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REVIEW

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# Biocontrol and molecular characterization of *Bacillus velezensis* D against tobacco bacterial wilt

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## Abstract

Natural rhizosphere bacteria has the potential to act as an alternative of chemical pesticides for sustainable agriculture. In the current study, tobacco rhizosphere *Bacillus velezensis* D exhibited great antibacterial effect against *Ralstonia solanacearum*, and significantly enhanced the tobacco resistance against bacterial wilt in pot experiments. Then *Bacillus velezensis* D was labeled with *gfp* marker and found to stably colonize in tobacco root, the colonization density of strain D in root still remained $5.33 \times 10^4$ CFU/gat 30 days post-inoculation. Subsequently, field trials for two years (2021–2022) showed the control effects of the strain D on the tobacco bacterial wilt were 12.26% and 36.37%, respectively, indicating the application of *B. velezensis* D could improve plant resistance to *R.solanacearum*. In order to further study the antibacterial activities of strain D, effects of the crude extracts on the swimming ability, cell viability and the morphology of *R. solanacearum* were analyzed. The results showed that the crude extracts reduced the motility of *R. solanacearum*, and caused cell wall rupture and cell death. Furthermore, MALDI-TOF-MS and HPLC-QTOF-MS analysis indicated that lipopeptides (fengycin and iturin) and polyketides (bacillaene) were detected in the crude extracts of strain D. Based on these findings, we speculated that *Bacillus velezensis* D firstly colonized in tobacco root, then produced antibacterial substances at ecological sites to exert antagonistic effects, inhibiting motility traits of *R. solanacearum* and damaging the cell well. Hence, *Bacillus velezensis* D could be used as a potential biological control agents against tobacco bacterial wilt.

**Keywords** *Ralstonia solanacearum*, *Bacillus velezensis* D, Lipopeptides, Antibacterial activity, Swimming motility

## Background

Tobacco bacterial wilt, a major devastating disease, is caused by *Ralstonia solanacearum* and lead to serious economic losses worldwide (Peeters et al. 2013). Studies have shown that during the infection process, *R. solanacearum* could produce multiple virulence factors such as extracellular polysaccharides (EPS) and cell wall degrading enzymes (CWDE), which eventually lead to typical wilting symptoms in host plants (Milling et al. 2011; Genin and Denny 2012). In addition, other factors like motility and biofilm formation ability were also essential for the virulence by influencing the

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colonization and infection behavior of *R. solanacearum* (Yao and Allen 2007).

At present, the bacterial wilt was mainly controlled by chemical method. However, continuous application of chemical agents is easy to cause the generation of fungicide-resistant pathogens and environmental pollution. In recent years, using the effective biocontrol strains has become the hotspot and proved to be a promising strategy for the management of plant diseases (Spadaro and Gullino 2004). Plant growth-promoting rhizobacteria (PGPR) has been widely studied due to its potential to exert plant growth promotion and enhance its tolerance against biotic and abiotic stresses, and are found in the rhizosphere, root surface as well as inside the root tissues. Various biocontrol agents developed from PGPR, such as *Bacillus* spp., *Pseudomonas* spp., *Streptomyces* spp., and *Paenibacillus* spp., played the important roles in managing the *Ralstonia solanacearum* (Ramesh et al. 2009; Xue et al. 2009; Gao et al. 2013; Abo-Elyousr et al. 2019). For example, the *B. cereus* BC1AW and *P. putida* PP3WT strains, obtained from the rhizosphere of healthy plants, were able to reduce the severity of bacterial wilt in tomato genotypes King Kong 2, and increase the shoot dry weight of plants as well (Kurabachew and Wydra 2013). And the volatile organic compounds (VOCs) produced by *P. fluorescens* WR-1 showed an inhibitory effect against the tomato wilt pathogen *R. solanacearum* (Raza et al. 2016a, b). Among them, *Bacillus* species has attracted more attention due to its spores with strong stress resistance, which is beneficial for the biocontrol stability of the *Bacillus* sp (Chowdhury et al. 2015; Fan et al. 2018; Ye et al. 2020).

*Bacillus* spp., typical plant-growth promoting rhizobacteria, were reported to effectively enhance plant growth and control soil-borne diseases by producing antimicrobial substances and activating an induced systemic resistance (Erlacher et al. 2014; Chowdhury et al. 2015; Fan et al. 2018; Boka et al. 2019). For instance, *B. velezensis* SQR9, isolated from the rhizosphere of cucumber, was able to induce plant systemic resistance by producing antibiotics (Wu et al. 2018). *Bacillus velezensis* GUMT319, isolated from healthy tobacco rhizosphere, has high biocontrol effects on tobacco black shank through producing enzymes with biocontrol activity and successfully colonizing tobacco roots (Yu et al. 2015; Ding et al. 2021). At present, most *Bacillus* species have been developed into commercial products,such as the microbial pesticide 'Baikang' by the *Bacillus subtilis* B908, which was jointly developed by Yunnan Agricultural University and China Agricultural University, and control the rice sheath blight, tobacco black shank and so on (Zhang et al. 2006). The *Bacillus velezensis* FZB42 has been registered and industrialized by the company ABiTEP GmbH of Germany (Chen et al. 2007).

Recently, biocontrol is widely used for prevention and control the tobacco bacterial wilt by using beneficial mircobes, like bacteria, fungi and bacteriophages. And the antibacterial mechanisms of these beneficial microbes were mainly including secretion of antibacterial substances, competition with bacteria for niche and nutrients, and induced resistance. In this study, the *Bacillus velezensis* D was isolated from the rhizosphere soil of healthy tobacco in the Tongzi tobacco field with a high incidence of tobacco bacterial wilt, in the Gui zhou Province of China, and exhibited a strong antibacterial effect on *R. solanacearum*. However, it was unclear how the *Bacillus velezensis* D affect the growth and infection characteristics of *R. solanacearum*. In order to clarify the biocontrol mechanism, its ability to colonize tobacco, biocontrol efficacy on tobacco bacterial wilt in pot and field experiments as well as the effects on the growth and infection characteristics of *R. solanacearum* were investigated. This work provides a theoretical basis for the use of D strain as a replacement for bactericide and supplements.

## Results

### Antibacterial activity of rhizosphere bacteria against *R. solanacearum*

A total of 36 rhizosphere bacteria were isolated and obtained from fifteen rhizosphere soil samples. Preliminary screening showed that eight strains displayed antagonistic activities against *R. solanacearum* (Fig. 1a). Among them, the positive control *Bacillus velezensis* FZB42 produced the largest zone of inhibition (20.40 mm), followed by the D (20.00 mm), which was not significantly different from that of the FZB42. While OR1-39 inhibited the pathogen at a lower level (7.00 mm) (Fig. 1b). The above results indicated that the strain D exhibited better antibacterial effect against *R. solanacearum*.

To identify strain D, the 16S rDNA and *gyrB* genes of D were amplified and sequenced. Phylogenetic tree analysis based on 16S rDNA indicated that strain D was a member of *Bacillus* sp (Fig. 1c). And the *gyr*B sequences amplified from strain D (GenBank Accession ID: OP785086) had a match to the reference strain *Bacillus velezensis* Y3 (GenBank Accession ID: MW879356) (Fig. 1d). Thus, taking into account the 16S rDNA and *gyr*B gene sequences, strain D was identified as a *Bacillus velezensis* strain.

