UNIVERSITI PUTRA MALAYSIA

EPIDEMIOLOGY OF Cucumber Mosaic Virus ON GINGER AND TURMERIC, AND ITS SUPPRESSION USING SILVER NANOPARTICLES

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By

MUHAMMAD BUHARI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

January 2021

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DEDICATION

This thesis is dedicated to my parents and my teachers.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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January 2021

Chairman : Associate Professor Ganesan a/l Vadamalai, PhD
Faculty : Agriculture

Plant viruses have hampered vegetable crops production worldwide, causing huge economic losses. Viral diseases have also been reported in ginger with two viruses, *Ginger mosaic virus* and *Ginger chlorotic fleck virus.* Virus-like symptoms, such as mosaic, stripping and stunted growth pattern, were observed on the ginger and turmeric crops in the States of Selangor, Pahang and Perak of Malaysia and there was no prior study to unfold the pathogen inciting these symptoms. In principle, for disease management to be successful and feasible, proper and accurate identification of causal organisms must first be achieved. Consequently, a total of 60 ginger leaf samples, 20 from each State and 45 turmeric leaf samples, 15 from each State were collected. Enzyme-linked immunosorbent assay (ELISA) was first employed to index the virus, then nucleic acid extracted using cetyl trimethyl ammonium bromide (CTAB), after which reverse transcription polymerase chain reaction (RT-PCR) was conducted using CMV coat protein (CP) gene-specific primers and primers for GCFV. The expected amplicon of ~500 bp, which encodes 120 amino acids of the coding sequence of CMV CP gene was obtained, which was cloned and sequenced. In ginger plants, 23 % of the total samples were positive for CMV across the three States from the ELISA and RT-PCR assays, with 30 % of the samples in Selangor and 20 % of the samples gotten from each of Pahang and Perak States being detected as CMV-positive. Only one turmeric sample from Selangor was positive for CMV. The ginger and turmeric CMV isolates found in Malaysia, have 100% nucleotide similarity amongst themselves and shared 96% sequence homology with CMV cucumber isolate from Thailand (AJ810264) and tomato CMV isolate from China (KX525736) respectively. The ginger CMV and turmeric CMV isolates obtained from this study, were phylogenetically grouped into CMV subgroup I B. Electron microscopic analysis has confirmed the CMV isolates, when the virus was purified, negatively stained using uranyl acetate and viewed under high resolution electron microscope. GCFV was however not detected from the two crops in this study by

RT-PCR assay. Pathogenicity test was conducted on the ginger and turmeric host plants by mechanical inoculation of the CMV-positive samples; ginger (TM3 isolate) and turmeric (TMR isolate). The inoculated test plants (100 %) showed similar symptoms of mosaic, stunting and stripping as observed in the field and confirmed CMV positive by CP gene through RT-PCR assay. CMV was also detected in three-month-old plants grown from CMV-infected rhizomes with 90 and 100 % infections in ginger and turmeric plants respectively. Host range studies conducted through a sap transmission technique showed nine plant species from six plant families as potential hosts for CMV isolated from ginger. The efficacy of silver nanoparticles (AgNPs) against CMV in ginger and turmeric plants was investigated by real time PCR. The virus concentration was significantly reduced by AgNPs ($p<0.01$) in the two plants from one-month post inoculation (MPI) up to 5 MPI. Thus, AgNPs has provided an avenue for the control of CMV infection in ginger and turmeric plants. The virus causing the symptoms of mosaic, stripping and stunting observed on ginger and turmeric plants, was unravelled, and identified as CMV, and its pathogenicity and host range were also conducted and reported in this study. Finally, silver nanoparticles were shown to suppress the CMV multiplication in ginger and turmeric plants.

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EPIDEMIOLOGI *Virus Mosaic Cucumber* (CMV) PADA HALIA DAN KUNYIT, DAN PENGAWALAN MENGGUNAKAN NANOPARTIKEL PERAK

Oleh

MUHAMMAD BUHARI

Januari 2021

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Virus tumbuhan menjejaskan pengeluaran tanaman sayuran di seluruh dunia, menyebabkan kerugian ekonomi yang besar. Penyakit virus juga telah dilaporkan pada tanaman halia di mana, dua virus, *Ginger mosaic virus* dan *Ginger chlorotic fleck virus* (GCFV) didapati menjangkiti tanaman halia. Simptom penyakit virus, seperti mozek, penjaluran dan pertumbuhan yang terbantut, telah diperhatikan pada tanaman halia dan kunyit di Negeri Selangor, Pahang dan Perak di Malaysia dan tidak ada kajian sebelumnya untuk mengenalpasti patogen yang menyebabkan symptom tersebut. Pada prinsipnya, untuk pengurusan penyakit yang berjaya, pengenalpastian organisma penyebab yang betul dan tepat mesti dicapai terlebih dahulu. Sebanyak 60 daun tumbuhan halia, 20 dari setiap negeri dan 45 daun tumbuhan kunyit, 15 dari setiap negeri telah dikutip berdasarkan keterlihatan symptom penyakit virus. Uji imunosorben berkait enzim (ELISA) digunakan untuk mengindeks virus terlebih dahulu, kemudian asid nukleik diekstrak menggunakan cetly trimetil amonium bromida (CTAB), kemudian *reverse transcription polymerase chain reaction* (RT-PCR) menggunakan primer spsifik untuk gen kot protin CMV (CP) dan primer khusus untuk GCFV. Amplikon yang dijangakan ~ 500 bp, yang menyandikan 120 asid amino dari urutan kot gen CMV CP telah diperolehi, diklon dan diuraikan. Pada tanaman halia, 23% daripada jumlah sampel dari ketiga-tiga negeri adalah positif untuk CMV dari ujian ELISA dan RT-PCR, dimana 30% sampel dari Selangor dan 20% dari Negeri Pahang dan Perak masing-masing dikesan positif dengan CMV. Hanya satu sampel kunyit dari Selangor yang positif untuk CMV. Isolat CMV dari halia dan kunyit yang ditemui di Malaysia mempunyai 100% persamaan nukleotida di antara mereka dan berkongsi 96% homologi dengan isolat CMV timun dari Thailand (AJ810264) dan isolat CMV tomato dari China (KX525736) masing-masing. Analisis filogenetik menunjukkan isolat CMV halia dan CMV kunyit yang diperolehi dari kajian ini dikelompokkan ke dalam subkumpulan CMV IB. Analisis mikroskop elektron telah mengesahkan isolat CMV,

