kept at 22°C with cycles of 16 h light:8 h darkness in a growth chamber for 3 weeks. Then, plants were sown in 170 cm^3 individual plastic pots with soil mix (Tref Substrates BV, Moerdijk, Netherlands) and grown for 1 week in a greenhouse under natural light. Two potato clones with different levels of resistance to bacterial wilt, 13001.79 (susceptible, referred here as 13001.79 S) and 09509.6 (partially resistant, referred here as 09509.6 R) were selected from the National Institute for Agricultural Research (INIA, Uruguay) germplasm collection. The potato cultivar *S. tuberosum* cv. Chieftain (Chieftain) was used as a susceptible control.

Plant ionome analysis

Plant ionome was characterized by using ICP-OES (7300 DV, PerkinElmer,Waltham, MA, USA) as previously described (21, 53), with simultaneous measurements of Ca, copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), sulfur (S), and zinc (Zn). Briefly, plant tissues including leaves, stems, and roots were collected from one-month-old plants by sampling five replicate plants for each genotype. Plant tissues were washed with tap water and incubated in the drying oven at 80°C until tissues were crushable. Samples were weighed out to exactly 10 mg and

placed in a 1.5 mL microcentrifuge tube. A hole was poked in the center of the lid of each tube using a sterile syringe, and 200 µL of concentrated nitric acid (Optima, Fisher Scientific International Inc., Pittsburgh, PA, USA) was added for acid digestion. Sample tubes were placed in a heat block at 99°C for 30 min. After digestion, 800 µL of ultrapure metal-free water was added to each sample and mixed. To avoid ICP-OES machine clogging, sample tubes were spun down. Blanks of nitric acid were digested and diluted with metal-free water. Metal concentrations were determined by comparing intensities to a standard curve created from certified metal standards (Spex Certiprep, Metuchen, NJ, USA). Phosphorus concentration was used to normalize other metal concentrations because different plant tissues were not dried homogeneously, and dry weight could not be used for normalization (28).

Metal quantification was also performed in xylem sap samples collected from 3-month-old plants of the selected genotypes. Sap was obtained by cutting the stem of each plant 5 cm above the soil surface and allowing the xylem content to exude from the cut stem due to root pressure. Plants were watered the day before sampling to create less negative water potential in the soil and facilitate water uptake. Xylem sap from each plant was collected in 50 mL conical tubes for 12–24 h, filter-sterilized using a 0.22 µm membrane filter, and frozen at −20°C (10, 54). For mineral element composition analysis by ICP-OES, 200 µL of each sample was mixed with 800 µL of ultrapure metal-free water into a clear 1.5 mL microcentrifuge tube. Blanks of ultrapure metal-free water were analyzed in parallel.

Quantification of bacterial growth

To analyze the effect of Ca on pathogen multiplication, bacterial growth was measured in CPG and MM medium with or without supplementation with $CaCl_2$ at a final concentration of 1.0 mM. This value was selected to mirror the concentration of Ca observed in the xylem sap of the resistant genotype (Table S1).

Bacterial cells were scraped from TZC plates, suspended in phosphate buffered saline (PBS) and adjusted to an OD600 of 0.1. Sterile polystyrene 96-well plates (Deltalab SL, Barcelona, Spain) containing 190 µL per well of each medium condition were inoculated with 10 µL of cell suspension. Plates were incubated at 28°C with shaking and OD600 nm was measured every hour until 66 h and every 6 h until 78 h in CPG and MM, respectively, using a spectrophotometer (Infinite 200 PRO, Tecan). Experiments were repeated two times with 16 replicates for each condition.

Biofilm quantification in 96-well plates

To analyze the effect of Ca on biofilm formation, a microtiter plate assay was conducted using CPG broth supplemented with $CaCl_2$ (1.0 mM) as previously described (46). GMI1000 cell suspension was prepared as described above for growth curve experiments. Sterile polystyrene 96-well plates (Costar, Kennebunk, ME, USA) containing 190 µL per well of CPG or CPG supplemented with Ca were inoculated with 10 µL of cell suspension and incubated at 28°C without shaking. After 24 h, planktonic growth was quantified by transferring the total volume of the liquid media containing suspended cells of each well to a new plate and measuring the OD600 using a Cytation 3 Image Reader spectrophotometer (BioTek Instruments Inc., Winooski, USA). To quantify biofilm growth, the original 96-well plate was gently rinsed one time with Milli-Q water using a multichannel pipette, and the adhering cells were stained with 230 µL of 0.1% w/v crystal violet for 20 min, and then rinsed again with Milli-Q water. Finally, 230 µL of 95% ethanol was added to each well, and plates were incubated at room temperature on an orbital shaker at 150 rpm for 5 min to allow crystal violet dissolution. Biofilm growth was estimated by measuring OD590 using a Cytation 3 Image Reader spectrophotometer (BioTek Instruments Inc., Winooski, USA). Blanks of medium were included for each condition. Experiments were repeated three times with six replicates for each condition.

Assessment of biofilm structure by confocal laser scanning microscopy

The effect of Ca on biofilm structure was evaluated by confocal laser scanning fluorescence microscopy using the reporter strain GMI1000 Pps-GFP (49). Briefly, bacterial suspensions were prepared from overnight cultures in CPG and CPG supplemented with $CaCl_2$ 1.0 mM and adjusted to a concentration of 10^7 cfu/mL using the same medium. Then, 300 µL of suspensions were placed into each well of an 8-well chambered cover glass (Nunc Lab-Tek II, Thermo Scientific, Rochester, USA) and incubated at 28°C without shaking. Biofilm formation was visualized every day for 3 days with a confocal laser scanning microscope (TCS SP5, Leica), using a 60X oil immersion objective with 405 nm excitation wavelengths. Images were acquired with Leica LAS AF Lite software version 2.6 (Leica Microsystems, Wetzlar, Germany). A biomass quantification analysis was performed with Comstat 2 software (55) using an automatic threshold and a non-connected biomass filtering. Blanks of medium were included for each condition. Experiments were repeated three times with three replicates for each condition.

