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First report of *Enterobacter* sp. causing bacterial wilt on patchouli in Aceh, Indonesia

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Abstract. *Zulfadli, Wasistha NI, Oktarina H, Khairan, Sriwati R. 2023. First report of* Enterobacter *sp. causing bacterial wilt on patchouli in Aceh, Indonesia. Biodiversitas 24: 3815-3820.* Patchouli (*Pogostemon cablin* Benth), is an essential commodity in Indonesia, with Aceh Jaya being one of its main producing regions. Several incidences of wilt disease symptoms have been reported in patchouli plantations in Ranto Sabon, Sampoianet Sub-district, Aceh Jaya District. This study aimed to isolate and identified the bacterial pathogen responsible for wilt disease in patchouli in Aceh Jaya. Various tests, including morphology characterization and molecular characteristics using 16s PCR primer, hypersensitive test, and pathogenicity test were conducted to identify the cause of disease. The isolation result revealed that *Enterobacter* sp. was responsible for invading the patchouli plant. Further testing was carried out on various culture media including Nutrient Broth, Tryptic Soy, Kings B, Sulfide Indole Motility, Sucrose Potato Agar, and Yeast Potato Agar. The highest colony density was found in Tryptic Soy media (2.002) after 120 hours of inoculation. The results of hypersensitivity and pathogenicity tests showed that *Enterobacter* sp. caused symptoms similar to those of natural infection. Among all the media, Sucrose Potato Agar media had the fastest incubation period, with 7 and 48 days after inoculation. Therefore, this is the first study to report the presence of *Enterobacter* sp. associated with bacterial wilt in patchouli in Aceh.

Keywords: Bacterial wilt, *Enterobacter*, patchouli, sucrose potato agar

INTRODUCTION

Patchouli, scientifically known as *Pogostemon cablin* Benth, belongs to the Lamiaceae family and is renowned for its medicinal and aromatic properties (van Beek et al. 2017). The oil derived from patchouli leaves is an essential raw material used in various industries, including perfume, cosmetics, food, pharmaceutical, antimicrobial, radical capture activity, and others. Swamy and Sinniah (2015) reported that the demand for patchouli oil continuously increasing globally, making it an important commodity. Aceh patchouli is considered to be a superior type of patchouli compared to two other species, such as *P. heyneanus* (Javanese patchouli) and *P. hortensis* (soap patchouli). It contains a higher amount of patchouli oil between 2.5% and 5%, while the other species only contain 0.5% (Haryono 2015; Pharmawati and Candra 2015). Indonesia is the world's largest producer of patchouli oil, with almost 90% of production, and Aceh alone accounts for 40%. Other essential oil-producing provinces in the country include Aceh, North Sumatra, West Sumatra, Bengkulu, Java, Kalimantan, and Sulawesi (Pharmawati and Candra 2015; Astuti et al. 2022). In Aceh Province, the center of production is in Aceh Jaya District, which has a suitable climate for patchouli growth. Patchouli farmers in Ranto Sabon, Sampoiniet Sub-district, Aceh Jaya District have reported wilt disease symptoms, but the cause is unknown. Bacterial wilt disease is a major obstacle faced by patchouli growers due to its difficulty in controlling (Xie et al. 2017). This plague causes significant economic losses by reducing the yield and quality of plant products. Symptoms of wilt disease caused by *Ralstonia* sp. in patchouli plants can result in losses of up to 60-80%, and have affected almost all production centers in Indonesia (Nasrun et al. 2007). *Ralstonia solanacearum* is a pathogen that causes bacterial wilt disease in various plant species. Wilt disease is characterized by wilted leaves, brown vascular tissue, and even plant collapse (Jiang et al. 2017). The incidence of bacterial wilt can be exacerbated by the presence of nematodes, particularly when they are found together with *R. solanacearum* in the same field.

Patchouli farmers in Ranto Sabon Village have suffered over 50% decrease in production due to disease attacks, which causes wilting and necrosis on the stem. In recent years, other bacterial wilt disease-causing organisms, such as members of the *Enterobacter*, *Erwinia*, and *Kosakonia* genera have been reported to cause similar wilt symptoms in various plants (Sarkar and Chaudhuri 2015; Jeevan et al. 2022; Zhang et al. 2022). *Enterobacter* has been reported to cause disease in a wide variety of plants, there has been no prior report on its involvement in patchouli wilt disease.

