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Morpho-physiological and molecular characteristics of bacteria causing stalk rot disease on corn in Gorontalo, Indonesia

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Abstract. *Suriani, Patandjengi B, Muis A, Junaid M, Mirsam H, Azrai M. 2023. Morpho-physiological and molecular characteristics of bacteria causing stalk rot disease on corn in Gorontalo, Indonesia. Biodiversitas 24: 1749-1758.* Stalk rot disease was observed in corn in Gorontalo with typical symptoms, such as soft rot on the stalk, leaf wilting, and plant death. This study aimed to characterize the bacteria causing stalk rot disease in corn. Samples of infected plants were collected and identified morphologically, physiologically, and molecularly. The results showed that nine bacterial isolates were isolated from infected plants. All nine isolates showed positive hypersensitive responses on tobacco leaves. In comparison, only two bacterial isolates (BGO1 and BGO4) were positive on pathogenicity tests on corn. However, the BGO4 isolate caused the highest disease incidence with a faster incubation period. The BGO4 isolate was gram-negative with white-gray colored colonies. Physiological characterization of BGO4 also showed: positive catalase and indole, oxidase negative, fermentative oxidation, caused soft rot on potato, non-fluorescent, and sensitive to erythromycin. In addition, it can grow at 37-40°C and 5% NaCl, producing protease and lecithinase enzymes. The BGO4 also isolates infected rice, corn, sorghum, foxtail millet, celery, and *Aloe vera*. Morpho-physiology characteristics and diagnostic amplification of DNA by PCR using the *Dickeya*-specific primers (ADE1/ADE2) showed that the isolate belongs to the genus *Dickeya*. Further molecular characterization by analysis of the 16S rDNA using universal primer 27F/1497R successfully amplified the DNA band of BGO4 isolate measuring ±1300 bp. Phylogenetic analysis showed that it was in the same group as *Dickeya zeae* strain MS32 from Taiwan, strain DZ15SB01 (Thailand), and strain HNJF02 (China), with the coefficient of genetic distance ranging from 0.001 to 0.002. This study is the first report of *D. zeae* infecting corn in Gorontalo.

Keywords: Corn, *Dickeya zeae*, pathogen characterization, stalk rot

INTRODUCTION

Corn is one of the priority crops in Indonesia because it is used as food and feed. The challenge is the future strategy to meet the demand for corn as a feed, food, and energy raw materials. In addition, the increase in corn production in Indonesia is 5.21% per year (Panikkai et al. 2017), with the productivity in corn planting centers ranging around 5-9 t/ha. Therefore, the government continues to make various efforts to increase national corn production, especially in corn production centers such as East Java, South Sulawesi, Gorontalo, North Sumatra, and Lampung. For example, leading agro development in Gorontalo Province selects superior commodities in the corn processing industry in the food agriculture sector and coconut in the plantation sector as stated in the Regulation of the Minister of Industry No. 98/MIND/PER/8/2010 concerning Roadmap for Leading Industry Development of Gorontalo Province (Podungge et al. 2019). Another effort made by the Gorontalo government to increase corn farming is the establishment of cooperative institutions in corn production centers.

The development of corn farming is constrained by plant pest organisms that reduce the quality and quantity of production (Mirsam et al. 2021). So far, the major diseases of corn that have been reported as damaging in Indonesia are downy mildew, leaf blight, and leaf rust. However, recent infectious diseases have been observed that cause the plant to die completely, with symptoms of soft rot on the stalk that emit an unpleasant odor. This disease is generally found in the rainy season. According to Subedi et al. (2016), stalk decay after flowering is more prominent in reducing production than decay at the vegetative phase. Infected plants wilt with the yellowing of the leaves from the top; over time, the entire leaf turns yellow (Ahamad et al. 2015). External symptoms on the stalk include maceration and basal internodes. In addition, it causes soft rot and discoloration of infected tissue (Kumar et al. 2017a). In severe conditions, a foul odor occurs, and the plant collapses, so the potential for yield loss is quite significant. The disease is caused by the bacteria *Dickeya zeae*, formerly known as *Erwinia chrysanthemi* pv. *zeae* (Martinez-Cisneros et al. 2014; Ahamad et al. 2015; Subedi et al. 2016; Guan et al. 2020; Prokić et al. 2020). In

addition to infecting corn plants, *D. zeae* was also reported to infect several host plants throughout the world, including pineapple, taro corn, rice, banana, clivia, and sugarcane (Zhang et al. 2014; Hu et al. 2018; Zhang et al. 2018; Boluk et al. 2021; Yang et al. 2021). The existence of this pathogenic bacteria in Indonesia was previously reported by Aeny et al. (2020), infected pineapples in Lampung, Indonesia.

The *D. zeae's* ability to infect various dicotyledons and monocotyledons shows this bacterium has various virulence factors. *D. zeae* was reported as a pectolytic bacterium that produces pectic enzymes. These enzymes are important in plant tissue macerations, causing soft rot symptoms. However, several other factors also trigger the virulence and development of this bacterium, including the flow of irrigation water, fertilization with high nitrogen, and rainfall (Kumar et al. 2017a). Therefore, the aim of this study was to characterize the bacteria that cause corn stalk rot in Gorontalo Province, Indonesia.

MATERIALS AND METHODS

Exploration and isolation of the pathogen

A sampling of infected plants with corn stalk rot disease was conducted in Suka Makmur Village, Patilanggio Sub-district, Pohuwato District, Gorontalo Province, Indonesia. The sampling location was selected purposively, namely Pohuwato District, the highest maize-producing area in Gorontalo Province, Indonesia. Sampling was done by selecting maize based on specific criteria for symptoms of stalk rot disease caused by the bacterial infection (Figure 1). Symptomatic samples were taken and covered with tissue to retain moisture. The collected samples were then stored in separate plastic bags and immediately transported to the laboratory using a cooling box.