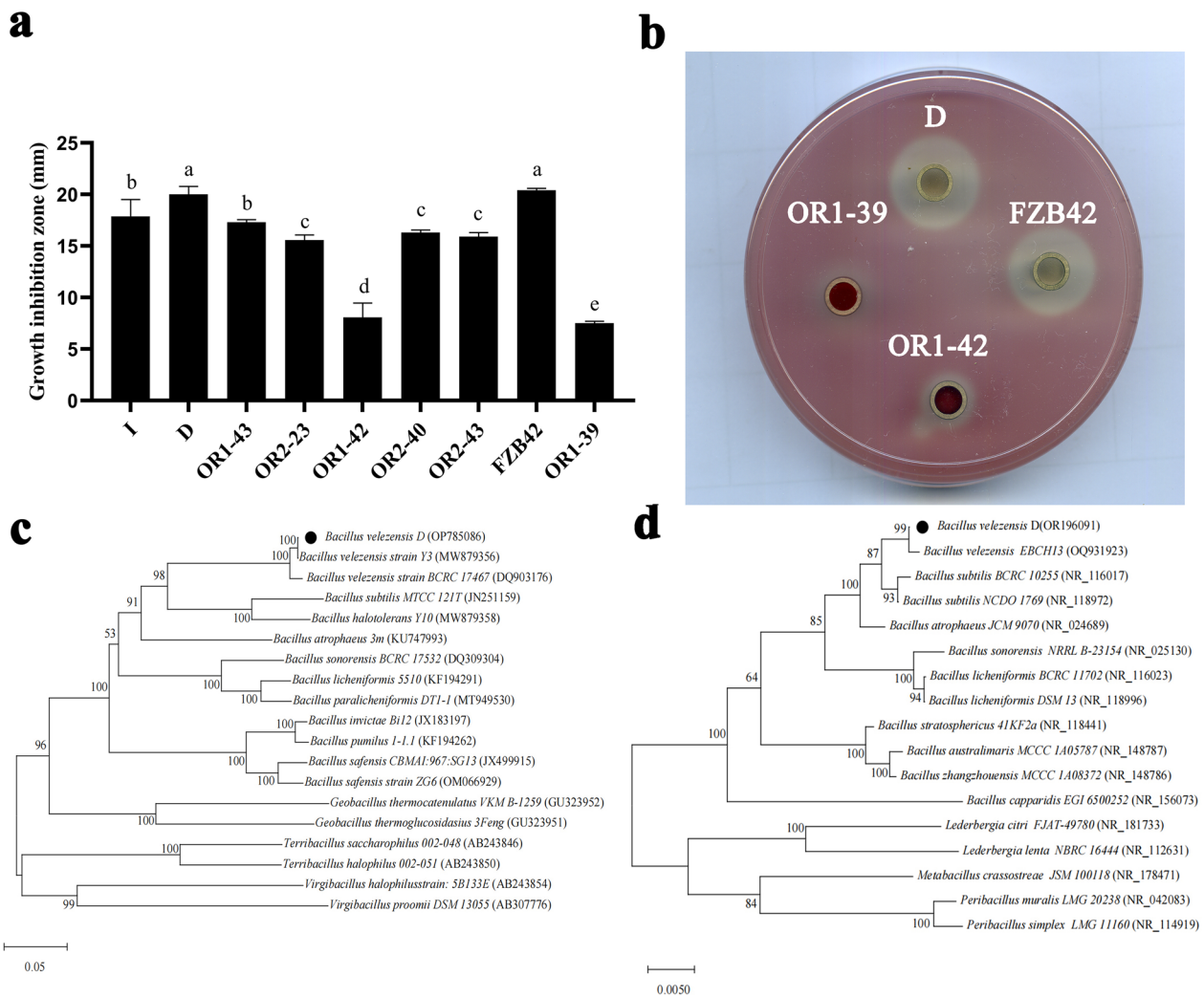


**Fig. 1** *Bacillus velezensis* D exhibited the best antibacterial activity against *R. solanacearum*. **a, b** Inhibition zone of rhizosphere bacteria against *R. solanacearum*. Antagonistic activities of rhizobacteria strains against *R. solanacearum* were analyzed through oxford cup method. 5 mL *R. solanacearum* suspensions ($1 \times 10^8$ CFU/mL) were mixed with 1 L TTC medium and poured in plates. Sterile oxford cups (0.6 cm) impregnated with 150 µL rhizosphere bacterial suspensions ($1 \times 10^8$ CFU/mL) were placed on the surface of solid medium. Finally, the diameters of inhibition zones were measured after incubating at 30℃ for 48 h. Sterile LB medium were used as control. Phylogenetic tree based on the 16S rDNA **(c)** and *gyrB* **(d)** sequences of *B. velezensis* D with Mega 7.0 software using neighbor-joining method. Data are presented as the mean ± SD based on three biological replicates, P-values were determined by LSD post-hoc test. Different letters represent significant difference

### Colonization analyses of *Bacillus velezensis* D in tobacco and rhizosphere soil

To further investigate the colonization of *B. velezensis* D in tobacco, we monitored the populations of strain D colonized on the root, stem, and leaf of tobacco. After 14 days post-inoculation, green fluorescent protein (GFP)-labeled *B. velezensis* strain D were investigated using confocal laser scanning microscopy. The strain D mainly colonized in clusters or singlets of the root elongation zone (Fig. 2a), and relatively fewer GFP-labeled D cells were detected in stem and leaf tissues. And this was further confirmed by quantitative measurement of bacterial population. The results showed that the D cell counts in the tobacco roots peaked at approximately $8.27 \times 10^4$ CFU/g at 20th day post-irrigation, and it remained at a range of $5.33 \times 10^4$ CFU/g at 30th day (Fig. 2b). Whereas, the D cell counts in the stems and leaves were relatively less than that of roots, the population density of the strain D in tobacco stems and leaves was only $2.10 \times 10^4$ CFU/g and $8.00 \times 10^2$ CFU/g until 30 days post-inoculation (Additional file 1: Figure S1).

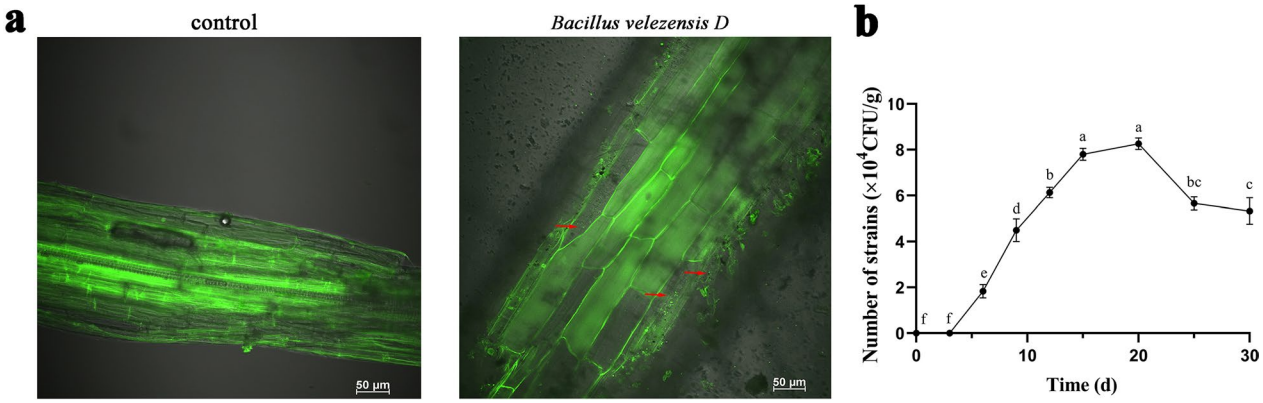


**Fig. 2** Colonization analyses of strain D in tobacco roots. **a** Visualization of D colonizing tobacco plants at 14th day after inoculation under laser confocal microscopy. The GFP-labeled D cell suspensions ($1 \times 10^8$ CFU/mL) were added to the roots of 55-day-old tobacco (50 mL per plant). Sterile water was used as negative control. After inoculation for 14 days, the roots were collected and cut into 1 cm in length. Finally, the strain D colonized in tobacco roots were observed with the laser confocal microscopy. The D in tobacco roots was indicated by red arrow; scale bars represent 50 μm. **b** The population density of the D-*gfp* in tobacco roots. After inoculation for 0, 3, 6, 9, 12, 15, 20, 25, and 30 days, the roots were collected and homogenized in sterile water, and colony numbers were counted with dilution coated plate method. Data are presented as the mean ± SD based on three biological replicates; *P*-values were determined by LSD post-hoc test

