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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Pakistan Journal of Biological Sciences

ISSN 1028-8880
DOI: 10.3923/pjbs.2021.588.598

Research Article
Variability of *Lecanicillium* spp. Mycoparasite of Coffee Leaf Rust Pathogen (*Hemileia vastatrix*) in Indonesia

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Abstract

Background and Objective: Coffee leaf rust disease caused by *Hemileia vastatrix* resulted in high yield loss and difficult to control. Several chemical fungicides have been used to control this disease. However, the effectiveness of chemical control is low, so it is necessary to find other methods such as biological control. *Lecanicillium* spp. is well-known as mycoparasite on *H. vastatrix* uredospores but the study in Indonesia is still limited. This study aimed to collect and investigated the genetic variability of *Lecanicillium* spp. at various coffee plantations in Indonesia. **Materials and Methods:** Samples of *Lecanicillium* spp. were collected from 20 districts in 7 provinces throughout Indonesia. Morphology of colony and conidia were identified by visual examination and by viewed under the light microscope. Genetic variability was conducted using Rep-PCR and clustered with UPGMA. **Results:** Morphological observation in this study revealed all isolates collected from uredospores of *H. vastatrix* were similar with *Lecanicillium* spp. Genetic variability analysis clustered the 80 isolates into eight clusters with their specific characters. **Conclusion:** Morphological identification in this study showed that 80 isolates of mycoparasite on *H. vastatrix* belong to *Lecanicillium* spp. Further study using the molecular technique is needed to identity the species of *Lecanicillium*.

Key words: Biological control, coffee, *Hemileia vastatrix*, leaf rust disease, *Lecanicillium* spp., mycoparasite, variability

Citation: Setiawati, R., A. Wibowo, A. Widiastuti and A. Priyatmojo, 2021. Variability of *Lecanicillium* spp. Mycoparasite of coffee leaf rust pathogen (*Hemileia vastatrix*) in Indonesia. Pak. J. Biol. Sci., 24: 588-598.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Coffee (*Coffea* spp.) is one of the world's main plantation commodities on which many countries rely as a source of foreign exchange[1]. Indonesia is the fourth largest coffee producer in the world with a planted area of 1.2 million ha and total production of 639,305 t in 2017[2].

Coffee productivity in Indonesia is still relatively low when compared to other coffee producing countries. The main obstacles in the development of coffee in Indonesia are coffee leaf rust disease caused by *Hemileia vastatrix* (Berkeley and Broome). The loss caused by this disease may reach 70% in susceptible varieties[3].

Conventional control over this disease is carried out with the application of fungicides[4]. Several types of fungicidal active ingredients recommended by the Pesticide Commission in Indonesia are cyproconazole, hexacazanole, triadimefon, benomyl, copper oxychloride, mancozeb, copper hydroxide, copper oxide, difenoconazole and propiconazole. This application only reduces the rate of plant damage on coffee leaf rust disease by about 20%[5]. The use of the systemic fungicide triadimefon should be limited because it can affect plant physiology, especially photosynthetic[6].

Unfortunately, this application method not only causing pathogen resistance to fungicides but also causing phytotoxicity, resulting in the emergence of a new physiological race of *H. vastatrix* and residue in the surrounding environment[4,7]. Therefore, it is necessary to use alternative disease control in coffee using biological control agents to control coffee leaf rust disease[8].

Lecanicillium spp. (formerly *Verticillium* spp.) is well-known as entomopathogens that have been commercially developed as a biopesticide. This fungus produces toxic secondary metabolites, namely bassianolidae and dipicolinic acid, which are insecticidal[9-11]. However, recently is has been discovered that this entomopathogen is also known as mycoparasite in some pathogenic fungi such as *Sphaerotheca fuliginea, S. macularis* f. sp*. fragariae*, *Penicillium digitatum and Phytium ultimum*[11-15]. Several species of the *Lecanicillium* also have been reported to parasitize *H. vastatrix* uredospores[4,16-21].

Lecanicillium spp. was able to control *H. vastatrix* in the laboratory and in the greenhouse[18]. *In vitro*, this reduced uredospores of *H. vastatrix* up to 59.3% and the rate of crop damage due to *H. vastatrix* decreased by 68.2%. Gomez-De La Cruz *et al.*[22] also stated that *Lecanicillium* spp. showed mycoparasitism in coffee leaf rust uredospores, 120 hrs after inoculation with an average of 68%. The potential of *Lecanicillium* spp. as a biological control agent of *H. vastatrix* needs to be further investigated as an environmentally friendly control effort.

The purpose of this study was to collect and find out the genetic variability of *Lecanicillium* spp. associated with *H. vastatrix* isolated from a various coffee plantations in Indonesia.

MATERIALS AND METHODS

Study area: The study was carried out at the Laboratory of Plant Disease, Department of Crop Protection, Faculty of Agriculture, Universitas Gadjah Mada, Special Region of Yogyakarta, Indonesia since August, 2019-October, 2020.

Sample collection: Samples of *Lecanicillium* spp. were collected by purposive sampling at coffee plantations in seven provinces in Indonesia including West Java, Central Java, Special Region of Yogyakarta, East Java, Bali, West Nusa Tenggara (NTB) and East Nusa Tenggara (NTT) (Fig. 1). Coffee leaf samples showing lesions and containing uredospores of *H. vastatrix* with colonies of *Lecanicillium* spp. on top were plucked from the coffee plant. The leaves are put in a paper bag and store in a cooler box before taken to the laboratory for isolation.

Isolation of *Lecanicillium* spp.: Isolation of *Lecanicillium* spp. was done aseptically by taking white mycelium using a needle preparation and growing it into a petri dish containing 2% Water Agar (WA) medium. After incubation for 3-5 days, purification was carried out by inoculating the growing mycelium on the WA into a petri dish containing Potato Dextrose Agar-L (PDA added 1-2 drops of 25% lactic acid). Incubation was carried out at room temperature (25-27°C) for 7 days.