The stalk tissue between the diseased and healthy area was taken about 1 cm, surface sterilized using 75% ethanol for 30 seconds, 2% NaOCl for 1 minute, and rinsed with sterile distilled water. The sterilized sample was macerated using a 10% glycerol solution. The formed suspension was stored in an Eppendorf tube. Moreover, the suspension was cultured on Nutrient Agar (NA) media using the streak plate method. Then, a loop of suspension was carefully scratched in a quadrant pattern and then incubated at 30°C for 24 h. Finally, the dominant colony was selected and re-cultured on NA media to obtain a pure bacterial strain.

Preparation and measurement of the optical density of the bacterial suspension

The bacterial culture was re-cultured using Nutrient Broth (NB) media and incubated using a rotary shaker at 160 rpm for 24 hours. Bacteria growing on NB media were measured for their optical density using a spectrophotometer at a wavelength of 600 λ to obtain a cell concentration of 10^8 CFU/mL. Bacterial suspension with a 108 CFU/mL concentration was further analyzed on hypersensitive reaction, potato spoilage, pathogenicity, and host range tests.

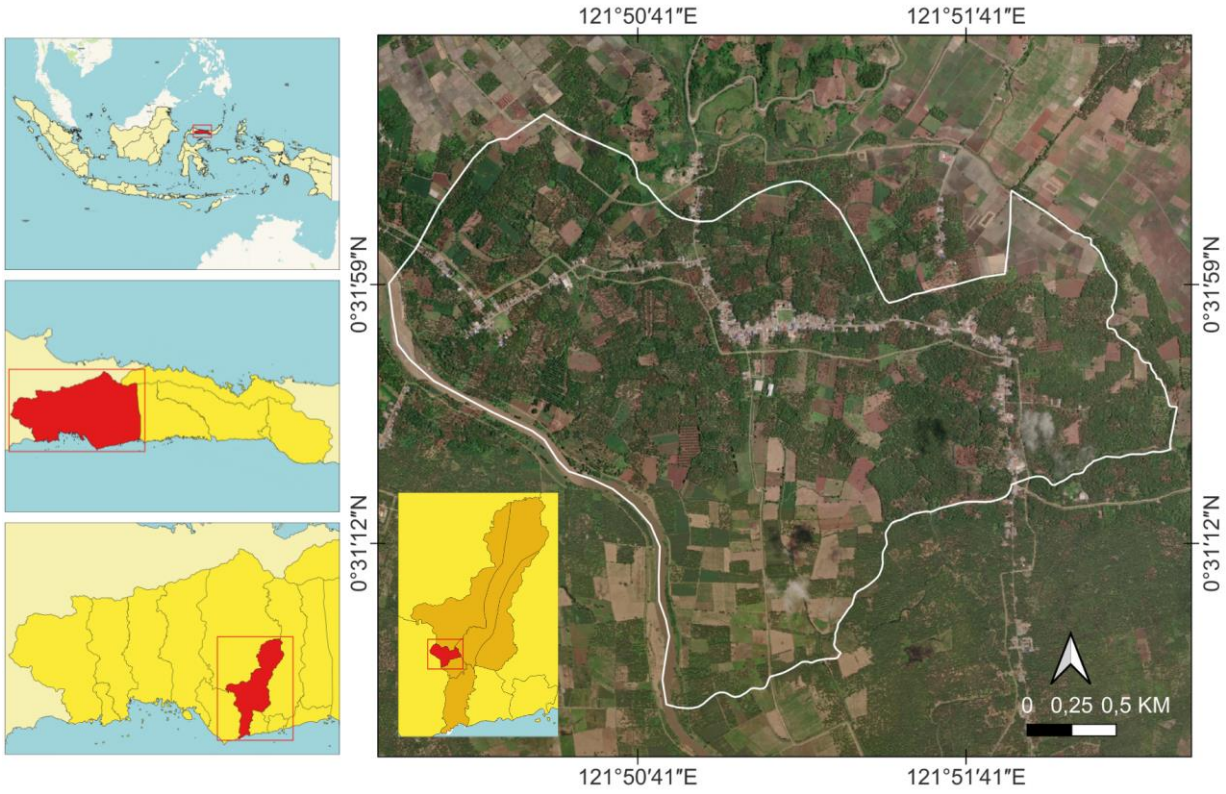

Figure 1. Sampling locations and disease symptoms found in Suka Makmur Village, Patilanggio Sub-district, Pahuwato District, Gorontalo Province, Indonesia

Hypersensitive reaction test

The hypersensitivity reaction test was carried out to determine the pathogenic and non-pathogenic bacterial isolates, according to Klement et al. (1990). A total of 1 mL of bacterial suspension with 10^8 CFU/mL concentration was injected underside the tobacco leaves. The necrosis symptoms were observed 24, 48, and 72 hours after injection.

Pathogenicity test on corn

The pathogenicity test on corn was carried out to confirm whether the bacterial isolates were pathogenic or non-pathogenic. The pathogenicity test was conducted in a greenhouse using polybags. The seeds of the NK7328 variety were treated with hot water at 55°C for 20 min, then airdried for 20 min on sterile tissue paper. The tested seeds were planted in polybags with sterile soil and combined media (1:1). The test was replicated three times. Corn was inoculated ten Days After Planting (DAP) following the method of Ahamad et al. (2015). Bacterial suspension with 10^8cfu/mL concentration was injected as much as 1 mL into the second segments from the base of the corn stalk. In addition to the negative control, corn plants were injected with sterile distilled water. The parameters observed were disease incidence and incubation period (when symptoms first appeared). The disease incidence rate was measured according to the following formula (Equation 1):

$$DI = \frac{A}{B} x\ 100\% \quad \text{..... (1)}$$

DI : Disease Incidence (%)

A : Number of plants infected with stalk rot

B : Number of plants inoculated for each bacterial isolate

Identification of the bacterial isolate based on morphological characteristics

Morphological characterization was carried out after obtaining pure isolates of tested bacteria. The tested bacterial isolate was identified based on the characteristics of the shape, elevation, margin, and colony color (Holt et al. 1994). Furthermore, the cell shape was observed using a compound microscope with a magnification of 1000x**.**

