Research Article

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Microbial diversity in coastal *Casuarina equisetifolia* forest and its potential in counteracting bacterial wilt infections

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Abstract

BACKGROUND: *Casuarina* wilt is a destructive soil-borne disease caused by *Ralstonia solanacearum* species complex (RSSC). Recent large-scale outbreaks of *Casuarina* wilt in the coastal regions of Guangdong Province, China, suggest that the originally resistant *Casuarina* clones become susceptible to RSSC infection. This study aimed to investigate the microbial diversity of environmental microorganisms and its potential in biocontrol of this devastating disease.

RESULTS: The results unveiled the dominant and common microbial species in *Casuarina equisetifolia* tree tissues, the rhizosphere soils and seawater in the vicinity of *Casuarina equisetifolia* forest belt. We also found a range of bacterial species with potent antimicrobial activities against *Ralstonia pseudosolanacearum*. Both the *Casuarina* endophyte A1-5, identified as *Bacillus velezensis,* and a combination biocontrol agent named CEP consisting of three mutually compatible soil isolates belonging to *Citrobacter farmeri*, *Enterobacter aerogenes*, and *Pseudomonas mosselii*, respectively, could effectively control the *R. pseudosolanacearum* infections on *Casuarina* and tomato. The active substance of strain A1-5 that inhibits the growth of *R. pseudosolanacearum* was purified and identified as surfactin C.

CONCLUSION: The findings unveiled the microbial diversity and their specific distributions in the Guangdong coastal *Casuarina equisetifolia* forest areas, and present useful clues and resources for developing new strategies to prevent and control the *Casuarina* bacterial wilt.

Supporting information may be found in the online version of this article.

Keywords: *Casuarina* bacterial wilt; microbial diversity; *Casuarina* endophyte; rhizosphere microorganism; biocontrol bacterium

1 INTRODUCTION

Casuarina , which is a designation with a total of four *Casuarina* genera and 96 species, is indigenous to southeast Asia, Malaysia, northern Australia, Oceania.[1,2] *Casuarina* is an important coastal afforestation tree species, mainly distributed in the tropical and subtropical regions. it has excellent wind resistance, drought resistance, salt tolerance, and fast growth characteristics. Since 1897, several *Casuarina* species including *Casuarina equisetifolia, Casuarina glauca*, and *Casuarina cunninghamiana*, have been introduced to and planted in the tropical and subtropical regions of China.[3–5] Among them, *Casuarina equisetifolia* is the main species planted in the coastal regions to withstand typhoon attack. At present, it has become an important shelter forest tree species in the southeast coastal areas of China, including Guangdong, Guangxi, Fujian and Hainan provinces accounting for about 300 000 ha.[3,4,6]

The major threat of *Casuarina* plantation is the infectious diseases caused by various microorganisms, including the *Casuarina* wilt caused by *Ralstonia solanacearum* species complex (RSSC),[1,7] the powdery mildew caused by *Erysiphe robusta*,[8] the canker disease caused by *Phomopsis* sp.,[9] the sooty mould caused by *Capnodsum* sp., the root rot disease caused by *Fusarium* sp.,[10] and the smut disease caused by *Trichosporium vesiculosum*.[11] Among them, *Casuarina* wilt is the most destructive disease that threatens *Casuarina* plantation.[1,7] The disease was initially reported in the *Casuarina* seedlings of Mauritius.[12] In China, *Casuarina* wilt initially appeared in Hailing Island, Guangdong Province.[13] then the disease was found in Hainan, Guangxi, Fujian and other provinces.[1,11]

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RSSC is a gram-negative, aerobic, which belongs to β-Proteobacteria.[14] As a soil-borne pathogen, it commonly infects plants through the root vascular system, spreads in the xylem of plant stems and produces massive extracellular polysaccharides which block water transportation resulting in plant wilt and death.[15–17] The pathogen has a wide host range all over the world and can infect more than 450 plant species in about 50 families.[4,18] Given the wide geographical range, variations in host specificity and genome, *R. solanacearum* is considered as a species complex known as RSSC, a heterogeneous group of related strains.[19] Due to its remarkable survival characteristics, including strong ability to live inside plants and soil (especially deep soil), water flow (it can live for 40 years in 20–25 °C water), and weeds, and so forth, the bacterial wilt is very difficult to prevent and control.[20] Traditionally, control of RSSC infection mainly relies on crop rotation and breeding of disease-resistant varieties, which however are restrained by their wide host range and limited resources of resistant germ plasms.[21] In addition, genome sequence analysis showed that RSSC strains could evolve to overcome plant resistance.[22] Given that RSSC strains are soil-borne bacterial pathogens, biological control using soil microorganisms might represent a promising alternative to control plant wilt diseases.[23] It has been shown that rhizosphere predatory protozoa can reduce the incidence of tomato bacterial wilt by reducing pathogenic bacteria through direct and indirect effects.[24] Interestingly, transplanting the rhizosphere microbiome of resistant eggplant varieties to susceptible ones could significantly reduce the disease incidence.[25] Recently, KEGG enrichment analysis showed that phenylpropanoid, flavonoid, plant hormone signal transduction and MAPK signal transduction pathways may play a key role in the resistance of *Casuarina* to bacterial wilt.[26] The compounds isolated from ginger, tomato, and mangosteen pericarp could inhibit the growth and pathogenic process of RSSC.[27–29] However, research on biocontrol of *Casuarina* wilt has not yet been reported.

Casuarina wilt outbreak in Guangdong Province was initially reported in 1960–1970s, which caused massive death in the *Casuarina* plantations. The disease spread was constrained by screening and large-scale plantation of resistant *Casuarina* clones in the 1980s.[30] However, an outbreak of *Casuarina* wilt has occurred again in recent years,[22] suggesting that RSSC may have adapted or evolved to overcome the resistance of *Casuarina* clones. In this article, microbial isolates were collected from the *Casuarina* trees, rhizosphere soil and seawater samples from different coastal regions of Guangdong Province. Genetic analysis unveiled the preferred habitats of various microorganisms. Further antagonistic assays unveiled a range of bacterial isolates with potent antimicrobial activity against the *Casuarina* pathogens, as well as the mutually-compatible potential biocontrol agents. We then tested the biocontrol potency and potential of a *Casuarina* endophyte belonging to the species of *Bacillus velezensis* as well as a biocontrol combination consisting of three mutually-compatible soil bacterial species. Moreover, the active antimicrobial compound from the endophyte *Bacillus velezensis* was purified and characterized. The findings from this study aid to our understanding of the microbial diversity within and at the vicinity of the coastal *Casuarina* forest belts, and provide useful clues and resources for the prevention and control of the devastating *Casuarina* wilt disease.

2 MATERIALS AND METHODS

2.1 Media, plasmids and growth conditions

YEB medium was used for isolation of microorganisms from *Casuarina* trees and soil samples, which contains the following components: yeast extract 5.0 g/L, tryptone 10.0 g/L, KCl 5 g/L, glucose 5 g/L., and $MgSO_4{\cdot}7H_2O$ 0.5 g /L. LB medium (tryptone 10.0 g /L, yeast extract 5.0 g/L, NaCl 10.0 g/L, pH 7.0) was used for isolation of microorganisms from seawater samples. CPG medium contains tryptone 10.0 g/L, glucose 5 g/L, and acid hydrolyzed casein 1 g/L. TTC medium was prepared by supplementing CPG with 2, 3, 5-triphenyltetrazole chloride (TTC) at a final concentration of 0.5‰, and was used for maintenance and routine culture of *Ralstonia pseudosolanacearum* strains, which are natively resistant to polymyxin B. The enhanced green fluorescent protein (eGFP) fluorescence expression construct pBBRI-mcs2-eGFP was generated by cloning the 720 bp eGFP coding sequence in the site between *BamHI* and *HindIII* of the plasmid vector PBBRI-mcs2 containing a kanamycin resistance gene.[31] LB medium was sterilized at 121 °C for 20 min, YEB and CPG media were sterilized at 115 °C for 30 min and solidified by adding agar powder 15 g/L when necessary. Polymyxin B and kanamycin were added at a final concentration of 25 μg/mL and 100 μg/mL, respectively, when necessary. All the bacterial strains used in this study were cultured at 28 °C.

2.2 Sample collection and bacterial isolation

The environmental microorganisms used in this study were isolated from the *Casuarina equisetifolia* forest belts of five coastal cities including Shanwei, Huizhou, Yangjiang, Maoming and Zhanjiang in the Guangdong Province of China in March 2018 and October 2018, respectively. These cities are the major coastal regions of *Casuarina* plantation in Guangdong Province. *Casuarina* (wilt and healthy trees) roots and stems, rhizosphere soils, and seawater near the forest belt were collected for bacterial isolation. The root and stem samples were cut into 4–5 cm long segments about 1–2 cm in thickness, which were surface-sterilized in 95% alcohol for 30 s. The segment was then surface-disinfected on an alcohol flame briefly, cleaned twice with sterilized deionized water, and placed in a centrifuge tube containing 15 mL sterilized water for 5 h with gentle shaking at 200 rpm. Soil samples were treated by adding 5 g into a tube with 10 mL sterilized water, placed in a shaker at 28 °C and oscillated at 200 rpm for 2 h. The aqueous solutions including seawater about 200 μL were diluted when necessary and spread on a YEB or LB (for seawater samples) plates with three repeats for each sample, and placed upside down in an incubator at 28 °C for 36–48 h. Single colonies were collected and purified on the same fresh plates. The purified isolates were prepared as stock solutions in YEB or LB liquid medium containing 20% glycerol and kept in a –80 °C refrigerator till further use.