Purification of *Lecanicillium* spp. with single-spore isolation: *Lecanicillium* spp. single spore isolation was carried out by cutting using 0.5 cm cork borer of 7 days culture on PDA and adding with 1 mL sterile distilled water in 1.5 mL tube. The suspension was homogenized by Thermolyne Mixer vortex (Haverhill, MA, USA) for about 2 min. The suspension obtained was taken using a loop needle and scratched on the new PDA medium. The streaks were incubated for approximately 12-24 hrs at 25-27°C. The germinated fungal spore then took by examination through a light microscope using a sterile needle and transferred into new PDA plates. Pure cultures were identified by visual examination

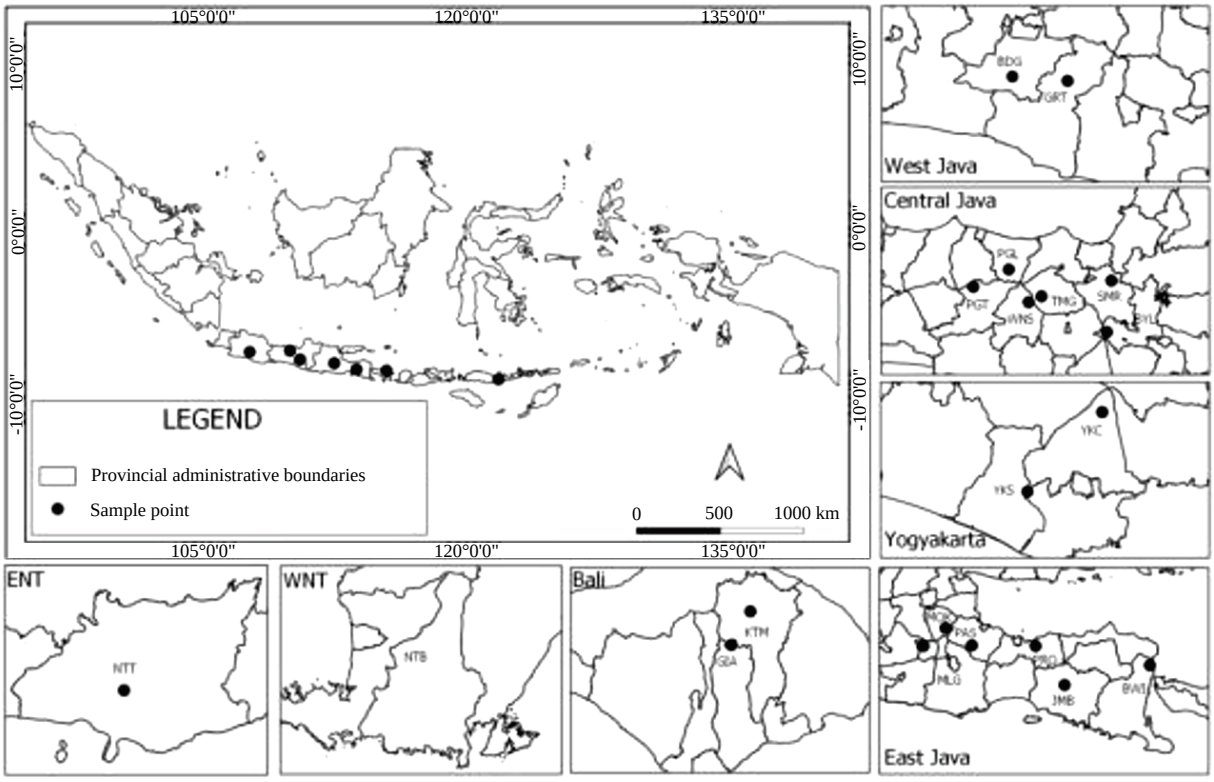

Fig. 1: Sampling sites *Lecanicillium* spp. associated with *Hemileia vastatrix*

Map resource: RBI Map 2010

(macroscopic) and viewed under the CX31 microscope (Olympus Co., Tokyo, Japan) (microscopic).

Morphological analysis: Morphological characteristics of the isolates were observed based on culture growth on PDA which incubated at 25-27°C for 21 days[18,23]. The observation of culture morphology including colony color, colony texture and colony diameter visually. Fungal colony colors were observed from the lower side of the culture plate and compare with the Munsell Soil Color Charts after three weeks of incubation. Microscopic features were observed by slide cultures on PDA, included conidiophores, crystal and conidia size. Fifty conidia were observed for shape and size measures. The examination was done using CX 31 microscope (Olympus Co., Tokyo, Japan), an image captured by DP 22 microscope camera (Olympus Co., Tokyo, Japan) and viewer cellSens imaging software (Olympus Co., Tokyo, Japan).

DNA extraction: Mycelia of 80 sample isolate for DNA extraction were grown on Potato Dextrose Broth (PDB: potato 200 g: Dextrose 20 g: Water to a final volume of 1,000 mL) in Erlenmeyer and shaken for 7 days at 25-27°C. Each DNA was extracted using the Cetyl Trimethyl Ammonium Bromide (CTAB) according to Xia *et al.*[24] with modification. 0.5 g of mycelia were broken done by grinding by a mortal in a porcelain with the addition of 700 µL of 2% CTAB (2% PVP, 100 mM Tris-HCl pH 8, 25 mM EDTA, 2 M NaCl and 200 mM beta-mercaptoethanol/BME CTAB) solution and a little quartz sand. The mixture was then put into an Eppendorf tube 1.5 mL, then heated in a water bath at 60°C, for 30 min (shaken every 10 min) and centrifuged at 5000 rpm for 5 min. The supernatant was recovered into a 1.5 mL microtube, then added with CIAA (chloroform: isoamyl alcohol 24:1,v/v)) added until the tube was full, followed by centrifugation for 10 min at 12,000 rpm. The top supernatant fraction was recovered into a new microtube, to which, absolute ethanol was added and stored overnight at -20°C. Subsequently, the fraction was centrifuged for 10 min at 12,000 rpm. The resultant supernatant was removed and the pellet was washed in 70% ethanol and further centrifuged for 10 min at 12,000 rpm. The pellet was air dried for 2-3 hrs and re-suspended in 20-50 µL of pure water.

Table 1: Primer sets for rep-PCR used in this study

Primers	Sequences	PCR cycle	No. of cycle	Reference
		95°C 7 min		
		94°C 1 min		
BOX A1R	5′-CTACGGCAAGGCGACGCTGACG-3′	53°C 1 min	30	25
		72°C 8 min		
		72°C 16 min		
ERIC 1	5′-ATGTAAGCTCCTGGGGATTCAC-3′	95°C 7 min		
ERIC 2	5′-AAGTAAGTGACTGGGGTGAGCG-3′	94°C 1 min		
		52°C 1 min	30	25
		72°C 8 min		
		72°C 16 min		

PCR amplification: The PCR was carried out in a total volume 25 µL, comprised of 12.5 µL DNA Taq polymerase (My Taq HS Red Mix, Bioline, London, United Kingdom), 1.5 µL each primer (100 µM) 1 µL DNA template and sterile water in a total volume of 25 µL. Amplification was conducted using T100 Thermal Cycler (Biorad, California, United States). The rep-PCR using BOX A1R and ERIC with the PCR program conditions in Table 1. Reactions were performed in BioRad T100'tm Thermal Cycler (Bio-Rad, Hercules, CA, USA). The PCR products were then visualized using 1.5% agarose gel (0.45 g of agarose (Molecular Biology Grade, Vivantis) in 30 mL of TBE 1X). Agarose gel was then electrophoresed at 100 V for 25 min using BioRad Mini ReadySub-Cell gt Horizontal Electrophoresis Cell (Bio-Rad, Hercules, CA, USA). Agarose gel was then stained with a solution of ethidium bromide for 10 min and washed with water. The result was then visualized under UV light. The DNA bands were then captured using a digital camera[25].