Identification of the bacterial isolate based on physiological characters

The physiological characterization of bacteria was carried out by several tests, including: the gram test using 3% KOH solution and bacterial gram staining (Mu'minah et al. 2015; Begum et al. 2017); the oxidase test used Sigma strip oxidase contains plastic strips with a paper zone saturated with a solution of N,N-dimethyl-1,4-phenylene diamine and alpha-naphthol, catalase test (Sudewi et al. 2020); oxidation fermentation test, tolerance test for temperature conditions of 37-40°C and acid using 5% NaCl, fried egg colony test on PDA media (Thakkar et al. 2016); sensitivity test to 15 µg erythromycin antibiotic (Kanzil et al. 2015), and fluorescent pigment production using King's B media (Nepali et al. 2018). In addition, enzyme activity tests were conducted on the ability of bacteria to produce enzymes: protease, lecithinase, and pectolytic enzymes, following the methods of Boluk et al. (2021), Thakur et al. (2021) and Kumvinit and Akarapisan (2019). In addition, protease and lecithinase enzyme activity tests were carried out using skim milk agar media and NA media enriched with 5% egg yolk emulsion. At the same time, the pectolytic enzyme test was carried out using Himedia Crystal Violet Pectate Medium. First, a well was made using a 3 mm cork borer on each test medium. Next, 50 µL of 24-hours-old bacterial suspension was added to each plate well and then incubated at 28°C for 24-48 hours. The formation of clear zones around the bacterial colonies observed the activities of protease and lecithinase enzymes.

The soft rot on potato tubers test was carried out according to the modified method of Azadmanesh et al. (2016). First, a sterile toothpick was dipped into a bacterial suspension with a concentration of 10^8 CFU/mL and incubated at 25°C for 24 hours. Then, toothpicks were injected into the potato tubers to a depth of 1 cm and placed in a sterile petri dish lined with wet cotton. Next, soft rot symptoms in potato tubers were observed 24, 48, and 72 hours after injection.

The ability of bacteria to degrade tryptophan to indole was conducted with Kovac's reagent, which contained para-dimethyl amino benzaldehyde in a test tube. The formation of a red ring layer on the surface of the bacterial suspension identified a positive indole test.

Host range test

The host range test was carried out on seven tested plant species following the method of Aeny et al. (2020). The tested plants were rice, sorghum, wheat, foxtail millet, *Aloe vera*, celery, and corn. Next, a 48-hour-old bacterial suspension with a 10^8 CFU/mL concentration was injected into the stem. Disease incidence was observed every day for five days and calculated using equation 1. The test was conducted in a completely randomized design consisting of seven host plants as the treatment and repeated three times.

Identification of bacterial isolate based on the polymerase chain reaction

DNA extraction

DNA extraction was performed using ZymoBiomics Quick-DNA™ Fungal/Bacterial Miniprep Kit. The working principle of the kit was to add 50-100 mg bacterial cells suspended in water to a ZR BashingBead™ Lysis Tube. The ZymoBIOMICS lysis system was achieved by bead beating with the innovative ultra-high-density BashingBeads. The DNA was then isolated and purified using Zymo-Spin™ Technology.

Amplification of bacterial DNA

Amplification using specific primers was done to confirm the tested bacteria's genus before sequencing the 16S rDNA. The specific primers used were ADE1 (5'-GATCAGAAAGCCCGCAGCCAGAT-3') and ADE2 (5'-CTGTGGCCGATCAGGATGGTTTTGTCGTGC-3') with ±420 bp target amplicon. Furthermore, to determine species, the partial 16S rDNA gene fragment was amplified using the universal primers 27F (5-

AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3) with ±1500 bp target amplicon. PCR was performed using a SensoQuest Thermal Cycler (Germany) machine with the following program: 1 cycle of initial denaturation at 94°C for 2 minutes, 35 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 62°C for 45 seconds, extension at 72°C for 2 minutes, and final extension at 72°C for 3 minutes (ADE1/ADE2 primers); 1 initial denaturation cycle at 95°C for 3 minutes, 35 cycles consisting of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, primer extension at 72°C for 1 minute, and the final extension at 72°C for 3 minutes (27F/1492R primers). Bacterial DNA was electrophoresed at a voltage of 110 V for 50 minutes. The electrophoresis results were visualized with a UV transilluminator. While the photos were taken using the camera.

Analysis of sequencing of the 16S rDNA

The positive amplicon from the universal primer was sent to FirstBase (Malaysia) for sequencing. First, the sequence result was analyzed using the basic local alignment search tool (BLASTN 2.13.0+) with an optimization program to obtain the sequence of DNA bases contained in the National Center for Biotechnology Information (NCBI) site. Next, the nucleotide sequence results were analyzed using multiple alignments Clustal on the software Bioedit Sequence Alignment Editor 7.2 version. Finally, the kinship relationship between isolates was constructed using software Molecular Evolutionary Genetic Analysis Software 10.0 version (MEGAX) with bootstrap 1000 times repetitions.

Data analysis

Data analysis was carried out at the stage of testing the host range. Observational data were analyzed statistically with a One-way Analysis Of Variance (ANOVA). If there was a treatment effect, then proceed by Least Significant Difference (LSD) at the 5% significance level (0.05)

RESULTS AND DISCUSSION

Symptoms of corn stalk rot

Symptoms of corn disease were soft and slimy rot on the stalk, wilted plant, yellowing of leaves, and an unpleasant odor from the infected part of the stalk. Infected corn showed death, but some only showed symptoms of wilted leaves; first to second rootstock segments turned into a wet brown color with clear boundaries. It was also observed that the disease became more serious during the rainy season, which reduced the productivity of maize. A total of nine isolates were isolated from the infected samples, namely BGO1, BGO2, BGO3, BGO4, BGO5, BGO6, BGO7, BGO8, and BGO9.