2.3 Bacterial characterization and phylogenic analysis

The purified bacterial colonies were grouped according to the origin of samples and their colony morphological features including colony colour, shape, size, margin, elevation, texture, opacity and other features that can be distinguished by the naked eye. Representative strains were identified by 16S rDNA, *gyrA* and *gyrB* sequence analysis using the universal primers 27F/1492R (27F: 5′-CAGAGAGTTTGATCCTGGCTCAG-3′; 1492R: 5′-TCCTACGGGTACCTTGTTACGACTT-3′); gyrA-F/gyrA-R (gyrA-F:5′-ATTCACGCTATCACTGACTTATTC-3′; gyrA-R:5′-ATGGGAGACAAAGTAGAA CCGAG-3′); and gyrB-F/gyrB-R (gyrB-F:5′-GAAGTCATCATGACCGTTCTGCAYGCNGGNGGNAA RTTYGA-3′; gyrB-R:5′-AGCAGGGTACGGATGTGCGAGCCRTCNACRTCNGCRTCNGTCAT-3′).[32–34] The polymerase chain reaction (PCR) products were sequenced and the data were searched using the Blast program in the National

Centre for Biotechnology Information (NCBI). The 16S rDNA, *gyrA* and *gyrB* gene sequences of other *Bacillus* spp. strains with known sequevar information were retrieved from NCBI RefSeq database, and aligned using MAFFT v7.520. Phylogenetic trees were constructed using IQ-TREE v2.1.2 with the GTR+G model, and the reliability of tree topology was evaluated with 1000 ultrafast bootstrap replicates.

2.4 Antagonistic and compatibility analysis

For qualitative assay, environmental microbial isolates and bacterial pathogens were activated on YEB medium or TTC medium (for *R. pseudosolanacearum*), respectively, and cultured in an incubator at 28 °C. In the antagonistic assay, bacterial pathogens were cultured with shaking to an optical density measured at 600 nm (OD_{600}) of 1.2 in corresponding liquid medium, and the environmental microbial isolates were grown overnight in YEB plates. Aliquots of 100 μL of fresh pathogenic cells or other bacterial cells were added to the prewarmed 1% agarose solution at about 45 °C, mixed gently and poured into CPG or other medium plates as indicated. Single colonies of the environmental microbial isolates were inoculated by toothpicks on the earlier bioassay plate containing corresponding pathogenic cells. The plates were kept at 28 °C for 24–48 h, and the isolates producing inhibition zones on the pathogen lawns were recorded.

Semi-quantitative assay was performed by punching 5 mm wells about 2 cm in distance in the corresponding plate containing pathogenic cells or other targeted bacterial cells as described earlier. The fresh antagonistic bacterial fluid at $OD_{600} = 1.5$ was added to each well. Upon the bacterial fluid being absorbed in the solid medium, the plates were placed in an incubator at 28 °C for 24–48 h. The experiment was repeated three times and the diameters of inhibition zones were measured.

2.5 Thermal stability assay

Overnight antagonist cells in YEB liquid medium was evenly spread on YEB solid plate, incubated in an incubator at 28 °C for 2 days. The bacteria were removed with filter papers, the remaining agar medium was cut into pieces, soaked in ethyl acetate overnight with gentle shaking, and the organic solvent was dried by rotary evaporation. The remaining residues were dissolved with 5 mL of methanol and kept in a fridge as the stock solution. For thermal stability assay, the stock solation was diluted by adding sterilized water at a ratio of 1:9 in a capped plastic tube, which was placed at 30, 60, 70, 80, 90, and 100 °C respectively for 30 min. An aliquot of 2 μL of each heat-treated solution was added to the semi-quantitative bioassay plate containing *R. pseudosolanacearum*. The experiment was repeated three times and the inhibition zone diameters were recorded 48 h after incubation at 28 °C.

2.6 Niche colonization assay

The fluorescent marker plasmid PBBR1-mcs2-eGFP was transformed into the endophyte strain A1-5 by electric shock transformation. The bacterial solution was evenly spread on the YEB + Kan plate, and the green fluorescent marker strain A1-5-eGFP was obtained by detection with inverted fluorescence microscope and PCR analysis. Strain A1-5-eGFP was cultured overnight, the bacterial cells were collected by centrifugation, washed with sterilized water for three times, and resuspended in sterilized water to $OD_{600} = 1.0$. *Casuarina* seedlings were inoculated by adding 5 mL bacterial dilution per plant to the rhizosphere soil evenly, and the control group was set up by adding the same volume of sterilized water. On the third and fifth day post-inoculation, the roots, stems and leaf-like branchlets of each seedling were taken, cut into thin slices with a scalpel and placed on glass slides for observation with a fluorescence microscope. The experiment was repeated three times with 2–3 replicates.

For soil niche colonization assay, 1 g of moisture soil was added to a 14 mL centrifuge tube, autoclaved at 121 °C for 30 min. Overnight bacterial cultures were adjusted to $OD_{600} = 1.0$, and the wilt pathogen and biocontrol agent or CEP combination (a biocontrol agent consisting of three mutually compatible soil isolates belonging to *Citrobacter farmeri*, *Enterobacter aerogenes*, and *Pseudomonas mosselii*) or YEB liquid medium were mixed at a 1:1 ratio. To each sterilized centrifuge tube containing soil, 180 μL of the diluted bacterial mixture were added and shaken for 15 min at 150 rpm. The tubes were placed in the incubator at 28 °C and a small portion of samples were taken out at 24, 48 and 72 h, respectively. The soil sample was added to 1 mL of sterilized water, and incubated at 28 °C for 15 min with constant shaking. The tubes were then taken and allowed to stand for about 10 min. The supernatants were taken and diluted accordingly and spread on the TTC medium containing polymyxin B, which was used to distinguish the drug-resistant wilt pathogens and the drug-sensitive biocontrol bacteria. The plates were placed in an incubator at 28 °C for 48 h before counting colony-forming unit (CFU) numbers of the wilt pathogen. The experiment was repeated three times with 2–3 replicates.

2.7 Purification and characterization of active antibacterial compounds

The active antimicrobial compounds from strain A1-5 were prepared using solid fermentation method. An aliquot of 100 μL overnight bacterial culture about $OD_{600} = 0.5$ was spread evenly on the YEB agar plates, which were kept in a 28 °C incubator for 2 days. After removal of bacterial cells from plates using filter paper, the agar medium in the plates was cut into small pieces, which were placed in a glass container and extracted twice by adding sufficient ethyl acetate to cover the agar slices and shaking vigorously for over 2 h. The extracts were combined and the organic solvent was evaporated. The residues were weighted and dissolved in pure methanol and kept in a refrigerator for further purification.

Silica gel (Agela Bio, 200–300 mesh) column chromatography was conducted by adding sample and eluted with three gel volumes of dichloromethane and methanol mixture at a ratio of 100:0, 90:10, 80:20, 50:50, and 0:100, consecutively under a pressure of about 100 psi. The elutes were dried by evaporation, dissolved in methanol and assayed against *R. pseudosolanacearum*. The active fractions were combined, separated by Sephadex-LH 20 gel chromatography by eluting with methanol at a flow rate of about 3 mL/min. Elute fractions about 30 mL each were collected and assayed against *R. pseudosolanacearum*, and the active fractions were dried in an evaporator, weighed, and stored in a refrigerator at −20 °C.

High performance liquid chromatography (HPLC, Agilent 1260; Agilent Technologies, Santa Clara, CA, USA) was performed with an YMC brand C18 reversed-phase semi-preparative column, coupled with an ultraviolet (UV) detector using absorption wavelengths of 210, 230, 254, and 290 nm. The mobile phase was methanol (chromatographic grade) and pure water supplemented with 0.1% volume of trifluoroacetic acid at a flow rate of 3 mL/min. Fractions were collected every 2 min, bioassayed and dried with a vacuum desiccator.

Nuclear magnetic resonance (NMR) analysis was conducted by dissolving the purified antimicrobial component in dimethyl sulphoxide (DMSO) with an appropriate amount of deuterium. The solution was added into a NMR tube, sealed, labelled and sent to the South China Sea Institute of Oceanology, Chinese Academy of Sciences for proton (^{1}H) NMR detection of hydrogen spectrum and carbon-13 (^{13}C) NMR detection of carbon spectrum with operating frequency at 700 MHz and 151 MHz, respectively.

2.8 Biocontrol trial against *Casuarina* wilt

Pathogenic bacterial strains and biocontrol agent or combination were cultured overnight in 200 mL YEB medium with shaking at 28 °C for 24 h. The pathogen and biocontrol cultures were adjusted to OD_{600} = 0.8, respectively, using sterilized water and mixed at a ratio of 1:1. In the case of CEP combination, members were grown separately and equal volume was taken to prepare combination mixture before mixing with bacterial pathogen. *Casuarina* or tomato seedlings with root damaged by cutting a small portion of root tips were soaked in the bacterial mixture or sterilized water for 40 min (Casuarina), then planted in pots and maintained in a growth room at 28 °C. Plant growth status and disease symptoms were observed and recorded daily. The experiment was repeated at least three times with seven plants for each treatment. Plant wilt disease severity was recorded daily based on the percentage of wilted leaf-like branchlets following infection as described previously.[22,31,35]

In addition to co-inoculation, we also tested the effect of pre-treatment with biocontrol agents against the wilt pathogens. Two days after inoculation with the biocontrol agent A1-5 or CEP as described earlier, the plant roots were wound again and immersed for 40 min in the fresh culture dilutions of *R. pseudosolanacearum* YJA2 at OD_{600} = 0.8. The seedlings were planted in pots and maintained in an incubator at 30 °C, and the disease symptoms were recorded daily.[31,35]