Phylogenetic analysis: The band patterns were analyzed by summarizing them into table with notification of 0-1 (0 for no band, 1 for the present band). This was made using Microsoft Excel to place the band arrangement in columns and isolate numbers in rows to construct the dendrogram[26]. Dendrogram representing the genetic variability among *Lecanicillium* spp. were generated from the similarity matrices by applying unweighted pair-group arithmetic (UPGMA) mean methods in numerical taxonomy and multivariate analysis system (NTSYSpc version 2.10e).

RESULTS

Study area: Samples collection of *Lecanicillium* spp. was conducted at 20 districts in 7 provinces throughout Indonesia with various altitudes (Table 2). Altitude varied from 669-1620 m above sea level. Its host was *H. vastatrix* with varied varieties origin of coffee, namely *Coffea arabica* L., *C. canephora* Pierre and *C. liberica* var *dewevrei*.

Symptoms of rust on coffee leaves are shown in Fig. 2a, b. In general, a symptom of rust on coffee leaves occur on the lower leaf surface. The initial symptom is in the form of chlorotic spots and will continue with the formation of a light yellow necrotic lesion which gradually turns dark yellow like rust. These lesions form orange pustules, which are masses of *H. vastatrix* uredospores (Fig. 2a). *Lecanicillium* spp. is one of the mycoparasite associated with *H. vastatrix* caused coffee rust disease. This fungal infection is characterized by initial symptoms of white colonies on *H. vastatrix* uredospores that form on the surface of the coffee leaves. The occurrence of these parasites causes less infection of *H. vastatrix* (Fig. 2b).

Morphological feature: Morphological characteristics of *Lecanicillium* spp. were observed on PDA after 21 days of incubation. Colonies reached 32-68 mm diameter in 21 days at 25-27°C. Texture colony of isolates was mostly wholly (Fig. 3a) and cottony (Fig. 3b), dense colony with aerial mycelia. Only a few isolates were velvety (Fig. 3c). The color of the aerial view of the isolates was white but the color of the reverse view varied, such as pale yellow, yellow, white, light brown and dark brown. Conidiophores are single or in groups of 2-4 phialides (verticillate) (Fig. 3d, e). Conidia formed in heads at the apex of phialides, 1.7-2.2×2.7-4.4 µm. Conidia produced by *Lecanicillium* spp. isolates were cylindrical or ellipsoidal (Fig. 3f). Crystals present with octahedral in shape (Fig. 3g).

Phylogenetic analysis using rep-PCR: Phylogenetic analysis was grouping 80 *Lecanicillium* spp. isolates the polymorphic bands ranging between 100-5000 bp were observed. UPGMA analysis using BOX A1R and ERIC primers showed that there were 8 clusters of *Lecanicillium* spp. at a coefficient value of 0.80 (Fig. 4). The data of Table 3 showed cluster 1 consisting of 26 isolates, cluster 2 consisting of 17 isolates, cluster 3 consisting of 14 isolates, cluster 4 consisting of 9 isolates, cluster 5 consisting of 4 isolates, cluster 6 consisting of 3 isolates cluster 7 consisting of 5 isolates and cluster 5 which only consisted of 2 isolates, namely DIY 4 and NTB 3.

