RESEARCH ARTICLE

CHARACTERIZATION OF *Dickeya fangzhongdai* KPJ 1, THE CAUSATIVE AGENT OF SOFT ROT OF *Aglaonema* 'MARIA' AND ITS BIOLOGICAL CONTROL

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ABSTRACT

***Aglaonema* 'Maria' is a popular indoor plant, which is vulnerable to soft rot, a bacterial disease that causes serious losses in nurseries and is a major barrier in producing export-quality planting materials. Biocontrol agents are the best eco-sustainable alternatives for agrochemicals in managing plant diseases. This study was focused on isolating the causative agent of soft–rot disease of *Aglaonema* 'Maria' and evaluating the biocontrol potency of *Bacillus velezensis* strain DCJ 2 (DCJ 2) *in situ* under different delivery methods. The causative bacterium isolated from infected *Aglaonema* 'Maria' plants, coded as KPJ 1 was subjected to morphological, and biochemical, molecular and physiological characterization. The tests were performed in duplicate, indicating that the bacterium was positive for all of the tests that should be positive, except the oxidase test, which should be negative. The bacterium was tested to be gram-negative. Molecular characterization revealed that the KPJ 1 is *Dickeya fangzhongdai.* A pot experiment with healthy plants of *Aglaonema* 'Maria' was carried out to evaluate the biocontrol potency of DCJ 2 on KPJ 1 using a Completely Randomized Design with eight treatments and six replications. T1 and T2 represented untreated controls whereas the plants inoculated with only the pathogen (KPJ 1) considered as the negative controls [T3 (drench) and T4 (foliar spray)]. T5 and T6 represented the plants treated sequentially with KPJ 1 and DCJ as a soil drench. Similarly, foliar spray was performed in the T7 and T8. The data were analyzed using SAS software (version 9). Among both inoculum application protocols T5 and T6 (soil drench) showed the lowest disease severity (p=0.05). In conclusion, the antagonistic bacterium DCJ 2 demonstrated potent activity against soft-rot disease in *Aglaonema* caused by *D. fangzhongdai* which could be effectively used as an eco-friendly biological control agent in *Aglaonema* nurseries.**

Keywords: *Aglaonema* 'Maria', *Dickeya fangzhongdai* KPJ 1, *Bacillus velezensis* DCJ 2, soft-rot, biocontrol agent, soil drench, foliar spray

INTRODUCTION

Aglaonema species, usually Chinese evergreen, are herbaceous perennials grown in shady environments as potted indoor plants. *Aglaonema* 'Maria' belongs to the family Araceae, which has many horticulturally important genera. The most well-known species is *Aglaonema* 'Maria' which is commercially important as a foliage plant. It has a high value in the export market. Foreign exchange earned by Sri Lanka exporting foliage plants including *Aglaonema* 'Maria' was more than USS Mn 14 in 2022 (Subhashini *et al*., 2024). Over the years, epidemics of soft rot were observed on *Aglaonema* 'Maria' plants in export plant nurseries in Sri Lanka. The commercial production of *Aglaonema* 'Maria' is hampered by the 'soft-rot' disease caused by *Dickeya* species namely *Dickeya fangzhongdai* KPJ 1. The genus *Dickeya* which was previously known as *Erwinia chrysanthemi* has diverse isolates that cause soft-rot disease on diverse crops and ornamental plants species worldwide including economically important crops and ornamental plants, namely chrysanthemums, maize, *Euphorbia*

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pulcherrima, Dieffenbachia spp., *Philodendron* spp., bananas*, Saintpaulia ionantha*, *Aglaonema,* Dahlia, and carnations (EPPO, Data Sheet, 1982) where they cause typical wilting, black leg and soft-rot symptoms (Alic *et al*., 2018). High humidity and warm temperatures created ideal conditions that led to a high frequency of soft rot disease (Sudarsono *et al*., 2018). In warm humid climates, tissues become soft and rotted with a foul smell. Infected leaves show rot symptoms which are characterized by irregular water-soaked lesions. Phytopathogenic *Dickeya* species were previously classified as *Erwinia chrysanthemi* and then members of this species were elevated to the genus *Pectobacterium* (*P. chrysanthemi*) (Wei *et al.,* 2021; Gardan *et a*l., 2003; Burkholder *et al*., 1953). The genus *Dickeya* becomes reclassified from *Erwinia chrysanthemi* primarily based on host range, biochemical, and molecular analysis (Balamurugan *et al*., 2020). *Pectobacterium chrysanthemi* species was extended to the genus level and renamed *Dickeya* by Samson *et al.,* in 2005 and currently incorporates six genomic species: *Dickeya chrysanthemi*, *D. paradisiaca D. dadantii*, *D. dianthicola, D. zeae* and *D. dieffenbachia*. *D. solani,* which was a new *Dickeya* sp. found to be widespread on potatoes in Europe (Czajkowski *et al.,* 2014). The recently described species *Dickeya fangzhongdai*, is a newly emerging bacterial pathogen, that causes bleeding cankers in pear trees (Tian *et al*., 2016), soft rot disease in taro (Huang *et al*., 2021) peduncle soft rot on banana (Yang *et al*., 2022), and soft rot of onion (Ma *et al*., 2020). Further, soft rot disease of *Belamcanda chinensis* caused by *Dickeya fangzhongdai* (Yang *et al*., 2023), soft rot of *Banxia* (*Pinellia ternata*) (Wang *et al*., 2021); soft rot disease on *Dendrobium nobile* (Balamurugan *et al.,* 2020) are some more to mention. However, scientists have discovered that there are 12 *Dickeya* spp. distributed worldwide (Wolf *et al*., 2021).