Bacterial growth and biofilm formation in microfluidic chambers

Pathogen growth was analyzed under flow conditions in microfluidic chambers (MCs) mimicking conditions inside the plant vascular system. MC design and fabrication were performed as previously described (56). Briefly, the MC consisted of a molded polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning, Midland, USA) body sandwiched between a cover glass and a supporting glass microscope slide. Molded PDMS fabrication involved photolithography and deep reactive-ion etching of a silicon wafer followed by replica molding of the wafer surface features with PDMS. MC used contained two parallel channels (80 µm wide by 3.7 cm long by 50 µm deep) each with two entries, one for media and other for bacterial inoculation, and an outlet to media and bacteria flow out. Both channels allowed analysis of two different conditions simultaneously. MC assays were performed in CPG, MM, and CPG and MM supplemented with 1.0 mM of $CaCl_2$ to analyze Ca effect under flow conditions. Firstly, channels were filled with media using an automated syringe pump (Pico Plus, Harvard Apparatus) and flow rate was maintained at 0.1 µL/min. Bacterial suspensions of strain GMI1000 were prepared in media, adjusted to an OD_{600} of 0.5 and injected in an MC to observe attached cells. When attached cells reached more than ~10 cells/100 μm^2, bacterial inlets were clamped, and the MC was monitored for 3–4 days. The MC was mounted onto an inverted microscope (Eclipse Ti-U, Nikon) and observed with a 40X objective using phase contrast and Nomarski differential interference contrast (DIC) optics. Bacterial growth and attachment over time were recorded using time-lapse video imaging microscopy. Images were acquired automatically every 30 s with a Nikon DS-Q1 digital camera (Nikon) controlled by NIS-Elements software version 3.0 (Nikon). Each condition was evaluated at least three times in independent experiments.

Assessment of twitching motility on agar plates

Bacterial suspensions were adjusted in PBS to a concentration of 10^5 cfu/mL and 10 µL were spotted in quadruplicate onto CPG and CPG supplemented with 1.0 mM of $CaCl_2$ agar plates. After 2 days of incubation at 28°C, colony peripheral fringe morphology was assessed using a graduated scale under a microscope (Eclipse E200, Nikon) (46, 57). Images were acquired with a Nikon DS-Q1 digital camera (Nikon) controlled by NIS-Elements software version 3.0 (Nikon). Each condition was replicated on four plates, for a total of 16 evaluations per condition. Experiments were repeated two times independently.

Plant inoculation assays

To analyze Ca effect on plant resistance, 1-month-old potato plants of the susceptible cultivar Chieftain and 09509.6 R grown in individual pots were watered with 40 mL of saline solution (SS) supplemented with $CaCl_2$ 20 mM every 3 days during the whole

experiment. A set of plants receiving non-supplemented SS served as control for Ca treatment. Plant watering was carried out by pouring solutions (SS or SS+$CaCl_2$) onto the soil near the stem. Plants were arranged in a completely randomized design and maintained at 24/20°C day/night with 65% relative humidity and a photoperiod of 16 h light: 8 h darkness in a growth chamber (BJPX-A500, Biobase).

Plant inoculation was performed 13 days after Ca or SS watering started. Bacterial suspension was prepared from overnight liquid cultures on CPG and spectrophotometrically adjusted to a concentration of 10^7 cfu/mL of SS. Plants were soil inoculated by drenching 40 mL of the bacterial suspensions into each pot to reach a final density of 10^6 cfu/g. Roots were wounded before inoculation as described previously (58). After bacterial inoculation, the temperature in the growth chamber was changed to 28/20°C day/night to stimulate disease development. Disease progression was visually recorded until 28 days after inoculation using an ordinal scale ranging from 0 (asymptomatic plant) to 4 (all leaves wilted) (59). Sets of non-inoculated plants for both watering treatments were also included in the assay. Experiments were performed using six plant replicates for each genotype (Chieftain and 09509.6 R) per treatment. Ca concentration in plant tissues was assessed by sampling one leaf of each plant 1 day before and 12 days after inoculation. Samples were processed as described above for ICP-OES analysis.

Statistical analyses

Data are represented as mean ± standard error of mean (SEM). Statistical analysis was carried out with unpaired Student's *t*-test and one-way analysis of variance (ANOVA). Multiple comparisons were performed using the Tukey's multiple range test. Model residuals were used to check for the assumptions of normality and homogeneity of variances. Data from replicate trials of experiments were combined when there were no significant effects among trials. All statistical analyses were done using Infostat (60).

RESULTS

Potato ionome profiles

In this study, the mineral profiles of plant tissues and xylem sap from susceptible and resistant potato plants were analyzed by ICP-OES. Ionome profiling was first performed in roots, stems, and leaves of susceptible (13001.79 S and cv. Chieftain) and resistant 09509.6 R potato genotypes. The most abundant mineral elements were K, Ca, Mg, and S, regardless of the tissue or genotype analyzed. In addition, Fe, Mn, Na, and Zn were detected in low concentrations and Cu was not detected (Table S1). Interestingly, comparison of ionome profiles of resistant and susceptible genotypes revealed a significantly higher Ca concentration for the resistant genotype in the stems ($P = 0.0007$) but not in other tissues (Fig. 1). The mineral content was also evaluated in xylem sap collected from the resistant and susceptible potato clones showing K, P, S, Mg, and Ca as the most abundant elements. Interestingly, in these samples, also significantly higher concentrations of Ca were consistently found in the xylem sap of the resistant compared to the susceptible genotype ($P = 0.011$) (Table S1). Taking into account these results, Ca was selected for further evaluation of its effect on pathogen virulence traits and plant resistance.

Effect of calcium on pathogen growth, biofilm formation and twitching motility

Growth curves in rich and minimal media supplemented with $CaCl_2$ were performed to evaluate the effect of this element on pathogen growth (Fig. 2). Ca concentration for media supplementation was set at 1.0 mM based on the concentration of this mineral element in the xylem sap of the resistant genotype (Table S1). In CPG medium, Ca supplementation did not produce any effect in bacterial growth and no differences were found in growth profile ($P > 0.05$) (Fig. 2A). However, in MM Ca supplementation decreased GMI1000 growth in comparison with the non-supplemented media (Fig. 2B).