Table 2: List of *Lecanicillium* spp. isolates

Sample code	Field code	District	Province	Host	Varieties origin	Altitude	Coordinates
BDG 1	JBR 1	Bandung	West Java	*H. vastatrix*	*C. arabica*	1286	7°09'25.4"S 107°33'52.5"E
BDG 2	JBR 2	Bandung	West Java	*H. vastatrix*	*C. arabica*	1286	7°09'25.4"S 107°33'52.5"E
BDG 4	JBR 3	Bandung	West Java	*H. vastatrix*	*C. arabica*	1286	7°09'25.4"S 107°33'52.5"E
BDG 5	JBR 4	Bandung	West Java	*H. vastatrix*	*C. arabica*	1286	7°09'25.4"S 107°33'52.5"E
BDG 6	JBR 5	Bandung	West Java	*H. vastatrix*	*C. arabica*	1286	7°09'25.4"S 107°33'52.5"E
BDG 8	JBR 6	Bandung	West Java	*H. vastatrix*	*C. arabica*	1286	7°09'25.4"S 107°33'52.5"E
BDG 15	JBR 7	Bandung	West Java	*H. vastatrix*	*C. arabica*	1286	7°09'25.4"S 107°33'52.5"E
GRT 3	JBR 8	Garut	West Java	*H. vastatrix*	*C. arabica*	1264	7°21'53.8"S 107°48'40.4"E
GRT 7	JBR 9	Garut	West Java	*H. vastatrix*	*C. arabica*	1264	7°21'53.8"S 107°48'40.4"E
GRT 10	JBR 10	Garut	West Java	*H. vastatrix*	*C. arabica*	1264	7°21'53.8"S 107°48'40.4"E
YKC 1A	DIY 1	Sleman	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	930	7°35'56.9"S 110°26'55.7"E
YKC 2A	DIY 2	Sleman	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	930	7°35'56.9"S 110°26'55.7"E
YKC 1R	DIY 3	Sleman	Special Region of Yogyakarta	*H. vastatrix*	*C. canephora*	930	7°35'56.9"S 110°26'55.7"E
YKP 1A	DIY 4	Sleman	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	743	7°36'51.2"S 110°25'35.4"E
YKP 3A	DIY 5	Sleman	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	743	7°36'51.2"S 110°25'35.4"E
YKP 1R	DIY 6	Sleman	Special Region of Yogyakarta	*H. vastatrix*	*C. canephora*	743	7°36'51.2"S 110°25'35.4"E
YKP 2R	DIY 7	Sleman	Special Region of Yogyakarta	*H. vastatrix*	*C. canephora*	743	7°36'51.2"S 110°25'35.4"E
YKS 1	DIY 8	Kulon Progo	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	857	7°38'51.4"S 110°10'58.2"E
YKS 2	DIY 9	Kulon Progo	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	857	7°38'51.4"S 110°10'58.2"E
YKS 3	DIY 10	Kulon Progo	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	857	7°38'51.4"S 110°10'58.2"E
YKS 6	DIY 11	Kulon Progo	Special Region of Yogyakarta	*H. vastatrix*	*C. arabica*	857	7°38'51.4"S 110°10'58.2"E
PGT 1	JTH 1	Banjarnegara	Central Java	*H. vastatrix*	*C. arabica*	1039	7°17'48.7"S 109°48'43.4"E
PGT 2	JTH 2	Banjarnegara	Central Java	*H. vastatrix*	*C. arabica*	1039	7°17'48.7"S 109°48'43.4"E
PGT 3	JTH 3	Banjarnegara	Central Java	*H. vastatrix*	*C. arabica*	1039	7°17'48.7"S 109°48'43.4"E
PGT 4	JTH 4	Banjarnegara	Central Java	*H. vastatrix*	*C. arabica*	1039	7°17'48.7"S 109°48'43.4"E
PJW 2	JTH 5	Banjarnegara	Central Java	*H. vastatrix*	*C. arabica*	1326	7°13'15.9"S 109°49'28.6"E
PJW 5	JTH 6	Banjarnegara	Central Java	*H. vastatrix*	*C. arabica*	1326	7°13'15.9"S 109°49'28.6"E
PGL 2	JTH 7	Batang	Central Java	*H. vastatrix*	*C. arabica*	948	7°06'43.1"S 109°51'21.3"E
PGL 4	JTH 8	Batang	Central Java	*H. vastatrix*	*C. arabica*	948	7°06'43.1"S 109°51'21.3"E
PGL 12	JTH 9	Batang	Central Java	*H. vastatrix*	*C. arabica*	948	7°06'43.1"S 109°51'21.3"E
PGL 14	JTH 10	Batang	Central Java	*H. vastatrix*	*C. arabica*	948	7°06'43.1"S 109°51'21.3"E
PGL 18	JTH 11	Batang	Central Java	*H. vastatrix*	*C. arabica*	948	7°06'43.1"S 109°51'21.3"E
PGL 20	JTH 12	Batang	Central Java	*H. vastatrix*	*C. arabica*	948	7°06'43.1"S 109°51'21.3"E
BOY 3	JTH 13	Boyolali	Central Java	*H. vastatrix*	*C. arabica*	1620	7°30'06.4"S 110°27'38.6"E
BOY 4	JTH 14	Boyolali	Central Java	*H. vastatrix*	*C. arabica*	1620	7°30'06.4"S 110°27'38.6"E
BOY 6	JTH 15	Boyolali	Central Java	*H. vastatrix*	*C. arabica*	1620	7°30'06.4"S 110°27'38.6"E
BOY 7	JTH 16	Boyolali	Central Java	*H. vastatrix*	*C. arabica*	1620	7°30'06.4"S 110°27'38.6"E
BRG 1	JTH 17	Semarang	Central Java	*H. vastatrix*	*C. arabica*	1554	7°10'54.1"S 110°21'36.8"E
BRG 4	JTH 18	Semarang	Central Java	*H. vastatrix*	*C. arabica*	1554	7°10'54.1"S 110°21'36.8"E
JMB 1	JTH 19	Semarang	Central Java	*H. vastatrix*	*C. arabica*	836	7°19'00.2"S 110°22'15.7"E
TMG 1	JTH 20	Temanggung	Central Java	*H. vastatrix*	*C. arabica*	1488	7°19'33.1"S 110°01'58.5"E

Table 2: Continued

Sample code	Field code	District	Province	Host	Varieties origin	Altitude	Coordinates
TMG 4	JTH 21	Temanggung	Central Java	*H. vastatrix*	*C. arabica*	1488	7°19'33.1"S 110°01'58.5"E
TMG 5	JTH 22	Temanggung	Central Java	*H. vastatrix*	*C. arabica*	1488	7°19'33.1"S 110°01'58.5"E
TMG 6	JTH 23	Temanggung	Central Java	*H. vastatrix*	*C. arabica*	1488	7°19'33.1"S 110°01'58.5"E
WNS 1	JTH 24	Wonosobo	Central Java	*H. vastatrix*	*C. arabica*	1495	7°23'30.3"S 109°57'41.8"E
WNS 5	JTH 25	Wonosobo	Central Java	*H. vastatrix*	*C. arabica*	1495	7°23'30.3"S 109°57'41.8"E
BWI 1	JTM 1	Banyuwangi	East Java	*H. vastatrix*	*C. arabica*	1323	8°00'01.7"S 114°17'10.0"E
JMB 1	JTM 2	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
JMB 2	JTM 3	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
JMB 3	JTM 4	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
JMB 4	JTM 5	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
JMB 9	JTM 6	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
JMB 10	JTM 7	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
JMB 13	JTM 8	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
JMB 14	JTM 9	Jember	East Java	*H. vastatrix*	*C. arabica*	722	8°03'53.5"S 113°38'32.3"E
MLG 1	JTM 10	Malang	East Java	*H. vastatrix*	*C. liberica* var *dewevrei*	669	7°94'10.8"S 112°21'54.2"E
MLG 3	JTM 11	Malang	East Java	*H. vastatrix*	*C. liberica* var *dewevrei*	669	7°94'10.8"S 112°21'54.2"E
MLG 4	JTM 12	Malang	East Java	*H. vastatrix*	*C. liberica* var *dewevrei*	669	7°94'10.8"S 112°21'54.2"E
MOK 3	JTM 13	Mojokerto	East Java	*H. vastatrix*	*C. arabica*	1174	7°40'56.0"S 112°35'8.00"E
MOK 4	JTM 14	Mojokerto	East Java	*H. vastatrix*	*C. arabica*	1174	7°40'56.0"S 112°35'8.00"E
PAS 1	JTM 15	Pasuruan	East Java	*H. vastatrix*	*C. arabica*	1431	7°45'48.0"S 112°38'2.00"E
PAS 3	JTM 16	Pasuruan	East Java	*H. vastatrix*	*C. arabica*	1431	7°45'48.0"S 112°38'2.00"E
PAS 4	JTM 17	Pasuruan	East Java	*H. vastatrix*	*C. arabica*	1431	7°45'48.0"S 112°38'2.00"E
PRO 1	JTM 18	Probolinggo	East Java	*H. vastatrix*	*C. arabica*	905	7°56'34.0"S 113°29'04.0"E
PRO 2	JTM 19	Probolinggo	East Java	*H. vastatrix*	*C. arabica*	905	7°56'34.0"S 113°29'04.0"E
GIA 5	BAL 1	Gianyar	Bali	*H. vastatrix*	*C. arabica*	957	8°19'42.0"S 115°17'29.0"E
GIA 6	BAL 2	Gianyar	Bali	*H. vastatrix*	*C. arabica*	957	8°19'42.0"S 115°17'29.0"E
KTM 2	BAL 3	Bangli	Bali	*H. vastatrix*	*C. arabica*	1141	8°10'42.5"S 115°15'56.2"E
KTM 7	BAL 4	Bangli	Bali	*H. vastatrix*	*C. arabica*	1141	8°10'42.5"S 115°15'56.2"E
SEM 3	NTB 1	Lombok Timur	West Nusa Tenggara	*H. vastatrix*	*C. arabica*	857	8°18'50.2"S 116°29'16.3"E
SEM 4	NTB 2	Lombok Timur	West Nusa Tenggara	*H. vastatrix*	*C. arabica*	857	8°18'50.2"S 116°29'16.3"E
SEM 1R	NTB 3	Lombok Timur	West Nusa Tenggara	*H. vastatrix*	*C. canephora*	857	8°18'50.2"S 116°29'16.3"E
END 1A	NTT 1	Ende	East Nusa Tenggara	*H. vastatrix*	*C. arabica*	891	8°43'49.2"S 121°43'34.8"E
END 3A	NTT 2	Ende	East Nusa Tenggara	*H. vastatrix*	*C. arabica*	891	8°43'49.2"S 121°43'34.8"E
END 4A	NTT 3	Ende	East Nusa Tenggara	*H. vastatrix*	*C. arabica*	891	8°43'49.2"S 121°43'34.8"E
END 5A	NTT 4	Ende	East Nusa Tenggara	*H. vastatrix*	*C. arabica*	891	8°43'49.2"S 121°43'34.8"E
END 6A	NTT 5	Ende	East Nusa Tenggara	*H. vastatrix*	*C. arabica*	891	8°43'49.2"S 121°43'34.8"E
END 9A	NTT 6	Ende	East Nusa Tenggara	*H. vastatrix*	*C. arabica*	891	8°43'49.2"S 121°43'34.8"E
END 1R	NTT 7	Ende	East Nusa Tenggara	*H. vastatrix*	*C. canephora*	891	8°43'49.2"S 121°43'34.8"E
END 2R	NTT 8	Ende	East Nusa Tenggara	*H. vastatrix*	*C. canephora*	891	8°43'49.2"S 121°43'34.8"E