## Biological control of *Bacillus velezensis* D against tobacco bacterial wilt

### *Evaluation of Bacillus velezensis D against tobacco bacterial wilt in pot experiments*

The biocontrol efficacy of *B. velezensis* D against tobacco bacterial wilt were evaluated by pot experiments. Fourteen days after inoculation of *R. solanacearum*, most of bottom leaves in control plants completely withered seriously. However, only small amounts of bottom leaves in *B. velezensis* D and chemical groups turned yellow and withered (Fig. 3a). The disease severities were also investigated. Compared with control, the plants in *B. velezensis* D and chemical groups exhibited less severe disease symptoms, with disease index of 30.33 and 12.33 respectively (Fig. 3b). And the control efficiency of *B. velezensis* D and chemical bactericide reached to 57.47% and 82.71% (Fig. 3c). These results indicated that the strain D had potential to control tobacco bacterial wilt.

To further investigate the effectiveness of the *B. velezensis* D against tobacco bacterial wilt, the biomass of *R. solanacearum* in soil after treatment with strain D were measured using RT-qPCR method. Firstly, the relationship of log (*R. solanacearum* DNA content) and Ct value was obtained as follows: $y = -2.526 \times + 20.32$ ($R^2 = 0.9966$), there was a negative correlation between the Ct value and the log value of *R. solanacearum* DNA quantity (Additional file 1: Figure S2). On this basis, the DNA contents of the *R. solanacearum* in tobacco rhizosphere soil were calculated, the DNA of *R. solanacearum* in rhizosphere soil treated with D and chemical bactericide were 1.01 ng/g and 0.42 ng/g, respectively, which were significantly lower than control (3.32 ng/g) (Fig. 3d). The results indicated that pre-treatment with D could retard the growth of *R. solanacearum* in rhizosphere soil.

### *Evaluation of Bacillus velezensis D against tobacco bacterial wilt in field trials*

Subsequently, the efficacy of *B. velezensis* D against the tobacco bacterial wilt in the field was conducted for two consecutive years (2021–2022). Field experiment results showed that *B. velezensis* D effectively controlled tobacco bacterial wilt. Disease index of strain D-treated groups was significantly lower than control (Table 1). In 2021 year, the disease index of D and chemical agent (chloruric copper sulfate wettable powder)-treated groups were 42.81 and 39.57, which were lower than control. And the control efficiency of strains D and chemical agent reached to 12.26% and 18.90%, respectively (Table 1). In 2022 year, the disease index of D and chemical agent-treated groups was 15.85 and 13.73, while the disease index of control was 24.91. Control efficiency of strains D and chemical agent were 36.37% and 44.88%, respectively (Table 1). The results suggested that the *B. velezensis* strian D could control tobacco bacterial wilt effectively.

### *Crude extracts of strain D inhibited motility of R. solanacearum*

To further investigate the effects of strain D against *R. solanacearum*, the antibacterial activities of crude extracts were detected. The crude extracts of stain D showed inhibitory activity against *R. solanacearum* (Fig. 4a). Subsequently, the minimum inhibitory concentration (MIC) of crude extracts was evaluated, and was determined as 1200 μg/mL (Additional file 1:

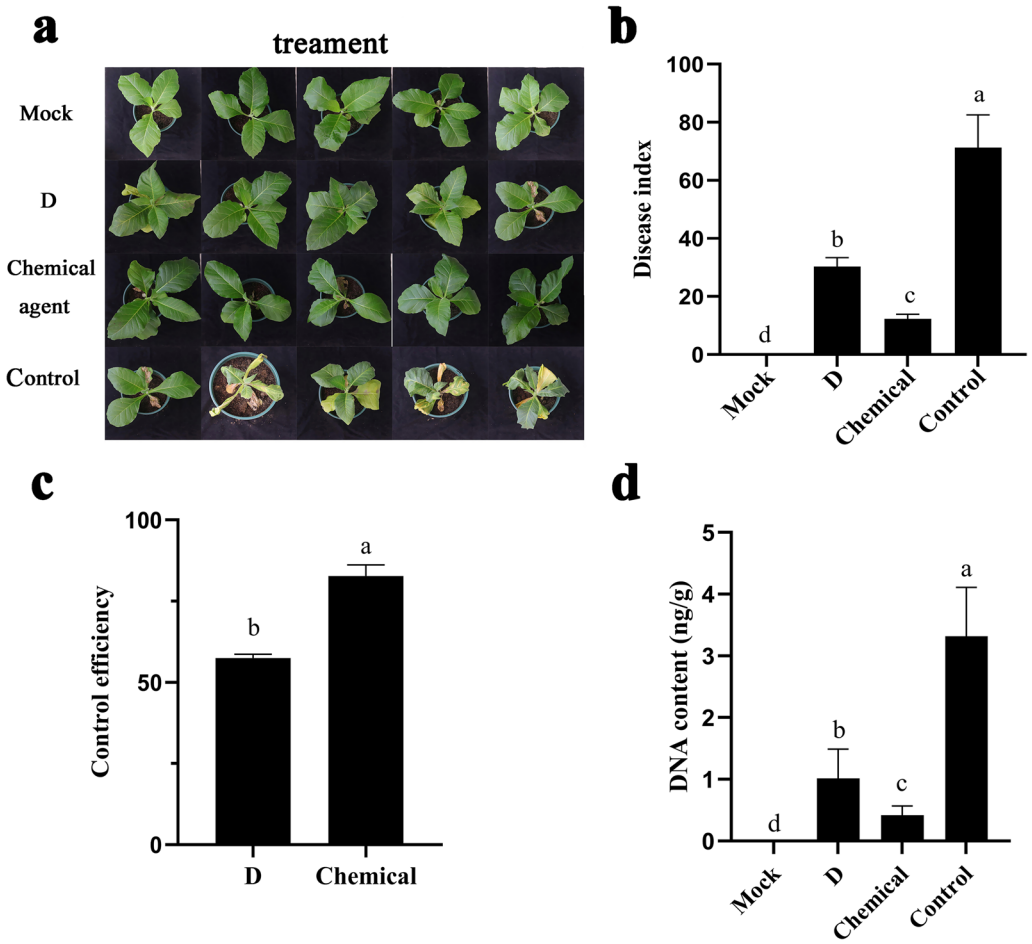


**Fig. 3** Control efficiency of *B. velezensis* D against tobacco bacterial wilt. **a** Symptoms of tobacco bacterial in different groups at 14 d after inoculation with pathogen. **b** Disease indexes of groups at 14 d post-*R. solanacearum* treatment. **c** Control efficiency of groups at 14 d post-*R. solanacearum* treatment. **d** Concerntration *R. solanacearum* DNA in rhizosphere soil of groups at 14 d post-*R. solanacearum* treatment. D represents *B. velezensis* D, Chemical represents chemical bactericide (52% chloruric copper sulfate wettable powder). Data are presented as the mean ± SD based on three biological replicates; *P*-values were determined by LSD post-hoc test. Different letters represent significant difference