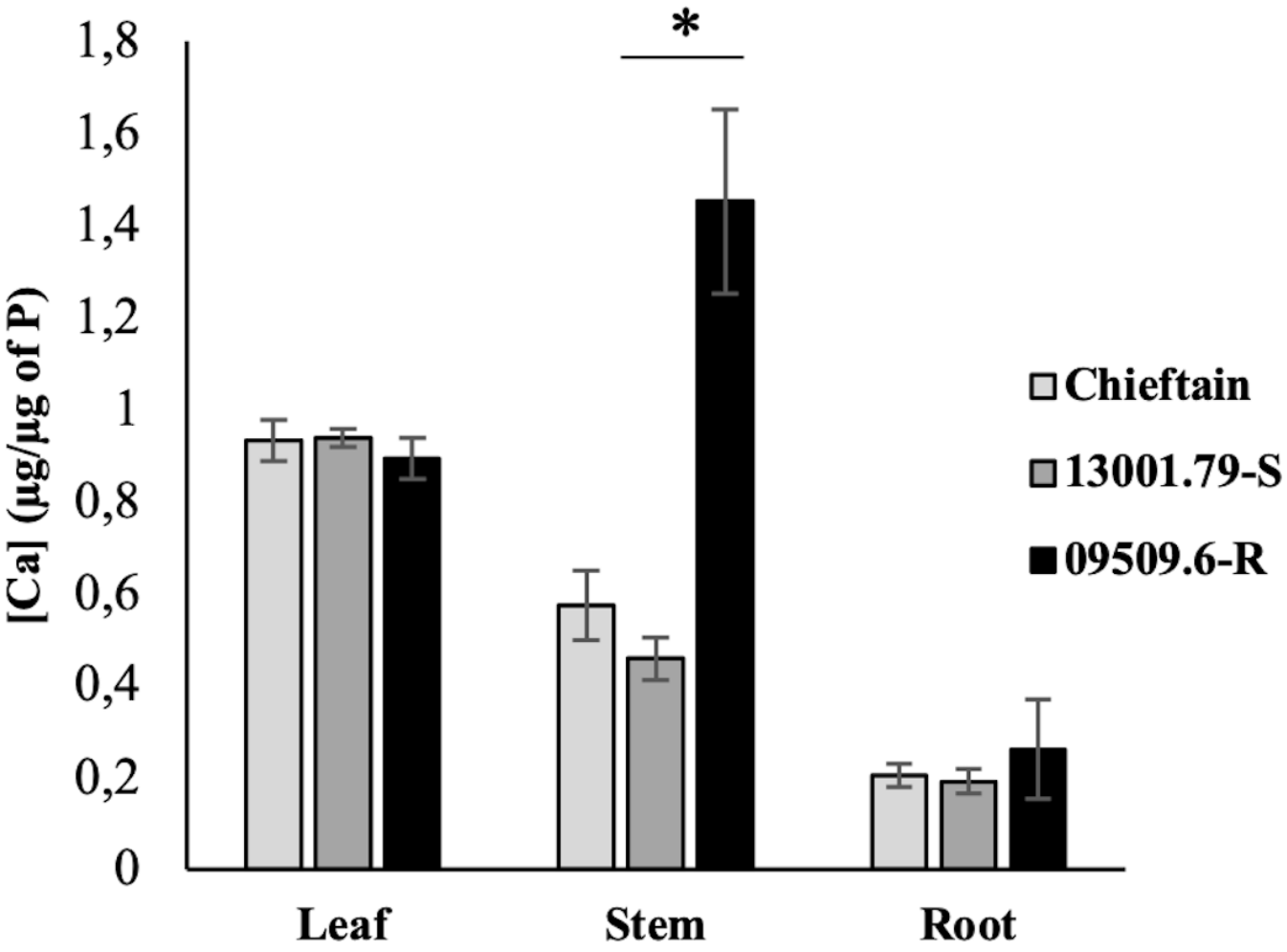


FIG 1 Calcium quantification in leaves, stems, and roots of potato plants. Interspecific potato breeding lines with different levels of bacterial wilt resistance including 13001.79 S and 09509.6 R genotypes and susceptible control (cv. Chieftain) were analyzed by ICP-OES. Each column represents calcium content ($n = 3$). * indicates significantly different concentrations according to Tukey's multiple comparison test ($P < 0.001$). Vertical bars represent standard errors of the means.

Significant differences were found between both conditions throughout the entire culture period (78 h) ($P < 0.0001$).

When Ca was added to CPG, a significant reduction in biofilm formation was observed in 96-well plate assays compared with non-supplemented medium ($P = 0.0003$) (Fig. 3). In contrast, no significant differences ($P > 0.05$) in planktonic growth were observed between both conditions (Fig. 3A). A biomass quantification of biofilms formed in both formulations supported these results. GMI1000 biofilms formed in CPG accumulated ~7 times more biomass than those formed in CPG supplemented with Ca ($P < 0.001$) (Fig. 3B through F).

The effect of Ca on cell twitching motility was determined by the colony fringe on agar plates. GMI1000 colonies grown on CPG supplemented with Ca (mean = 109 µm) had a 1.5-fold decrease fringe size compared with colonies grown on non-supplemented medium (mean = 171 µm) ($P < 0.001$) (Fig. 4A and B).

Growth of *R. pseudosolanacearum* under flow conditions

In this study, growth of *R. pseudosolanacearum* was evaluated for the first time by using MCs, mimicking pathogen growth conditions in the vascular system of the host plant. In this system, only attached cells to the MC channel can be observed. Cells flowing with the medium are not visible.