Bacterial identification generally be done using two methods, including morphological and molecular tests. Molecular identification involves examining the bacterial genome based on DNA base sequences using polymerase chain reaction (PCR) techniques and analyzing genetic relationships with phylogenetic trees. The 16S rRNA gene sequence, which is present in all bacterial genetic material and highly conserved, is universal. Also, the gene sequence can be exploited to identify unknown bacterial species and analyze phylogenetic relationships with distant taxa (Ludwig 2007). The objective of this study was to isolate and identified pathogenic bacteria causing disease in patchouli plants in Aceh, Indonesia.

MATERIALS AND METHODS

Sample collection

Samples of patchouli plants that showed signs of wilting were collected from planting areas in Ranto Sabon Village, Sampoiniet Sub-district, Aceh Jaya District, Aceh, Indonesia.

Pathogen isolation from infected plants

Diseased plant samples were brought to the Plant Disease Laboratory, Faculty of Agriculture, Syiah Kuala University. Isolation was done from the symptomatic plants and pathogenicity test was conducted by inoculating bacteria into healthy patchouli plants (Zhang et al. 2022). To obtain the bacterial suspension, the base of the stem was immersed in sterile distilled water, then the suspension was spread on a petri dish containing media using T streak plate method.

Pathogenicity and hypersensitivity tests

Pathogenicity and hypersensitivity tests were conducted at the Atsiri Research Center, Universitas Syiah Kuala, in a greenhouse. A bacterial suspension with a concentration of 10^8 cfu/mL was used for the tests. Bacteria was cultured on six growth media, such as sucrose peptone agar media, Kings B, sulfide indole motility, nutrient broth, tryptic soy, and yeast potato agar. To perform hypersensitivity test, 1 mL of a bacterial inoculum suspension was injected into the veins of lower surface of tobacco leaves (Garc 2019). Daily observations of hypersensitivity reactions were recorded to tobacco leaves.

For pathogenicity test, about 10^8 cfu/mL bacterial suspension was injected into healthy patchouli plant grown in glass house. The suspension was injected at the base of a healthy stem approximately 2 cm from the soil surface. Daily observations were made during the incubation period to record the first wilting symptom. Furthermore, re-isolated isolates obtained from the test results were used for morphological and molecular identification (Bhunjun et al. 2021).

Morphological characterization of pathogen

To characterize the pathogen, various observations were made including colony morphology. Morphological observation included colony color (such as buff, white, red, black, etc.), shape (irregular, circular, and rhizoid), elevation (convex, raised, umbonate, and flat), and edge (filamentous, entire, serrate, undulate, and lobate). Additionally, microscopic examination was conducted to identify the shape of bacteria (such as cocci, bacilli, spirilla, and cylindrical) and their types (gram-positive and negative). Gram staining was carried out 24 hours after isolation (Maulidia et al. 2021). To obtain a more accurate identification, the samples were sent to PT. Genetika Science Indonesia.

DNA extraction, PCR, and sequencing

Molecular identification was performed by PT. Genetika Science Indonesia using QuickDNA™ Fungal/Bacterial Miniprep Kit technique to isolate bacterial DNA (Zymo Research, D6005). The concentration of extracted DNA was quantified using nanodrop technique. This DNA was referred to as a template for amplification with primer 16s (27F/1492R) using (2x) MyTaq HS Red Mix (Bioline, BIO-25048). PCR master mix of 25 µLwas produced, consisting of 9.5 µL dd H_2O, 12,5 µL MyTaq HS Red (2x), 1 µL of each 10 µM 27 Forward Primer (5'–AGAGTTTGATCMTGGCTCAG–3') and 1492 Reverse Primer (5'–GGTTACCTTGTTACGACTT– 3'), and 1 µL and DNA template. Amplification was performed through 35 independent cycles at a temperature of 95°C, with initial denaturation for 3 minutes and 15 seconds, and annealing for 30 seconds. Also, initial extension was carried out at a temperature of 72°C for 45 seconds, followed by a final extension for 3 minutes. The amplification results (1 µL) were visualized using electrophoresis gel (0.8% TBE agarose). A ladder of 1 Kb (2.5 µL) was positioned in the agarose solution to identify the intended length of the target DNA, which was visualized using a UV transilluminator. Bi-directional sequencing was performed to sequence both the top and bottom strands of the initial double-stranded DNA fragment in separate reactions. The resulting sequences were then compared using the BLASTN program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to examine their similarity. Relatedness analysis with phylogenetic trees was performed by aligning the sample sequences with the NCBI database using the Molecular Evolutionary Genetics Analysis (MEGA) tool version X, relatedness analysis with phylogenetic trees was performed (Augusta et al. 2018).