Pathogenicity characteristics of bacterial isolates

All nine bacterial isolates showed a positive hypersensitive response 24-48 hours after inoculation. The symptoms of tobacco leaf damage were clearly observed in the inoculated leaf area in the form of yellow necrosis. Meanwhile, the pathogenicity test showed that only two BGO1 and BGO4 isolates caused stalk rot on corn. However, the BGO1 isolate suspension caused brown color discoloration, dryness, and death ten days after inoculation. Therefore, it can be suspected that the death of maize inoculated with BGO1 was not caused by bacterial infection; other factors, such as microbial infection by the fungus, could cause it. Isolate BGO4 had a shorter disease incubation period than other isolates, ranging from 1 to 4 Days After Inoculation (DAI), with a disease incidence of up to 83.33% (Table 1).

Pathogenicity test results showed wilting in lower leaves, and infected corn stalk tissue became brown and softer than healthy tissue (Figure 2). The damage to tissue structure was caused by the activity of bacterial pectolytic enzymes, which damage the binding material of plant cells. Nevertheless, symptoms persisted, and some tested plants died at 3 DAI. Rotting of stem tissue was a symptom of death in infected corn, while the plants inoculated with sterile distilled water did not show any symptoms of damage.

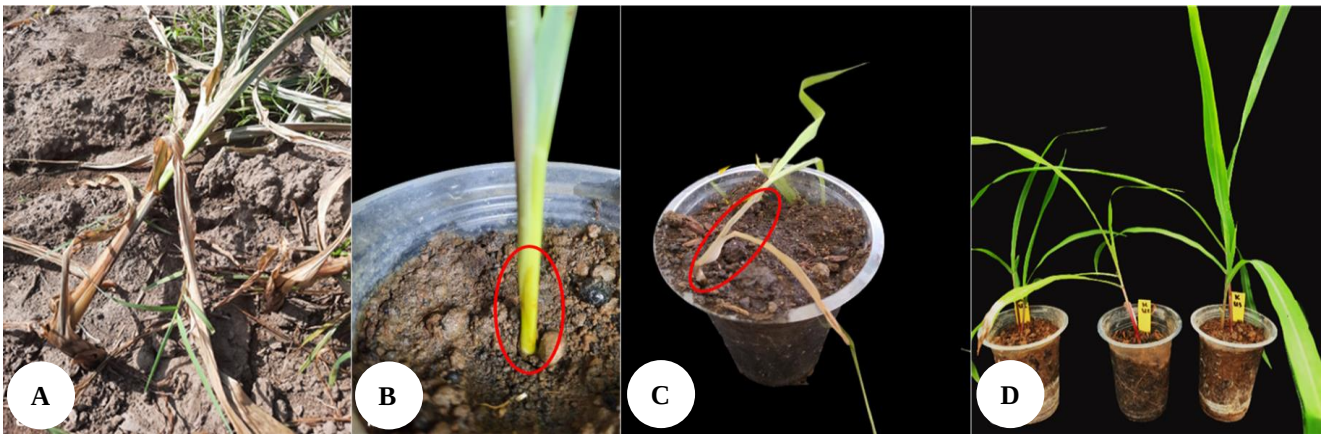

Figure 2. Corn plants infected with stalk rot disease in the crop (A); Necrotic symptoms develop around the point of inoculation (B); Symptoms of plant death 3-6 days after inoculation (C); Plants that remain healthy in the control treatment (D)

Morphological characters of BGO4 isolate

The re-isolation of BGO4 isolate from the pathogenicity test was then identified based on morphological characteristics. On NA medium, BGO4 isolate had white-gray colored colonies with round shape, elevation convex, and entire margin. At 2 to 3 days after incubation, the colony shape turned nearly round to an irregular margin. On Kings B media, bacterial colonies were a white-cream color, shiny, round, and convex shapes, and did not produce fluorescent pigment. In addition, the bacterial cells were bacilli with rounded ends. Based on the morphological characteristics, the BGO4 isolate was suspected of belonging to the genus *Dickeya*.

Physiological characteristics of BGO4 isolate

Physiological characteristics of the BGO4 isolate showed catalase positive, indole positive, and oxidase negative and caused soft rot on potato tubers. In addition, isolate BGO4 was oxidative fermentative by changing the color of OF medium to yellow, both covered and uncovered. The bacteria formed colonies like fried eggs on PDA media and were able to grow under relatively high-temperature conditions at 37-40°C and 5% NaCl media (Table 2).

The sensitivity to antibiotics showed that the BGO4 isolate was sensitive to erythromycin 15µg. A positive result was indicated by forming a clear zone around the antibiotic paper disc. The bacterial growth colonies on media without antibiotics were evenly distributed on the surface (Figure 3.A). Another physiological characteristic tested in this study was the activity of protease and lecithinase enzymes. Isolate BGO4 could grow on the skim milk agar media and NA media, enriched with 5% egg yolk emulsion, forming clear halo zones (Figure 3.B). This showed that bacteria could produce protease and lecithinase enzymes.

The production of pectolytic enzyme was indicated by the ability of bacteria to form clear zones on CVP media 72 hours after incubation (Figure 3.C). The clear zone formation indicated that the BGO4 isolate could degrade sodium poly pectate in the media. In addition, the pectolytic activity of bacteria was capable of rotting potato tubers. The indole test showed a direct red color change in the BGO4 isolate surface when it was dropped with Kovac's reagent, indicating the formation of indole (Figure 3.D).

Host range of BGO4 isolate

In the host range test, maize and celery showed soft rot symptoms at 1 DAI with 4.76% and 3.33% disease incidence, respectively. Meanwhile, the other five tested plants showed stalk rot symptoms at 2 DAI to 4 DAI, except wheat, which showed no symptoms till the end of observation. Although the initial infection in *Aloe vera* was found at 2 DAI and increased to 5 DAI with 73.33% disease incidence, this was not significantly different from the disease in celery and corn. The initial symptom in corn, sorghum, and foxtail millet was necrosis that developed around the inoculation site. These symptoms indicated stem tissue decay which causes plants to wilt and die. Meanwhile, the symptoms of infection in celery were leaf drooping and wilting (Figure 4).