In the last few years, outbreaks of soft rot were observed on *Aglaonema* 'Maria, plants in several export foliage nurseries in Sri Lanka having water-soaked lesions with a dark brown to black margin and a rotten smell. Hence, urgent attention was needed to identify the etiological agent of this disease and formulate control strategies. Chemical controlling is largely practiced by nurseries, but the large-scale application of toxic chemicals may cause environmental hazards, lead to the development of resistance in pathogen populations, and damage to non-target organisms (Li *et al*., 2020). Thus, there is a need for new solutions to curb plant diseases that provide effective control while minimizing negative consequences for human health and the environment (Elizebath *et al*., 1999). Biocontrol is one of the most effective and promising approaches for the control of soft rot and other plant diseases (Cui *et al*., 2019; Grady *et al*., 2019; Li *et al.,* 2020).

Biological control is an alternative to the breeding of resistant crops and chemical control of plant diseases and has been increasingly tested to control *Pectobacterium* and *Dickeya* pathogens, particularly using broad-spectrum antagonistic bacteria such as *Bacillus* and *Paenibacillus*, which produce multiple antimicrobial compounds against broad range of phytopathogens (Ying *et al*., 2022; Hossain *et al*., 2023; Liu *et al*., 2023).

Further, *Myxococcus* sp. strain BS showed to be a promising candidate to control various plant pathogenic bacteria, including *Pectobacterium carotovorum, Pseudomonas solanacearum, Erwinia amylovora, Dickeya chrysanthemi and Dickeya fangzhongdai* (Li *et al.,* 2018). Some bacteriophages isolated from variable host-range profiles are currently being utilized against putative novel *Dickeya* spp. (Alic *et al.,* 2017). Among biocontrol agents, many *Bacillus* species including *B. subtilis, B. amyloliquefaciens,* and *B. velezensis* (Wang *et al*., 2020) were employed to suppress the growth of bacterial and fungal plant pathogens. *Bacillus* species are among the most exploited beneficial bacteria as biopesticides. The most remarkable trait of *Bacillus* spp. is the ability to produce a wide variety of bioactive compounds valuable for agricultural applications, including metabolites with antimicrobial activity, surface-active, efficient colonization of plants and implicated in the induction of plant

defense responses (Akarapisan *et al*., 2020; Bonaterra *et al*., 2022). *B. velezensis* can also trigger systemic resistance in plants (Rabbee *et al*., 2019). Plant growth-promoting rhizobacteria (PGPR) has been a sought after niche for bacterial biocontrol agents. PGPR such as *Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus velezensis,* and *Paenibacillus polymyxa* were reported to produce different antibiotics, such as surfactin, bacillomycin, fengycin, iturin, 2,4-diacetylphloroglucinol, polymyxin and fusaricidin, which strongly inhibit the growth of pathogenic fungi and bacteria (Mekonnen *et al*., 2021). The principal mechanisms underlying plant growth promotion by PGPR in addition to disease control include stimulation of phytohormones, production of siderophores, antibiosis, solubilization and mobilization of phosphates, induction of systemic resistance against pathogens and inhibition of ethylene synthesis, etc. (Wang *et al*., 20; Mekonnen *et al*., 2022; Teixeira *et al*., 2021; Rabbee *et al*, 2019).

Bacillus velezensis is an endospore-forming bacterium that possesses the ability for rapid replication and tolerates adverse environmental conditions. It is widely distributed in various environments namely plant rhizosphere, plants, soil, water so forth and has the potential as a biopesticide against a broad spectrum of microbial pathogens of plants (Soad *et al*., 2005; Martínez–Álvarez *et al*., 2016; Grady *et al*., 2019; Wang *et al*., 2020; Hasan 2022; Xu *et al*., 2022). Generally, *Bacillus* spp. and *Pseudomonas* spp. are the most extensively studied beneficial microorganisms in the rhizosphere and *B. velezensis* can stimulate resident rhizosphere-beneficial microorganisms (Sun *et al*., 2022). Some *Bacillus velezensis* strains are: SK71 (Akarapisan *et al*., 2020); QST713 (Pandin *et al*., 2018); 9D-6 (Grady *et al*., 2019); FZB42T (Luo *et al*., 2019); ZF2 (Xu *et al*.,2020); HNH9 (Hasan *et al*., 2020 and2022); SQR9 (Sun *et al.,* 2021); CE 100 9 (Maung *et al*., 2022) and A6 & P42 (Chandrashekar *et al*., 2023). Beneficial plant−microbiome interactions including *B. velezensis* improve plant fitness through growth promotion, stress alleviation, and defense against pathogens through various mechanisms (Sun *et al*., 2022). Therefore, this study focused on the isolation and characterization of the causal organism associated with soft rot in *Aglaonema* 'Maria', the evaluation of the biocontrol efficacy of *Bacillus velezensis* DCJ 2 and the investigation of the effectiveness of different delivery methods of application of the bacterial inoculum for the control of soft rot disease.