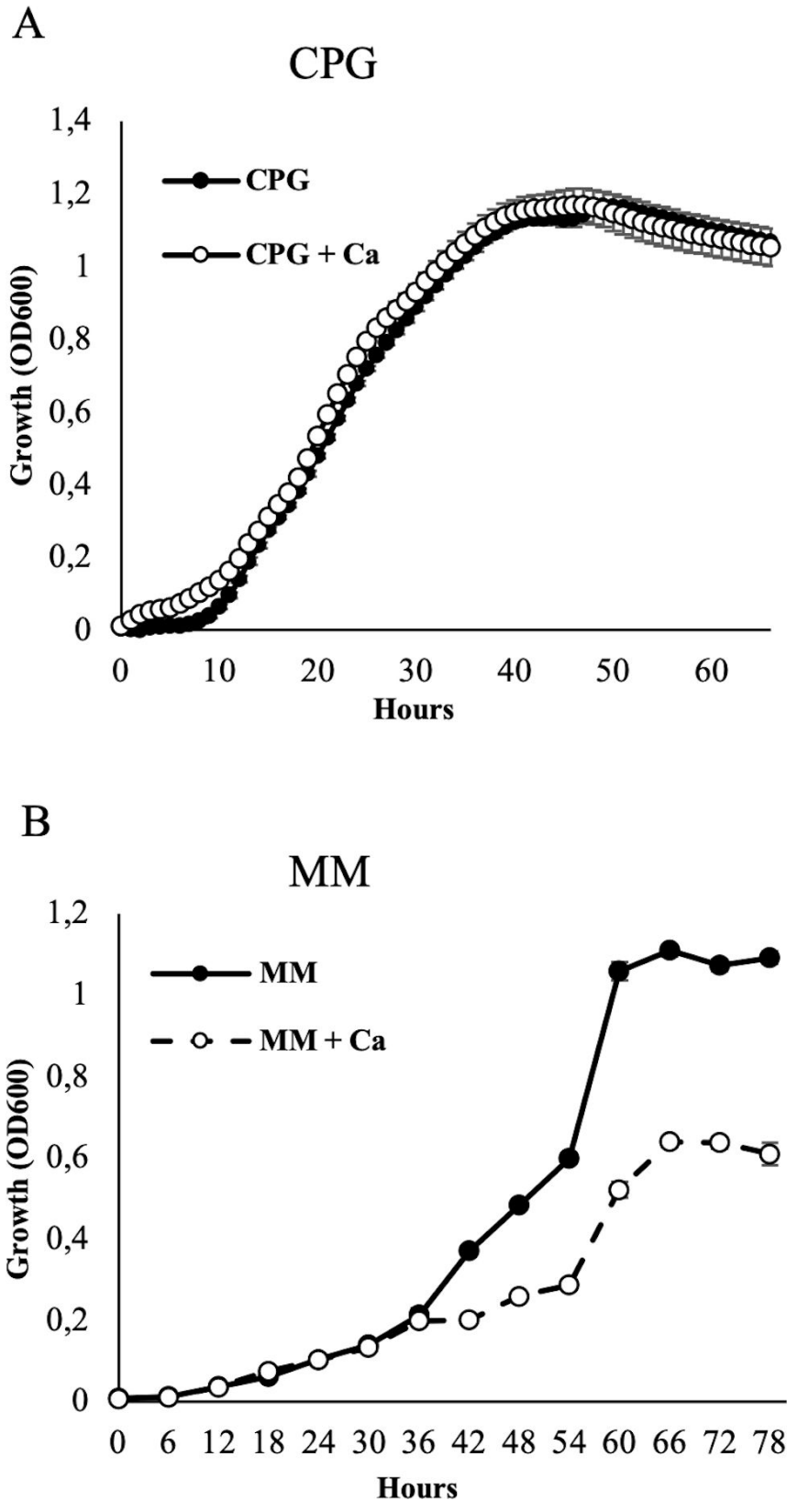


FIG 2 Growth curves of *R. pseudosolanacearum* in rich medium (CPG) (A) and MM (B) alone or supplemented with $CaCl_2$ 1.0 mM (CPG+Ca and MM+Ca, respectively). Growth curves were carried out in 96-well plates with shaking and bacterial concentration was estimated by measuring OD600 nm. Vertical bars represent standard errors of the means ($n = 16$).

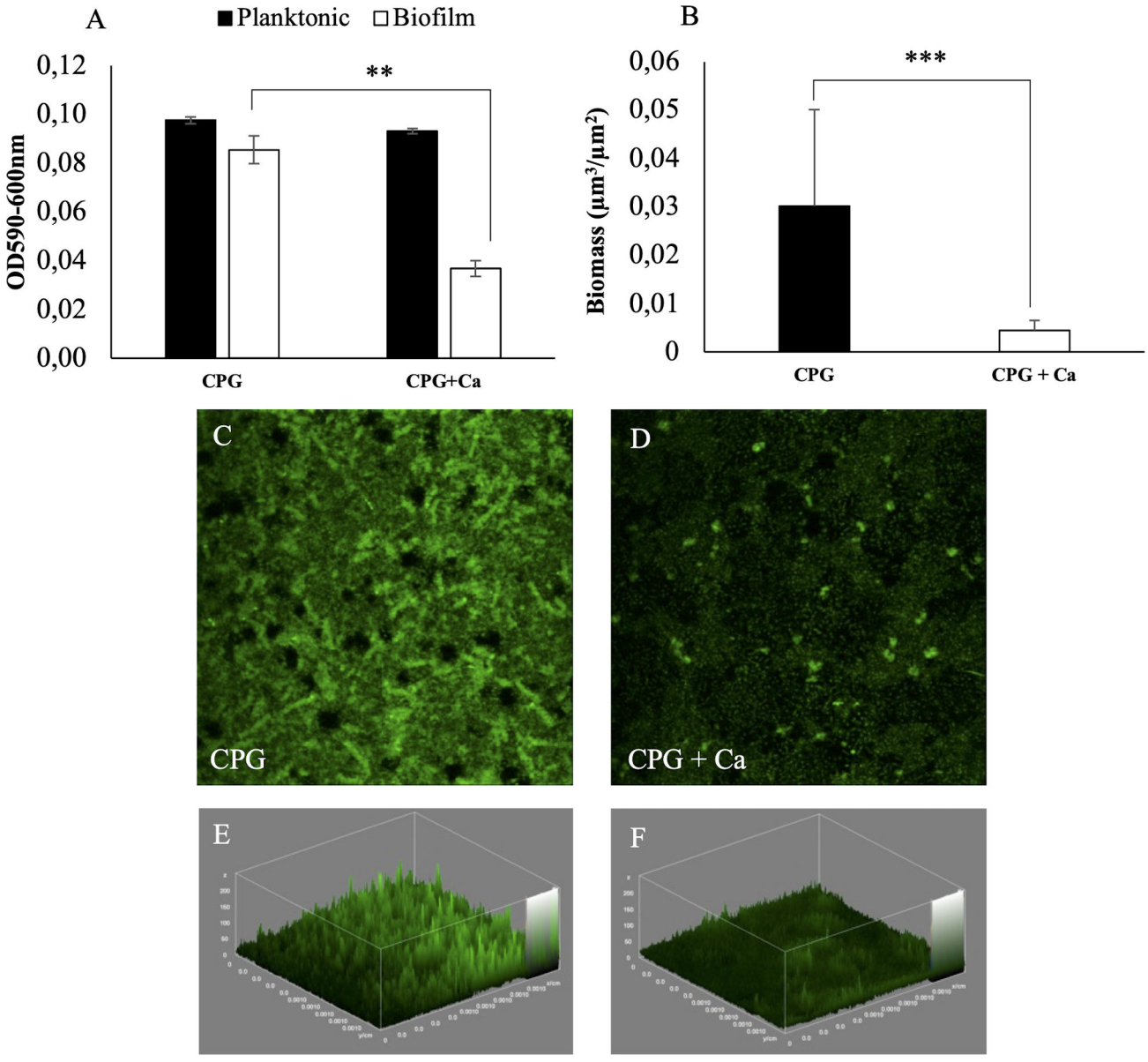


FIG 3 Calcium effect on biofilm formation of *R. pseudosolanacearum* in rich medium (CPG). (A) Biofilm formation assessed in 96-well plates with CPG and CPG supplemented with $CaCl_2$ 1.0 mM (CPG+Ca). ** represents significantly different according to ANOVA and Tukey's multiple comparison test (P = 0.003). Vertical bars represent standard errors of the means (n = 6). (B) Biofilm biomass quantification by confocal laser scanning microcopy (CLSM) three days after inoculation in CPG and CPG supplemented with $CaCl_2$ 1.0 mM (CPG+Ca). *** represents significant differences in biofilm biomass according to Student t-test comparison (P = 0.0001). Vertical bars represent standard errors of the means (n = 9). Two-dimensional (C and D) and three-dimensional (E and F) images of biofilm formed in CPG (C and E) or CPG supplemented with Ca (D and F).

Growth of GMI1000 strain in MC was analyzed first in rich (CPG) and minimal (MM) media without Ca supplementation. After 8 h of inoculation, a higher number of attached cells with smaller size and rounded morphology were observed in MM compared to rich medium. In CPG, a higher motility was observed, and cells were more elongated compared to MM. Cells attached in CPG increased over time and in MM remained in an equal concentration after 48 h (Fig. 5; Video S1).

