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CERCOSPORA CANESCENS: CAUSE OF LEAF SPOT DISEASE ON LETTUCE CROP IN BALI PROVINCE, INDONESIA

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SUMMARY

Cercospora leaf spot disease on lettuce (*Lactuca sativa* L.) caused losses in Tabanan Regency, Bali, Indonesia. Cercospora leaf spot in lettuce results from the pathogenic fungi *Cercospora canescens*. The preventive research aimed to identify the fungi on the morphological and molecular basis by using universal primers for PCR of ITS 1 and ITS 4. The study used Koch's Postulates method to isolate the pathogenic fungal isolates from lettuce plants and test for pathogenicity. Identification of fungi continued macroscopically, microscopically, and molecularly. The macroscopic and microscopic observations showed that the cause of cercospora leaf spot on lettuce is the fungal disease caused by pathogenic fungi *Cercospora* spp. The isolate creates a dull white and robust mycelium structure, sideways growth, branched and septate hyphae, branched conidiophores, and dark lanceolate conidia. Through molecular identification, it helped recognize that fungi *Cercospora canescens* isolate Cer11-18 (Accession Number: MN400290.1) is the foremost cause of a withered lettuce crop. It is also the first research on the cercospora leaf spot disease on lettuce caused by *Cercospora* spp. in Tabanan Regency, Bali, Indonesia.

Key words: *Cercospora canescens,* DNA barcoding, lettuce (*Lactuca sativa* L.), leaf spot disease, molecular study

Key findings: This research identified the pathogenic fungi *Cercospora canescens* being a prime cause of the cercospora leaf spot disease on lettuce crops morphologically and molecularly.

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INTRODUCTION

Lettuce (*Lactuca sativa* L.) is one of the essential vegetable commodities in supporting national food security. Moreover, lettuce is one of the horticultural plants with commercial value and prospects found easily worldwide. Lettuce is a native annual plant of the Mediterranean region belonging to the family Asteraceae, also known as 'daisy' (Ning *et al.,* 2019). Lettuce is often grown as a vegetable for its leaves that are consumed in raw form or cooked; however, its production package is still unknown to most farmers (Kuang *et al.,* 2008). Therefore, lettuce cultivation is necessary (Meilanisari, 2017), and according to BPS (2017), its production has been increasing annually because of its rising demand (Abima *et al.,* 2017; Hamed *et al*., 2022).

Horticultural crops have a crucial role in supplying nutritious foods for the society. Lettuce contains vital nutrients, such as, fiber, pro-vitamin A, calcium, and potassium (Panikkai *et al.,* 2017). Based on the harvest area data and seasonal horticulture production in Tabanan Regency in 2017, lettuce production has the sixth position, with 2.395 tons in an area of 189 ha. In Tabanan Regency, Indonesia, the Baturiti Subdistrict is one of the production centers of lettuce; however, in 2018, lettuce production in this area has also decreased (Food Crops Agriculture and Horticulture Service, Tabanan Regency, 2018).

The study of the plant microbiome has inspired much in food safety and crop cultivation systems. The insight into the microbial communities inhabiting plants and their environment in the field can shed light on pre-harvest transmission routes and the interactions of microbes that attack plants (Williams *et al.,* 2013; Allard *et al.,* 2020; Chaudhry *et al.,* 2021). In Indonesia, diseases caused by phytopathogens are considerably the chief cause of decreased vegetable yields, apart from bacterial, viral, and pest attacks (Bebber *et al.,* 2014; Siaka *et al.,* 2014; Lim *et al.,* 2023). Several fungal infections have posed unprecedented challenges to ecosystems, which ultimately have a vast impact on global food security (Wiradana *et al.,* 2018; Singh *et al.,* 2023). Some other relevant diseases of the lettuce crop are wet rot disease caused by the bacteria *Erwinia carotovora*, leaf spot caused by the pathogenic fungi *Cercospora janseana*, soft rot from the bacteria *Erwinia carotovora*, and stem rot from *Rhizoctonia solani* (Setyowati *et al.*, 2003).