**Table 1** Control effects of the *B. velezensis* D on tobacco bacterial wilt in field trials

| Treatment | Year | | | |
|---|---|---|---|---|
| | 2021 | | 2022 | |
| | Disease index | Control efficacy (%) | Disease index | Control efficacy (%) |
| Control | 48.79a | 0 | 24.91a | 0 |
| D | 42.81b | 12.26b | 15.85b | 36.37b |
| Chemical bactericide | 39.57c | 18.90a | 13.73c | 44.88a |

*P*-values were determined by LSD post-hoc test. Different letters represent significant difference

Figure S3). On this basis, the effects of 150 μg/mL (1/8 MIC) crude extracts on the motility of *R. solanacearum* were then measured. The 1/8 MIC crude extracts effectively inhibited the motility of *R. solanacearum*. The diameter of *R. solanacearum* (1.85 cm) treated with the crude extracts was significantly reduced by 2.65 times than that of control group (4.92 cm), and the inhibitory efficiency reached 62.40% (Fig. 4b). Moreover, the expression levels of *che*A, *che*W, *fli*A, and *lec*M genes associated with bacterial motility were also analyzed. The results of qRT-PCR analysis showed that all four genes in *R. solanacearum* were down-regulated in response to crude extracts of strain D, especially for two genes (*cheA* and *cheW*) (Fig. 4c).

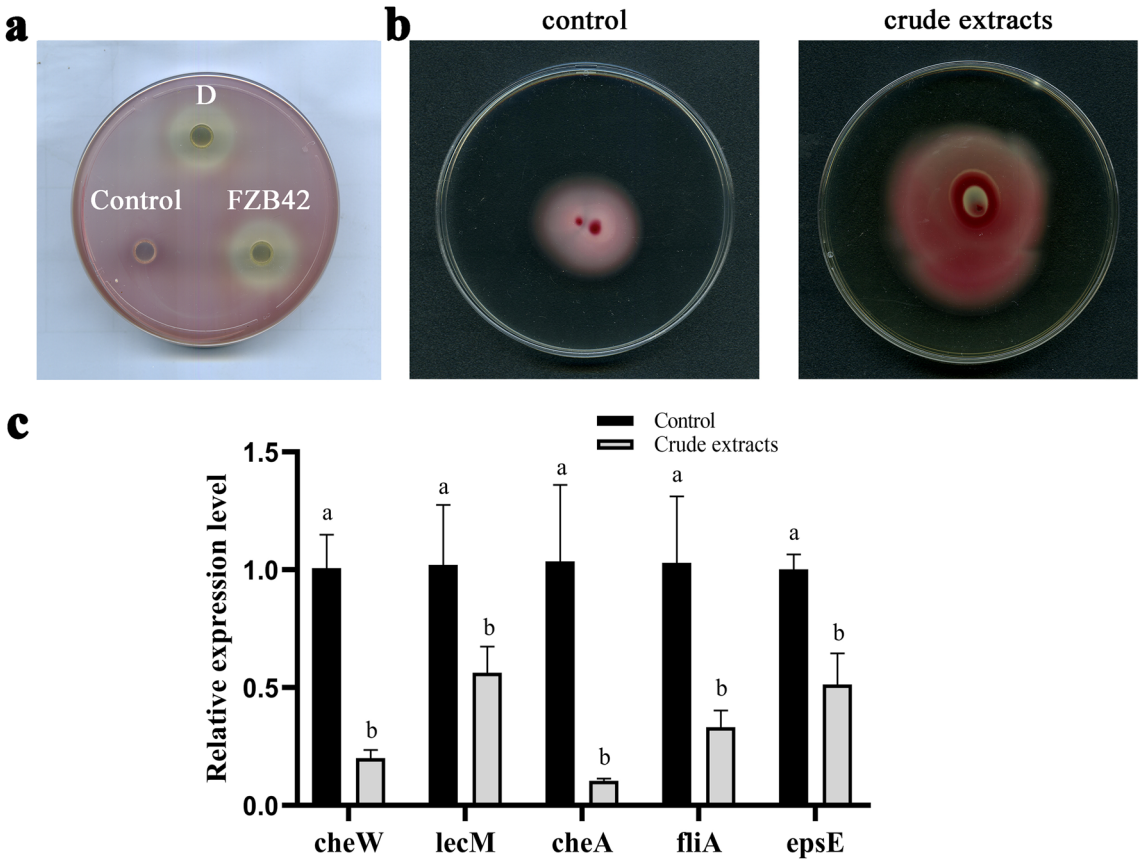


**Fig. 4** Crude extracts of *B. velezensis* D exhibited the antibacterial activity and inhibited motility of *R. solanacearum*. **a** Antagonistic activities of crude extracts against *R. solanacearum* were analyzed through oxford cup method. 5 mL *R. solanacearum* suspensions ($1 \times 10^8$ CFU/mL) were mixed with 1 L TTC medium and poured in plates. Sterile oxford cups (0.6 cm) impregnated with 150 µL crude extracts (150 µg/mL) were placed on the surface of solid medium. Finally, the diameters of inhibition zones were measured after incubating at 30℃ for 48 h. Sterile LB medium were used as control. **b** The motility of *R. solanacearum* was observed in semisolid medium supplemented with sterile water, referred as a control. **c** The expression levels of target genes relating to motility of *R. solanacearum* at 24 h. *serC* was used as reference genes for normalization. Data are presented as the mean ± SD based on three biological replicates; *P*-values were determined by LSD post-hoc test. Different letters represent significant difference

### *Crude extracts caused morphological abnormalities in R. solanacearum cells*

Scanning electron microscope (SEM) analysis revealed that crude extracts of strain D caused morphological changes in *R. solanacearum* cells, when compared to the untreated control. The cells in control samples were in rod shape, whereas the crude extracts-treated *R. solanacearum* cells were largely disrupted and abnormal, such as swelling, and even rupturing of the cell wall (Fig. 5a). Accordingly, the conductivity of *R. solanacearum* cells gradually increased from 0 to 6 h in response to crude extracts, and the conductivity was 16.06 ms/cm, which increased by 1.6 times compared with that of the *R. solanacearum* at 0 h (Fig. 5b). According to the above results, we speculated that crude extracts of the strain D could cause *R. solanacearum* cytoplasmic exosmosis by destroying the cell wall.

### *Crude extracts caused cell death of R. solanacearum*

SYTO9 can penetrate the intact cell membrane and bind to nucleic acids to yield bright green fluorescence, while PI entered cells with damaged membranes and showed red fluorescence. In the study, the *R. solanacearum* cells in control group (sterile water) had complete cell membrane, and emitted green fluorescence (Fig. 6a), and the survival rate was 100%. In contrast, part of the crude extracts-treated *R. solanacearum* cells showed red fluorescence (Fig. 6b), and the survival rate was significantly reduced to 61.33% (Fig. 6c). These results confirmed that the crude extracts of *B. velezensis* D could cause cell death in *R. solanacearum*.