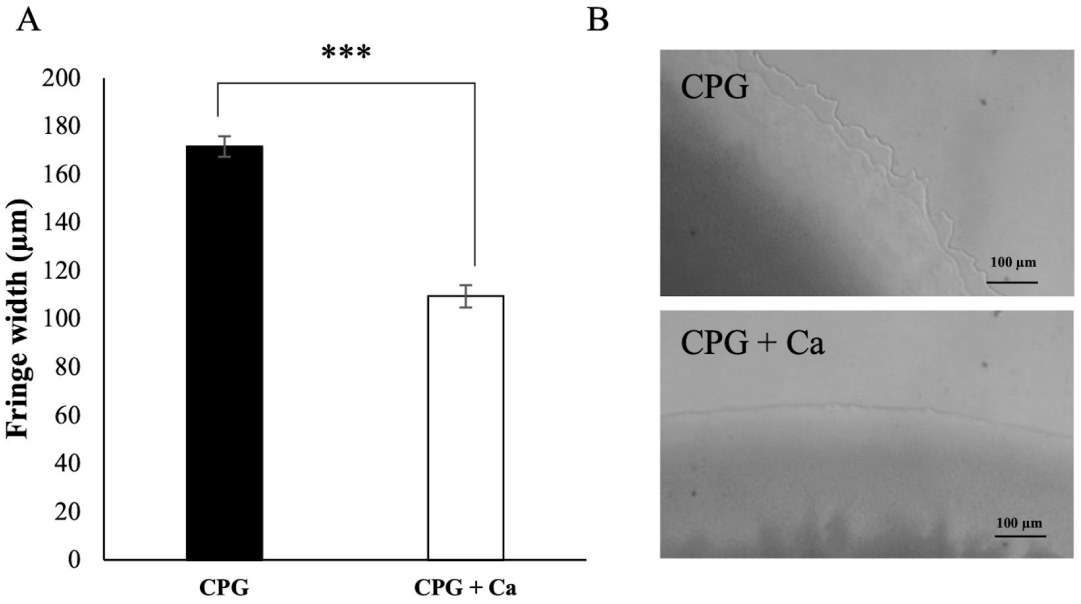


FIG 4 Calcium effect on *R. pseudosolanacearum* cell twitching motility. (A) Colony fringe width of *R. pseudosolanacearum* cultured on CPG and CPG supplemented with $CaCl_2$ 1.0 mM (CPG+Ca). *** represents significant differences in colony fringe width according to Student *t*-test comparison ($P < 0.0001$). Vertical bars represent standard errors of the means ($n = 16$). (B) Representative micrographs of colony fringes of *R. pseudosolanacearum* cultured on agar plates with treatments mentioned in A.

Effect of calcium on pathogen growth and biofilm formation under flow conditions

Ca effect was evaluated under flow conditions by comparing GMI1000 growth in CPG and CPG supplemented with Ca. In non-supplemented CPG medium, bacteria grew faster than in CPG supplemented with Ca, and the formation of cell masses adhered to walls of the chamber was observed only in the first condition (Fig. 6A). Biofilm

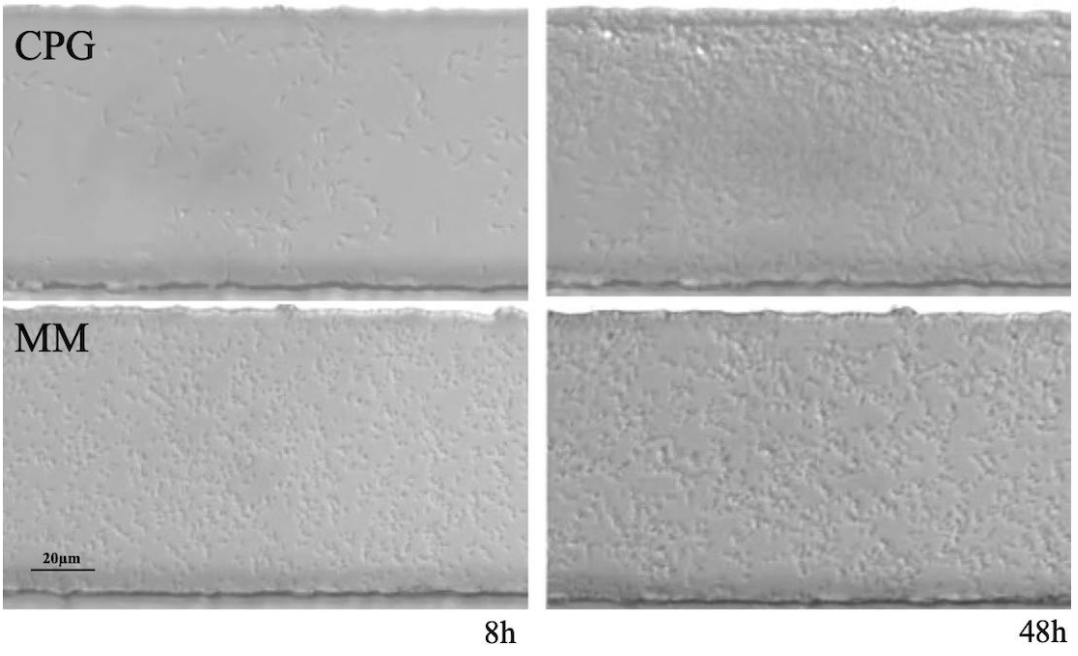


FIG 5 *R. pseudosolanacearum* growth in CPG and MM under flow conditions in MCs. Image was captured 8 and 48 h after inoculation. Scale bar is shown at the bottom left of the figure.