3 RESULTS

3.1 Characterization of the bacterial isolates from *Casuarina equisetifolia* trees

To understand the bacterial endophytes associated with *Casuarina equisetifolia*, a total of 51 *Casuarina equisetifolia* tree trunk samples were collected from the five coastal cities in Guangdong Province of China, including Huizhou, Shanwei, Yangjiang, Maoming and Zhanjiang, which cover the coastal lines over 2000 km (Supporting Information Fig. S1). In general, each city contained two sampling areas each about 2 km^2, except Zhanjiang with four sampling areas as this region was more intensively affected by the wilt disease than the other four cities. The collected samples included 31 from the diseased *Casuarina equisetifolia* trees showing the typical bacterial wilt symptoms, that is, brown bark and xylem tissues vertically from bottom to the top part of the tree trunk,[22] and 20 from healthy trees without wilt symptoms. A total of 644 representative bacterial isolates were isolated according to their colony morphologies from the trunk tissue samples of *Casuarina equisetifolia* trees. Among them, 47 isolates were from Huizhou city, 238 from Shanwei city, 57 from Yangjiang city, 56 from Maoming city, and 246 from Zhanjiang city (Table 1). Genetical analysis based on 16S rDNA sequences identified that these bacterial endophytes belong to 15 bacterial genera including *Arthrobacter*, *Bacillus*, *Burkholderia*, *Brachybacterium*, Enterobacter, *Escherichia*, *Fictibacillus*, *Gammaproteobacteria*, *Glutamicibater*, *Janibacter*, *Klebsiella*, *Pantoea*, *Paraburkholderia*, *Stenotrophomonas*, and *Xanthomonas* (Supporting Information Table S1). Among them, *Bacillus* and *Enterobacter* were the common bacterial genera presented in the *Casuarina equisetifolia* trees from all the five coastal cities (Table S1). In contrast, eight unique genera, including *Gammaproteobacteria*, *Paraburkholderia*, *Arthrobacter*, *Brachybacterium*, *Glutamicibater* and *Xanthomonas*, were found only in Shanwei, Yangjiang, and Maoming, respectively (Table S1). The largest genus of *Casuarina equisetifolia* endophyte is *Bacillus*, accounting for about 41%, which was followed by *Enterobacter* 19%, *Pantoea* 11%, *Stenotrophomonas* 9%, *Xanthomonas* 3%, *Klebsiella* 3%. The other nine genera including *Arthrobacter*, *Brachybacterium*, *Burkholderia*, *Escherichia*, *Fictibacillus*, *Paraburkholderia*, *Janibacter*, *Gammaproteobacteria*, *Glutamicibacter* were added up to 14% (Fig. 1(A)).

We then compared the bacterial genera isolated from the healthy and diseased *Casuarina equisetifolia* trees. A total of 8 and 12 bacterial genera were isolated from the healthy and diseased tree trunk tissues, respectively (Table S1). It was noted that among the eight bacterial genera found in the healthy *Casuarina equisetifolia* trees, *Brachybacterium*, *Fictibacillus* and *Janibacter* were the unique genera presented in the healthy trees (Table S2). However, 7 out of the 12 bacterial genera, that is, *Arthrobacter*, *Burkholderia*, *Gammaproteobacteria*, *Glutamicibater*, *Paraburkholderia*, *Stenotrophomonas* and *Xanthomonas* were presented in the diseased trees but not in the healthy trees (Table S2).

We then examined the six most frequently isolated bacterial genera to understand their relative proportions in the healthy and diseased *Casuarina equisetifolia* trees (Fig. 1(A)). Among the *Bacillus* isolates, which accounted for 41% of the total bacterial isolates, 30% of them were isolated from the diseased *Casuarina equisetifolia* trees, and 11% were isolated from the healthy *Casuarina equisetifolia* trees. In *Enterobacter*, about 14% of the isolates were from the diseased *Casuarina equisetifolia* trees, and 5% were from the healthy *Casuarina equisetifolia* trees. In *Pantoea*, 8% were isolated from the diseased *Casuarina*, and about 3% of the strains were isolated from the healthy *Casuarina*. In *Stenotrophomonas*, 4.5% were isolated from the diseased *Casuarina* and healthy *Casuarina* trees, respectively. In *Klebsiella*, 1% were isolated from the diseased *Casuarina*, and 2% were isolated from the healthy *Casuarina* samples (Fig. 1(A)).

3.2 Characterization of the bacterial isolates from the soil of *Casuarina equisetifolia* rhizosphere

Similarly, we set to characterize the bacterial isolates from the soil samples of *Casuarina equisetifolia* rhizosphere, which were collected and isolated in the same way as the bacterial endophytes described in the previous section. A total of 1195 representative bacterial isolates were obtained from the soil samples collected at the *Casuarina equisetifolia* rhizosphere in the five coastal cities of Guangdong Province (Table S3). The 16S rDNA analysis identified that these isolates represent 21 bacterial genera including *Arthrobacter*, *Bacillus*, *Brachybacterium*, *Burkholderia*, *Ensifer*, *Enterobacter*, *Escherichia*, *Flexibacter*, *Halomonas*, *Leifsonia*, *Lysinibacillus*, *Microbacterium*, *Paenibacillus*, *Paraburkholderia*, *Pantoea*, *Rhizobium*, *Pseudomonas*, *Sinorhizobium*, *Sphingomonas*, *Stenotrophomonas*, and *Streptomyces* (Table S3). Among them, there were three common genera, that is, *Bacillus*, *Stenotrophomonas* and *Streptomyces*, which were presented at the rhizosphere soil samples of *Casuarina equisetifolia* forest belts in the five coastal cities investigated in this study (Table S3). Interestingly, three unique genera including *Paenibacillus, Leifsonia*, and *Arthrobacter* were found only in the Zhanjiang city. (Fig. S1 and Table S3).

Table 1. The bacterial isolates collected from the Guangdong coastal regions of China

Location	Plant isolates	Soil isolates	Seawater isolates	Subtotal	*Ralstonia pseudosolanacearum*
Huizhou	47	303	51	401	0
Shanwei	238	199	45	482	4
Yangjiang	57	59	35	151	11
Maoming	56	121	18	195	9
Zhanjiang	246	513	66	825	7
Total	644	1195	215	2054	31

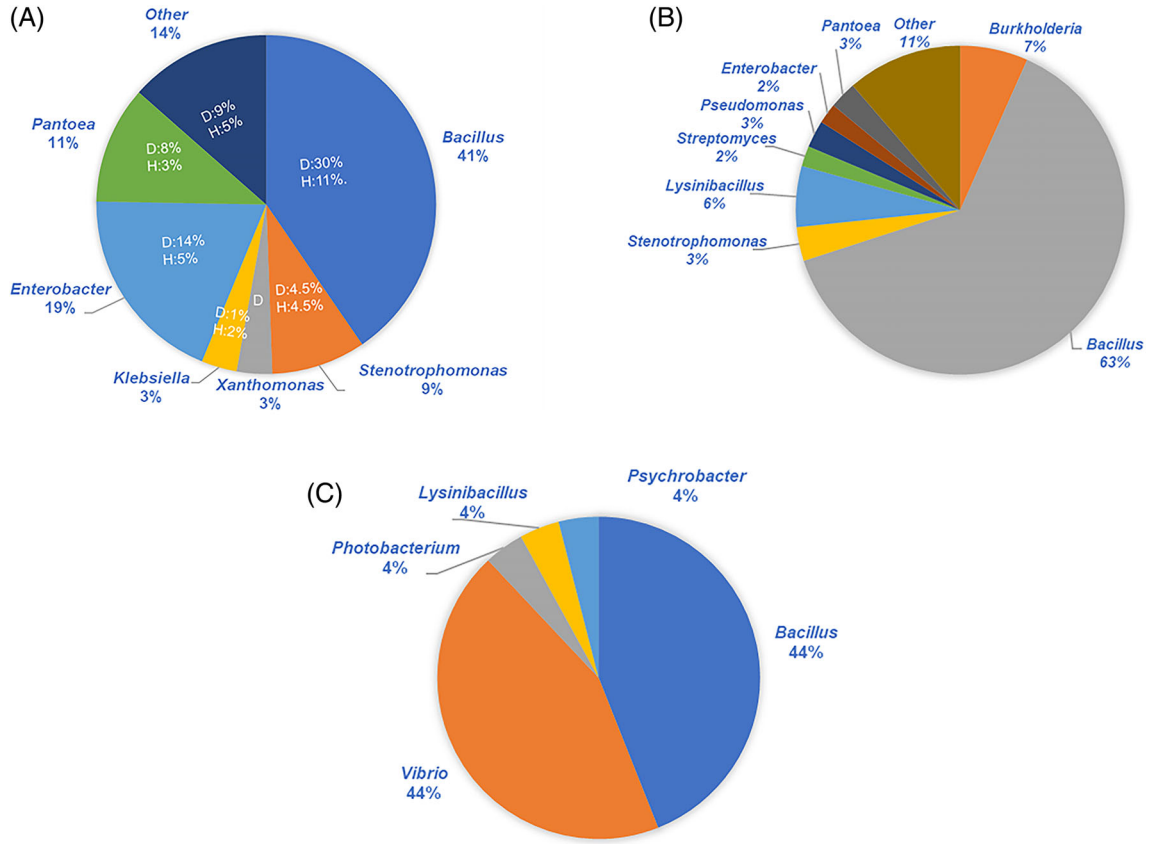

Figure 1. Microbial diversity in *Casuarina equisetifolia* trees, rhizosphere soils and nearby seawater. (A) Proportions of endophytic bacteria of different genera, and their proportions in healthy (H) and diseased (D) trees. Other groups include *Arthrobacter* sp., *Brachybacterium* sp., *Burkholderia* sp., *Escherichia* sp., *Fictibacillus* sp., *Paraburkholderia* sp., *Janibacter* sp., *Gammaproteobacteria* sp., and *Glutamicibacter* sp. (B) Proportions of different soil bacterial genera. Other groups include *Arthrobacter* sp., *Brachybacterium* sp., *Citrobacter* sp., *Halomonas* sp., *Leifsonia* sp., *Flexibacter* sp., *Rhizobium* sp., *Microbacterium* sp., *Sinorhizobium* sp., *Paenibacillus* sp., Paraburkholderia sp., *Sphingomonas* sp., and *Ensifer* sp. (C) Proportions of different seawater bacterial genera.