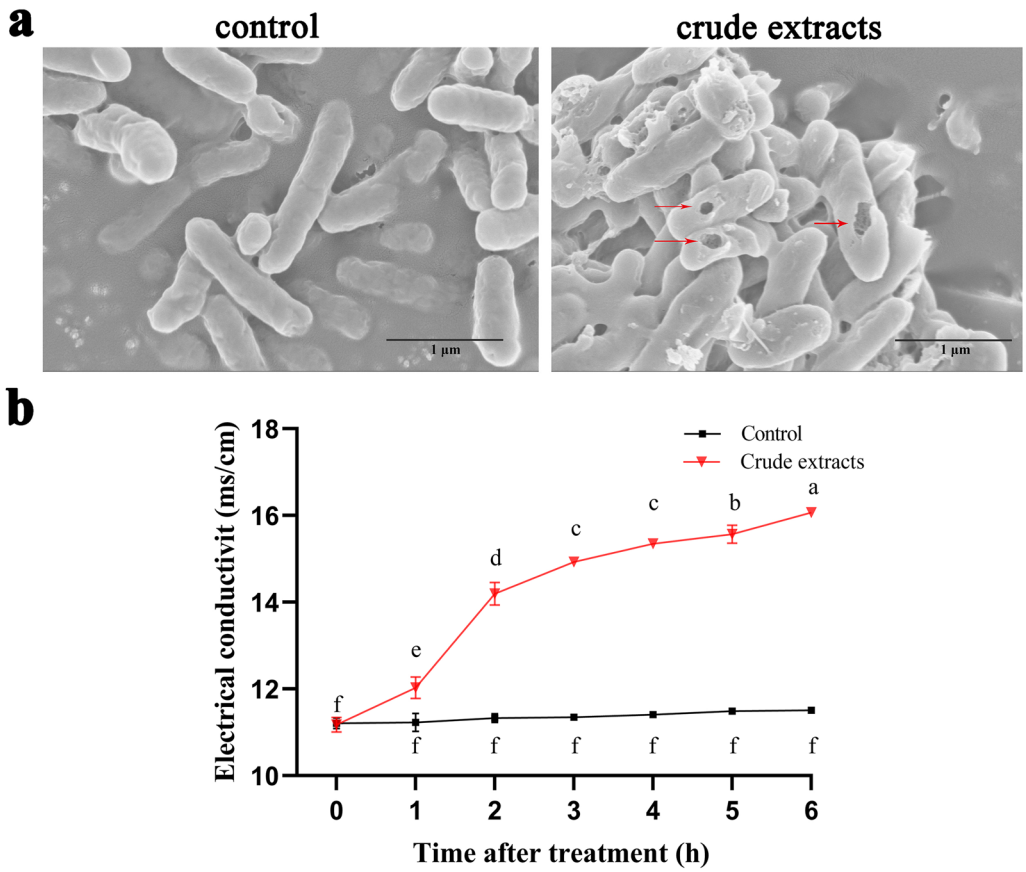


**Fig. 5** Effects of crude extracts on *R. solanacearum* cell morphology and conductivity. **a** crude extracts of *B. velezensis* D at 150 μg/mL caused abnormalities in the morphology of the *R. solanacearum* cells observed by SEM. The cell suspensions of *R. solanacearum* without the lipopeptides treatment was used as a control. Red arrow indicated the holes on the cell wall surface, and scale bars represent 1 μm. **b** The effect of crude extracts at 150 μg/mL on the *R. solanacearum* conductivity. Cell suspensions of *R. solanacearum* without the crude extracts treatment were used as control. Data are presented as the mean ± SD based on three biological replicates; *P*-values were determined by LSD post-hoc test. Different letters represent significant difference

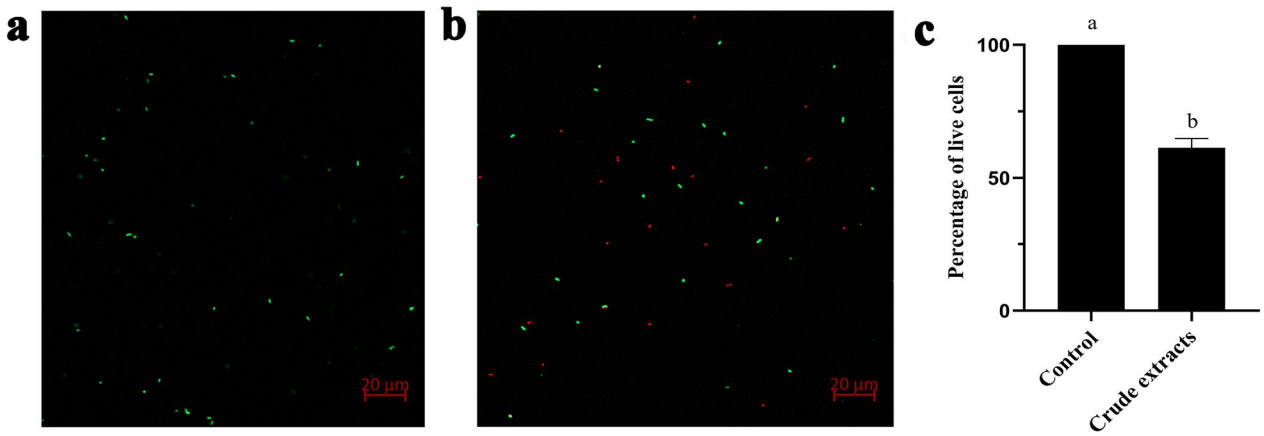


**Fig. 6** Effects of crude extracts on cell viability of *R. solanacearum*. Cell viability of *R. solanacearum* was detected with PI and SYTO9 after treatment with sterile water (**a**) or 150 μg/mL crude extracts produced by strain D (**b**). Live bacterial cells with intact membranes showed green fluorescence, whereas cells with damaged membranes showed red fluorescence. Bars = 20 μm. **c** The percentage of live cells supplemented with crude extracts after incubation for 15 min. Sterile water was used as a control. Data are presented as the mean ± SD based on three biological replicates; *P*-values were determined by LSD post-hoc test. Different letters represent significant difference

### *Identification of lipopeptides and polyketides compounds in the crude extracts of stain D*

To further clarify the antibacterial substances existing in the crude extracts of stain D. The MALDI-TOF-MS and UHPLC-QTOF-MS/MS analysis were used to identify the lipopeptide compounds and polyketides, respectively. Two kinds of lipopeptides including fengycin A and iturin families were produced by strain D. The molecular mass of iturin in the range m/z 1030.4–1059.4 was similar to previous published molecular mass (De et al. 2018), compound at m/z 1030.68 and 1043.58 represented a H adduct of iturin, and the mass spectra of m/z 1051.56 and 1065.56 represented Na adducts of iturin, respectively (Fig. 7a). While the molecular mass of fengycin A was in the range of m/z 1421.8–1449.8 representing a H adducts of C13 ~ C15 fengycin A, respectively (Fig. 7b), the mass agreed with previous studies (Ghoreishi et al. 2023; Liao et al. 2016). Besides the iturin and fengycin A, bacillaene (m/z value of 583) which belongs to polyketides was also detected in the crude extracts (Fig. 7c).

## Discussion

*Bacillus* strains have been confirmed to effectively control tobacco bacterial wilt disease (Liu et al. 2013; Yuan et al. 2014). In this study, the *B. velezensis* strain D was isolated from the rhizosphere of healthy tobacco in the Tongzi field with a high incidence of tobacco bacterial wilt, in the Guizhou Province of China. The control efficiency of D against tobacco bacterial wilt was greater than 50% in pot experiments, which is similar to that by using 20% thiodiazole copper against *R. solanacearum* (56.39%) (Inoue et al. 2022), meanwhile, the application of *B. velezensis* D could improve plant resistance to *R. solanacearum* in field trials. Hence, *B. velezensis* D has the potential to act as a biological control agent (BCA) for tobacco bacterial wilt in Guizhou tobacco-growing areas.