Bacteria culture test

A total of seven media, including Tryptic Soy Agar, Sulfide Indole Motility, Nutrient Broth, Kings B and Yeast Potato Agar, Sucrose Peptone Agar, and Aquadest (controls) were used for bacterial tests. All of these media were placed in a 100 mL Erlenmeyer flask and sterilized for 30 minutes at 121°C in an autoclave, and placed in a Laminar Air Flow (LAF) to cool down to room temperature. Subsequently, bacterial suspension was streaked onto each of the seven solid media and incubated at 28°C for 24 hours. A single bacterial isolate was obtained from each medium cultured in the seven liquid media. The optical density of the cells was measured at 1st, 24th, 48th, 72nd, 96th, 120th, and 144th hours of incubation time. A total of 5 mL bacterial suspension in each test medium was evaluated for absorbance using a UV-

Vis spectrophotometer at 600 nm wavelength to obtain the optical density value.

RESULTS AND DISCUSSION

Morphology of pathogen

The result showed that the isolated bacteria were Gram-negative and bacilli bacteria. Colonies were irregular, milky white in color, slimy surface, convex shape and wavy margin (Figure 1). Based on the morphology of the pathogen, it was identified as a species of *Enterobacter*. Gram staining is a crucial method for the classification of bacteria, and most Gram-negative species are known to possess pathogenic traits attributed to the presence of endotoxins and complex lipopolysaccharide (LPS) in their outer cell membrane. LPS can be toxic in certain environmental conditions (Aruwa 2017). Morphologically, *Enterobacter* sp. is a Gram-negative bacilli bacteria, motile, lacking spores, and facultative anaerobic. The colonies of *Enterobacter* sp. are generally pale white, round, smooth, and translucent (Wu et al. 2020).

Molecular identification

To further identify the bacterial species, the 16S rRNA gene sequence was obtained using PCR and electrophoresis with gel agarose 1%. The molecular identification results exhibited that the DNA band obtained from the samples had a size of 1049 bp (Figure 2). The successful isolation procedure was indicated by the appearance of the DNA band.

Table 1 shows the results of BLAST at GenBank National Centre for Biotechnology Information (NCBI). There was 99.9% similarity with 10 bacteria strains from the genus *Enterobacter*, with a query cover of 99-100%. Phylogenetic tree reconstruction indicated that the isolated bacteria had a high similarity to *Enterobacter* sp. The base sequences of the bacterial isolates were compared to data from National Center for Biotechnology Information (NCBI) using Neighbor-Joining (NJ) method (Figure 3).

Based on the phylogenetic tree analysis, bacteria isolate showed a high similarity of 99% to *Enterobacter* sp. This indicated that a new strain could be classified within the same group as the bacterial isolates collected in GenBank, provided that gene sequence homology of 16s rRNA reached up to 97-99%. When the gene sequence homology was less than 97%, it is classified as a different group of bacteria (Stackebrandt and Goebel 1994).

The pathogen isolated from wilted leaves of patchouli in Aceh Jaya showed similarity with *Enterobacter* sp. It is a gram-negative, bacilli bacteria, classified as facultative anaerobic, and motile. The locomotion form in bacteria is flagella which are evenly distributed over the cell surface (Wu et al. 2020).