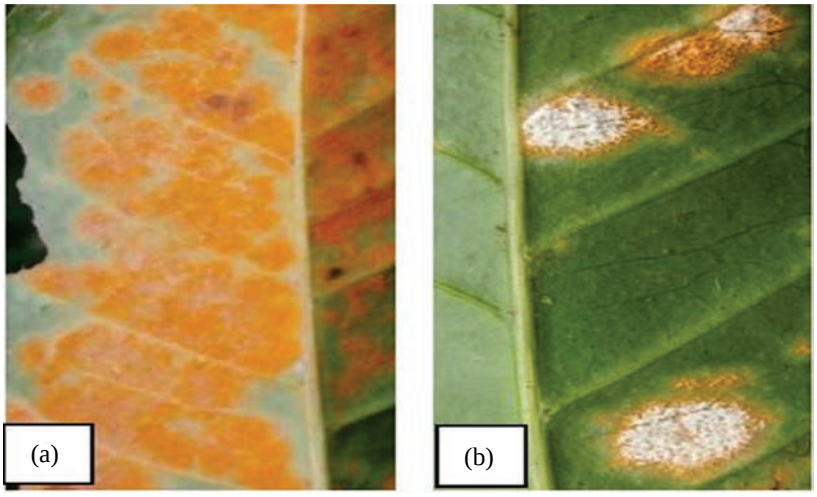

Fig. 2: Rust symptom on coffee leaf caused by *Hemileia vastatrix* (a), The white colonies fungus, Lecanicillium spp. associated with Hemileia vastatrix (b)

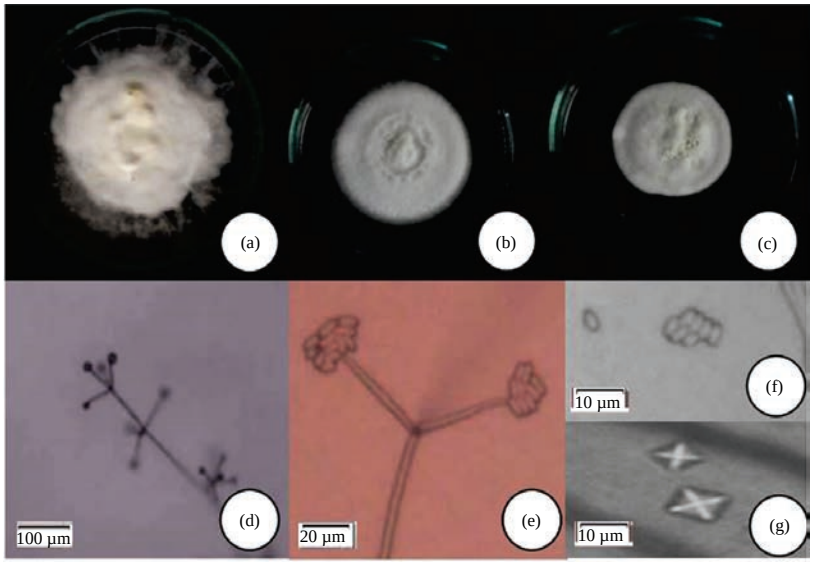

Fig. 3: Morphology of *Lecanicillium* spp.

Texture colony wholly (a); cottony (b); velvety (c); 21d on PDA; Conidiophores and phialides. (d-e); Conidia (f); Octahedral Crystal (g)

Table 3: List of *Lecanicillium* spp. isolates clusters based on UPGMA

Cluster	Number of isolate *Lecanicillium* spp.	Field code of *Lecanicillium* spp.
I	26	JBR 1, JBR 2, JBR 3, JBR 4, JBR 5, JBR 6*, JBR 8, JBR 9, JBR 10, DIY 2, DIY 3, DIY 9, DIY 11, JTH 1, JTH 2, JTH 3, JTH 4, JTH 14 JTH 16, JTH 23, JTH 24, JTH 25, JTM 2, JTM 18, NTT 9, NTT 10
II	17	JTH 5, JTH 6, JTH 7, JTH 8, JTH 9, JTH 10, JTM 12, JTM 13, JTM 14, JTM 15, JTM 16, NTB 1, NTB 2, NTT 2, NTT 4, NTT 5*, NTT 6
III	14	JBR 7, JTM 1*, JTM 3, JTM 4, JTM 5, JTM 6, JTM 7, JTM 8, JTM 9 JTM 11, BAL 1, BAL 2, BAL 3, BAL 4
IV	9	DIY 1, JTH 17, JTH 18, JTH 19, JTH 20, JTH 21, JTH 22, JTM 10*, JTM 12
V	4	JTH 11, JTH 12*, NTT 1, NNT 3
VI	3	JTH 13, JTH 15*, JTM 17
VII	5	DIY 5*, DIY 6, DIY 7, DIY 8, DIY 10
VIII	2	DIY 4, NTB 3*