These bacterial isolates predominantly were *Bacillus*, reaching up to 63%, which were followed by 7% *Burkholderia*, 6% *Lysinibacillus*, 3% *Stenotrophomonas*, 3% *Pantoea*, 3% *Pseudomonas* and 2% for each of *Streptomyces* and *Enterobacter*, respectively (Fig. 1(B)). Interestingly, comparison of the data in Tables S1 and S3 showed that 7 out of the 21 bacterial genera found in the *Casuarina equisetifolia* trunk tissues were not presented in the rhizosphere soil, including *Fictibacillus*, *Gammaproteobacteria*, *Glutamicibater*, *Janibacter*, *Klebsiella*, *Paraburkholderia*, and *Xanthomonas*, whereas the remaining eight genera, that is, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Brachybacterium*, *Enterobacter*, *Escherichia*, *Pantoea*, and *Stenotrophomonas*, could be found in both tree trunk and rhizosphere soil. (Table S1), only *Brachybacterium* was presented in the healthy but not in the diseased tree trunk tissues (Table S2).

3.3 Characterization of the bacterial isolates from the coastal seawater

To understand the potential influence of marine microorganisms on the microbial flora of coastal *Casuarina equisetifolia* forest belt, the seawater samples were collected in the vicinity of a forest. A total of 215 representative bacterial isolates were collected based on colony morphology. Genetic analysis based on 16S rDNA sequence led to the identity of five bacterial genera, that is, *Bacillus*, *Lysinibacillus*, *Photobacterium*, *Psychrobacter*, and *Vibrio* (Table S4). Among them, *Bacillus* and *Vibrio* were commonly found in the five regions, while the rest three bacterial genera showed a distinct pattern of distribution associated with their geographical locations with *Lysinibacillus* and *Photobacterium* being common in the eastern cities Shanwei and Huizhou; *Lysinibacillus* and *Psychrobacter* being common in the middle

neighbouring cities Huizhou and Yangjiang, whereas the seawater of Maoming and Zhanjiang in the south coast contained only *Bacillus* and *Vibrio* (Fig. S1 and Table S4). Among them, *Bacillus* and *Vibrio* are the predominant bacterial genera with each accounting for about 44%. The remaining three genera recorded 4% each (Fig. 1(C)).

Comparison of the bacterial genera from tree trunk, rhizosphere and seawater showed that not all the identified marine bacterial genera could be found in the tree trunk tissues or the rhizosphere soil. Among them, it is only *Bacillus* that was commonly encountered in the *Casuarina equisetifolia* trees, rhizosphere soils and seawater samples from the five coastal regions (Tables S1, S3 and S4). In contrast, *Lysinibacillus* was locally presented in the rhizosphere soil samples from Maoming and Zhanjiang, and in the seawater of Shanwei, Huizhou and Yangjiang (Tables S3 and S4), whereas *Vibrio*, *Photobacterium* and *Psychrobacter* were found only in the seawater samples (Table S4).

3.4 Bacterial antagonistic activity against the *Casuarina* wilt pathogens

A total of 96 endophyte isolates, 221 rhizosphere microorganisms and 44 marine bacterial isolates were selected for testing their potential antagonistic activity against *Casuarina* wilt pathogenic strain YJA2 according to their colony morphology, genera identity and native region. The initial qualitative screening led to the identification of 72 bacterial isolates showing bactericidal activity, which were subjected to second round of semi-quantitative assay. Considering the notorious genetic and phenotype variations of the RSSC species, the initially identified 72 potential biocontrol agents were assayed semi-quantitatively against the four representative *R. pseudosolanacearum* strains isolated from four coastal regions including strains SWA6, MMB5, YJA2, XWI2 from Shanwei, Maoming, Yangjiang and Zhanjiang, respectively (Fig. S2).[22] Most of these isolates showed antimicrobial activity against at least three *R. pseudosolanacearum* strains, but there were also 21 isolates that could only antagonize one or two *R. pseudosolanacearum* strains (Table S5). Among these isolates, only ten showed high antagonistic activities with inhibition zone diameter $>$ 1 cm against all the four *R. pseudosolanacearum* pathogenic strains, including three endophytes, five soil microorganisms from rhizosphere samples and two marine bacterial isolates (Table 2 and Fig. 2). Genetic analysis based on 16S rDNA sequence similarity showed that these ten isolates were most similar to *Bacillus velezensis, Bacillus* sp., *Bacillus arachidis, Citrobacter farmeri*, *Pseudomonas mosselii*, *Enterobacterm aerogenes, Bacillus aryabhattai*, *Paenibacillus kribbensis*, *Bacillus siamensis* and *Bacillus* sp., respectively (Tables 2 and S6).

Strain A1-5 shared a high 16S rDNA sequence similarity to both *Bacillus velezensis* and *Bacillus amyloliquefaciens* (Table S6). For validation, we conducted phylogenic analyses based on the sequence of 16S rDNA, *gyrA* and *gyrB*. The results showed that strain A1-5 is closely clustered with *Bacillus amyloliquefaciens* only in the phylogenic tree of 16S rDNA, whereas in both the phylogenic trees of *gyrA* and *gyrB*, strain A1-5 is located next to *Bacillus velezensis* strains (Fig. S3). Together, we concluded that strain A1-5 is most likely to be a member of *Bacillus velezensis*.

3.5 Antagonistic effect of selected bacterial isolates against *R. pseudosolanacearum* pathogens

Given that RSSC infection is through the plant vascular system, we thought that endophytes might hold a great promise as biocontrol agents against the *Casuarina* wilt disease. Towards this end, we set to evaluate the biocontrol-related properties of two endophytes, that is, isolates A1-5 and A7-5, which showed strong antagonistic activities against four *R. pseudosolanacearum* strains from different coastal regions (Table 2 and Fig. 2). Considering that *Casuarina* trees normally grow at the sandy coastal regions with high temperatures in sunny days, the thermal stability of the antibacterial compounds produced by these endophytes were tested. The prepared endophyte culture extracts were placed in five temperature gradients, that is, 30, 60, 70, 80, 90 and 100 °C, for 30 min prior to assay of their inhibitory activity against *R. pseudosolanacearum* YJA2. The results showed that only isolate A1-5 still had strong antagonistic activity after treatment up to 100 °C, whereas the antagonistic activities of endophyte A7-5 was not thermal stable at 60 °C and above (Fig. 3).

We then evaluated the survival potential of endophyte A1-5 against other microorganisms ，as well as the mutual compatible potentials of the potential biocontrol agents. To this end, we conducted a mutual antagonistic assay using strain A1-5 with four isolates showing strong antibiotic-producing ability, including A9-7, B1-1, B1-16, and A4-28 from soil and seawater samples, which were tentatively identified as *Enterobacter aerogenes*, *Citrobacter farmeri*, *Pseudomonas mosselii*, and *Bacillus* sp., respectively (Table 2). The results showed that the endophyte A1-5 could withstand the antibiotics produced by other microorganisms and showed strong inhibitory effect against other tested isolates (Table S7). The marine isolate A4-28 could generate decent inhibition zones on isolates B1-1 and B1-16 but had no effect on the other three isolates (Table S7). These results suggest that strain A1-5 could win an upper hand against its microbial competitors and is suitable to be used as a sole biocontrol agent, whereas isolates B1-1 identified as *Citrobacter farmeri*, A9-7 identified as *Enterobacter aerogenes*, B1-16 identified as *Pseudomonas mosselii*, which did not interfere with each other in growth but could inhibit the growth of *R. pseudosolanacearum* (Fig. 2), may be used as a combination biocontrol agent for the control of the bacterial wilt. Interestingly, these three mutual compatible biocontrol bacterial species were all isolated from *Casuarina equisetifolia* rhizosphere soil samples, which for the convenience of discussion were referred as CEP combination in subsequent experiments.

3.6 Purification and characterization of the antibacterial substances from strain A1-5

To purify the active antimicrobial compounds from strain A1-5, the bacterium was grown in YEB plates for 2 days prior to extraction using ethyl acetate. A total of 25.54 g of residues were obtained after evaporation of the organic solution from the extracts, which were dissolved in methanol and separated by silica gel column chromatography by eluting with dichloromethane and methanol at a ratio of 100:0, 90:10, 80:20, 50:50, and 0:100, separately. The elutes were assayed against *R. pseudosolanacearum* and the active fractions were combined into three portions of similar volume designated as BV-1, BV-2 and BV-3. Bioassay results showed that BV-2 was the most active fraction in antagonizing *R. pseudosolanacearum*, followed by BV-1 and BV-3 (Fig. S4(A)). Removal of the organic solvents by evaporation obtained 10 g BV-1, 2.7 g BV-2, and 3 g BV-3 residues. BV-2 was further separated by gel column chromatography eluted with methanol, and 16 × 30 mL fractions were collected for bioassay. The results showed that the active compounds were mostly in two fractions named as BV-2-2 (0.49 g) and BV-2-3 (0.40 g) (Fig. S4(B)). HPLC analysis found that compared with fraction BV-2-3, fraction BV-2-2 contained less impurities (Fig. S5(A),

Table 2. The bacterial isolates showing strong antagonistic activity against *Ralstonia pseudosolanacearum*

Sample	Isolate	Location	Tentative species[†]	Sensitive *R. pseudosolanacearum* strains[‡]
Tree trunk	A1-5	Yangjiang	*Bacillus velezensis*	SWA6, MMB5, YJA2, XWI2
Tree trunk	A7-5	Shangwei	*Bacillus* sp.	SWA6, MMB5, YJA2, XWI2
Tree trunk	C3-5	Maoming	*Bacillus arachidis*	SWA6, MMB5, YJA2, XWI2
Soil	B1-1	Maoming	*Citrobacter farmeri*	SWA6, MMB5, YJA2, XWI2
Soil	B1-16	Maoming	*Pseudomonas mosselii*	SWA6, MMB5, YJA2, XWI2
Soil	A9-7	Shanwei	*Enterobacter aerogenes*	SWA6, MMB5, YJA2, XW12
Soil	G5-1-2	Zhanjiang	*Bacillus aryabhattai*	SWA6, MMB5, YJA2, XWI2
Soil	K2-9-1	Zhanjiang	*Paenibacillus kribbensis*	SWA6, MMB5, YJA2, XWI2
Seawater	C4-17	Maoming	*Bacillus siamensis*	SWA6, MMB5, YJA2, XWI2
Seawater	A4-28	Yangjiang	*Bacillus* sp.	SWA6, MMB5, YJA2, XWI2

[†] Tentative species is based on 16S rDNA sequence similarity more than 96%.
[‡] Sensitive strain was specified as the one showing an inhibition zone > 1 cm in diameter.