MATERIALS AND METHODS

Isolation of the causative agent

The pathogenic bacterium was isolated from soft-rot lesions of nine samples of *Aglaonema* 'Maria' collected from an export-oriented foliage nursery located in Katana in the Gampaha District of the Western Province (7.24145^0 N and 79.88779^0 E) as per the method described by Lee *et al*., 2006 and Safi *et al*., 2020. Samples were placed separately in paper bags, appropriately labeled and immediately transported to the Plant Pathology Laboratory of National Plant Quarantine Services. The diseased leaves and stems were washed in running water and air-dried in a laminar air flow. Small segments of freshly invaded water-soaked tissues from the leading edge of lesions were cut into smaller pieces and placed in one milliliter of sterile water to release bacteria from the infected tissues. Next, one loop-full of the suspension was streaked onto Nutrient Agar (NA), Potato Dextrose Agar (PDA) and The NGM medium which is composed of nutrient agar plus glycerol and manganese chloride tetrahydrate. This medium is supplemented with $CaCl_2$. $2H_2O$, $MgCl_2$. $6H_2O$, and $MnCl_2$ $4H_2O$ to a final concentration of 2 mM to enhance the production of pigment (Lee *et al*., 2006) and plates were incubated for 3-4 days at 28^o C. The bacterial isolate obtained was coded as KPJ 1. Individual colonies were sub-cultured on NA plates, that displayed the best performance over other media. Bacterial isolates were further purified with repeated streaking onto fresh media. The isolates were stored in 30% glycerol at -80 °C for future use. In the meantime, an adequate number of

pure cultures were prepared from the purified single colonies for physiological, biochemical, and molecular characterization.

Morphological, physiological, and biochemical characterization of etiological agent KPJ 1

Pure cultures of the strain KPJ 1 were used for the identification of its morphological, physiological, and biochemical properties according to Bergey's Manual of Determinative Bacteriology and Vasundhara *et al*., 2017. Gram reactions were determined according to standard microbiological procedures. A series of biochemical and physiological tests and morphological characterizations were performed for identification and confirmation of the pathogen, that included treatment with 3% KOH, pathogenicity on carrot slices, growth at 37 ^{0}C, observation of cell shape under the inverted light microscope (Olympus IX73P1F; Mag. 60x10), nitrate reduction, gelatin liquefaction, oxidative fermentative test, indole production test, methyl red test, citrate utilization and sugar fermentation tests, sensitivity to erythromycin (50 µg/ml) and oxidase test. All the tests were conducted utilizing 2-day-old cultures of bacteria grown on NA. Selective media such as Crystal Violet Pectate (CVP) and NGM, were also used to investigate colony morphology. The pathogenicity of the isolate KPJ 1 was confirmed by performing Koch's postulates by re-inoculating a 50 -100 µl bacterial suspension having an inoculum density of 10^{8} CFU/mL amended with 0.05% Tween 20 (Sigma), onto mid veins of selected leaves of the *Aglaonema* plant in a pot using an injection cylinder with a needle. Similarly, the control plant was treated with sterile distilled water containing Tween 20. Immediately after inoculation, the pot was covered with transparent polyethylene to ensure a high humid atmosphere compatible with bacterial growth. Development of disease symptoms were observed by comparing with the control.

Molecular biological characterization of the isolate KPJ 1

For molecular biological confirmation of identity, bacterial genomic DNA was extracted as per the procedure described by Kate Wilson (2001) using the cetyl trimethyl ammonium bromide **(**CTAB) method. PCR was initially performed with a set of universal primers (27F/800R) targeting the 16SrRNA gene. Then it was further confirmed by the use of species-specific primers (Table 1).

DNA quantification and quality measurements were performed using the Genova Nano spectrophotometer (Genway, UK). All DNA samples recorded absorbance values between 1.8-2.0 at 260 nm and 280 nm wavelengths and DNA quantities greater than 50 ng/ µL were selected for Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction

Firstly, the analysis of 16S rDNA sequences of the pathogen associated with soft rot disease in *A.* 'Maria' was performed to confirm its identity. Furthermore, several more colonies were assessed using ADE1 and ADE2, and 5A/5B PCR (table 1) primers which are specific to the *Dickeya* genus and noted the production of the expected sizes of amplicons, 420 bp and 500 bp, respectively. Genus-specific oligonucleotide primer pairs namely 5A/5B designed by Chao *et al*., 2006 from the sequences of pT8-1, idg (a gene for blue-pigment synthesis), and pecS (a gene for regulation of pectinase, cellulose, and

Table 1: Primers used for the identification of KPJ 1

Primer designation	Primer sequence	Marker gene	Amplicon size (bp)	Reference
5A/5B	5′ GCGGTTGTTCACCAGGTGTTTT 3′ 5′ ATGCACGCTACCTGGAAGTAT 3′	pT8-1, idg, and pecS genes	500	Chao *et al.,* 2006
ADE1 / ADE2	5'-GATCAGAAAGCCCGCAGCCAGAT-3′ 5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3′	*pelADE* fragments	420	Nassar *et al.,* 1996
27F/800R	5'-AGAGTTTGATCMTGGCTCAG-3' 5'-TACCAGGGTATCTAATCC-3′	16SrRNA	733	Weisburg *et al*., 1991

pigment production) and ADE1 and ADE2 primer pair designed by Nassar *et al*., 1996 were used. Appropriate primers were employed to determine the expected amplicon sizes with Eppendorf™ Mastercycler™ Nexus Thermal Cycler, Germany as in the table 1.

The PCR reaction (50 µl) was composed of 10 µl 2x PCR buffer (Promega, Madison, USA), 1 µL of each primer (10 µM), 2 µL of $MgCl_2$ (25 mM) 1 µL of dNTP (10 mM), 1 µL of DNA (>50 ng/µL), 0.25 µL of Go Taq DNA polymerase (5U/µl). Nuclease-free water was added to volume up to 50 µL. PCR conditions for each primer are shown in Table2.