In horticultural crops, the well-documented devastation caused by emerging fungal diseases has thrived. Several mature elms' mortality was due to the Dutch elm disease caused by a strain of the fungus *Ophiostoma novo ulmi*. Meanwhile, millions of American chestnuts incurred attacks from chestnut blight caused by *Cryphonectria parasitica*. Lettuce cultivation problems persist because of a pathogen, such as, fungi, decreasing productivity up to 30%–35% and about 10%–20% after the harvest period. In lettuce, one of the diseases due to *Cercospora* spp. significantly decreased its productivity. Cercospora (Mycosphaerellaceae, Ascomycota) is a hyphomycete genus with insufficient morphological features for identification (Bakshi *et al*., 2015), and species delimitation requires further research with a molecular approach (Groenewald *et al*., 2013).

Cercospora spp. caused significant plant infections and foliar diseases in vegetables and field crops. Previous assumptions regarding host specificity in contagions of this genus resulted in morphological descriptions of species numbering up to >3000. However, its identification becomes challenging in the early detection of this species in field crops. For example, *Cercospora apii sensu lato* is a complex species consisting of *Cercospora* classes morphologically indistinguishable from *C. apii* sensu stricto (Crous and Braun, 2003). *C. apii* is the cause of Cercospora leaf spot (CLS) on celery (*Apium graveolens*); however, it also exists in 80 different host genera, such as, *Beta vulgaris* (Groenewald *et al*., 2013).

With the high demand for lettuce in Indonesia and abroad, farmers often experience crop failure because of the leaf spot attack by *Cercospora* spp. The Internal Transcribed Spacer (ITS) region can be an option as a universal mushroom barcode (Schoch *et al*., 2012). Species identification

execution can be done using DNA code based on a stem with the area method ITS (Schoch *et al*., 2012). Moreover, DNA code based on stem use can compare unknown order with an order data basis, such as, the international Sequence Database in the Gen Bank used to identify the various species (Raja *et al.*, 2017; Adeniyi *et al.*, 2018). Therefore, molecular fungi identification was mainly the means to recognize the fungi species (Ezeonuegbu *et al*., 2022). Based on the above discussion, the presented study aimed to identify fungal pathogens that cause black spots on lettuce plants using a DNA Barcoding approach with sequences on ribosomal DNA coding genes.

MATERIALS AND METHODS

Fungal pathogenic isolation

In the lettuce (*Lactuca sativa* L.) crop, the pathogen isolation used the *direct plating* technique (Suciatmih *et al.,* 2014). In the pertinent research, the tested fungus isolation started from lettuce with dark brown leaf spots. The *potato dextrose agar* (PDA) served as the isolation media. Isolation began by first cleaning parts of the plants for isolation under running water, then cutting portions of the lettuce with symptoms in sizes of 5 cm × 5 cm. The lettuce cuts acquired sterilization using alcohol (70%) for 1 min and Bayclin (5.25% sodium hypochlorite) for 2 min, then rinsed three times with *Aquades* and later dried on tissue (3–6 pieces).

Further cutting the lettuce to a smaller size (2 cm × 2 cm) continued, then placing them on the solid PDA media on a Petri dish and attained incubation in a dark space at room temperature (27 °C–28 °C). After 3–5 days of incubation period, the fungi appeared and received sterilization. Colony morphology with the same appearance, size, and color fell under the same-isolates category. Each colony underwent separation into specific isolates (Minarni, 2021). Later, the fungi isolate samples gained identification based on morphological characteristics and checking against the Common Fungus Introduction Book (Gandjar *et al.*, 1999). The different fungi colonies' disinfection continued, with their separation carried out using loose needles and grown.

Identification of pathogenic fungi

Determination of fungi that cause leaf spots on lettuce ensued using the 'Postulate Koch test' and macroscopic and microscopic observations and molecular identification using the internal transcribed spacer (ITS) area that consists of ITS1 and ITS4 and 5.8S rRNA with the following steps (Pit and Hocking, 1997):

Postulate Koch test

The gathered pure culture from the isolated result of inoculated 14-day planted lettuce helped identify the same symptoms in the field. Planting of all tested plants on polybag media ensued in the greenhouse. The daily observation of symptoms continued regularly for 30 days. The symptoms' comparison with the disease in the field followed. After that, re-isolation of pathogenic fungi on infected lettuce occurred to get a pure culture. Then, the pure culture incurred observing macroscopically and microscopically (Kowalski and Cramer, 2020). If the same fungus emerged as the isolated one on the lettuce, then a conclusion may be that the fungus is pathogenic that causes leaf spots on lettuce plants.