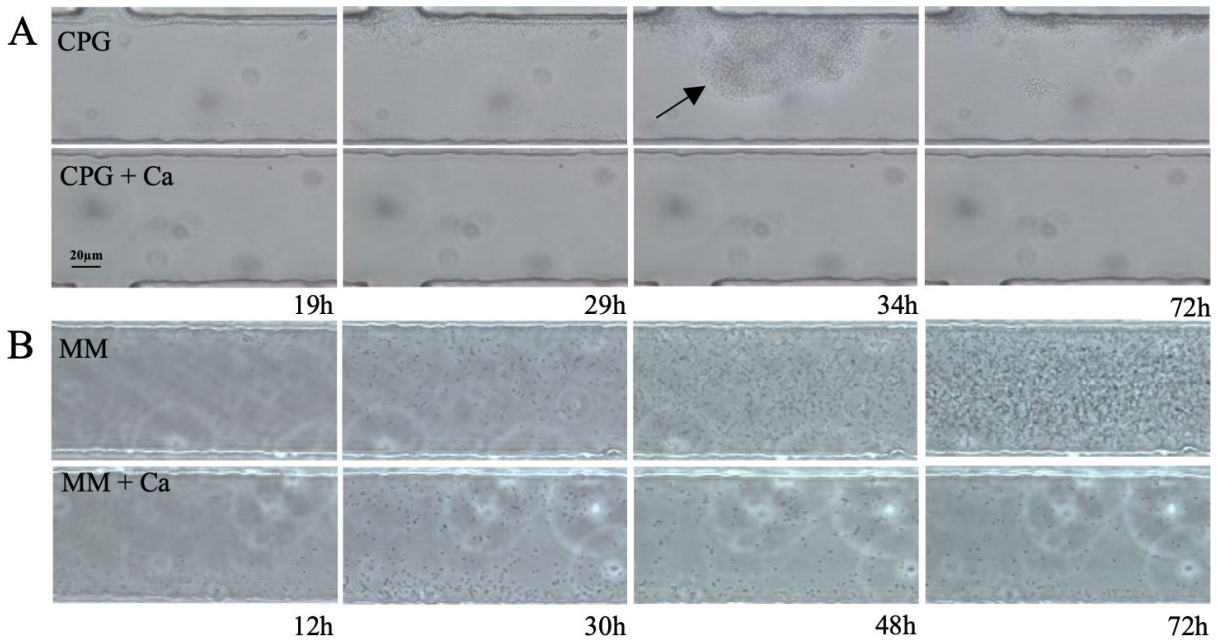


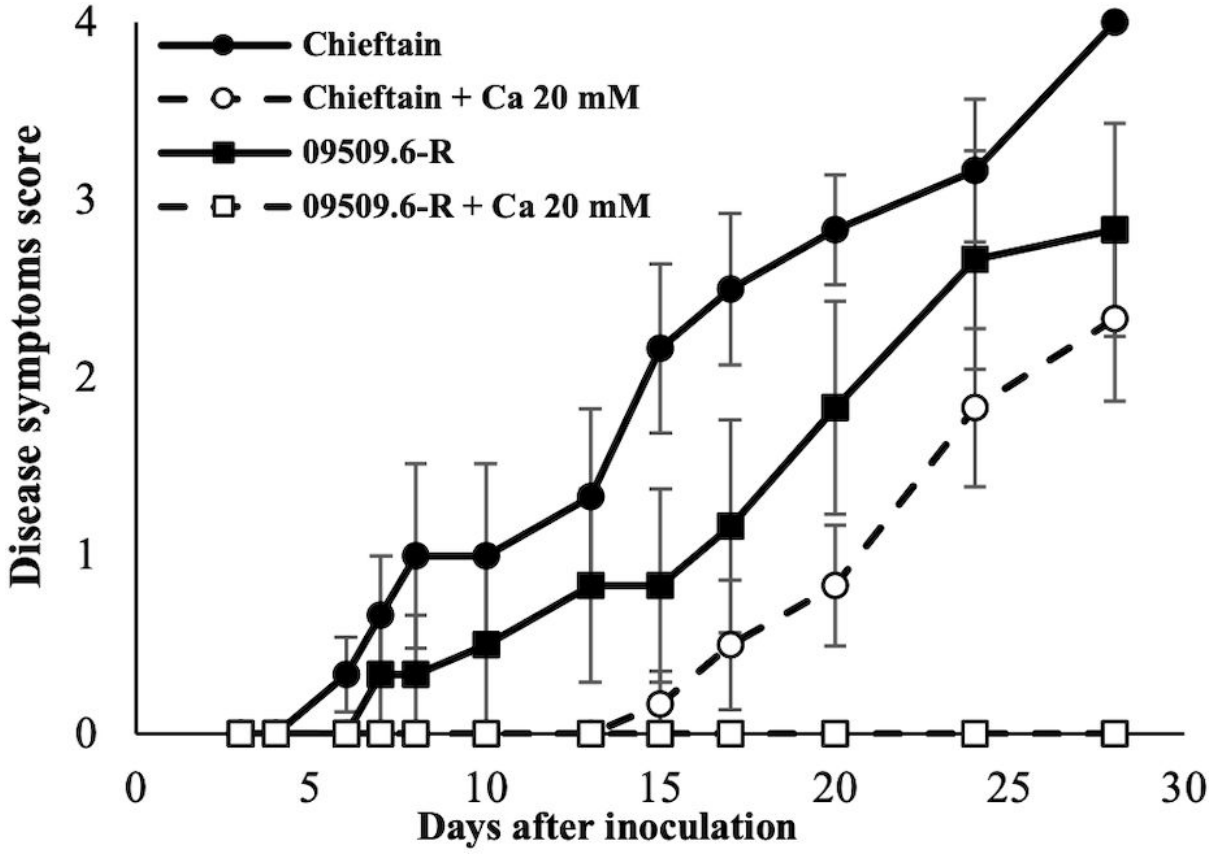
FIG 6 Calcium effect on *R. pseudosolanacearum* growth under flow conditions in MCs. (A) *R. pseudosolanacearum* growth in CPG and CPG supplemented with $CaCl_2$ 1.0 mM (CPG+Ca). Images were captured 19, 29, and 34 h after inoculation. Dark arrow shows biofilm formation. (B) *R. pseudosolanacearum* growth in MM and MM supplemented with $CaCl_2$ 1.0 mM (MM+Ca). Images were captured 12, 30, and 72 h after inoculation.

formation was observed around 30 h after assessment started and was followed by biofilm detachment after 35–72 h. Biofilm structures in this condition were not strongly attached, and MC flow could detach easily new cell aggregates. Cells in MM were observed more strongly attached but grew slower over time compared to CPG medium (Fig. 6B; Video S2). An increase of attached cells in non-supplemented MM, 48 h after assessment started, was observed; however, no biofilm formation was observed in this condition. Attached cells were observed along the channel, in MM supplemented with Ca, but in lower concentration, compared to the non-supplemented condition, 48 h after assessment started.

Calcium amendment effect on plant resistance

To analyze Ca effect on plant resistance, plants watered with SS or SS supplemented with $CaCl_2$, were inoculated with *R. pseudosolanacearum* and disease progress was compared. Calcium treatment showed a drastic enhancement on bacterial wilt resistance in clone 09509.6 R, with all plants remaining asymptomatic 28 days after inoculation. In the susceptible control (cv. Chieftain), a delay in symptoms appearance and a significant reduction in disease progress was also observed (Fig. 7).

Quantification of Ca in plant leaves prior and after inoculation was performed to evaluate Ca leaf accumulation changes depending on pathogen infection and plant resistance (Fig. 8). In plants of susceptible genotype that received Ca supplementation, bacterial infection induced an increase in Ca accumulation (35%) (P = 0.02) (Fig. 8A, Chieftain SS+Ca). Interestingly, in plants of resistant genotype that did not receive Ca supplementation, bacterial infection induced a bigger increase in Ca accumulation (57%) (P = 0.002) (Fig. 8A, 09509.6 R SS). No significant differences were found in Ca accumulation after inoculation in plants of cv. Chieftain watered with SS and resistant genotype watered with SS+Ca (Fig. 8A). Percentage changes of Ca concentrations were evaluated considering plant resistance, 09509.6 R relative to cv. Chieftain with the same treatment (SS or SS+Ca) in healthy and infected plants (Fig. 8B). In non-inoculated plants, the resistant clone 09509.6 R showed higher Ca accumulation levels compared to the susceptible cv. Chieftain (65% and 47% in plants treated with SS or SS+Ca respectively)



Genotype	Treatment	AUDPC
Chieftain	SS	49.67 C
Chieftain	SS + Ca	16.5 AB
09509.6-R	SS	31.5 BC
09509.6-R	SS + Ca	0 A

FIG 7 Bacterial wilt progress curves after soil inoculation with *R. pseudosolanacearum* in plants treated with SS and SS supplemented with $CaCl_2$ 20 mM (SS+Ca). Vertical bars represent standard errors of the means (n = 6). Each data point represents the average wilting rating using a scale ranging from 0 (asymptomatic plant) to 4 (all leaves wilted). Values of area under the disease progress curve (AUDPC) for the average wilting rating followed by the same letter were non-significantly different. Significant effects involving genotypes and treatments were found using ANOVA and Tukey's multiple comparison test (P = 0.0003).