Table 1. The analysis results of test sequences used the NCBI BLAST method

Description	Max score	Total score	Query cover	E value	Per. ident	Acc. no.
Bacterium strain BS1781 16S Ribosomal RNA Gene, partial sequence	1932	1932	100%	0.0	99.90%	MK824969.1
Enterobacter sp. XBGRY1 16S Ribosomal RNA Gene, partial sequence	1932	1932	100%	0.0	99.90%	KJ184859.1
Enterobacter sp. JBIWA003 chromosome, complete genome	1932	15417	100%	0.0	99.90%	CP074170.1
Enterobacter sp. strain INCA-FRr16 16s ribosomal RNA Gene, partial sequence	1932	1932	100%	0.0	99.90%	MT793107.1
Enterobacter sp. E4M-U 16S ribosomal RNA Gene, partial sequence	1932	1932	100%	0.0	99.90%	GQ478275.1
Enterobacter sp. 3 – 1t 16s ribosomal RNA Gene, partial sequence	1932	1932	100%	0.0	99.90%	EU543690.1
*Enterobacte*r sp. BS1961 16S ribosomal RNA Gene, partial sequence	1930	1930	99%	0.0	99.90%	MK82549.1
Enterobacter roggenkampii strain 218hs4-14 16S ribosomal RNA Gene, partial sequence	1930	1930	99%	0.0	99.90%	OM267708.1
Enterobacter sp. XBGRY 16S ribosomal RNA Gene, partial sequence	1930	1930	99%	0.0	99.90%	KJ84970.1
Enterobacter asburiae str. AEB30, complete genome	1927	15278	100%	0.0	99.81%	CP046618.1

Figure 1. Bacterial species identification. A. Bacterial mass in aquadest, B. Bacterial colony on sucrose peptone media, and C. Bacilli bacteria at 40x magnification

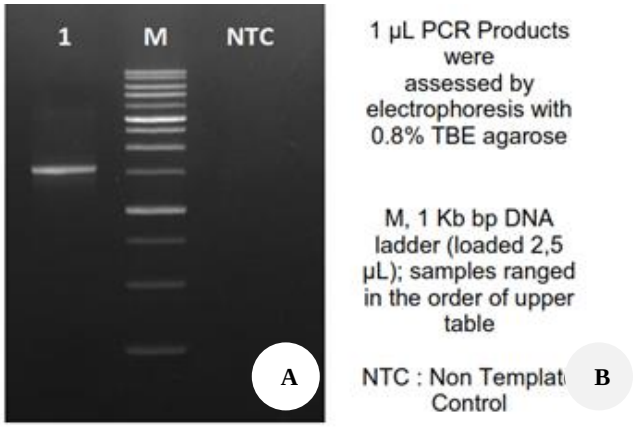

Figure 2. Gene sequence 16S rRNA using PCR and electrophoresis with gel agarose 1% (A) DNA ribbon as a marker of 1 kb DNA ladder, (B) amplicon of bacteria isolate A1 aligned with DANN marker 1049 bp

Enterobacter bacteria, are known to survive and thrive in soil, plants, and even humans (Liu et al. 2013). *Enterobacter* sp. is increasingly being recognized as a plant pathogen and pathogenic in various crops including tomatoes in India and China, chili pepper in Mexico, strawberry in China, and more others (Sarkar and Chaudhuri 2015; Wu et al. 2020; Ji et al. 2022; Guo et al. 2023) .

Hypersensitivity and pathogenicity test

The bacteria obtained from infected tobacco plants were subjected to a hypersensitivity test, which showed the presence of necrotic symptoms, as depicted in Figure 4. These symptoms indicated that the tested isolates were pathogenic. The hypersensitive reaction exhibited by plants was considered a form of physical defense against pathogen (Balint-Kurti 2019).