Sign* was represent to selected isolate

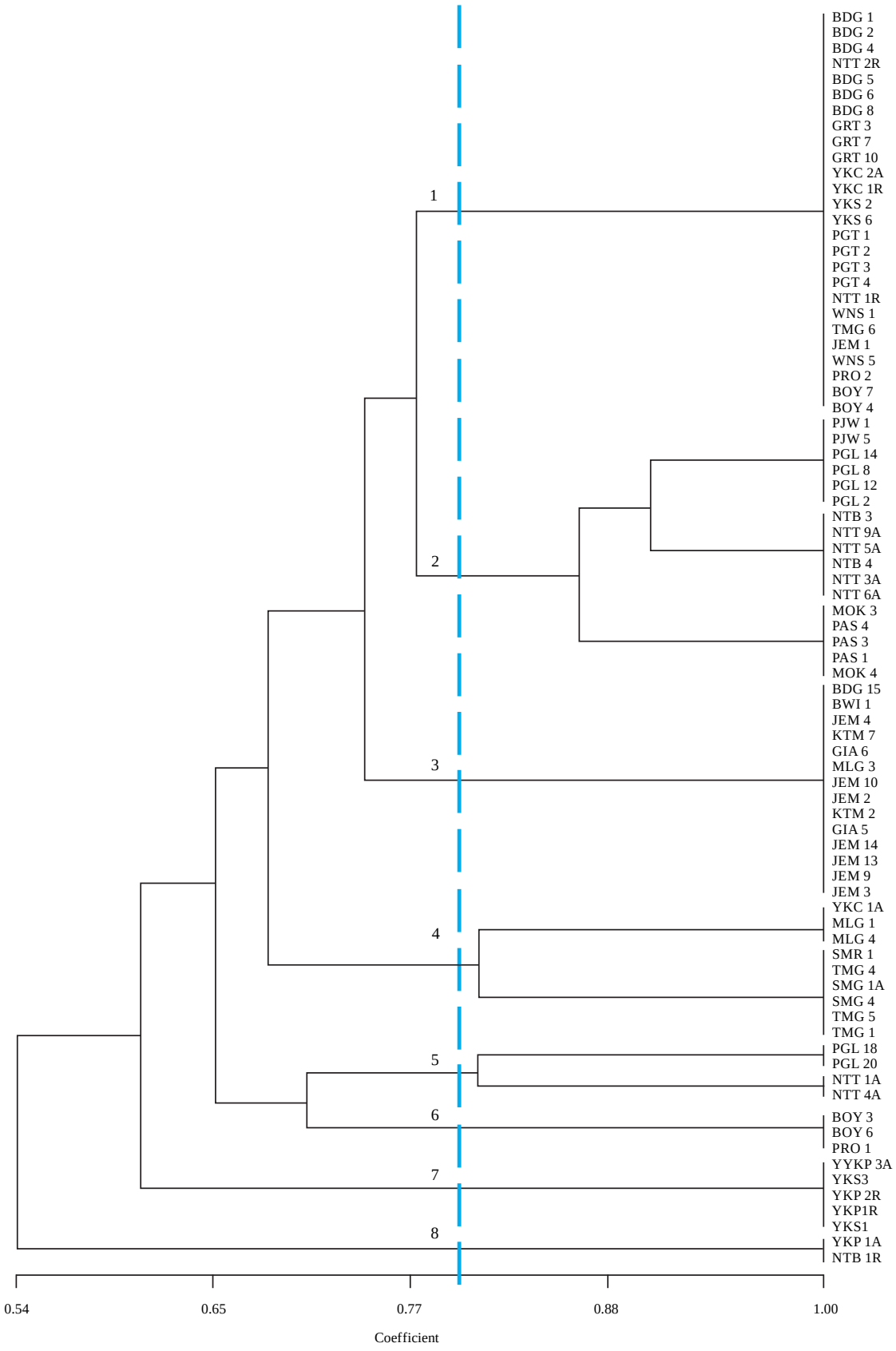

Fig. 4: Dendrogram of *Lecanicillium* spp. isolates clusters based on UPGMA

Table 4: Colony and conidia are characteristic of each *Lecanicillium* spp. cluster

Cluster	Field code	Sample code	Colony color		Colony texture	Growth rate (mm/day)	Conidial shape	Conidia size (µm)		Octahedral crystal
			Aerial view	Reverse view				Length	Width	
I	JBR 6	BDG 8	White	White	Wholly	1.089	Cylindrical	3.244	2.046	Present
II	NTT 5	NTT 6	White	Pale yellow	Cottony	3.089	Cylindrical	2.669	1.735	Present
III	JTM 1	BWI	White	Light brown	Cottony	2.750	Cylindrical	2.718	1.725	Present
IV	JTM 10	MLG 1	White	Yellow	Wholly	2.875	Cylindrical	3.642	2.090	Present
V	JTH 12	PGL 20	White	White	Wholly	1.982	Cylindrical	4.174	2.026	Present
VI	JTH 15	BOY 6	White	White	Wholly	2.053	Cylindrical	4.439	2.169	Present
VII	DIY 5	YKP 3	White	Pale yellow	Velvety	1.982	Cylindrical	2.906	1.731	Present
VIII	NTB 3	NTB 1	White	Dark brown	Wholly	2.607	Ellipsoidal	3.406	2.032	Present

Eight *Lecanicillium* spp. isolates were randomly selected as representatives from each cluster based on the phylogenetic tree to determine their genetic variation. The data of Table 4 showed that the 8 clusters had variation macroscopic and microscopic morphology. Cluster 1 was represented by JBR 6 isolate that had a white aerial view and reverse view colony. The texture colony was whole with a slow growth rate. Cluster 2 was represented by NTT 5 isolate that had cottony colony texture and a small size of conidia. Cluster 3 was represented by JTM 1 isolate that had light brown reverse view colony color, cottony with the medium growth rate. Cluster 4 was represented by JTM 10 isolate that had yellow reverse view colony color, wholly with a medium growth rate. Cluster 5 was represented by JTH 12 isolate that had a white aerial view and reverse view colony color, wholly with a medium growth rate. Cluster 6 was represented by JTH 15 isolate that had a big size of conidia and a fast growth rate. Cluster 7 was represented by DIY 5 isolate that had velvety colony texture and medium growth rate. Cluster 8 was represented by NTB 3 isolate that had a white aerial view and dark brown reverse view colony color. This cluster also had an ellipsoidal shape of conidia and a wholly texture colony. All isolates produced crystal with octahedral shape.