(P = 0.001 and P = 0.006 respectively). The highest Ca accumulation (107%) was observed for infected plants of the resistant clone 09509.6 R in comparison with cv. Chieftain (P = 0.0009) treated with SS. Resistant genotype also showed higher Ca accumulation levels compared with cv. Chieftain treated with SS+Ca (53%) (P = 0.02) in infected plants.

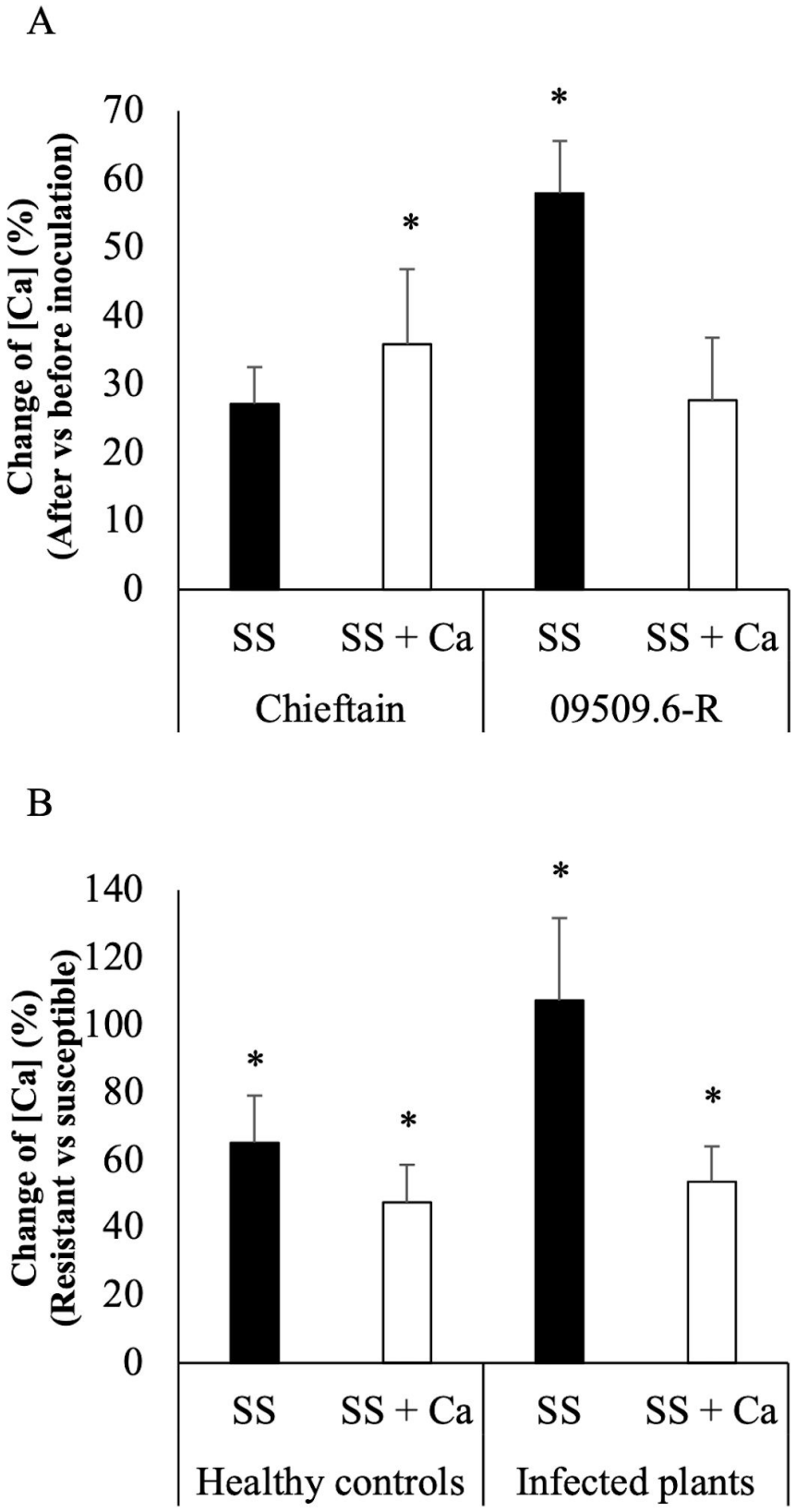


FIG 8 Relative changes of calcium concentration in leaves of 09509.6 R and susceptible control (cv. Chieftain) genotypes. Plants were treated with SS and SS supplemented with $CaCl_2$ 20 mM (SS+Ca). After 13 days of treatment, plants were inoculated with *R. pseudosolanacearum.* Calcium concentration was

(Continued on next page)

FIG 8 (Continued)

measured in leaves sampled one day before and 12 days after inoculation by ICP-OES. (A) Percentage changes of Ca concentrations of infected plants relative to healthy plants. * represents significant differences in Ca concentration according to Student *t*-test comparison ($P < 0.05$). (B) Percentage changes of Ca concentrations of resistant genotype relative to susceptible genotype. * represents significant differences in Ca concentration according to Student *t*-test comparison ($P < 0.05$). Each column represents the average of percentage of change of calcium concentration and vertical bars represent standard errors of the means ($n = 6$).

DISCUSSION

The present study advances our understanding of the relationship between potato plant ionome and resistance to bacterial wilt caused by *R. pseudosolanacearum*. The focus on the role of calcium (Ca) is especially enlightening, given the pervasive, yet poorly understood, role of minerals in plant-microbe interactions (61). We carried out different *in vitro* experiments that clearly showed that Ca hinders the growth, biofilm development, and twitching motility of the reference RSSC bacterial strain, *R. pseudosolanacearum* GMI1000. The effect of Ca on plant response to bacterial wilt was also verified through greenhouse experiments on potato plants.