Table 2: PCR reaction process for each primer

Primer	5A/5B		27F/800R		ADE1/ADE2	
	PCR conditions					
Steps	**Temp**	**Time**	**Temp**	**Time**	**Temp**	**Time**
Initial denaturation	95 °C	2 min	94°C	5 min	95 °C	2 min
Denaturation	95 °C	15 sec	94°C	45 sec	95 °C	15 sec
Primer annealing	55 °C	20 sec	55°C	45 sec	55 °C	45 sec
Extension	72 °C	45 sec	72°C	1 min	72 °C	45 sec
Final extension	72 °C	7 min	72°C	10 min	72 °C	7 min
No. of cycles	35		35		25	

Agarose Gel electrophoresis for analysis of PCR products

The PCR amplified products were electrophoresed in 1X TAE buffered agarose gel (1%) at 80 V for 45 minutes in Blue gel electrophoresis system (Major science, MBE-150, UK) and amplified genomic fragments were stained using 1.2% Ethidium bromide. The bands were visualized through the gel documentation system with UV transilluminator (Vilber Lourmat, France) supported by vision-capt software.

Sequencing and analysis of sequenced results

After validation, the amplified products of PCR were sequenced by outsourcing to Macrogen, Korea. The BLASTn search tool (http://www.ncbi.nlm.nih.gov/blastn) was utilized for the comparison of the nucleotide sequences with the GenBank nucleotide database. MEGA version 11 (Tamura *et al*., 2021) was utilized for phylogeny and molecular evolutionary analyses and bootstrap values were set as 1,000 replications for analysis. Ultimately, all the sequences were deposited in the National Centre for Biotechnology Information (NCBI) Database for future annotations.

Selection of a biocontrol agent

A preliminary *in-vitro* screening was performed in this study by adopting a dual-culture assay described by Anith *et al*., 2021 with some modifications to investigate the antagonistic potential of 22 isolates, obtained from the *Aglaonema* 'Maria' rhizosphere to ascertain their comparative biocontrol efficiency against *Dickeya fangzhongdai* KPJ 1. The strain *Bacillus velezensis* DCJ 2 (GenBank accession numbers: OR542034, OR476022, OR568536) was found to be the best isolate indicating maximum growth inhibition of *Dickeya fangzhongdai* KPJ 1 (Subhashini *et al*., 2024) and hence was tested in pot experiments in the current study. The method described by Dong *et al*., 2021 with a few modifications was adopted for the isolation of the biocontrol agent. A composite sample of 10 g of soil obtained from the rhizosphere soil of *Aglaonema* 'Maria' plant was suspended in an Erlenmeyer flask containing 90 ml of sterile distilled water. This was shaken for 10 min and then serially diluted up to 10^{-8}. Then, 50 µL of each dilution was taken and streaked evenly on NA plates in duplicates and incubated upside down at 28°C for 24h. Single bacterial colonies growing on the media were selected according to morphology, color, transparency, and other characteristics, and subcultured for further testing. Similarly, rhizosphere soil of several *Aglaonema* 'Maria' plants was tested for the isolation of a promising bioagent.

Investigating the biocontrol–potential of DCJ 2 and assessing the effectiveness of different delivery methods for the control of soft rot disease

A pot experiment was conducted using one-month-old same-size healthy plants which were maintained in pots having the dimensions of 15 cm (diameter) X 45 cm (height), filled with 2.5 kg of soil. Sandy loam field soil was sieved using Tokyo Sekiya Testing Sieve with a pore size of 2.00 mm to remove root debris, air dried, and filled into the pots. The soil used for pot experiments was collected from a field site with a history of *Aglaonema* plants being grown. Plants were watered daily and fertilization was done monthly with Yaramila® fertilizer which is commonly utilized by foliage nurseries to encourage the healthy growth of plants. In this study, all pots were laid out in the greenhouse in a complete randomized design (CRD) with eight treatments (Table 3). Each treatment had six replicates.

cultures were harvested and dissolved in sterile water and adjusted to 10^8 CFU/ml using a spectrophotometer (Eppendrof-Biospectrometer). As per the growth curve studies, one-day-old bacteria were found in the exponential growth stage. Two delivery methods namely foliar spray and soil drenching were adopted in the experiment. One month after planting the surrounding rhizosphere soil was drenched with approximately 100 ml of the prepared inoculum. For the foliar application method, 100 ml of bacterial inoculum was applied onto the surface of all leaves of *Aglaonema* 'Maria' plants. Control treatments of healthy plants were inoculated either by spraying or soil drenching with sterilized distilled water. Different treatments with six replications arranged in a complete randomized design are shown in Table 3. Plants maintained in field soil without bacterial inoculation were set as unimmunized controls. Normally, plants were watered at two-day intervals.