A large number of studies have confirmed that *Bacillus* could colonize in plant rhizosphere soil, roots, and above-ground tissues, and the colonization ability of *Bacillus* has become an important factor to exert its biological control function (Jin et al. 2019). In this study, the colonization dynamics of the *B. velezensis* D in tobacco roots was studied, and the *B. velezensis* D was detected in tobacco roots at the sixth day after irrigation. The quantity of D cells in tobacco roots retained to $5.33\times10^4$ CFU/g until the 30th day after inoculation, indicating that *B. velezensis* D could rapidly colonize the tobacco roots (Yunyan87). The stable colonization of *B. velezensis* D was due to the its good natural relationship with tobacco. Compared to other heterologous biocontrol bacteria, *B. velezensis* D was more likely to preempt advantageous ecological sites for rapidly colonization. In recent years, evaluating the colonization ability and its dynamics of biocontrol bacteria has become an important reference for screening and developing biocontrol *Bacillus* (Ying et al. 2010). Liu et al. found that colonization amount of *Bacillus* PTS in tomato rhizosphere soil was 20 CFU/g 30 days after irrigation root inoculation (Liu et al. 2014), however, *Bacillus subtilis* B2-GFP was found to be colonize the tobacco (LY1306) rhizosphere soil with $2.71\times10^6$ CFU/g 70 days after irrigation root inoculation (Lei et al. 2022). In the study, the quantity of *B. velezensis* D in tobacco (Yunyan87) roots were $5.33\times10^4$ CFU/g until the 30th day after inoculation. Comparative analysis showed that different species of *Bacillus* had different rhizosphere colonization abilities in different varieties of the same plant or different plants, this difference may be due to different types and contents, which were produced by plant root, affected the colonization ability of the biocontrol strain in rhizosphere soil.

Swimming motility allows the *R. solanacearum* to efficiently invade and colonize host plants (Tans-Kersten et al. 2001, 2004), and eventually contribute to virulence in the early stages of *R. solanacearum* invasion (Tans-Kersten et al. 2004). It was reported that the *fliC* deletion mutant from *R. solanacearum* significantly reduced the pathogenicity to tomato using the irrigation inoculation method (Tans-Kersten et al. 2004), and volatile organic compounds (VOCs) produced by *Bacillus amyloliquefaciens* SQR-9 significantly inhibited the motility traits, biofilm formation and tomato root colonization by *R. solanacearum* (Raza et al. 2016a), furtherly, Yang found that the motility of *R. solanacearum* treated with 7,8-dithydroxycoumarin at 50 mg/L final concentration was only 0.29 times that of the control group (Yang et al. 2016). However, there were relatively fewer reports about the effects of *Bacillus* sp. on the virulence of *R. solanacearum* virulence. In this study, the crude extracts of strain D at 150 μg/mL final concentration effectively inhibited the motility of *R. solanacearum*, and expression levels of four genes related to motility of *R. solanacearum* were all significantly down-regulated. These above results might suggest that the crude extracts can arrest the colonization ability of *R. solanacearum* in tobacco roots by restricting the movement of *R. solanacearum* invading plant roots.

Further study of the mechanism of action of crude extracts showed that it showed strong antibacterial activity against *R. solanacearum* by altering the integrity of the cell wall and permeability of cell membrane, leading to cell death of *R. solanacearum*. Additionally, lipopeptides and polyketides have been confirmed as an important mode of action for *Bacillus* (Mnif and Ghribi 2015). In the present study, fengycin A, iturin and bacillaene were detected in the crude extracts of strain D. Previous

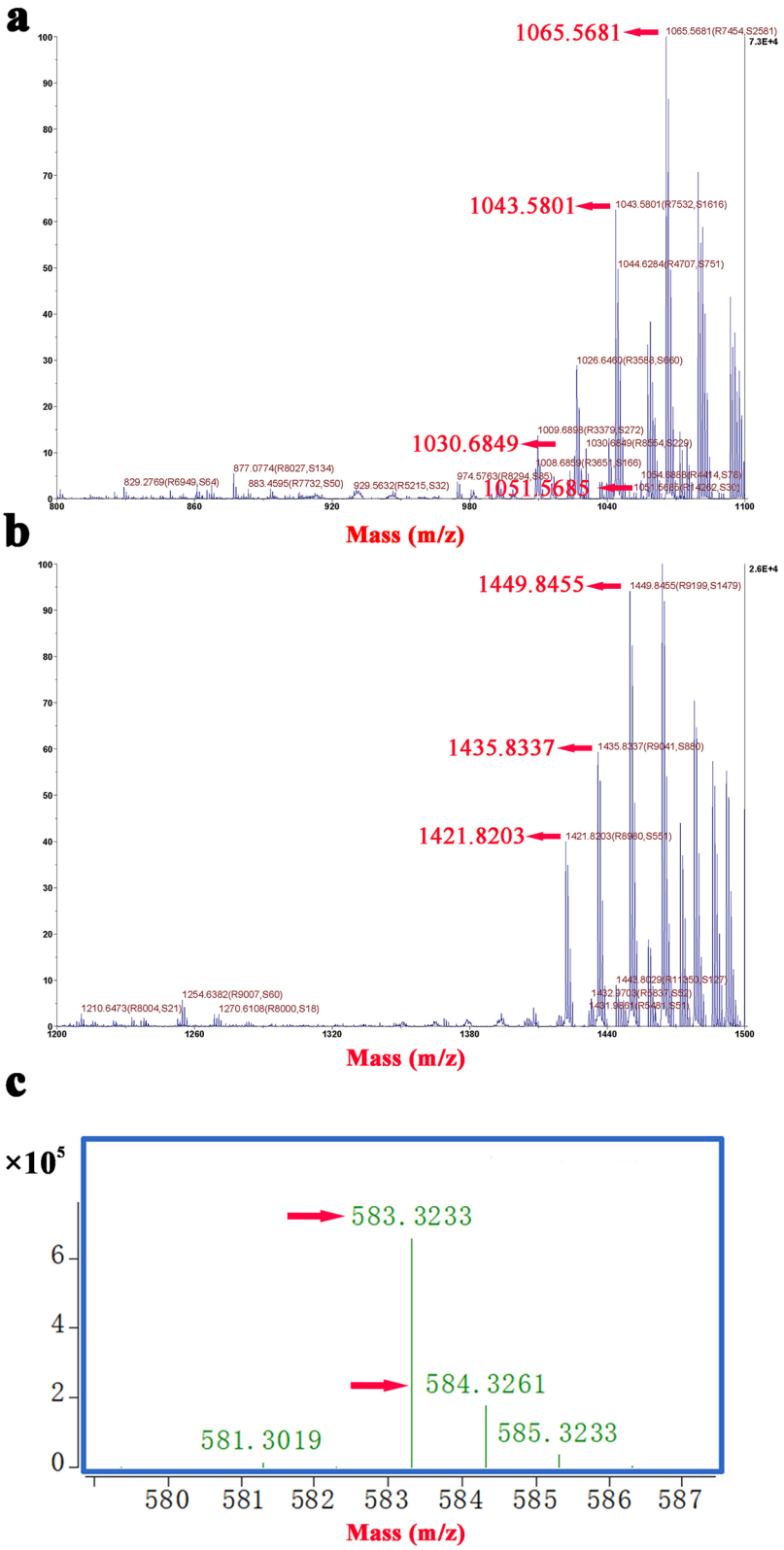


**Fig. 7** Identification of antibacterial components produced by *B. velezensis* D. MALDI-TOF mass spectra of lipopeptides produced by strain D for the presence of antibiotic groups iturin (**a**) and fengycin A (**b**). **c** UHPLC-QTOF-MS/MS mass spectra of bacillaene produced by strain D

studies have demonstrated that iturins displayed strong antifungal activities against a broad range of microorganisms but had limited antibacterial activity (Ambrico and Trupo 2017; Calvo et al. 2019), while fengycin was considered specific against filamentous fungi. However, in recent years, some researchers confirmed that fengycins showed great antibacterial activity against *R. solanacearum* which cause bacterial wilt of banana and tomato plants (Villegas-Escobar et al. 2018; Chen et al. 2019). Moreover, bacillaene plays important roles in microbial biofilm formation (Li et al. 2021) and microbial competition for finite resources (Ghoul and Mitri 2016), it inhibited bacterial growth by inhibiting prokaryotic protein synthesis, and was reported to have better antagonistic effects on the pathogenic bacteria such as *Escherichia coli* (Patel et al. 1995), *E. faecium* (Huan et al. 2019), and *Erwinia amylovora* (Chen et al. 2009). Based on the above findings, we speculated that the antibacterial activity of the crude extracts could be due to the lipopeptide compounds and bacillaene produced by *Bacillus velezensis* D.