Macroscopic and microscopic identification

Morphological identification proceeded in two steps: macroscopic and microscopic. The macroscopic identification observed growing pathogens on the PDA media in a Petri dish, which includes the color of the fungi colony, the colony shape and surface, how fungi grow, and 'full-plate' age (Sugiarta *et al.,* 2021)**.** Microscopic identification continued following the 'slide culture' technique (Sibero *et al.,* 2017) by taking sterilized isolated fungi using loose needles; then, placing on a glass object attained a drop of aquades, afterward, covered with a glass cover for the isolate's viewing under a microscope (Barnett and Hunter, 1998). Microscopic identification took place to

observe the colony shape, color, amount (one or more), and conidia position, whether found as microconidia or not, color, and conidia formation and then checked on the reference book written by Tsuneo Watanabe (2002), "*Pictorial Atlas of Soil and Seed Fungi" (2nd edition)*, CRC Press, Florida (Nahor, 2018).

Molecular identification

DNA extraction

Total *Cercospora* spp. DNA genome extraction employed the Fast DNA SPIN Kit (*MP Bio Thermo Scientific*). Taking one piece of fungi culture aged five days continued placing into a lysing matrix tube with 1 ml buffer Kit CLS-Y. Fungi and buffer underwent homogenizing with Super FastPrep-1 (MPBio Thermo-scientific) and then centrifuged at 12000 rpm speed for 10 min. Taking a supernatant (±800 µl) and placing in a 2 ml micro-tube, the addition of a binding matrix equal to the supernatant volume followed, then mixed and incubated at room temperature for 5 min. Half of the supernatant volume and binding matrix (±700µl) incurred transferring to a tube spin filter, then centrifuged at 12000 rpm for 1 min.

The same step attained repetitions toward the rest of the solution after discarding the emulsion in the bottom of the tube. The pellet incurred the adding with 500 µl SEWS-M and homogenization with micro-type. The pellet gained centrifuging at 12000 rpm for 1 min, with the supernatant discarded and the tube replaced with the new one. Without adding anything, the pellet underwent re-centrifuged at 12000 rpm speed for 2 min. Changing the tube with a new tube size of 1.5 ml, DNA was eluted with 100 µl DES and incubated at 55 °C for 5 min, with the DNA re-centrifuged again at 12000 rpm for 1 min, discarding the spin filter consisting of the residue. Storing the clear liquid DNA at a temperature of -20 °C for long-term storage or at 4 °C for short-term storage followed (Hartati *et al.,* 2021).

DNA amplification with PCR

DNA amplification employed the PCR technique. The first step of PCR comprised preparing the 'master-mix.' Filling each PCR micro tube ensued consisting of 12 µl free-water, master mix PCR (5 µl), primer ITS1 (1 µl), primer ITS4 (1 µl), and template DNA (1 µl). Hence, the total of each mix of PCR solution was 20 µl. The transfer of the mix reagent in the PCR tube used a micropipette, followed by the amplification using a PCR machine (PTC 100, M.J. Research). First denaturation continued: 95 °C (90 s) for 35 cycles, each cycle consisting of denaturation at 95 °C (90 s), annealing at 55 °C (30 s), extension at 72 °C (90 s), and a final extension at 72 °C (5 min). Finally, the maintained PCR resulted at a final temperature (4 °C).

DNA visualization continued by electrophoresis through a horizontal electrophoresis tank using 1% agarose gel, putting the solution into each agar well on the electrophoresis machine. Before running, add the TAE (*Triacetate*) solution to the electrophoresis machine until the agarose gel submerges. Electrophoresis ran for 30 min at 110 volts (running time depended on the gel concentration and voltage). After electrophoresis, the agarose gel's submergence in ethidium bromide solution at a concentration of 0.12 µg/ml for 15 min continued. Next, visualization of the result of electrophoresis of DNA used the *Digi-Doc-Imaging System,* with the results saved on the computer. On visualization, the DNA profile between gene loci would be visible as a bright ribbon (Nishizawa *et al.,* 2010; Wicaksono *et al.,* 2017).

Sequencing ITS region and DNA sequences analysis

The data timing and assembling employed the Chromas Pro-version 1.5, with the assembled data processed under BLAST using the genome data previously registered on NCBI at

https://blast.ncbi.nlm.nih.gov/Blast.cgi. Constructing the phylogenetic tree resulted in aligning all sequences and the compared sequences. The data's reanalysis continued by aligning the sequences using MEGA V.5.0 (Tamura *et al.*, 2011) and a bootstrap value of 10,000 times (Sibero *et al.,* 2018).