DISCUSSION

Recently, biological control using *Lecanicillium* spp. has been reported to manage some pathogenic fungi. Result of this study indicated that *Lecanicillium* spp. isolated from 7 provinces in Indonesia showed variations in macroscopic and microscopic morphology. Genus *Lecanicillium* formerly named *Verticillium*, can be identified by their morphological characteristics, despite it was very general. According to Zare *et al*.[27] genus *Verticillium* is divided into 2 groups, namely *Verticillium* section *Verticillium* and *Verticillium* section *Prostata.* Most entomopathogen and fungicolous were included in *Verticillium* section *Prostata*, while plant pathogenic fungi were included in *Verticillium* section *Verticillium*. In 2001, Zare and Gams[28] transferred a part of the species formerly classified in *Verticillium* sect. *Prostrata* into genus *Lecanicillium* and *Simplicillium*.

Results have indicated that *Lecanicillium* spp. is widespread. Those can be found on *H. vastatrix* in the coffee plantation centers throughout Indonesia regions at various altitudes.

Lecanicillium spp. also can be found associated with *H. vastatrix* on various varieties origin of coffee. These fungi formed white colonies on *H. vastatrix* uredospores. The occurrence of these parasites causes less infection of *H. vastatrix* (Fig. 2). According to Vandermeer *et al*.[29], this hyperparasite is capable of reducing spore viability and disease severity. Therefore, *Lecanicillium* spp. has the potential to be developed as a biological control agent of *H. vastatrix.*

The morphological characteristic of this mycoparasite isolated from *H. vastatrix* uredospores in Indonesia was generally similar to those reported in literature[30-33]. This fungus is characterized by verticillate branching of conidiophores. It arises in whorls on the upper portions of conidiophores. Conidia adhering in a globose slimy heads. All of them referred to the genus *Lecanicillium.*

Results based on UPGMA indicated that there are genetic variations among *Lecanicillium* spp. isolates. But this genetic variation is not related to the altitude of sample location or the distribution of the *Lecanicillium* spp. which tends to be random. Distribution of *Lecanicillium* spp. in clusters is thought to be influenced by environmental factors such as temperature, humidity, light and the interactions between them. Besides, the existence of the *H. vastatrix* physiological race is also thought to allow the formation of genetic variations of the mycoparasitic fungi. Recently, it was known as many as more than 49 races physiology of *H. vastatrix*[34,35]. This could be a further interesting research study.

Morphologically, the eight representatives of *Lecanicillium* spp. isolates are indistinguishable. An observation based on the morphology of fungi is less accurate because most of fungi cannot be distinguished

morphologically. A molecular identification technique needs to be done to identify fungal species through phylogenetic analysis. However, it can be used as supporting data for characteristics of the fungus[36]. Therefore, further molecular analyses based on DNA sequences are required for identifying these isolates.

CONCLUSION

Morphological identification in this study showed that 80 isolates of mycoparasite on *H. vastatrix* belong to *Lecanicillium* spp. These fungi were widespread and found in 7 provinces of Indonesia. Based on Rep-PCR *Lecanicillium* spp. had genetic variation divided into 8 clusters. This study will be beneficial for development of eco-friendly disease management of coffee rust disease and will be continued for sequencing at the species level.

SIGNIFICANCE STATEMENT

This study discovers the potency of the *Lecanicillium* spp. obtained from 7 provinces in Indonesia as mycoparasite of *Hemileia vastatrix*, the causal pathogen of coffee leaf rust disease. Thus, this research provides basic information for the further research to uncover the areas of the variation of *Lecanicillium* spp.

ACKNOWLEDGMENT

This study has been funded by the Agency for the Extension and Development of Agricultural Human Resources (BPPSDMP) Ministry of Agriculture of the Republic of Indonesia (contract number 1645/KP.320/J/3/14). We thank to Mr. Saipul Abbas and Ms. Tiya Farisa Agustin for technical support.

REFERENCES

1. Silva-Acuña, R., L.A. Maffia, L. Zambolim and R.D. Berger, 1999. Incidence severity relatiopship in pathosystem *Coffea arabica-Hemileia vastatrix*. Plant Dis., 83: 186-188.
2. Hartatri, D.F.S., L. Aklimawati and J. Neilson, 2019. Analysis of specialty coffee business performances: Focus on management of farmer organizations in Indonesia. Coffee Cocoa Res. J., 35: 140-155.
3. Prastowo, E., N.P. Erdiansyah and R. Arimarsetiowati, 2019. Leaf mineral composition of coffee infected by a *Hemileia vastatrix* fungus in Bondowoso, East Java. Coffee Cocoa Res. J., 35: 12-21.
4. Zambolim, L., 2016. Current status and management of coffee leaf rust in Brazil. Trop. Plant Pathol., 41: 1-8.
5. McCook, S. and J. Vandermeer, 2015. The big rust and the red queen: Long-term perspectives on coffee rust research. Phytopathology Rev., 105: 1164-1173.
6. Petit, A.N., F. Fontaine, P. Vatsa, C. Clément and N. Vaillant-Gaveau, 2012. Fungicide impacts on photosynthesis in crop plants. Photosynth. Res., 111: 315-326.
7. Júnior, J.H., L. Zambolim, C.E. Aucique-Pérez, R.S. Resende and F.A. Rodrigues, 2015. Photosynthetic and antioxidative alterations in coffee leaves caused by epoxiconazole and pyraclostrobin sprays and *Hemileia vastatrix* infection. Pestic. Biochem. Physiol., 123: 31-39.
8. Haddad, F., R.M. Saraiva, E.S.G. Mizubuti, R.S. Romeiro and L.A. Maffia, 2014. Isolation and selection of *Hemileia vastatrix* antagonists. Eur. J. Plant Pathol., 139: 763-772.
9. De Faria, M.R. and S.P. Wraight, 2007. Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control, 43: 237-256.
10. Lazreg, F., Z. Huang, S. Ali and S. Ren, 2009. Effect of *Lecanicillium muscarium* on *Eretmocerus* sp. nr. *furuhashii* (Hymenoptera: Aphelinidae), a parasitoid of *Bemisia tabaci* (Hemiptera: Aleyrodidae). J. Pest Sci., 82: 27-32.
11. Goettel, M.S., M. Koike, J.J. Kim, D. Aiuchi, R. Shinya and J. Brodeur, 2008. Potential of *Lecanicillium* spp. for management of insects, nematodes and plant diseases. J. Invertebr. Pathol., 98: 256-261.
12. Askary, H., Y. Carriere, R.R. Belanger and J. Brodeur, 1998. Pathogenicity of the fungus *Verticillium lecanii* to aphids and powdery mildew. Biocontrol Sci. Technol., 8: 23-32.
13. Benhamou, N. and J. Brodeur, 2000. Evidence for antibiosis and induced host defense reactions in the interaction between *Verticillium lecanii* and *Penicillium digitatum* the causal agent of green mold. Phytopathology, 90: 932-943.
14. Benhamou, N. and J. Brodeur, 2001. Pre-inoculation of Ri T-DNA transformed cucumber roots with the mycoparasite, *Verticillium lecanii*, induces host defense reactions against *Pythium ultimum* infection. Physiol. Mol. Plant Pathol., 58: 133-146.
15. Miller, T.C., W.D. Gubler, F.F. Laemmlen, S. Geng and D.M. Rizzo, 2004. Potential for using *Lecanicillium lecanii* for suppression of strawberry powdery mildew. Biocontrol Sci. Technol., 14: 215-220.
16. Kouvelis, V.N., R. Zare, P.D. Bridge and M.A. Typas, 1999. Differentiation of mitochondrial subgroups in the *Verticillium lecanii* species complex. Lett. Appl. Microbiol., 28: 263-268.
17. James, T.Y., J.A. Marino, I. Perfecto and J. Vandermeer, 2016. Identification of putative coffee rust mycoparasites via single-molecule DNA sequencing of infected pustules. Appl. Environ. Microbiol., 82: 631-639.