## Conclusions

In summary, our study showed that one of the antibacterial mechanisms of *Bacillus velezensis* D was that the strain D could successfully colonize in tobacco root and rhizosphere, then produced lipopeptide compounds and polyketides at ecological sites to exert antagonistic effects, inhibiting of motility traits of *R. solanacearum* and damaging the cell wall and membrane. Hence, *B. velezensis* D has the potential to act as BCA against tobacco bacterial wilt.

## Methods

### Microorganism strains and growth conditions

A total of 15 soil samples were collected from the rhizosphere of healthy tobacco during the June–July 2020 in the Tongzi tobacco field (28° 133 N and 106° 825 E) with a high incidence of tobacco bacterial wilt, in Guizhou Province of China. And the 36 rhizosphere bacteria were isolated from fifteen samples by the gradient dilution coating method (Shi and Sun 2017).

The *Ralstonia solanacearum* belonging to the physiological races 1, and the *Bacillus velezensis* FZB42 were both provided by Key Laboratory of Crop Biology of Anhui Province, Anhui Agricultural University. The *Ralstonia solanacearum* was maintained on liquid medium for growth of *Ralstonia solanacearum* (CPG) (acid hydrolyzed casein 1 g/L, peptone 20 g/L, glucose 5 g/L) at 30°C, while the strain FZB42 and rhizosphere bacteria were grown at 30°C in Luria–Bertani (LB) liquid medium (yeast extract 5 g/L; NaCl 10 g/L; tyrptone 10 g/L).

### Antagonism activity assay

Antagonistic activities of rhizobacteria strains and their lipopeptides against *R. solanacearum* were analyzed through the oxford cup method. Briefly, *R. solanacearum* was cultured in CPG liquid medium at 30°C for 48 h, then 5 mL *R. solanacearum* suspensions ($1\times10^8$ CFU/mL) was mixed with 1 L 2,3,5-triphenyl tetrazolium chloride (TTC) medium (peptone 10 g/L, glucose 5 g/L, acid hydrolyzed casein 1 g/L, 2,3,5-triphenyl tetrazolium chloride 10 g/L, agar 15 g/L), poured in plates and allowed to solidify. Sterile oxford cups (0.6 cm) impregnated with 150 μL bacterial suspensions of strain D ($1\times10^8$ CFU/mL) or crude extracts solution (150 μg/mL) were placed on the surface of solid medium. Sterile LB medium, and FZB42 were used as negative and positive controls, respectively. The diameters of inhibition zones were measured after incubating at 30°C for 48 h. The experiment was conducted with three replications.

### Phylogenetic analysis of the antagonistic strain

Bacterial DNA was extracted with the Ezup column genomic DNA extract kit (Hunan Accurate Biology). The 16S rRNA gene was amplified with the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) (forward) and 1492R (5′-GGTTACCTTGTTACPGACTT-3′) (reverse). The *gyr*B gene was amplified with the primers UP-1 (5′-GAAGTCATCATGACCPGTTCTGCAYGC-NGGNGGNAARTTYGA-3′) and UP-2 (5′- AGC AGGGTACPGGATGTGCPGAGCCRTCNACRTC-NGCRTCNGTCAT-3′) (Xie et al. 2020). The PCR products were sequenced and blasted against the NCBI database. A phylogenetic tree was established with Mega 7.0 software using the Neighbor-joining method.

### GFP labeling of antagonistic rhizobacteria

The pAD43-25 with *gfp* marker was transformed into the competent cells of the antagonistic rhizobacteria by electroporation as previously described (Wang et al. 2020). The competent cells (100 mL) were completely mixed with 50 ng pAD43-25 plasmid DNA and electroporated at 2.5 kV (2.5–5 ms) using an electroporation apparatus (MicroPulser, Bio-Rad) (Hao and Chen 2017). Subsequently, 100 μL cell suspensions were incubated in 900 μL LB medium and cultured at 37°C for 5 h with shaking at 100 rpm. Finally, the mixture (100 μL) was spread on LB plates containing 5 μg/mL chloramphenicol at 37°C for 24 h. The GFP-labeled cells were selected on the antibiotic plates for five generations, and further confirmed with laser confocal microscope (OlympusBX53, Japan).

### Colonization of the *Bacillus velezensis* D assay

The tobacco seeds (Yunyan 87) were grown in the seedling-raising pot and cultured at 28°C under a 14/10 h light/dark photoperiod and 75% relative humidity. Six-week-old tobacco seedlings were transplanted into the nutrient pots (16 cm × 17.5 cm) filled with the mixture of soil and vermiculite ($v_{soil}$: $v_{vermiculite}$ = 2:1). The GFP-labeled D suspensions ($1 \times 10^8$ CFU/mL) were added to the roots of 55-day-old tobacco (50 mL per plant) and sterile water were used as control. After inoculation for 0, 3, 6, 9, 12, 15, 20, 25, and 30 days, the roots, stems and leaves were collected, cut into pieces, and homogenized in sterile water (Sun et al. 2019). The homogenates were diluted gradiently and spread on LB plates containing 5 μg/mL chloramphenicol. Colony numbers were counted after incubating at 30°C for 36 h. Each treatment contained 81 plants.

After inoculation for 14 days, roots, stems and leaves tissues were collected and cut into 1 cm in length and observed with laser confocal microscopy (OlympusBX53, Japan). The experiment was repeated three times.

### Biocontrol assays of the *Bacillus velezensis D*

The control efficiency of D against tobacco bacterial wilt was evaluated using plot experiments and field trials. In the plot experiments, the suspensions of D ($1 \times 10^8$ CFU/mL) were applied to the roots of 55-day-old tobacco by soil drenching (50 mL per plant). Sterile water and chemical bactericide (52% chloruric copper sulfate wettable powder) were used as the negative and positive controls, respectively. Two days after treatment with strain D and chemical bactericide, *R. solanacearum* cell suspensions ($1 \times 10^8$ CFU/mL) were added to the tobacco root (15 mL per plant). Then the plants were cultured in glasshouse at 28°C and relative humidity 70%. After 14 days post-infection (dpi), the disease severity was recorded with a scoring range of 0–9 (National Standard of the People's Republic of China GB/T23222-2008). Each treatment consisted of three replicates, and ten plants were in per replicate.

On the basis of the pot experiment, the field control effects of strain D against *R. solanacearum* were carried out in tobacco field with a high incidence of tobacco bacterial wilt for two consecutive years in the Tongzi, in Guizhou Province of China. When tobacco seedings were transplanted, the strain D cell suspensions ($1 \times 10^8$ CFU/mL) were applied to the tobacco root (50 mL per plant). Sterile water and chemical bactericide (52% chloruric copper sulfate wettable powder) were used as the negative and positive controls, respectively. Each treatment was conducted with three replications and contained 90 plants. Then the disease severity of the tobacco bacterial wilt in different groups were investigated in the harvest period, Disease index and biocontrol efficacy were calculated according to the formulas below.

$$\text{Disease index} = \sum (\text{di} \times \text{li}) / \text{L} \times \text{N} \times 100$$

$$\text{Biocontrol efficacy} = (\text{I}_0 - \text{I}_i) \times 100\%$$

where di = the grade of disease severity, $I_I$ = the number of plants at different grades of disease, L = the number of all investigated plants, N = the highest grade of disease severity, $I_0$ = the disease index of control, and $I_I$ = the disease index of treatment groups.