Hypersensitivity test was performed on tobacco plant because they are known to produce chemical defense against pathogen (Schaad et al. 2001). It was also recorded that bacteria grew well in sucrose peptone agar media with the shortest incubation period of 7 days. The bacterial pathogen was inoculated into 3-month-old healthy plants to verify whether they exhibit similar symptoms as those observed in the field (Figure 5). Wilting and discoloration of young and old leaves, leading to plant death, were observed in the inoculated plants. Zhang et al. (2020) reported that wilt disease in patchouli was caused also by *R. solanacearum*, which exhibited several symptoms including wilted and yellowed leaves, drooping stems, and plant death. Meanwhile, Sarkar and Chaudhuri (2015) reported that tomatoes infected by *Enterobacter* expressed wilting in the apical system and necrosis on plants. It is difficult to differentiate wilting symptoms caused by abiotic and biotic factors.

Bacteria incubated in sucrose peptone agar exhibited the shortest period (48 days after inoculation) compared to those growing in the remaining media. In control plants, no symptoms of wilting were observed as only water was injected. Sucrose peptone agar is a selective media that supports the growth of desired bacteria while inhibiting that of unwanted ones. This media contains sucrose as an energy source and peptone, which provides the necessary nitrogen for bacteria growth. Sucrose, a disaccharide composed of glucose and fructose subunits, is metabolized by bacteria to produce the energy required for their metabolic processes and cell division, enabling them to proliferate. Tian et al. (2021) highlighted the importance of sucrose in bacterial multiplication, as it serves as a carbon source for their metabolism. Sucrose acts as a carbon source in the metabolic process of bacteria (Jung et al. 2013).

Bacterial culture test

Bacterial density was determined by measuring the absorbance at different time points including 1st, 24th, 48th, 72nd, 96th, 120th, and 144th hours after incubation. The results showed variations in bacterial density among the culture media used in this study (Table 2).

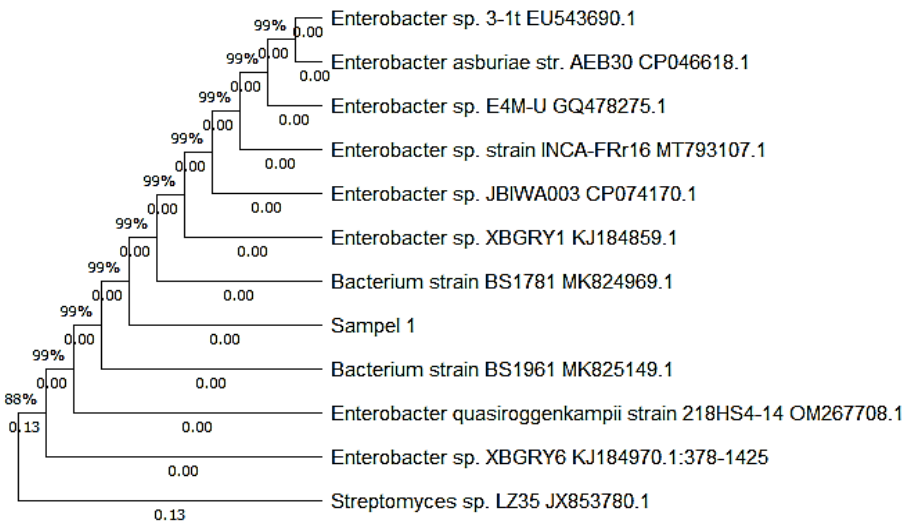

Figure 3. Phylogenetic tree of bacterial isolates identified as *Enterobacter* sp.

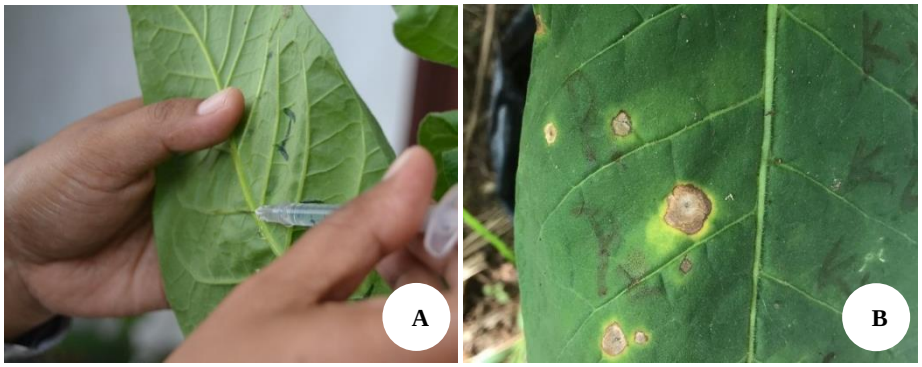

Figure 4. Hypersensitivity test. A. Bacterial suspension is injected into tobacco leaves, B. Necrotic symptoms appeared at 7 days after inoculation