18. Mahfud, M.C., Z.A.M. Ahmad, S. Meon and J. Kadir, 2006. *In vitro* and *In vivo* tests for parasitism of *Verticillium psalliotae* Treschow on *Hemileia vastatrix* BERK. and BR. Malaysian J. Microbiol., 2: 46-50.
19. Vandermeer, J., I. Perfecto and H. Liere, 2009. Evidence for hyperparasitism of coffee rust (*Hemileia vastatrix*) by the entomogenous fungus, *Lecanicillium lecanii*, through a complex ecological web. Plant Pathol., 58: 636-641.
20. Jackson, D., J. Skillman and J. Vandermeer, 2012. Indirect biological control of the coffee leaf rust, *Hemileia vastatrix*, by the entomogenous fungus *Lecanicillium lecanii* in a complex coffee agroecosystem. Biol. Cont., 61: 89-97.
21. Alavo, T.B.C., 2015. The insect pathogenic fungus *Verticillium lecanii* (Zimm.) Viegas and its use for pests control: A review. J. Exp. Biol. Agric. Sci., 3: 337-345.
22. De La Cruz, I.G., E. Pérez-Portilla, E. Escamilla-Prado, M. Martínez-Bolaños, G.L.L. Carrión-Villarnovo and T.I. Hernández-Leal, 2018. Selection *in vitro* of mycoparasites with potential for biological control on coffee leaf rust (*Hemileia vastatrix*). Mex. J. Phytopatol., 36: 172-183.
23. Pramunadipta, S., A. Widiastuti, A. Wibowo, H. Suga and A. Priyatmojo, 2020. Short communication: *Sarocladium oryzae* associated with sheath rot disease of rice in Indonesia. Biodiversitas J. Bio. Diversity, 21: 1243-1249.
24. Xia, Y., F. Chen, Y. Du, C. Liu, G. Bu, Y. Xin and B. Liu, 2019. A modified SDS-based DNA extraction method from raw soybean. Biosci. Rep., Vol. 39. 10.1042/BSR20182271.
25. Versalovic, J. and J.R. Lupski, 2002. Molecular detection and genotyping of pathogens: More accurate and rapid answers. Trends Microbiol., 10: 15-21.
26. Masanto, A. Hieno, A. Wibowo, S. Subandiyah, M. Shimizu, H. Suga and K. Kageyama, 2019. Genetic diversity of *Phytophthora palmivora* isolates from Indonesia and Japan using rep-PCR and microsatellite markers. J. Gen. Plant Pathol., 85: 367-381.
27. Zare, R., W. Gams and H.J. Schroers, 2004. The type species of *Verticillium* is not congeneric with the plant-pathogenic species placed in *Verticillium* and it is not the anamorph of 'Nectria' inventa. Mycol. Res., 108: 576-582.
28. Zare, R. and W. Gams, 2001. A revision of *Verticillium* section *Prostrata*. IV. The genera *Lecanicillium* and *Simplicillium* gen. nov. Nova Hedwigia, 73: 1-50.
29. Vandermeer, J., I. Perfecto and S. Philpott, 2010. Ecological complexcity and pest control in organic coffee production: Uncovering an autonomous ecosystem service. Bioscience, 60: 527-537.
30. Lu, L.M., B.P. Cheng, D.C. Du, X.R. Hu and A.T. Peng *et al*., 2015. Morphological, molecular and virulence characterization of three *Lencanicillium* species infecting Asian citrus psyllids in Huangyan citrus groves. J. Invertebr. Pathol., 125: 45-55.
31. Feng, K.C., B.L. Liu and Y.M. Tzeng, 2002. Morphological characterization and germination of aerial and subemerged spores of the entomopathogenic *Verticillium lecanii*. World J. Microbiol. Biotechnol., 18: 217-224.
32. Aiuchi, D., K. Inami, M. Sugimoto, R. Shinya, M. Tani, K. Kuramochi and M. Koike, 2008. A new method for producing hybrid strains of the the entomopathogenic *Verticillium lecanii* (*Lecanicillium* spp.) through protoplast fusion by using nitrate non-utilizing (nit) mutans. Micologia Aplicada Int., 20: 1-16.
33. Zare, R. and W. Gams, 2008. A revision of the *Verticillium fungicola* species complex and its affinity with the genus *Lecanicillium*. Mycol. Res., 112: 811-824.
34. Sarwar, S., Q. Firdous and A.N. Khalid, 2019. Importance of Molecular and Phylogenetic Analyses for Identification of Basidiomycetes. In: Recent Advances in Phylogenentics, Yousaf, Z. (Ed.)., IntechOpen, UK.
35. Gichuru, E., J.M. Ithiru, M.C. Silva, A.P. Pereira and V.M.P. Varzea, 2012. Additional physiological races of coffee leaf rust (*Hemileia vastatrix*) identified in Kenya. Trop. Plant Pathol., 37: 424-427.
36. Talhinhas, P., D. Batista, I. Diniz, A. Vieira and D.N. Silva *et al*., 2017. The coffee leaf rust pathogen *Hemileia vastatrix*: One and half centuries around the tropics. Mol. Plant Pathol., 18: 1039-1051.