Molecular characters of BGO4 isolate

The isolated bacteria were then confirmed by DNA amplification using specific primers for the genus *Dickeya*, i.e., ADE1/ADE2. The ADE1/ADE2 primers amplified DNA bands of the BGO4 isolate measuring at ± 420 bp (Figure 5.A). Furthermore, the DNA of isolate BGO4 was amplified again using primer 27F/1492R, the amplicon of which was used for sequencing analysis. Finally, using universal primer pairs 27F/1492R successfully amplified a DNA band of BGO4 isolate measuring at ±1300 bp (Figure 5.B).

Table 1. Hypersensitive reaction and pathogenicity test on corn of 9 bacterial isolates

Isolates	Hypersensitive reaction at (HAI)			Pathogenicity test on corn	
	24	48	72	DI (%)	IP (Day)
BGO1	(-)	(+)	(+)	16.67	10
BGO2	(+)	(+)	(+)	0	0
BGO3	(-)	(+)	(+)	0	0
BGO4	(+)	(+)	(+)	83.33	1 s/d 4
BGO5	(-)	(+)	(+)	0	0
BGO6	(+)	(+)	(+)	0	0
BGO7	(+)	(+)	(+)	0	0
BGO8	(-)	(+)	(+)	0	0
BGO9	(-)	(+)	(+)	0	0
Control	(-)	(-)	(-)	0	0

Note: (+): symptoms of necrosis and decay; (-): no signs of damage; DI: Disease Incidence; IP: Incubation Period (day); HAI: Hours After Inoculation

Table 2. Physiological characterization of BGO4 bacterial isolate from Gorontalo Province and reference *Dickeya zeae*

Characters	BGO4 isolate	*Dickeya zeae**
Gram reaction	-	-
Hypersensitive on tobacco	+	+
Oxidase	-	-
Catalase	+	+
Lecithinase activity	+	+
Protease	+	nd
Potato soft rot	+	+
Fluorescence on King B medium	-	-
Glucose metabolism	O/F	O/F
Grows at 37°C	+	+
Growth at NaCl 5%	+	v
Erythromycin sensitivity 15µg	+	nd
"Fried egg" like colonies on PD medium	+	+
Indole test	+	nd
Pathogenicity assay on corn	+	+

Note: * Prokić et al. (2020); (+): indicates positive; (-): indicated negative; v: indicated variable reaction; OF: Oxidative-Fermentative; nd: no data

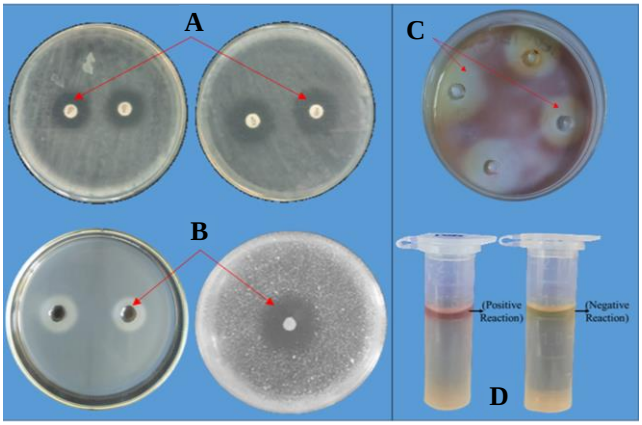

Figure 3. A. The formation of a clear zone indicated the sensitivity of the isolate to 15 g antibiotics after 24 hours of incubation. B. The activity of the lecithinase and protease enzymes was indicated by the formation of a clear zone. C. The pectolytic activity of bacteria on CVP media. D. The reaction of bacteria on indole test

Homology levels of BGO4 isolate DNA sequences with bacterial strains on the GeneBank

The results of the DNA sequence analysis of the BGO4 isolate based on the amplification of the 16S rDNA using BLASTN 2.13.0+ program showed a different sequence identity with each bacterial strain in GenBank. The sequence length ranges of BGO4 isolate with similar bacterial strains in GenBank ranged from 1346-504 bp. In addition, coverage query from BLASTN results also showed a very high percentage of the length of DNA sequence conformity between isolate BG04 and similar bacterial strains in GeneBank of ≥99% (Table 3).

Analysis of the BLASTN 2.13.0+ program showed that the DNA sequence of isolate BGO4 had the highest similarity with *D. zeae* (> 90% with an e value of 0.0). Isolate BGO4 had similarities to *D. zeae* strain DZ15SB01, HNJF02, MS32, MAFF311098, DZ-B1-1, DzM2, VR01P1 and *E. chrysanthemi* strains ECH279, ICMP4649, and A5264 with homology values ranging from 98.20%-99.20%. In addition, isolate BGO4 had a relatively far level of similarity with the outgroup comparison isolate from the *Pseudomonas aeruginosa* strain KSG bacteria group with a homology value of 84.90% (Table 4).

Phylogenetics of BGO4 isolate based on nucleotide base sequence

Phylogenetic analysis showed that isolate BGO4 was in the same group as *D. zeae* strain MS32 from Taiwan, strain DZ15SB01 from Thailand, and strain HNJF02 from China, with genetic distance coefficient values ranging from 0.001 to 0.002. In addition, isolate BGO4 also had a close kinship with bacteria *D. zeae* strain DzM2 from India, strain VR01P1 from Australia, strain MAFF311098 from Japan, and bacteria *E. chrysanthemi* strain A5264 from the USA, strain ECH279 from Malaysia, and strain ICMP4649 from New Zealand, with a fairly close genetic distance coefficient of 0.003. In contrast, isolate BGO4 had a fairly distant relationship with the KSG outgroup comparison strain from the *P. aeruginosa* bacteria group with a genetic distance coefficient of 0.085 (Figure 6).

Analysis of genetic diversity of BGO4 isolate with bacterial strains on GenBank

The genetic diversity of molecular-based bacterial specimens was further analyzed using the DNA sequence alignment method between BGO4 and bacterial specimens deposited at NCBI. The alignment of DNA sequences showed a very high genetic variation between bacterial strains. For example, the alignment of the DNA sequences of the BGO4 isolate with the bacterial strains in GenBank showed that the amplified 16S rDNA was generally in a region with high and stable conservation. In addition, there was also very high genetic variation in the nucleotide sequence or loci of BGO4 isolate155, 267, 275, 277, 280, 336, 353, 355, 357-359, 366-369, 371-373, 778, 786, 828, 834, 901, 905-907, 917, 920-922, 935, 937, 962, 970, 1034, 1036-1038, 1244, and 128 (Figures 7 and 8).