Figure 5. Wilt symptoms on patchouli in the field. A. Wilting in patchouli, B. Healthy patchouli and patchouli plant showing initial symptom

Table 2. The optical density of bacterial cultured in different media

Media	Optical Density							
	1	**24**	**48**	**72**	**96**	**120**	**144**	**Mean**
Tryptic Soy	0.858	1.643	1.988	2.315	2.461	2.490	2.262	2.002
Sulfide Indole Motility	0.331	1.271	1.902	2.889	2.254	2.342	2.363	1.907
Nutrient Broth	0.165	1.294	1.819	2.147	2.224	2.082	1.639	1.624
Sucrose Peptone Agar	0.130	1.132	1.419	1.489	1.792	1.929	2.060	1.422
Kings B	0.150	1.233	1.268	1.293	1.307	1.996	1.590	1.262
Yeast Potato Agar	0.193	0.641	0.850	0.900	1.477	1.100	2.505	0.881
Aquadest (control)	0.059	0.061	0.059	0.063	0.065	0.064	0.082	0.065
Mean	0.269	1.039	1.329	1.585	1.654	1.715	1.572	1.309

The experiment results indicated that *Enterobacter* grown in tryptic soy medium exhibited the highest optical density (OD) (2.002). It was significantly different from the pathogen grown in Yeast Potato Agar and aquadest (control). However, it was not different from *Enterobacter* grown in Sulfide Indole Motility, Nutrient Broth, Sucrose Peptone Agar, and Kings B. These results indicated that these media were suitable for the growth of *Enterobacter*.

Tryptic Soy showed the highest OD of *Enterobacter* at 1 hour compared to other media and reaches its peak (2.490 and 2.262) after 120 and 144 hours of inoculation. This indicated that this media provided sufficient nutrients for the growth of *Enterobacter*. Meanwhile, the sulfide indole motility medium exhibited high OD but only after 72 and 144 hours of inoculation (2.889 and 2.363). Nutrient Broth showed a higher OD at 96 and 144 hours (2.224 and 1.639), while sucrose peptone agar had its highest at 144 hours (2.060). Kings B showed the highest OD at 120 and 144 hours after inoculation (1.996 and 1.590), while the lowest result was demonstrated by yeast potato agar with OD of 1.477 and 1.005 at 96 and 144 hours after inoculation, respectively.

In conclusion, the tests conducted on the seven-culture media showed variations in the cell density of growing

bacteria. In the hypersensitivity test, tobacco plant showed necrotic symptoms on the tested leaves, which resembled the symptoms of *R. solanacearum* attack and other types of vascular diseases in the Postulates Koch's stage. Furthermore, OD measurements taken at 1, 24, 48, 72, 96, 120, and 144 hours after inoculation showed that tryptic soy had the highest average value of 2.002. The macroscopic and microscopic observations of bacterial morphology indicated that the bacterial colonies were irregular in shape, small in size, slimy, milky white, with wavy colony edges, convex and shiny colony surfaces, and Gram-negative rod shape. Based on the molecular identification of bacteria using the 16S rRNA PCR gene sequence, the appearance of DNA bands indicates the success of isolation procedure. BLAST results in the GenBank NCBI showed that 10 bacteria showed 99.9% gene similarity with 99-100% query coverage and generally belonged to the genus *Enterobacter*. The phylogenetic tree analysis indicated a phylogenetic relationship with *Enterobacter* sp. Based on the test results, the isolated pathogen was responsible for wilt disease in Aceh patchouli (*P. cablin* Benth.) in Ranto Sabon is a group of bacteria that share characteristics similar to *Enterobacter* sp. This is the first study to report the presence of *Enterobacter* sp. associated with the wilt of patchouli.

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