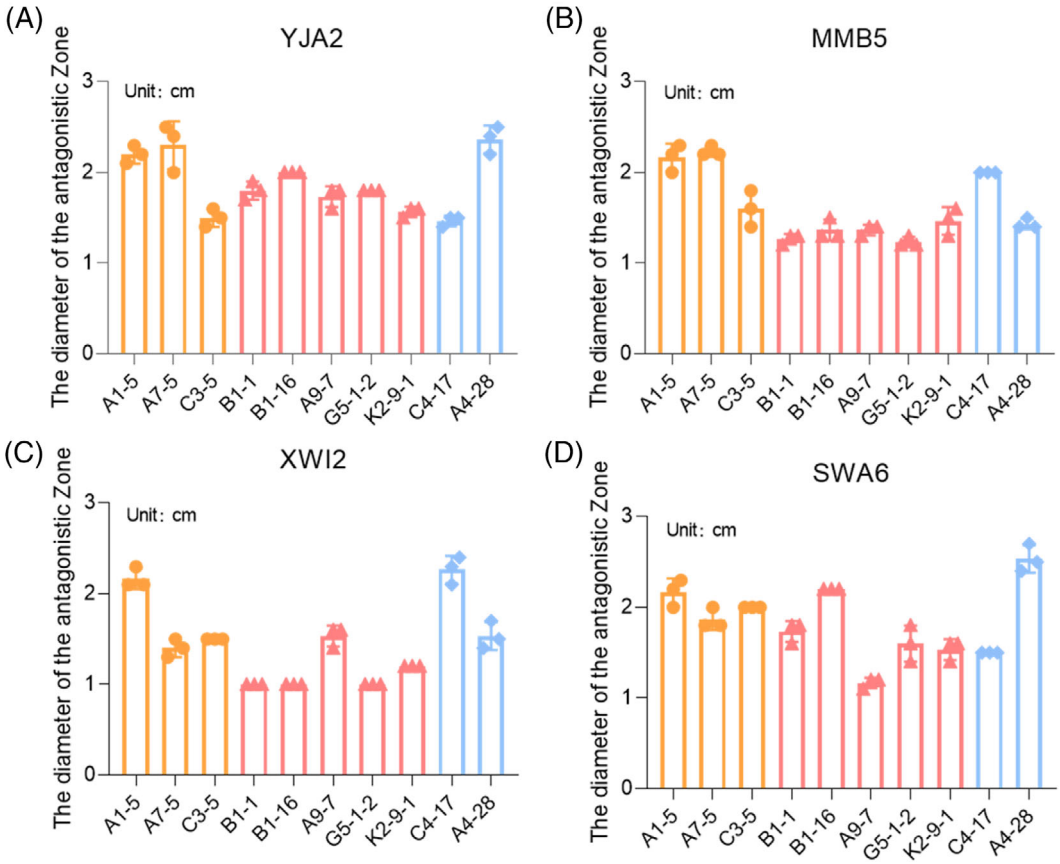

Figure 2. The antimicrobial activities of ten selected bacterial isolates against four representative *Ralstonia pseudosolanacearum* strains isolated from different coastal *Casuarina equisetifolia* forest belts. The experiment was repeated three times with three replicates each time. The data shown are the means with standard errors.

(B)). The fraction BV-2-2 was then used for further separation with a semi-preparative HPLC column, and nine distinct UV-absorbance peaks were obtained. Bioassay showed that there might be four antimicrobial active compounds contained in fractions BV-2-2-1, BV-2-2-4, BV-2-2-6, and BV-2-2-9, respectively (Fig. S5(C)). Purity analysis indicated that only fraction BV-2-2-9 of about 13 mg was of sufficient purity for structural analysis. The results of ^{1}H NMR and ^{13}C NMR analyses showed the molecule has 53 carbons, 93 hydrogens with a molecular mass of 1036, agreeable with a molecular formula of $C_{53}H_{93}N_7O_{13}$ (Fig. 4 and Table S8), which was identified previously as surfactin C from *Bacillus subtilis* and *Bacillus amyloliquefaciens*, respectively.[36–38]

3.7 Niche colonization and biocontrol potency of *Bacillus velezensis* A1-5 and the CEP combination

To test how well strain A1-5, which was isolated from the trunk tissue of *Casuarina equisetifolia* (Table 1), could invade and colonize at the different parts of *Casuarina equisetifolia*, the fresh culture

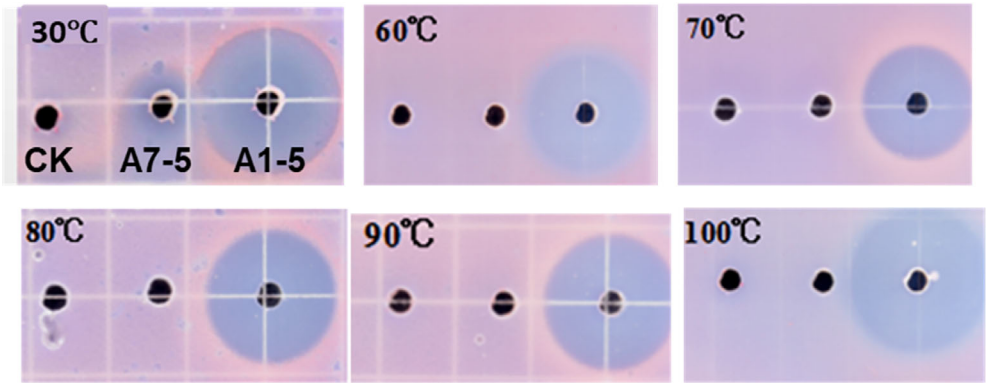

Figure 3. Thermal stability analysis of the antimicrobial compounds produced by two endophytes. The cell culture extracts of the endophytes were placed at various temperatures as indicated for 30 min before addition to the bioassay plates containing *Ralstonia pseudosolanacearum* cells. The experiment was repeated twice and the photographs show the representative bioassay plates.

solution of strain A1-5 expressing the *eGfp* gene was mixed with the soil in pots growing the *Casuarina equisetifolia* seedlings about 25–40 cm in height. On the third day after inoculation, the root, stem and needle-like branchlet tissues of *Casuarina equisetifolia* seedlings were observed with inverted fluorescence microscopy. The results showed that the endophyte could invade and colonize well in the root, stem and branchlet tissues of *Casuarina equisetifolia*. The endophyte cells were mainly distributed in the root hair cells and stem ductal cells, and partially in the stem epidermal cells (Fig. 5). In contrast, no fluorescence could be detected on the root and stem tissues of control *Casuarina equisetifolia* seedlings, which was mixed with the same amount of sterilized pure water (Fig. 5).

Given that the members of the CEP biocontrol combination were isolated from *Casuarina equisetifolia* rhizosphere soil samples, the CEP members as an individual or as a combination were then tested for their ability to antagonize the *Casuarina* wilt pathogen in a soil environment. The results showed that at 24 h post-inoculation, the CFU numbers of the wilt pathogen in all the treatments were significantly and similarly lower than the blank control without addition of biocontrol agent (Fig. 6(A)). However, the inhibitory effect of individual CEP member against the wilt pathogen were much weakened at 48 h and 72 h post-inoculation (Fig. 6(B),(C)). In contrast, addition of the CEP combination showed a steadily increasing inhibitory effect against the wilt pathogen along with the time post-inoculation, and hardily any *R. pseudosolanacearum* cell could be detected at 72 h post-inoculation (Fig. 6(A)–(C)).

To determine the biocontrol potency of strain A1-5 and CEP combination, *Casuarina equisetifolia* seedlings were co-inoculated with *R. pseudosolanacearum* YJA2 and the biocontrol agents. The results showed that co-inoculation of the biocontrol agent A1-5 with the wilt pathogen could significantly reduce the wilt disease severity. On day 14 post-inoculation, the wilt disease severity caused by strain YJA2 was as high as 95%, but when co-inoculated with the biocontrol agent A1-5, the disease severity was down to about 37% (Fig. 7(A)). In another set of experiments, the disease incited by strain YJA2 was about 84%, but in the case of co-inoculation with CEP, the disease severity was only about 26% (Fig. 7(B)). In both experiments, co-inoculation with the biocontrol agents, either strain A1-5 or CEP combination, could reduce the virulence of *R. pseudosolanacearum* YJA2 by about 58%.