Phylogenetic analysis

The continuing analysis ran the phylogenetic analysis using the software: ChromasPro, Molecular Evolutionary Genetics Analysis (MEGA 6.06), PAUP 4.0, and TreeGraph2.0.

RESULTS AND DISCUSSION

Isolate of pathogenic fungi

Based on the results obtained from the 'Postulate Koch Test' by inoculating the fungus *Cercospora* spp. on healthy lettuce, leaf spots were visible on healthy lettuce leaves. The basis of observations were the symptoms and the decay on the lettuce surface. The indications found in the field were the same as those on the Postulate Koch Test. Therefore, the study concluded that *Cercospora* spp. caused the leaf spot disease on lettuce (Figure 1). Of the eight lettuce genotypes subjected to Koch's Postulates, six sustained leaf spots, with two other lettuce genotypes attacked by late blight. *Cercospora* spp. also showed in several agricultural commodities, such as, beets, as well as, reducing the extraction of sugar content from the sap by interfering with the infected root system, being unable to hold sugar in high quantities and concentrations. At the same time, plants infected with *Cercospora* spp. can cause secondary infections from bacteria and viruses, resulting in higher losses to farmers (Hassanin *et al*., 2020; Papan *et al*., 2021).

Likewise, a study reported maize infected with the gray leaf spot disease caused by *Cercospora* spp., with symptoms in many large rectangular lesions on leaves, sheaths, and husks. When sensitive corn plants incur *Cercospora* spp. infections, many spots occur on the leaves, causing premature death with stems falling off, resulting in yield loss of 60%–80% (Dhami *et al.*, 2015). As many as 165 *C. zeina* isolates collected from corn fields exhibited gray leaf spot symptoms in several regions in China through single-spore isolation techniques and morphological and molecular identification (Duan *et al*., 2022). The number of these isolates is more than those obtained from this study; thus, further research is still necessary by expanding the sampling area in lettuce plantations in the Province of Bali.

Figure 1. A. Control, B. The mushroom was inoculated on lettuce seeds by spraying them on lettuce plants aged 14 days causing spots on the lettuce seeds.

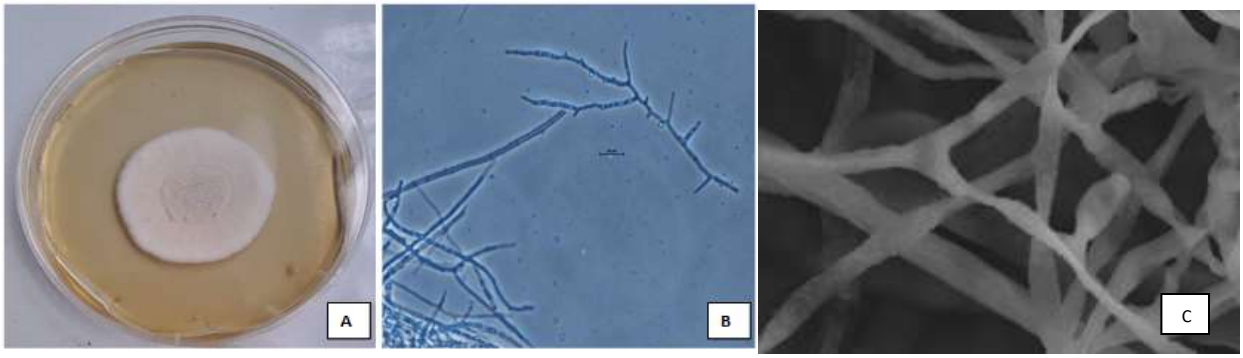

Figure 2. Macroscopic Characteristic: *Cercospora canescens*, A. fungal colonies, B. Microscopic Characteristic; C. Hyphae with SEM (Scanning Electron Microscope).

Table 1. Comparison of 18S rDNA gene similarity levels of *Cercospora* spp. isolates with multiple sequences in Gen Bank using the BLAST program.

Isolate	% Similarity	Accession Number
Cercospora canescens isolate Cer11-18	94%	MN400290.1
Cercospora canescens strain Cer74-18	91%	MK027100.1
Cercospora canescens genomic DNA containing 18S	90%	MN400241.1

Macroscopic and microscopic characteristics

Macroscopic characteristics' recordings revealed colony shape, color, and spread. Perceptible traits of *Cercospora spp*. were white, gray, and soft (Figure 2). The fungus Cercospora colony grew on the Petri dish 20 days after inoculation (HSI) (Sumartini, 2016). Based on fungi conidia observations, the shape of *Cercospora canescens* was lanceolate, septate with a length of 27.5–90 µm and width of 2.5–3.75 µm. *Cercospora canescens* fungus had dark conidiophores with three or more septates, saprophyte (Barnett, 1960). Microscopically observed were the shape, color, number, and position of conidiophores or sporangiophores (Ade, 2013).