Table 3. Information on the similarity of BGO4 isolate with DNA sequences in the GeneBank NCBI

Organisms	Strain codes	No. accession	Sequence length (bp)	Query cover (%)	Year	Origin
Dickeya zeae	DZ15SB01	KY817902	1503	99.00	2017	Thailand
Dickeya zeae	HNJF02	KY784647	1504	99.00	2017	China
Dickeya zeae	MS32	OM095445	1408	100.00	2022	Taiwan
Dickeya zeae	MAFF311098	AB713546	1370	99.00	2014	Japan
Dickeya zeae	DZ-B1-1	KJ438949	1414	99.00	2014	Mexico
Dickeya zeae	DzM2	MT037020	1365	99.00	2020	India
Dickeya zeae	VR01P1	MT995003	1400	99.00	2021	Australia
Erwinia chrysanthemi	ECH279	KF058032	1399	99.00	2013	Malaysia
Erwinia chrysanthemi	ICMP4649	EF530560	1346	99.00	2007	New Zealand
Erwinia chrysanthemi	A5264	EU821583	1358	99.00	2008	USA

Table 4. Matrix of DNA sequence identity for BGO4 isolate paired with isolates in GenBank

Codes	BGO4	A	B	C	D	E	F	G	H	I	J
BGO4	ID										
A	0.992	ID									
B	0.992	1	ID								
C	0.990	0.990	0.990	ID							
D	0.987	0.990	0.990	0.984	ID						
E	0.984	0.990	0.990	0.982	0.993	ID					
F	0.986	0.992	0.992	0.982	0.995	0.996	ID				
G	0.983	0.989	0.989	0.980	0.993	0.996	0.997	ID			
H	0.984	0.987	0.987	0.981	0.996	0.990	0.992	0.989	ID		
I	0.982	0.988	0.988	0.980	0.991	0.996	0.994	0.993	0.993	ID	
J	0.849	0.850	0.850	0.852	0.851	0.853	0.850	0.850	0.849	0.851	ID

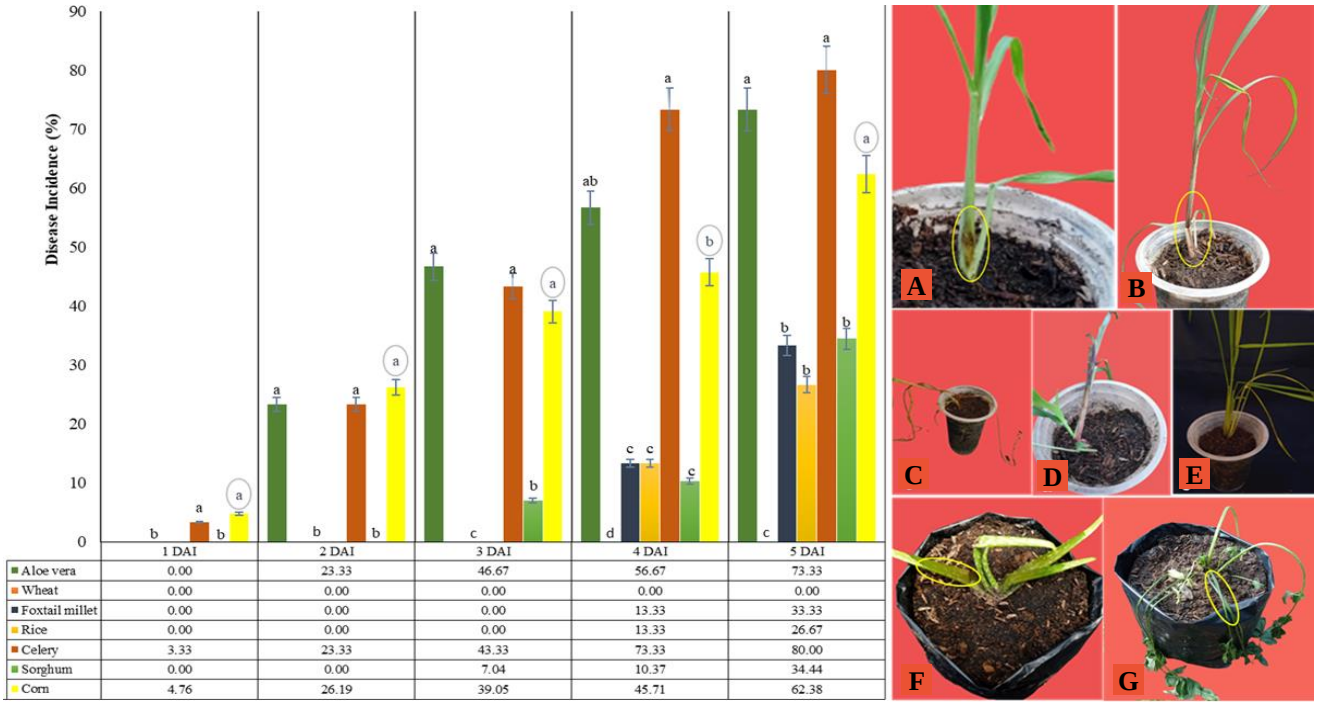

Figure 4. *Left*: The incidence of stalk rot disease from several plant species after inoculation with the BGO4 isolates; *Right*: Symptoms of necrosis in sorghum and foxtail millet (A-B); stalk rot symptoms in sorghum, foxtail millet, and rice after 2-5 DAI (C-E); soft rot on *Aloe vera* (F) and celery (G)