Table 3: Treatment structure of the pot experiment

	Pathogen application		Biocontrol agent application		Sterile distilled water application (SDW)	
	Soil drench	Foliar spray	Soil drench	Foliar spray	Soil drench	Foliar spray
T1 (Control)	-	-	-	-	✓	-
T2 (Control	-	-	-	-	-	✓
T3	✓	-	-	-	-	-
T4	-	✓	-	-	-	-
T5	(1)	-	(2)	-	-	-
T6	(2)	-	(1)	-	-	-
T7	-	(1)	-	(2)		
T8	-	(2)	-	(1)		

Note: Bacteria were applied at the rate of 108 CFU/ml; (1) - applied first, (2) - applied one hour after the (1st) application

T1: Plants established in field soil (FS) and drench with sterile distilled water (SDW), T2: Plants established in FS and foliar application with SDW, T3: Plants established in FS and inoculated plants as a drench with the bacterial suspension (BS) of causal organism KPJ 1 (BSKPJ 1 108 CFU/ml), T4: Plants established in FS and inoculated plants as a foliar application with KPJ 1 108 CFU/ml, T5: Plants established in FS and drenched with BSKPJ 1 108 CFU/m and after one hour drenched with the suspension of Bio Control agent, DCJ 2 108 CFU/ml (BSDCJ 2 108 CFU/ml), T6: Plants established in FS and drenched with BSDCJ 2 108 CFU/ml and after one hour BSKPJ 1 108 CFU/ml, T7: Plants established in FS and foliar application with BSKPJ 1 108 CFU/ml and after one hour BSDCJ 2 108 CFU/ml, T8: Plants established in FS and foliar application with BSDCJ 2 108 CFU/ml and after one hour BSKPJ 1 108 CFU/ml

Before using both bacterium for treatment, the bacteria which were preserved at -80 ^{0}C were revived in sterilized soil (*in vitro*). The inoculum was prepared with a day-old bacterial culture grown on NA. The bacterial

The bacterial population was estimated by re-isolation from treated plants and soil with serial dilution and plating. Colonies were estimated by colony counting after incubation for 48 h 28 ^{0}C. Colonies that grew similarly to

the colonies isolated previously were considered to be the same as the inoculated strain. Further, confirmation was carried out through PCR analysis.

Disease severity (DS) was assessed with the help of the following formula and the scale given in table four (Rahma *et al.,* 2020; Safi *et al.,* 2020) with some modifications. DS was measured from 23.02.2023 to 29.05.2023 at 7-day intervals. Soft-rot disease symptoms could be traced from water-soaked lesions on *Aglaonema* leaves. Scoring was made for such lesions when the disease progressed and the measurements of the disease severity were then calculated using the formula based on the score as follows: Disease Severity of the disease was assessed by using a 0-5 scale devised below where 0 means no visible symptoms on foliage while a rating up to 5 has a different percentage of symptoms. Percent Disease Severity (DS) was then worked out using the equation.

$$\text{Disease Severity \%} = \sum \frac{\text{nb x 100}}{\text{N x T}}$$

Where n = Grade, b = number of infected leaves in each grade, N = number of grades used in the scale, T = total number of leaves used for scoring

Statistical analysis

All data were subjected to analysis of variance (ANOVA) by using the General Linear Model (GLM) procedure of SAS. Data were analyzed using SAS software (version 9 SAS Institute, Cary, NC, USA) and treatment means were separated by using the least significant difference (LSD) test (p=0.05) among the treatments. All the data were analyzed with the LSD values, at $P \leq 0.05$, by using the statistical software mentioned above. Variances were stabilized with square root transformation of data.

Table 4: Disease rating scale used to calculate disease severity of soft-rot of *Aglaonema* 'Maria'

Leaf area infected (%)	Grade
0	0
0-10	1
11-25	2
26-50	3
51-75	4
76-100	5

RESULTS AND DISCUSSION

Morphological characterization of the etiological agent

Strain KPJ 1 is a gram-negative bacteria and young colonies growing on nutrient agar were initially creamy white with smooth surfaces with an entire margin and turned grayish blue with irregular margins resembling to feathery appearance after 4-5 days of incubation at 28 ° C. The cells were rod-shaped and motile. Exhibited positive reactions for biochemical and physiological tests namely 3% KOH, pathogenicity on carrot slices, growth at 37 ^{0}C, nitrate reduction, gelatin liquefaction, oxidative fermentative test, indole production test, methyl red test, citrate utilization and fermentation and gas production of glucose, sensitivity to erythromycin (50 µg/ml) except oxidase reaction test (Table 5). Besides, pectate degradation on crystal violet pectate medium (CVP) and the development of a brownish to blue color on the NGM medium that consists of nutrient agar supplemented with 1% glycerol and 2 mM $MnCl_2$ $4H_2O$ was also observed. The isolate thrived well on the NGM medium developing dark brownish to blue pigmentation around colonies which made *Dickeya fangzhongdai* easily recognizable from other *Dickeya* spp. (Lee *et al*., 2006).

PCR amplification, gel electrophoresis, sequencing of amplified PCR products and phylogenetic analysis of the 16S rRNA, pT8-1, idg, pecS and *pelADE* genes

PCR amplification of the 16S rRNA gene of the bacterial isolate produced a typical 733bp band (Figure 1) which was further purified and sequenced to identify the isolate KPJ 1 down to the species level. Similarly, PCR amplification was conducted targeting pT8-1, idg, and pecS and *pelADE* genes of *D. fangzhongdai* for reconfirmation of the isolate. PCR amplification of these genes produced bands at the expected level of 500 bp and 420bp, respectively (Figure 2 and 3). The resultant consensus sequences were

Table 5: Physiological and biochemical characteristics of strain *D. fangzhongdai* KPJ 1