We then tested the effect of pretreatment of plants with biocontrol agents before inoculation of the wilt pathogen. *Ralstonia pseudosolanacearum* YJA2 was inoculated 20 days after treatment of *Casuarina equisetifolia* seedlings with or without the biocontrol agents A1-5 or CEP combination, respectively. After inoculation of strain YJA2, the disease severity steadily increased and reached about 96% on day 14 post-inoculation, while pretreatment of either biocontrol agent A1-5 or CEP combination before inoculation of the pathogen showed disease severity at about 19%, decreasing the *Casuarina* bacterial wilt severity by about 77% (Fig. 8).

We also tested the biocontrol potential of strain A1-5 and CEP combination against the bacterial wilt infection on tomato by using the co-inoculation approach. On day 10 post-inoculation, the wilt disease severity incited by pathogen YJA2 in the absence of biocontrol agent was about 74% and 94%, respectively (Fig. S6 (A),(B)). In contrast, in the presence of biocontrol strain A1-5 and CEP combination, the wilt disease severity was merely about 12% and 30%, respectively (Fig. S1S6(A),(B)). These data indicate that similar to the case of *Casuarina equisetifolia*, application of the biocontrol agents A1-5 and CEP combination could significantly attenuate the pathogenicity of *R. pseudosolanacearum* YJA2 on tomato by about 62% and 64%, respectively.

4 DISCUSSION

Casuarina forest belt plays a critical role in withstand typhoon attack and protection of the ecosystems in the South China coastal regions, but recent outbreak of the bacterial wilt disease poses a great threat to *Casusrina* plantation.[4,22] Considering the wilt-resistant *Casuarina* clones selected and planted in 1980s has now became susceptible to RSSC infection,[22,30] in this study, we set to investigate the microbial resources in the *Casuarina equisetifolia* forest regions with an aim to identify effective biocontrol agents. A total of 2054 representative isolates were obtained from different samples in this study. According to the 16S rDNA results, there were 15 genera isolated from *Casuarina equisetifolia* sample, 21 genera isolated from soil samples and five genera isolated from seawater (Tables S1, S3, and S4). Among them, *Bacillus* appears to be most abundant genus and widely present in *Casuarina equisetifolia* trees, rhizosphere soils and seawater samples from the five coastal regions (Tables S1, S3, S4 and Fig. 1). In contrast, *Vibrio*, *Photobacterium* and *Psychrobacter* were found only in the seawater samples (Table S4). Interestingly, *Lysinibacillus* was locally presented in the rhizosphere soil samples from Maoming and Zhanjiang, which are in the southmost parts of the Guangdong coastal regions (Fig. S1), and in the seawater

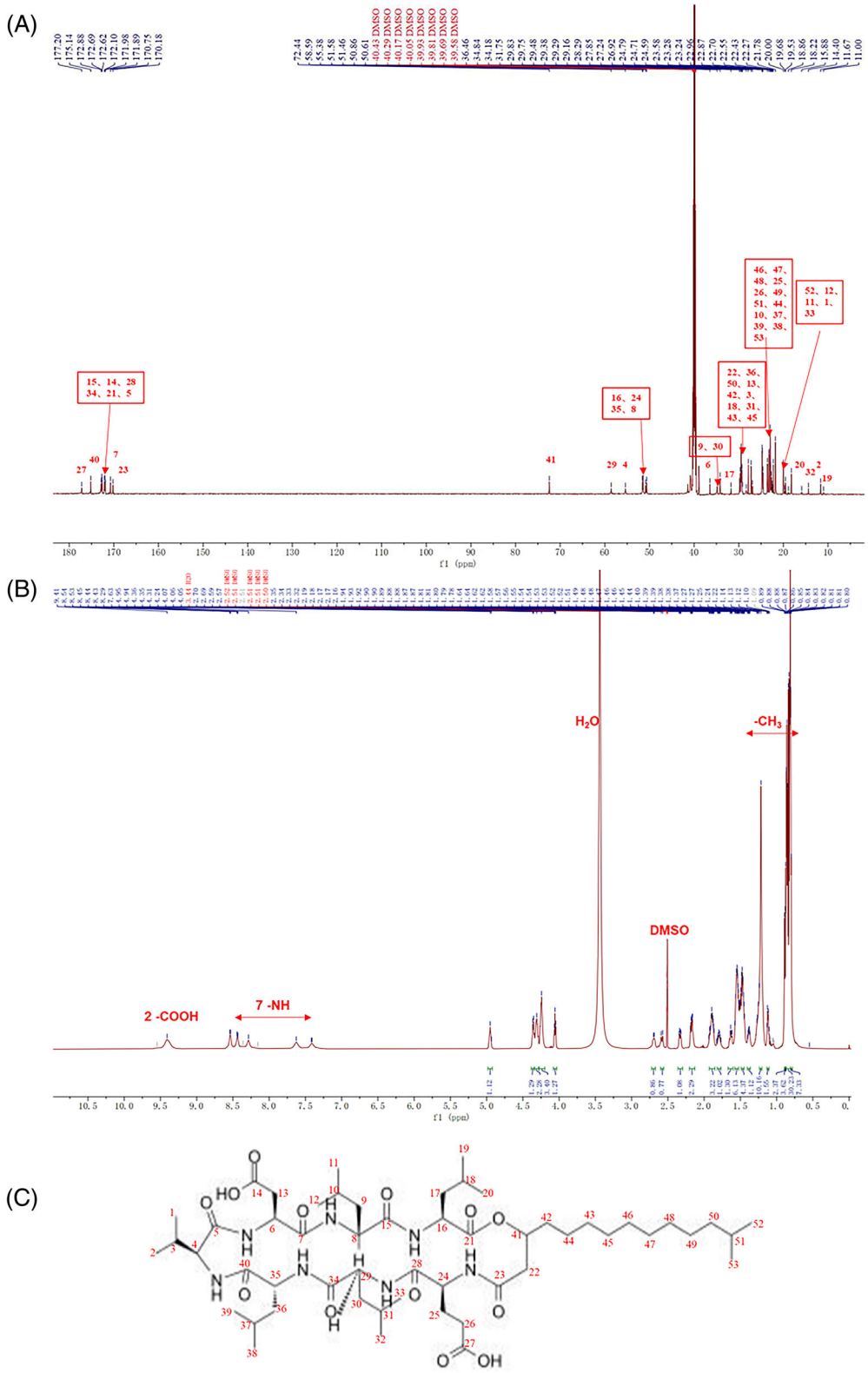

Figure 4. Characterization of the purified antibacterial compound from strain A1-5. (A) ^{13}C NMR spectrum of the active compound; the numbers in the figure represent this signal peak corresponding to the carbon in the compound. (B) ^{1}H NMR spectrum of the active compound. (C) Chemical structure of the active compound. To assign the ^{13}C NMR signals, each carbon in the structure of antibacterial compound was numbered.

of Shanwei, Huizhou and Yangjiang, which are at the northern areas relative to Maoming and Zhanjiang (Fig. S1, Tables S3 and S4). It is not clear at this stage whether this unusual distribution is related to ocean current flow direction or other factors. We also found that more bacterial genera were present in the diseased tree trunk tissues than the heathy tree tissues. In particular,

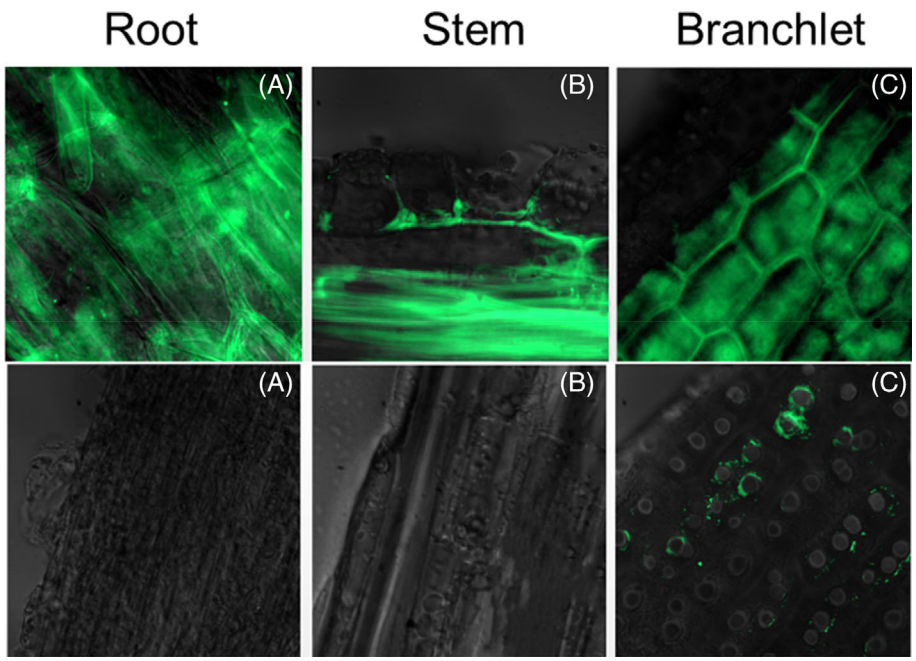

Figure 5. Colonization of endophyte strain A1-5 in *Casuarina equisetifolia* root (A), stem (B), and leaf-like branchlet (C) tissues. Strain A1-5 was labelled by expressing the *eGFP* gene carried in the expression construct pBBRI-mcs2-eGFP. Top panel, *Casuarina equisetifolia* seedlings inoculated with strain A1-5-eGFP. Bottom panel, control seedlings treated with sterilized pure water. The photographs were taken 3 days post-inoculation. The experiment was repeated twice with similar results.