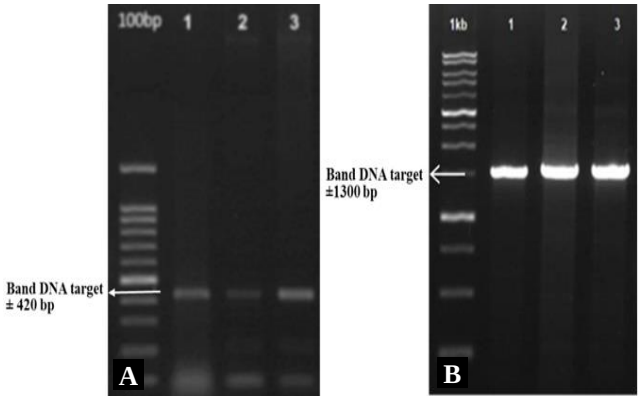

Figure 5. Visualization of DNA fragments from gene pelADE amplification with primers ADE1/ADE2 (a) and 16S ribosomal DNA amplification (b) of isolate BGO4 by Polymerase Chain Reaction (PCR) method. 1kb: Marker/DNA ladder; 1, 2, and 3, replications

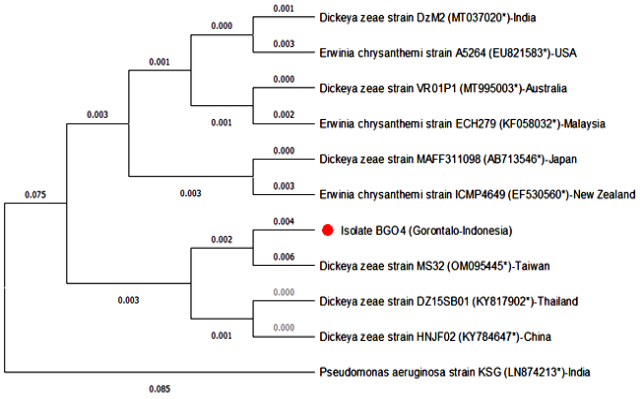

Figure 6. Phylogenetic tree of isolate BGO4 based on analysis of Neighbor-Joining Tree with Kimura 2-parameter model with distance matrix calculation of genetic bootstrap replications 1000 implemented in Bioedit 7.2 program and MEGAX. (●) Research isolate; (*) Accession number

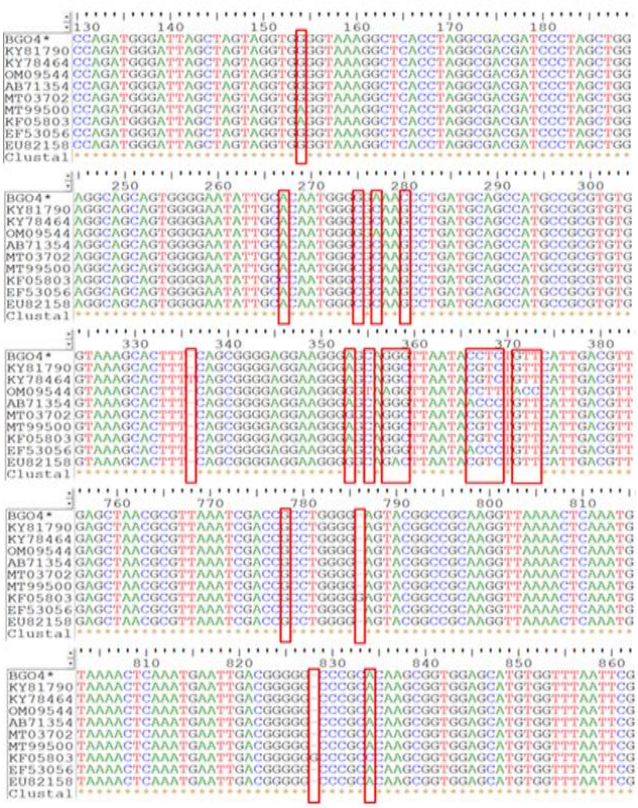

Figure 7. Alignment of nucleotide bases of isolate BGO4 with several bacterial strains in GeneBank at loci 130 to 862. The *(asterisk) indicates identical and highly conserved nucleotide bases; red boxes indicate areas of high genetic variation

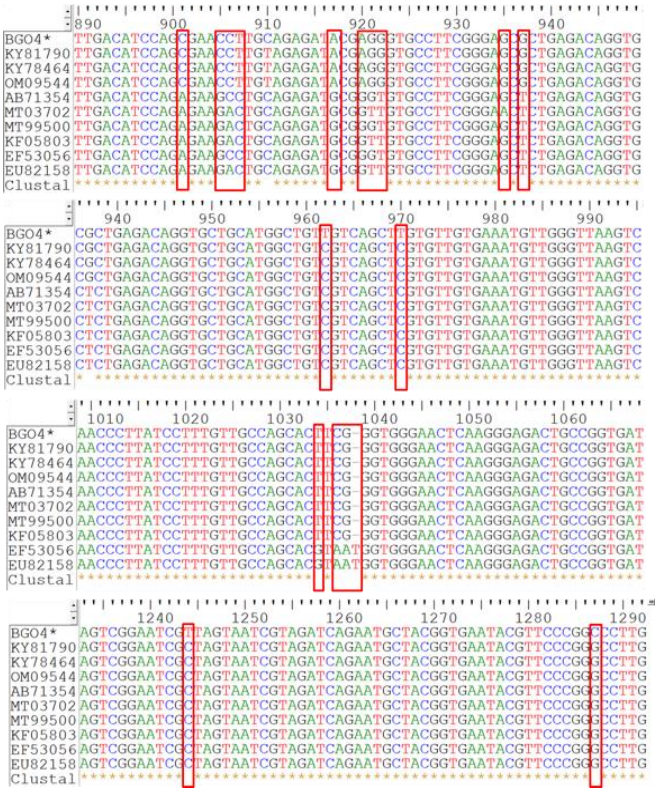

Figure 8. Alignment of nucleotide bases of isolate BGO4 with several bacterial strains on GeneBank at loci 890 to 1292. The *(asterisk) indicates identical and highly conserved nucleotide bases; red boxes indicate areas of high genetic variation

Discussion

The early symptoms of bacterial stalk rot disease in corn are wilting, softening, and rotted stalk tissues. During the survey in Gorontalo, the disease was in its early generative phase. In addition, symptoms of ear rot and drooping were found before physiologically ripe. The stalk rot disease caused by *D. zeae* bacteria plagues corn crops worldwide (Ahamad et al. 2015; Subedi et al. 2016; Jittikornkul et al. 2017; Kumar et al. 2017b; Prokić et al. 2020). Ahamad et al. (2015) reported that *D. zeae* infected hybrid, composite and local corn in India with different disease incidence rates in each region, ranging from 10-39%.