Environmental conditions that are always rainy will support the development and spread of this fungus. One of the factors causing the widespread proliferation of this fungus is ambient temperature. This fungus will grow optimally at a temperature of 28 °C–32 °C and when the temperature's appropriate environment will help the spread of spores in infecting the plants. Leaf spot disease can reduce yields by up to 50% (Nursanti *et al*., 2021). Infected lettuce plants, brought to the laboratory for isolation, sought to determine the pathogenic species that infect lettuce. At this stage, the direct plating method proceeded. Lettuce leaves previously applied with pathogenic fungi are cut into small pieces with a size of approximately 1 cm in 6–8 parts and then planted on PDA media. A pure fungal culture appears in Figure 2A.

Molecular analysis

Samples of Cercospora spp.

The data collection was from the molecular analysis. Based on the alignment order of the Gen Bank database, *Cercospora* spp. had 94% similarity with the isolate *Cercospora canescens* Cer11-18 (Gen Bank access no: MN400290.1) (Table 1). It is also evident in the resulting phylogenetic tree, with a 1000× bootstrap value of 94% closely related to *Cercospora canescens* (Figures 3 and 4). Results of the phylogenetic tree analysis using

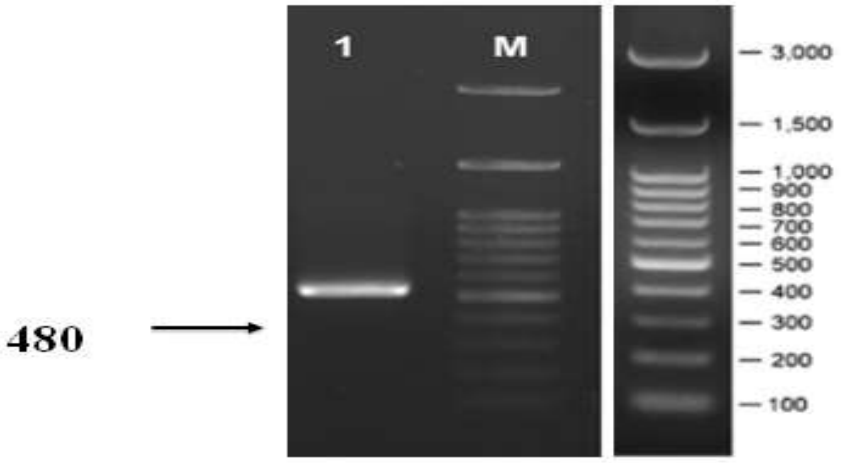

Figure 3. PCR amplification of ITS genes; 1= PCR product sample *Cercospora* spp.; M = 1 kb, ladder marker.

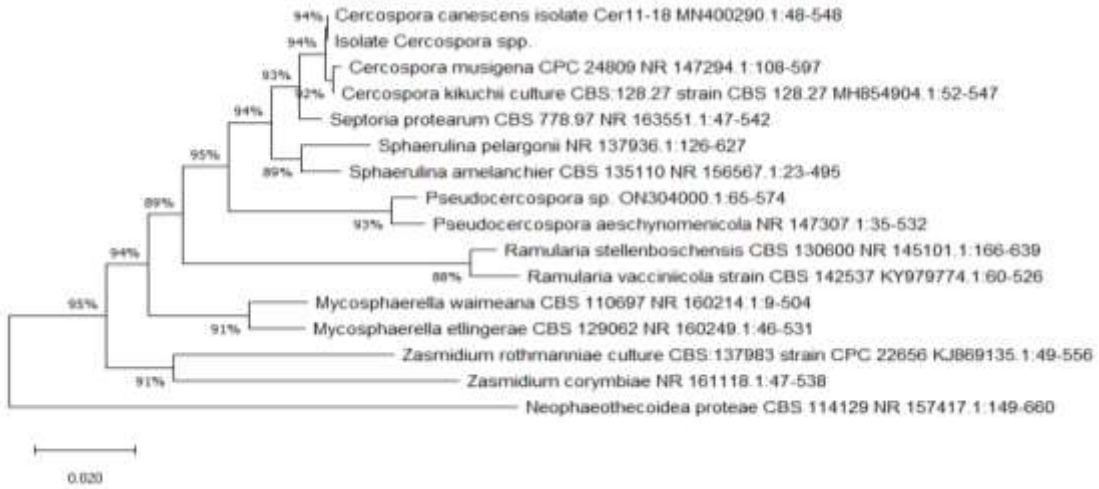

Figure 4. Phylogenic Tree from *Cercospora* spp. sample 94% related with *Cercospora canescens.*