Characteristic	Observation
3% KOH	+ Solution- become viscous and form a mucoid string
Gram stain	- Pink
Pathogenicity on carrot slicers	+ Rotten
Growth at 37 ^{0}C	
Cell shape observed under the microscope (60x10)	Rod
Oxidative Fermentative test	+ Green to yellow
Indole production test	+ Formation of the red color ring on the top of media
Gelatin liquefaction	+ Liquefied gelatin
Nitrate reduction	+ Color change pale yellow cherry red color
Citrate utilization	+ Color change to blue
Sensitivity to erythromycin (50 µg/ml)	+ Inhibition zones around the erythromycin discs
Oxidase test	-
Pectate degradation on CVP	+ Formation of shallow pits on medium
Blue pigment on NGM	+ Production of blue color pigment on the medium
Fermentation and gas formation of glucose	+ Media turn yellow to red and production of gas
Pathogenicity test	+ Observed soft rot lesions on the inoculated area

"+" indicates positive response; "–" indicates negative response

blasted against the publicly available gene database at NCBI to confirm their specificity to *D. fangzhongdai*. The causal organism was highly homologous to *D. fangzhongdai* strains sharing the same query coverage and percentage identity mentioned in the table suggesting a close genetic relationship of soft rot causal organism with *D. fangzhongdai.*

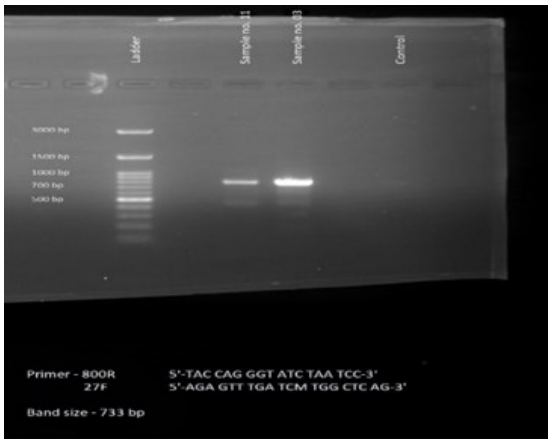

Figure 1: Agarose gel electrophoresis of bacterial pathogen DNA amplified with 27F/800R (733 bp) primers

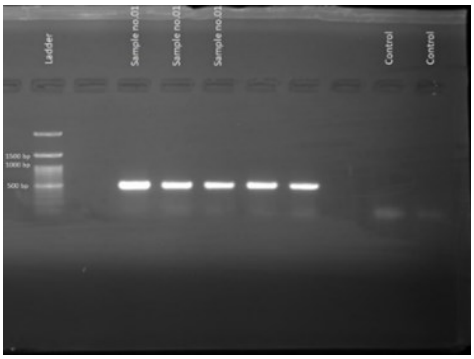

Figure 2: Agarose gel electrophoresis of bacterial pathogen DNA amplified with 5A/5B (500 bp) primers.

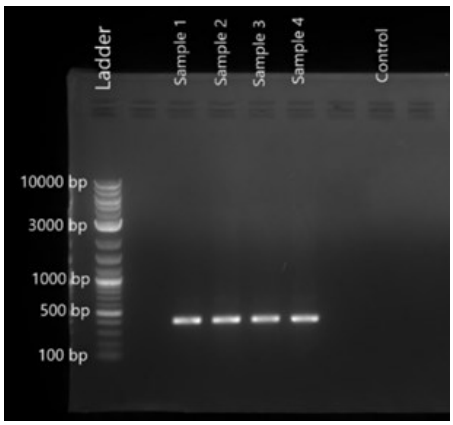

Figure 3: Agarose gel electrophoresis of bacterial pathogen DNA amplified with ADE1 / ADE2 (420 bp) primer.

Table 6: Identified organisms based on genomic DNA sequencing data

Primers	Identified organism	Accession No	Percentage identity	Query coverage (%)	E value
27F/800R	*Dickeya fangzhongdai* strain JS5	NR_151914.1	99.73	99	0
5A/5B	*Dickeya fangzhongdai* strain 643b	CP092458.1	100	100	0
ADE1/ADE2	*D. fangzhongdai* strain YZY-SG-17	OL855840.1	93.75	100	0

Phylogenetic analysis

Figure 4 exhibited the evolutionary relationships of the 16S rRNA sequence of KPJ 1 associated with soft rot disease of *Aglaonema* 'Maria' and 10 16S rDNA accessions available in the DNA database at NCBI. The evolutionary relationships were worked out using the Maximum likelihood method. The evolutionary analysis was conducted in MEGA11 (Tamura *et al*., 2021). Furthermore, the 16SrRNA sequence of strain KPJ 1 (GenBank accession number OR476021) showed 99% homology to that of *D. fangzhongdai* strain JS5 (GenBank accession number NR 151914.1) among other *Dickeya* spp. The scale bar represents 0.010 nucleotide substitutions per site.

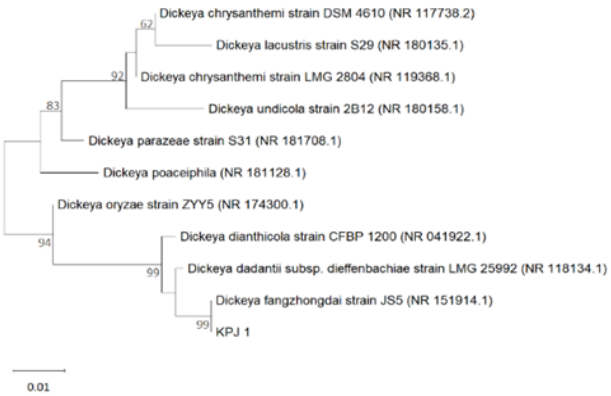

Figure 4: Maximum likelihood Phylogenetic tree constructed from 16SrRNA gene

The phylogenetic tree is based on comparative pT8-1, id g, and pecS genes of isolate KPJ 1 (GenBank accession number OR542035) on available reference sequences from GenBank. The phylogenetic tree showed that strain KPJ 1 clustered with *D. fangzhongdai Onc5 (*GenBank accession number *CPO80400.1)*, *D. fangzhongdai B16 (*GenBank accession number CPO87226.1*) and D. fangzhongdai 643b (CPO92438.1)* 100% identities to several records of *Dickeya fangzhongdai* deposited in NCBI GenBank based on BLAST analysis. Thus, *Dickeya* sp. KPJ 1 was identified as *D. fangzhongdai* (Figure 5).