Two of the nine bacterial isolates found in this study were capable of causing stalk rot disease, based on the pathogenicity test on corn. For example, isolate BGO4 could damage stems one day after inoculation. The same condition was reported by Kumar et al. (2015) stated the internodes of corn plants were damaged after 24 HAI of *D. zeae*, and the virulence of bacteria caused death in sweet corn plants at 4-5 DAP. Jittikornkul et al. (2017) also reported that *D. zeae* isolated from corn was able to cause stalk rot disease in corn 2 DAI. Kumar et al. (2017b) reported that pectolytic enzymes play an important role in the degradation of plant tissue.

The BGO4 isolate from Gorontalo was identified as *D. zeae* based on the characteristics of its colony growth. Previous studies reported that *D. zeae* colonies were circular and convex. While creamish-color colonies on King's B media and fried eggs colonies on PDA or NA media enriched with 2% glucose (Alic et al. 2017; Zhang et al. 2020; Mokrani and Nabti 2021). Prokić et al. (2020) reported that *Dickeya* colonies isolate from corn and were grown on NA media for 2-3 days with the characteristics of a grayish-white, shiny, non-mucous, round-shaped colony with irregular boundaries. In addition, the bacterium could also degrade potato tissue by softening the tissue at the inoculation site, and the presence of a bad smell indicates positive pectolytic activity. Muturi et al. (2018) stated that *Pectobacterium* and *Dickeya* are pectolytic bacteria widely reported to cause soft rot disease. One distinguishing characteristic of the genus *Dickeya* and *Pectobacterium* is the ability of *Dickeya* to produce indole. The genus *Pectobacterium* lacks the tryptophanase enzyme that breaks down the amino acid tryptophan into indole, so the indole test is negative (Kamau 2020). However, these two genera of bacteria have some characteristics in common, including: catalase activity, negative oxidase, the ability to utilize carbohydrates both fermentatively and oxidatively, and the production of the enzyme lecithinase (Czajkowski et al. 2015; Pratama et al. 2022). The BGO4 isolate can produce protease, and lecithinase enzymes play a role in the decomposition of plant cell walls or the maceration of plant tissues (Boluk et al. 2021). The characteristics of isolate BGO4 are similar to those of *D. zeae* isolated from maize stalk rot strains in Serbia (Prokić et al. 2020).

The BGO4 isolated from corn plants could infect five other tested plants. Therefore, rice, sorghum, barley, celery, and *Aloe vera* can potentially host *D. zeae* in Indonesia. The ability to infect several tested plants strengthens the suspicion that BGO4 isolate is a bacterium of the genus *Dickeya* which was previously reported to have many host plants (Hu et al. 2018; Li et al. 2020). Aeny et al. (2020) reported that *D. zeae* isolate from pineapple plants caused soft rot disease in lettuce, chicory, celery, dragon fruit, corn, leeks, tomatoes, green beans, long beans, *Aloe vera*, guava, pak-choy, chrysanthemums, and orchids. In Japan, *D. zeae* was isolated from maize, rice, calanthe orchids, and Setaria grass (Suharjo et al. 2014).

Morpho-physiology characteristics and diagnostic amplification DNA by PCR using the Dickeya-specific primers (ADE1/ADE2) showed the isolate belongs to the genus *Dickeya*. The species identity of this bacterium was confirmed using the partial sequence analysis of the 16S rDNA gene. The universal primer pair 27F/1492R detected the presence of the 16S-rDNA encoding gene in BGO4 isolates, where this gene is known to be the skeleton of the ribosome which is generally used to identify bacterial groups, highly conserved region, and plays an important role in protein synthesis (Jayaseelan et al. 2018). Furthermore, this gene is known to be a ribosomal framework generally used to identify all groups of bacteria. The 16S rDNA sequences are widely used to identify phylogenetic relationships between known and unknown bacteria (Beissner et al. 2012; Braun et al. 2021; Jones et al. 2021; Mirsam et al. 2022; Shah et al. 2022). Phylogenetic analysis of BGO4 isolates showed that this bacterium belongs to the *D. zeae* group with the highest similarity to the *D. zeae* strain DZ15SB01 from Thailand and the HNJF02 strain from China.

The alignment of the BGO4 DNA sequences with the bacterial strains in GeneBank showed very high genetic variation in the nucleotide sequence or loci. In addition, Zhang et al. (2022) reported the sequencing genomes of three *D. zeae* strains isolated from banana plants showed phenotypic diversity in antibiotic effects, colony pigments, and virulence levels between strains. Therefore, studies of the genetic diversity of bacterial strains need to be carried out to suppress the spread of new virulent strains.

In conclusion, it was observed that out of nine isolates, only BGO4 isolate was found to cause stalk rot disease in corn plants. Further molecular characterization with 16S rDNA analysis revealed that the isolate was identified as *D. zeae*. The isolate BGO4 was also able to infect six plant species that could be potential hosts for this bacterium in Indonesia. This study is the first report of *D. zeae* infecting corn in Gorontalo. This is preliminary information to prevent the spread of *Dickeya* bacteria in Gorontalo Province from making a control decision and reducing farmers' losses. Identifying this bacteria is important for determining the characteristics of the bacteria that can be used as a reference in control technology, such as the sensitivity of bacteria to the antibiotic erythromycin or biological agents that produce appropriate antibiotic compounds in controlling *D. zeae*. In addition, information on the host range of this bacterium can be used as a reference for agricultural and horticultural officers to anticipate this bacterial attack.

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