### Quantitative analysis of *R. solanacearum* in tobacco rhizosphere soil

To further investigate the biocontrol activity of *B. velezensis* D on tobacco plants, the population of *R. solanacearum* was detected in tobacco rhizosphere soil treated with D suspensions in the plot experiments. Sterile water and chemical bactericide (52% Chloruric copper sulfate wettable powder) were used as the negative and positive controls. Fourteen days after the challenge with *R. solanacearum*, soil DNAs of different groups were extracted by soil DNA extraction kit (TianGen, Beijing, China), and qRT-PCR was used to detect *R. solanacearum* in tobacco rhizosphere soil (Umesha and Avinash 2015). Each PCR tube contained 10 μL AceQ qPCR SYBR Green Master Mix, 1 μL 25 ng cDNA, 8 μL water, and 1 μL primer (UP-1 (5′-GAACPGCCAACPGGTGCPGAACT-3′) and UP-2 (5′- GGCPGGCCTTCAGGGAGGTC-3′). The thermal cycling conditions were as follows: 30 s at 95°C, 40 cycles for 10 s at 94°C, and 30 s at 60°C, after 95°C for 15 s,60 s at 60°C, last 95°C for 15 s. Each sample was replicated three times.

RT-qPCR amplification was performed on tenfold gradient dilutions of *R. solanacearum* DNA with primers, and the genomic DNA standard curve equation, of which the *R. solanacearum* DNA quantity was log and the Ct value was the y-axis, the formula was obtained.

### Preparation of crude extracts of stain D

Strain D was grown in the landy medium at 30°C and 200 rpm on a shaker for 36 h, then the crude extracts in fermentation liquid was prepared by hydrochloric acid precipitation (Ding et al. 2017). The extracts were concentrated and vacuum freeze dried, and the dried material was dissolved in sterile water (Chen et al.2019; Gao and Zhao 2022).

### Swimming motility of the *R. solanacearum* assay

*R. solanacearum* suspensions ($OD_{600}$ = 0.1) were cultured with crude extracts of stain D at final concentration 1200, 600, 300, 150, 75, and 37.5 μg/mL respectively, in CPG liquid medium by shaking at 30℃ for 0, 6, 12, 18, and 24 h to determine the minimal inhibitory concentration (MIC). Then Plates containing a semisolid medium (peptone 20 g/L, glucose 5 g/L, acid hydrolyzed casein 1 g/L, agar 3 g/L) supplemented with 1/8MIC crude extracts (150 μg/mL) were prepared. *R. solanacearum* suspensions ($OD_{600}$ = 1.0), collected from CPG liquid culture, were washed twice with sterile water at 6000 r/min for 5 min and resuspended in sterile water. An aliquot of 5 μL cell suspensions were drop-inoculated at the center of semisolid medium plates. Sterile water was used as control. The petri dish was incubated at 30℃, and colony diameters on each plate were observed and measured after 12 h. Each sample was replicated three times.

Moreover, *R. solanacearum* suspensions ($OD_{600}$ = 1.0) was cultured with 150 μg/mL crude extracts of stain D in CPG liquid medium by shaking at 30℃ for 12 h, the cells were collected to extract total RNA using the Trizol reagent (Sangon, Shanghai, China). Transcriptional expression levels of genes related to motility were analyzed by qRT-PCR. qRT-PCR was performed with AceQ qPCR SYBR Green Master Mix on an ABI 7300 Real-Time System (Applied Biosystems, Foster City, CA, USA). The primers used were listed in Additional file 2: Table S1. Each sample was replicated three times.

### The conductivity of the *R. solanacearum* assay

The conductivity of the *R. solanacearum* treated with the crude extracts was analyzed following previously described methods (Bandian et al. 2022; Mahboubi and Kazempour 2011). The cell suspensions of *R. solanacearum* ($OD_{600}$ = 1.0) were incubated with 150 μg/mL crude extracts at 30°C for 6 h. Sterile water was used as control. And the conductivity was detected every hour. Three parallel experiments were conducted.

### Live/dead cell viability assay

Cell suspensions of *R. solanacearum* ($OD_{600}$ = 1.0) were incubated with 150 μg/mL crude extracts in CPG medium by shaking under 30°C for 12 h, then the cell viability of *R. solanacearum* was detected by live/dead BacLight bacterial viability Kit l13152 (Invitrogen, Thermo Fisher Scientific, America). An aliquot of 1 mL cell suspensions was centrifugated, washed and resuspended in 1 mL 0.85% NaCl solution, and 10 μL propidium iodide (PI, excitation wavelength/emission wavelength 490 nm/635 nm) and SYTO9 (excitation wavelength / emission wavelength 480 nm/500 nm) were added to the cell suspensions to incubate at 25℃ for 15 min in the dark. Sterile water was used as a control. Each group contained three replicates, and samples were observed with a laser confocal microscope (OlympusBX53, Japan). Survival rates of *R. solanacearum* cells were calculated according to the formulas below.

$$\text{Survival rates} = \text{Number of PI} - \text{stained cells/Total number of fluorescent staining cells} \times 100\%$$

### Effects of crude extracts on the morphology of *R. solanacearum*

The effect of crude extracts on morphological changes of *R. solanacearum* was observed by scanning electron microscope. The cell suspensions of *R. solanacearum* ($OD_{600}$ = 1.0) were incubated with 150 μg/mL crude extracts in CPG medium by shaking at 30°C for 12 h. Bacterial cells were collected by centrifugation at 6000 r/min for 5 min, and then washed, fixed with 2.5% glutaraldehyde overnight. The suspensions were dehydrated with ethanol and acetone. The samples were analyzed with a scanning electron microscope (HITACHI S-4800, Japan). The cell suspensions of *R. solanacearum* ($OD_{600}$ = 1.0) without the crude extracts were used as control.

### Identification of the lipopeptides and polyketides in crude extracts of stain D

Lipopeptides were analyzed using MALDI-TOF-MS at Shanghai Applied Protein Technology Co. Ltd. And data was acquired in positive reflector mode from 800 to 4000 m/z. Polyketides were identified with UHPLC-QTOF-MS/MS analysis according to the previous study (Liang et al. 2018).

### Statistical analysis

Statistical data analyses were conducted using the Data processing system (DPS) software. Differences between different groups were assessed by one-way ANOVA followed by LSD post hoc test at a significance level of $P$ = 0.05.

**Abbreviations**

| | |
|---|---|
| *R. solanacearum* | *Ralstonia solanacearum* |
| RT-qPCR | Real time quantitative PCR |
| MIC | The minimum inhibitory concentration |
| dpi | Days post-infection |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00204-x.

**Additional file 1**: **Figure S1**. Colonization analyses of strain D in tobacco stems and leaves. **Figure S2.** The genomic DNA standard curve equation of *R. solanacearum*. **Figure S3.** The effect of the minimum inhibitory concentration of crude extracts and its different diluents on *R. solanacearum* growth.

**Additional file 2**: **Table S1**. Target genes relating to motility of *R. solanacearum*.

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**Author contributions**
JW, YP, SX, and TD designed the research. JW, XY, and CB performed the research. JW, YP, HW, and YW analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**
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## Declarations

**Ethics approval and consent to participate**
Not applicable.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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