In this work, the ionome of a commercial susceptible cultivar Chieftain and two advanced clones from the potato breeding program were analyzed; 13001.79 S and 09509.6 R (19). We found that Ca concentration of 09509.6 R was higher in xylem sap and stems in comparison with susceptible genotypes. Strains belonging to the RSSC typically grow in the sap inside xylem vessels to high densities (9), and this led us to hypothesize that resistance to this pathogen could be associated with a higher concentration of Ca in xylem sap and stem.

In contrast to other macronutrients, a high proportion of the total Ca in plant tissues is located in the apoplast. The main known functions of Ca in the apoplast are to maintain the integrity of the plasmalemma and to stabilize pectins, improving plant cell wall integrity (38). Moreover, Ca signaling is essential in pathogen associated molecular pattern (PAMP)-triggered immunity and effector-triggered immunity responses. Ca-dependent protein kinases phosphorylate different substrates to regulate various plant immune responses, including ROS production, transcriptional reprogramming of immunity genes and hypersensitive response (27, 62–64). Furthermore, many studies have demonstrated that the increase of Ca content in plant tissues decreases certain potato diseases, including blackleg and soft rot caused by *Pectobacterium* spp. and *Dickea* spp. (43, 65, 66). and late blight caused by *Phytophthora infestans* (67). Conversely, infection with *X. fastidiosa*, a xylem-limited pathogen, was shown to increase the concentration of Ca in the xylem and leaves (21, 22, 45). Moreover, Ca increases biofilm formation and twitching movement of this pathogen (46, 68), suggesting that in this pathosystem Ca in the xylem is increasing infection level, instead of protecting the host like in the above-mentioned examples.

A drastic reduction in bacterial proliferation was observed when growth was assessed in an MM supplemented with Ca compared to the same medium without supplementation. Our results suggest that Ca concentrations, comparable to those present in xylem sap and stems of the resistant genotype (~1.0 mM), inhibit pathogen growth compared to environments with lower Ca levels. This finding is interesting since MM is a nutrient-limited medium designed to simulate plant apoplast conditions, being generally used as an approximation to determine bacterial behavior inside the host (69). In contrast, no effect of Ca on pathogen growth in rich medium (CPG) was observed. This suggests that other nutrients in the rich media might mitigate the effect of Ca, potentially through ionic competition or through direct use in metabolic pathways that circumvent the Ca-mediated restrictions.

This work also explored additional dimensions to understand the role of Ca in regulating the pathogen behavior of *R. pseudosolanacearum*. Notably, the data reveal a significant impact of Ca on key attributes of virulence, such as biofilm formation and

twitching motility. Elevated levels of Ca appear to modulate these characteristics, which are essential for the pathogen's ability to infect and damage host tissues effectively (2, 70). These observations suggest that Ca serves as a crucial environmental cue for this pathogen, affecting not just growth but also influencing its virulence mechanisms, in agreement with previous studies (44, 57).

The use of MCs is a noteworthy methodological advancement, particularly for vascular pathogens as RSSC that are able to survive and multiply within the xylem vessels in continuous flow conditions (21, 71). In this environment, continuous sap flow may affect the direction of bacterial motility, change the shape of biofilms, increase nutrients concentration and decrease waste products and quorum sensing signals (9). Since this is the first time that MCs were used to study *R. pseudosolanacearum* growth under flow conditions, we first compared the behavior of strain GMI1000 in rich (CPG) and MM before evaluating the effect of Ca supplementation. Notably, distinct differences in cell morphology, motility, and attachment were observed in both conditions. In MM, cells had a rounded and smaller morphology with better attachment, resembling the behavior of a previously studied *phcA* mutant known for maintaining a low cell density condition (6, 52). These observations suggest that in nutrient-poor environments, the bacteria adapt for survival by assuming a low cell density condition, marked by a rounded morphology and greater attachment capabilities. Conversely, in rich medium, the bacteria exhibit elongated morphology and better motility, similar to conditions associated with high cell density (52). Interestingly, Ca supplementation under flow conditions reduced bacterial attachment and proliferation in both rich medium and MM. This underscores the significance of Ca in influencing pathogen behavior also under flow conditions as well as under batch conditions.

Our study highlights the role of Ca in enhancing resistance to bacterial wilt in potato plants. Ca treatment in our experiments led to a significant delay and reduction in disease progression in susceptible plants (cv. Chieftain) and a complete absence of symptoms in the partially resistant genotype (09509.6 R). Furthermore, we observed distinct genotype-specific responses in Ca accumulation, both before and following pathogen inoculation. In particular, the resistant genotype inherently accumulated higher levels of Ca, irrespective of Ca treatment, suggesting an innate capacity for enhanced Ca uptake that may contribute to disease resistance. These findings align with previous reports on other Solanaceous crops such as tomatoes and tobacco (44, 57, 72–77). These studies have consistently shown that Ca nutrition can markedly reduce the severity of bacterial infections. This is particularly relevant given the role of Ca in modulating various physiological functions within plants. Plants possess Ca sensors that play crucial roles in early responses to pathogens and other physiological functions such as cell division, nutrient uptake, and stress responses (78).

In conclusion, this research marks a significant step in understanding the complex relationship between the potato plant ionome and bacterial wilt resistance. The study convincingly argues for the role of Ca as both an inhibitor of bacterial growth and virulence and an enhancer of plant resistance, thereby providing a dual pathway for its inclusion in integrated disease management strategies. Moreover, the findings pave the way for future lines of research, from the molecular mechanisms by which Ca exerts these modulatory effects to the practical aspects of Ca amendments in agricultural settings. The hope is that such integrated perspectives will lead to more sustainable and effective ways to combat this devastating disease.

ACKNOWLEDGMENTS

L.D.L.F. acknowledges funding from the Alabama Agricultural Experiment Station (AAES) Hatch Program. M.V.F. and M.I.S. acknowledge funding from the Basics Sciences Development Program (PEDECIBA) from Uruguay. M.V.F. received a Ph.D. scholarship from the Academic Postgraduate Committee (CAP) from Universidad de la República (UdelaR).

We thank technical staff from the Biotechnology Laboratory in INIA (Uruguay) for assistance with *in vitro* plant propagation.

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FUNDING

Funder	Grant(s)	Author(s)
Programa de Desarrollo de las Ciencias Básicas (PEDECIBA)	N/A	María Inés Siri
Academic Postgraduate Committee (CAP)	PhD Scholarship	María Virginia Ferreira
AU | Alabama Agricultural Experiment Station (AAES)	N/A	Leonardo De La Fuente

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (AEM00242-24-s0001.pdf). Table S1; legends for Videos S1 and S2.
Video S1 (AEM00242-24-s0002.mov). *Ralstonia pseudosolanacearum* growth in CPG and MM under flow conditions in microfluidic chambers. The video corresponds to the experiment shown in Fig. 5 and encompasses a time lapse of 8 hours starting from inoculation, at a 5X speed.
Video S2 (AEM00242-24-s0003.mov). Calcium effect on *Ralstonia pseudosolanacearum* growth under flow conditions in microfluidic chambers.

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