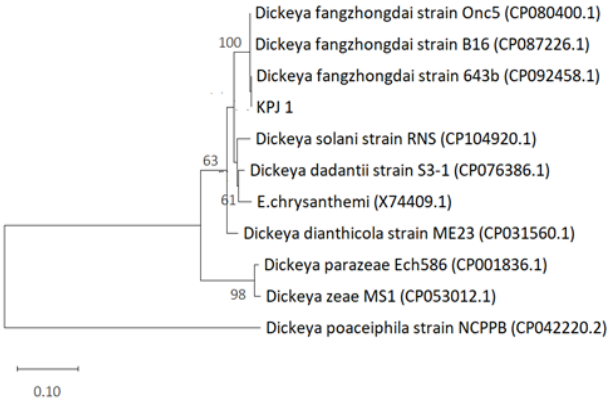

Figure 5: Maximum likelihood Phylogenetic tree constructed from pT8-1, id g, and pecS genes

The *pelADE* gene sequence of strain KPJ 1 (GenBank accession number OR568537) showed 98% homology to that of *D. fangzhongdai Onc5 (*GenBank accession number *CPO80400.1)*), *D. fangzhongdai 643b (CPO92438.1)* and *D. fangzhongdai YZY-SG-17(OL855840.1)* (Figure 6).

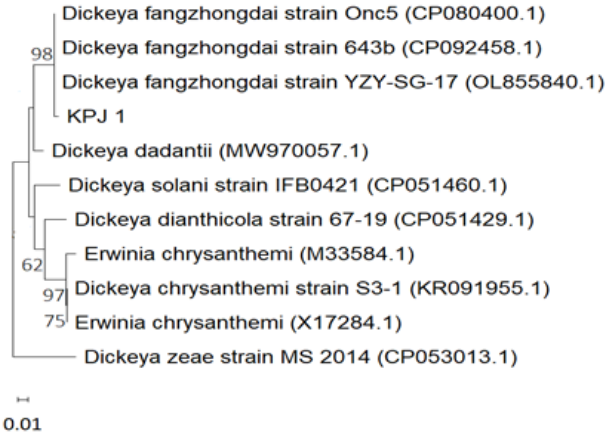

Figure 6: Maximum likelihood Phylogenetic tree constructed from *pelADE* gene

Based on the sequence homology of 16S rRNA, pT8-1, idg, and pecS and *pelADE* genes of KPJ 1, BLASTn analysis in the NCBI database unveiled the similarity between strain KPJ 1 and *Dickeya fangzhongdai* was 99%, 100% and 98%, respectively. Validation of the isolate's pathogenicity by inoculating the leaves of *Aglaonema* 'Maria' plants confirmed the pathogenic nature of the bacterium as typical soft-rot lesions ranging from 3-7 mm in diameter were observed within 24 hours of post-inoculation, fulfilling Koch's postulates. However, leaves inoculated with distilled water remained unchanged.

Appearance of zone of inhibition was noticeable around KPJ 1where three bands were streaked parallel to each other in two plates, with DCJ 2 in the center and KPJ 1 on either side of DCJ 2, and vice versa. Visualization of the clear zone around KCJ 1 after 24 h of incubation in dual culture plate assay indicating the inhibitory activity of *Bacillus velezensis* DCJ 2 against KPJ 1 (Figure 7).

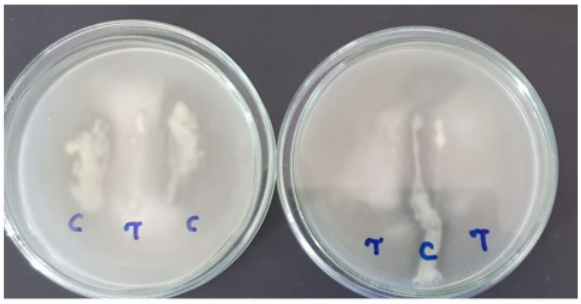

Figure 7: Dual culture assay of *Bacillus velezensis* (T) isolated from the *A.* 'Maria' rhizosphere against KPJ 1(C)

As per the results of the pot experiment, the application of both bacteria suspensions as a soil drench in either way (Treatment 5 and 6) exhibited a comparatively lower disease severity at p=0.05 as compared with inoculated plants with KPJ 1 as the soil drench or foliar spray (Table 7). However, there is no significant difference in disease severity between treatment 5 and 6 at p=0.05. Plants that were treated with bacterial suspension as a soil drench exhibited lower disease severity over foliar spray. This is expected as the pathogen infects the leaves and foliar spray delivers the pathogen to its niche. In addition, the results suggested that foliar spray with DCJ 2 to healthy plants of *Aglonema* 'Maria' before developing the pathogen can significantly reduce the disease severity (T8) which may be due to the niche colonization and competitive exclusion of the pathogen by the biocontrol agent. Eventually, this study revealed that the introduction of DCJ 2 to the *Aglaonema* 'Maria' plants by any means can successfully control the disease. Controls (T1 and T2) sprayed with sterilized distilled water (SDW) remained unchanged whereas pathogen-inoculated controls (T3 and T4) produced high disease severity. All treatments showed a significant rot reduction compared to the control. Thus, *B. velezensis* DCJ 2 which demonstrated potent antagonist activity against soft-rot in pot experiment could be effectively used as an eco-friendly biological agent to mitigate the bacterial soft rot caused by *Dickeya fangzhongdai* KPJ 1 in nurseries where *Aglaonema* 'Maria' plants are being mainly maintained in pots.