8 out of the 15 endophyte bacterial genera were presented in the diseased trees but not in the healthy trees, whereas only *Brachybacterium, Fictibacillus* and *Janibacter* were the unique genera presented in the healthy trees (Table S2). Interestingly, a previous study showed that the soil with high abundance of *R. solanacearum* had low bacterial species diversity,[39] suggesting that *R. solanacearum* may hold a competitive advantage over other soil microorganisms. In contrast, our results seem to be agreeable with the notion that invasion of the wilt pathogen may compromise the plant immunity and hence facilitate colonization of more diverse bacterial species than the healthy host plants. It is also not clear yet at this stage whether the eight bacterial genera found only in diseased plants play a role as potential helpers of *R. solanacearum* infection as reported for the soil-transmitted bacterial blight,[40] These interesting findings may serve as useful clues for further investigation of the pathogenic mechanisms of RSSC against *Casuarina equisetifolia*.

There was hardly any investigation on the possibility of using microorganisms for biocontrol of *Casuarina* bacterial wilt disease, except one study showed that *Frankia* sp. could produce antimicrobial compound(s) against *R. pseudosolanacearum* and reduce the bacterial wilt death rate of *Casuarina* seedlings by about 17–28%.[41] In this study, we identified two types of potent biocontrol agents for the control of *Casuarina* bacterial wilt. The first one is the endophyte isolate A1-5 isolated from the diseased *Casuarina equisetifolia*. trees, which was identified as a member of *Bacillus velezensis* (Fig. S3). Strain A1-5 showed excellent competitive properties suitable for survival and flourishing in the sandy environment, which is typically dry and hot during day time, in the coastal regions of south China, including production of thermal-stable antimicrobial compounds (Figs 3 and 4), and desirable colonization ability *in planta* (Fig. 5). The second type is the CEP combination consisting of soil microorganisms *Citrobacter farmeri* B1-1, *Enterobacter aerogenes* A9-7, and *Pseudomonas mosselii* B1-16. Importantly, each of the CEP members could produce anti-*R. pseudosolanacearum* compound(s), but did not affect the growth of other CEP members (Fig. 2 and Table S7), suggesting that they can form a mutually compatible team to antagonize *R. pseudosolanacearum*. Biocontrol assay showed that pretreatment of *Casuarina* either strain A1-5 or CEP combination and co-inoculation of these biocontrol agents with *R. pseudosolanacearum* could significantly reduce the *Casuarina* wilt disease severity by about 77% and 58% (Figs 7 and 8), respectively. These two types of biocontrol agents could also be used for protection of other plants against *R. pseudosolanacearum* infections. Co-inoculation of strain A1-5 and CEP combination could significantly attenuate the pathogenicity of *R. pseudosolanacearum* YJA2 on tomato by about 62% and 64% (Fig. S6), respectively.

Bacillus velezensis genome contains 12 gene clusters encoding for biosynthesis of secondary metabolites, and ten bioactive metabolites were characterized including amylocyclicin, bacilysin, bacillomycin-D, bacillibactin, bacillaene, difficidin, fengycin, macrolactin, plantazolicin and surfactin.[42] Among them, iturin and fengycin from *Bacillus velezensis* might play important roles in defense against tomato wilting pathogen *R. solanacearum*.[43] Surfactin is a family of bioactive molecules of which contain a heptapeptide carrying a fatty acid side chain with variable chain length.[37] Several *Bacillus* species are known to produce surfactin molecules including *Bacillus subtilis, Bacillus velezensis*, and *Bacillus amyloliquefaciens*.[42] Among them, *Bacillus subtilis* are known to produce six surfactin derivatives A to F.[36,37] In this study, HPLC analysis showed that *Bacillus velezensis* A1-5 produced about eight molecules with antimicrobial activities (Fig. S5). Among them, one was purified and characterized as surfactin C (Figs 4 and S5(C)). Intriguingly, expression of the lipopeptide biosynthesis genes, including *srfAB*, *ituC*, and *fenD* for synthesis of surfactin, iturin, and fengycin, respectively, were drastically induced when

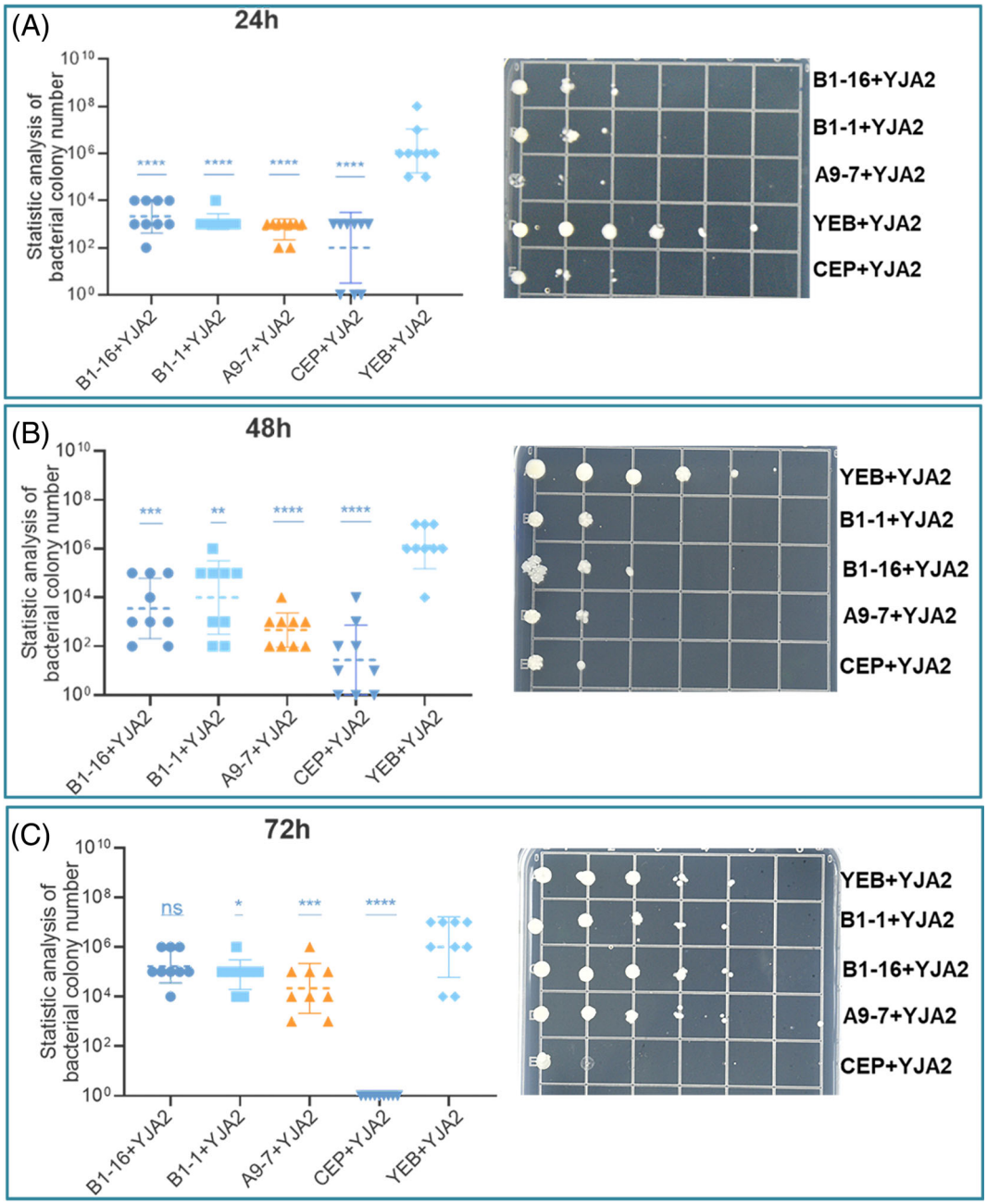

Figure 6. Impact of biocontrol agents on *Casuarina* wilt pathogen survival in soil. Panels show the cell numbers of strain YJA2 in the soil treated with CEP and its individual members, respectively, at 24 h (A), 48 h (B) and 72 h (C) post-inoculation. The experiments were repeated three times with three replicates, and YEB + YJA2 was used as an untreated control. Data are the means of three independent repeats and error bars are standard errors. Statistical significance: ****, *P*-value < 0.0001; ***, *P*-value = 0.0002 (B) or 0.0004 (C); **, *P*-value = 0.0211; *, *P*-value = 0.0211; ns, *P*-value = 0.1507 (ordinary one-way ANOVA).

Bacillus velezensis co-cultured with *R. solanacearum*.[43] Taken together, these findings suggest that *Bacillus velezensis* A1-5 could be a valuable resource for prevention and control of *Casuarina* wilt disease.

The CEP combination contains three soil bacterial species, which were tentatively identified as *Citrobacter farmeri*, *Enterobacter aerogenes*, and *Pseudomonas mosselii*, respectively. Among them, *Pseudomonas mosselii* BS011 from rice rhizosphere soil showed strong inhibitory activity against the rice blast fungus *Magnaporthe oryzae*.[44] To our knowledge, *Enterobacter aerogenes* has been not used previously for biocontrol of plant diseases. Interestingly, however, *Enterobacter aerogenes* is an endophytic bacterium and encode an AHL-lactonase.[45] There features should be very useful as *R. solanacearum* relies on AHL quorum sensing signals to regulate virulence gene expression and causes systemic infection through vascular system of host plants.[31] *Citrobacter farmeri* was isolated from bacterial wilt bananas,[46] but its biocontrol potential remains unknown until this study. *Pseudomonas mosselii*. is not clear yet whether that is effective against *R. solanacearum*. *Enterobacter aerogenes* were showed to produce