the Neighbor-Joining (NJ) method showed that *Cercospora* spp. has a high similarity with *Cercospora canescens*, with a bootstrap value of 94% (Figure 4). Based on the phylogenetic tree, it validated that the ITS gene can provide an informative picture of the molecular phylogenetics of Rajalawe banana at the species level. Purnamasari *et al.* (2012) stated that the Internal Transcribed Spacer (ITS) area is an area that can serve as a genetic marker because it has a relatively high sequence variation even within the same species.

In various studies, *Cercospora* spp. had been indicative as a pathogen causing leaf spot disease in different countries. A report in Thailand stated *Cercospora lactucae-sativa* caused the *Cercospora* (To-Anun *et al*., 2011). Moreover, in China, findings said Cercospora leaf spot was evident on cultivated and wild lettuce. Koohakan *et al*. (2008) have reported that, in Thailand, *Cercospora* spp. has infected outdoor NFT-cultivated lettuce, causing leaf spot disease. Further outcomes mentioned that leaf spot on hydroponic lettuce in Thailand

occurred for the first time in 2004, and currently, it has become a chief disease in commercial hydroponic cultivated lettuce. Leaf spot typically manifests in rainy seasons. In its first appearance, a leaf spot emerged on the bottom surface of the leaf and continuously spread to the top cover. Close row planting, high humidity, and poor ventilation cause leaf spot disease to grow well. The symptoms usually have the name of frog eye leaf spots. Infected plants will lose weight because the rotten leaf needs cutting and are unable to sell in the premium market because the product has low quality.

The use of ITS gene primers was also successful for molecular identification of *Cercospora* spp. isolated from maize plantations in China with gene diversity (H') ranging from 0.1421 to 0.3090 (Duan *et al*., 2022). Genetic differences can incur influences from biotic factors and their adaptability to the environment (Supartha *et al*., 2022). Biological mutations resulting in a higher genetic distance for an organism in each region can originate directly from organisms, including gene mutations, gene migration, sexual recombination, somatic recombination, and population size (Khademi *et al*., 2019). Abiotic factors largely influence genetic divergence between populations. For example, temperature and rainfall impacted the genetic diversity of *Caragana microphylla* (Huang *et al*., 2016). Latitude, temperature, and nutrient availability can all contribute to the microdiversity of species (Ren *et al*., 2013). The strain of *C. kikuchii* ARG_18_001 achieved successful isolation from purple soybean seeds in San Pedro, Argentina, obtaining a 33.1 Mb draft genome with 53% GC content and gene prediction, resulting in 14,856 model genes/14,721 protein-coding genes (Sautua *et al.,* 2019). Further research can progress to identify *C. canescens* isolates obtained in this study using the whole genome sequencing approach, as they are helpful as a source for future pathosystem studies.

Several control measures with a biocontrol approach can serve for further research on *C. canescens* attacks on lettuce crops. Previous studies revealed that the in vitro and in vivo efficacy of *Bacillus subtilis*, *Moringa oleifera,* and potassium bicarbonate can suppress the Cercospora leaf spot disease. Two concentrations of five and 10 g/l tested on a laboratory and field scale showed a substantial decrease in the linear development of Cercospora in sugar beets (Sehsah *et al*., 2022). In addition, this study also used the scanning electron microscope (SEM) to analyze the morphology of *C. canescens,* which showed the structural presence of the fungus and the formation of conidiophores and conidiospores.

CONCLUSIONS

Based on this research, it concludes that the pathogenic fungus that causes leaf spots on lettuce in Bali, Indonesia, is *Cercospora canescens*. However, further research is necessary to get other *Cercospora* species, which may also be responsible for the lettuce leaf spot. Likewise, efforts to manage this pathogenic fungus using a biocontrol approach are appropriate.

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