Table 7: Severity of soft-rot disease of *Aglaonema* 'Maria' after *Bacillus velezensis* DCJ 2 treatment

	Treatment	Mean Disease Severity (%)[1]
1	Plants drench with sterile distilled water (SDW)	0.00 (0.71^{e})
2	Foliar application with SDW	0.00 (0.71^{e})
3	Plants drench with the BS of the causal organism (KPJ1)[2]	12.64(3.37^{a})
4	Foliar application with BS of the causal organism (KPJ1)[2]	16.56(3.47^{a})
5	Soil drench with KPJ 1 and after one hour drench with BS of Bio Control Agent, DCJ 2[2]	**0.60(0.91^{de})**
6	Soil drench with DCJ 2[2] and after one hour KPJ 1[2]	**1.83(1.15^{d})**
7	Foliar application with KPJ 1[2] and after one hour DCJ 2[2]	8.28(2.21^{b})
8	Foliar application with DCJ[2] and after one hour KPJ 1[2]	3.36(1.52^{c})

CV= 1.96, means with the same letter do not differ significantly at P ≤ 0.05 using the LSD test

1 Average of six replications; 2 108 CFU/ml; BS-Bacterial Suspension; Transformed data are within parentheses.

Furthermore, without an appropriate management strategy, using an antagonist alone will not provide stable biological control against soil-borne diseases. This is because the introduced biocontrol agents compete for niches and nutrients with other native microbes for their survival (Wright *et al*., 2018; Cui *et al*., 2019; Balla *et al*., 2021 and Bamisile *et al*., 2021).

Soft rot of plants is a major issue that harms crops severely throughout the world and causes critical crop damage and reduces the quality of plant products to varying degrees based on the host, climate, cultivars, etc. (Akarapisan *et al.,* 2020). The present study was undertaken to investigate the biocontrol potential of the bacteria isolated from the rhizospheric soil of *Aglaonema* 'Maria' plants grown in a nursery located in Gampaha District against soft-rot disease.

Using beneficial bacteria like *B. velezensis* for biological control in agriculture is becoming more widespread nowadays (Akarapisan *et al* 2020). The production of antimicrobial compounds by *Bacillus* spp including strain DCJ 2 (Jiang *et al*., 2015; Singhe *et al*., 2016; Liu *et al*., 2022) may be not only the reason for the inhibition of I KPJ 1's growth, despite this theory not being tested in this investigation and but also due to inducing systemic resistance (ISR) against pathogens (Mekonnen and Kibret, 2021). Therefore, a combination of the above two factors has led to the control of the disease. In addition to pathogen control, numerous studies demonstrate that *Bacillus* species can boost plant growth by producing gibberellins, indole acetic acid, and cytokinins, among other plant growth hormones (Teixeira *et al*., 2021). Taking into account of beneficial characteristics like inducing systemic resistance and inhibitory ability against a broad spectrum of microbial pathogens and boosting plant growth, *B. velezensis* can be effectively employed as a potential biocontrol agent (Singhe *et al.,* 2016; Hassan *et al*., 2019; Tao *et al*., 2019; Wang *et al*., 2020; Teixeira *et al*., 2021; Liu *et al.,* 2022). The *Bacillus* spp. along with *Bacillus velezensis* possess greater advantages over other bacterial antagonist genera since they can withstand higher temperatures and are resistant to desiccation due to the formation of endospores and the ability to promote plant growth as well. Therefore, its potential for commercial development as a biocontrol agent is enhanced by the above-mentioned characteristics (Grady *et al*., 2019). In addition to its capacity to suppress disease and stimulate plant growth, the antagonist *B. velezensis* has other benefits, including non-toxicity to humans, animals, and the environment, as well as simplicity of application for farmers. (Whipps, 2001; Almoneafy *et al*., 2012).

CONCLUSIONS

Phylogenetic reconstructions, 16S rRNA sequence analysis, and morphological, biochemical, and physiological reactions, served as the basis for the identification of the pathogen KPJ 1 as *Dickeya fangzhongdai*. *B. velezensis* DCJ 2 showed high antagonistic activity against the pathogen in pot experiments displaying better ability in the biocontrol of the soft rot disease caused by *D. fangzhongdaiI.* In addition, the application of *B. velezensis* DCJ 2 as a soil drench proved to reduce soft-rot in *Aglaonema* 'Maria' significantly. Therefore, it might work well as a plant disease management technique in the future. However, to guarantee a precise dosage and reaction, it is still necessary to evaluate in different locations and different seasons.

AUTHOR CONTRIBUTION

MHAD conceptualized and designed the study. MHAD conducted the experiments. DDE and IRT were supported to perform the experiment. MHAD analyzed the data and CM instructed for the analysis MHAD wrote the original draft. CM and DMJB commented on and revised the manuscript.

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