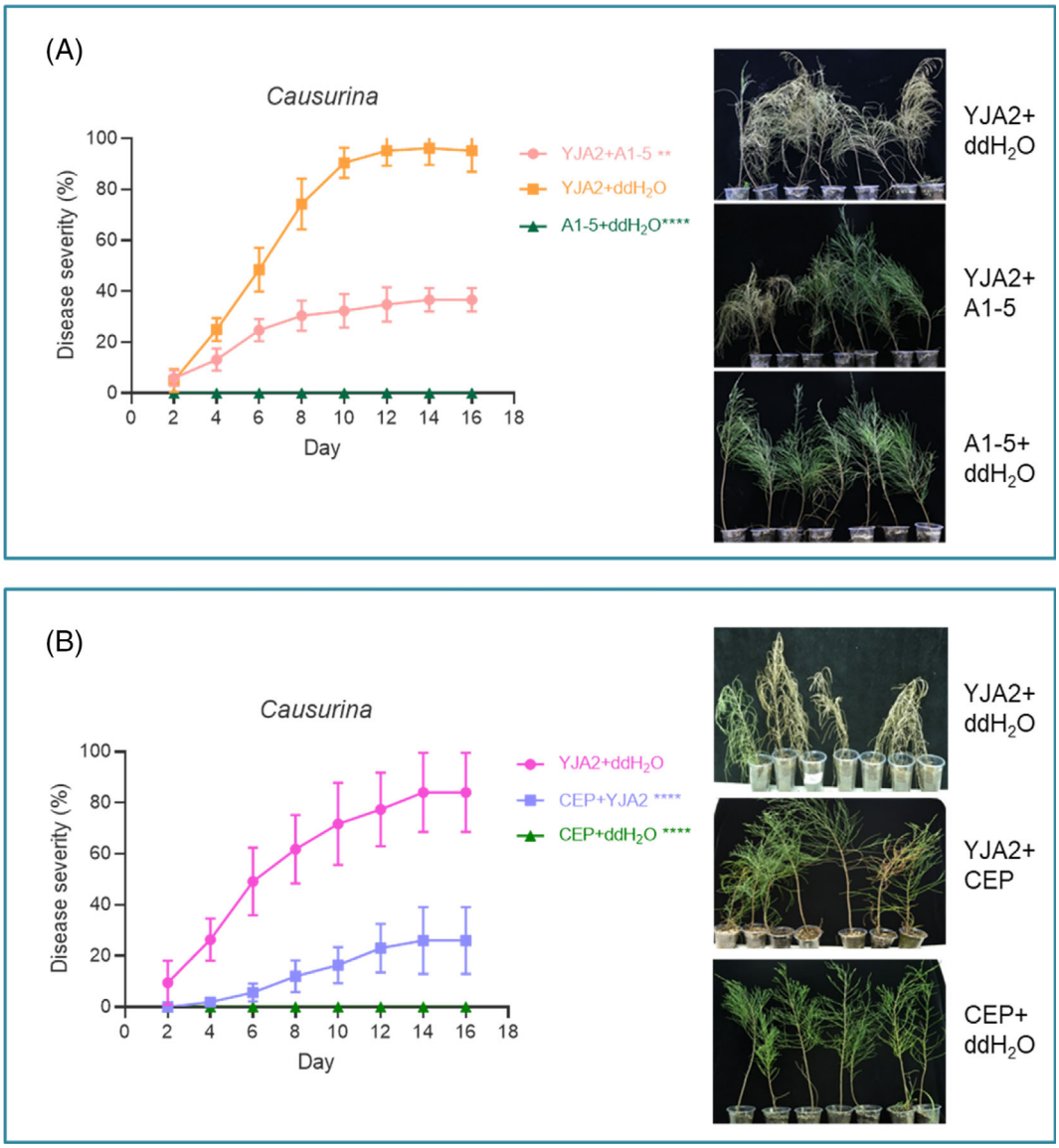

Figure 7. Biocontrol effect of endophyte strain A1-5 and combination biocontrol agents CEP on *Casuarina* bacterial wilt. (A) The effect of strain A1-5 on the *Casuarina* wilt caused by YJA2. (B) The effect of CEP on the *Casuarina* wilt caused by YJA2. The experiments were repeated three times with seven plants in each treatment. The photographs on the left of each panel show the representative plants in the biocontrol assay. In the analysis of disease severity, YJA2 + ddH_2O (double-distilled water) was set as the reference in the comparison with other groups. Data are the means of three independent repeats and error bars are standard errors. Statistical significance: **, *P*-value = 0.003. ****, *P*-value < 0.0001 (ordinary one-way ANOVA).

a few anti-fungal compounds by screening against *Phytophthora parasitica*.[47] Much remains to be done for understanding the biocontrol mechanisms of the CEP combination against *R. pseudosolanacearum* infection.

In summary, this study conducted a comprehensive investigation on the microbial diversity in the South China coastal *Casuarina* Forest belt over 2000 km. The results unveiled distinct bacterial genera associated with healthy and diseased *Casuarina* trees, particular geographical regions, and seawater. In addition, we also uncovered a range of bacterial isolates capable of producing antimicrobial compounds against the *Casuarina* wilt pathogens. These potential biocontrol agents could be valuable resources for further investigations. For example, the role of two antimicrobial endophytes isolated from the healthy *Casuarina* trees in protection against *R. pseudosolanacearum* infection is certainly worthy of in-depth investigation. They were capable of antagonizing four *R. pseudosolanacearum* strains (Table 2 and Fig. 2). Furthermore, we identified a potent biocontrol agent *Bacillus velezensis* A1-5 and the CEP biocontrol combination, which displayed highly promising potentials in the control of *Casuarina* and tomato wilt diseases in our glasshouse assay. Considering the potential impact of environmental variability and unavoidable interaction with native microbial communities and so forth, further studies are warranted to investigate the long-term efficacy of these biocontrol agents against the plant wilt infection under field conditions. Taken together, the findings from this study not only present valuable resources for the control of the plant bacterial wilt diseases, but may also provide useful clues for dissecting the pathogenic mechanisms of *R. pseudosolanacearum* in such an apparently complicated and highly competitive microbial ecosystems.

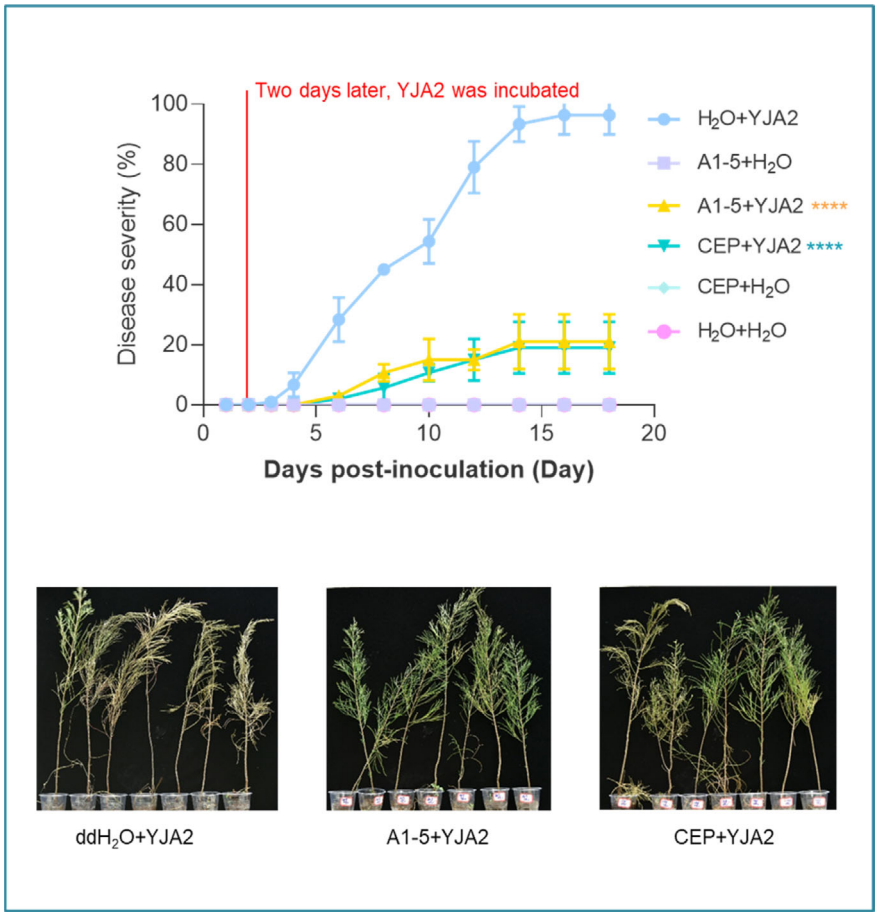

Figure 8. Effect of endophyte strain A1-5 and the combination biocontrol agent CEP pretreatment on biocontrol of *Casuarina* bacterial wilt. Two days after inoculation of strains A1-5 and CEP, the *Casuarina* seedlings were challenged with the pathogen. The experiments were repeated three times with seven plants in each treatment. The photographs on the bottom show the representative plants in the biocontrol assay. In the analysis of disease severity, YJA2 + ddH_2O (double-distilled water) was set as the reference in the comparison with other groups. Data are the means of three independent repeats and error bars are standard errors. Statistical significance: ****, *P*-value < 0.0001 (ordinary one-way ANOVA).

5 CONCLUSIONS

The results unveiled the dominant and common microbial species in *Casuarina equisetifolia* tree tissues, rhizosphere soils and the seawater, respectively, in the vicinity of the *Casuarina equisetifolia* forest belt. In particular, a *Casuarina* endophyte isolate A1-5, which was characterized as a member of *Bacillus velezensis*, and three mutually compatible soil isolates belonging to *Citrobacter farmeri*, *Enterobacter aerogenes*, and *Pseudomonas mosselii*, respectively (CEP), as a combination biocontrol agent for biocontrol analysis. The results showed that both strain A1-5 and the combination biocontrol agent CEP could effectively control the *R. pseudosolanacearum* infection on *Casuarina* and tomato, respectively. One active substance of strain A1-5 that inhibit the growth of *R. pseudosolanacearum* was purified and identified as surfactin C. Our findings may also provide novel insights into the pathogenic mechanisms of RSSC in the context of microbial competitions in soil environment.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

No competing interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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