dimana partikel virus yang tulen diwarnai secara negatif menggunakan uranyl asetat dapat dilihat di bawah mikroskop elektron resolusi tinggi. Bagaimanapun GCFV tidak dikesan dari dua spesis tanaman dalam kajian ini dengan ujian RT-PCR. Ujian patogenisiti dilakukan pada tumbuhan halia dan kunyit dengan inokulasi mekanikal sampel positif CMV; halia (isolat TM3) dan kunyit (isolate TMR). Pokok yang diinokulasi (100%) menunjukkan simptom yang sama seperti yang dilihat di ladang dan disahkan CMV positif melalui idetifikasi gen CP melalui RT-PCR. CMV juga dikesan pada tanaman berusia tiga bulan yang tumbuh dari rimpang yang dijangkiti CMV dengan 90 dan 100% jangkitan pada tanaman halia dan kunyit masing-masing. Kajian perumah yang dilakukan melalui transmisi sap menunjukkan sembilan spesies tumbuhan dari enam keluarga tumbuhan sebagai perumah yang berpotensi untuk CMV yang diasingkan dari halia. Keberkesanan nanopartikel perak (AgNPs) terhadap CMV pada tanaman halia dan kunyit dikaji mengunakan *Real-Time* PCR. Kepekatan virus dikurangkan dengan signifikan oleh AgNPs ($p < 0,01$) dalam kedua-dua tanaman dari satu bulan selepas inokulasi (BSI) hingga 5 BSI. Oleh itu, AgNPs boleh menjadi satu kaedah alternatif untuk mengawal jangkitan CMV pada tanaman halia dan kunyit. Virus yang menyebabkan simptom mozek, penjaluran dan pertumbuhan yang terbantut yang dilihat pada tanaman halia dan kunyit, telah dikenalpasti sebagai CMV, kajian patogenisiti dan potensi perumah telah dijalankan dan dilaporkan dalam kajian ini. Akhirnya, nanopartikel perak didapati menindas replikasi CMV dalam tanaman halia dan kunyit.

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LIST OF ABBREVIATIONS

°C	Degrees Celcius
µg	Micrcogramme
µL	Microlitre
µM	Micro molar
mg	Milligramme
aa	amino acid
AMV-RT	Avian myeblastosis virus reverse transcriptase
bp	base pair
cDNA	Complimentary deoxyribonucleic acid
CMV	*Cucumber mosaic virus*
CTAB	cetyl trimethylammonium bromide
dNTP	Deoxynucleotide triphosphate dATP, dCTP. dGTP, dTTP
g	Gramme
g	Centrifugal force
GCFV	*Ginger chlorotic fleck virus*
h	Hour
L	Litre
Min	Minute
mL	Millilitre
PCR	Polymerase chain reaction
PVP	Polyvinyl pyrrolidone
RNA	Ribonucleic acid
RT-PCR	Reverse transcription- Polymerase chain reaction
ppm	Parts per million
UV	Ultraviolet
U	Unit
rpm	Revolution per minute
TBE	Tris borate ethylene diamine tetra acetic acid
SDDW	Sterile double distilled water

SDS	Sodium dodecyl sulphate
v/v	Volume/volume percentage
w/v	Weight/volume percentage
dpi	Day post inoculation
mpi	Month post inoculation
s	Second
WPI	Week post inoculation
MPI	Month post inoculation
EDTA	Ethylene diaminine tetra acetic acid
M	Molar
SOC	Super optimal broth
LB	Luria Bertani
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
RFLP	Restriction fragment length polymorphism

CHAPTER 1

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.) of the *Zingiberaceae* family, are among the economically important vegetable crops in Malaysia. Ginger is of culinary and medicinal importance and it is among the most valued and widely cultivated spice crops in the world (Singletary, 2010; Amadi, 2012; Darshana et al., 2014). Like ginger plant, turmeric has cosmetic, medicinal preservative values besides its culinary function (Akam et al., 2010; Babu et al., 2011; Gul and Bakht, 2015; Abdul Haiyee et al. 2016).

Malaysia is the 13th world producer of ginger (FAOSTAT, 2015), cultivating a high-quality Bentong variety, with superior properties compared to many world gingers in terms of mellow taste, richness of gingerols, and long storability. Turmeric production in the country is about 2,640 tonnes and consumed locally (Suhaimi et al., 2014). Both ginger and turmeric have generated revenues to Malaysia at an approximately $ 24 million and $ 2 million annually respectively (DOA, 2018).

Fungal, bacterial and viral diseases have constituted a major threat to crop production, which can lead to an enormous yield loss (Koike et al., 2007). Fungi and bacteria constitute a nuisance in ginger and turmeric production, with rhizome rot induced by *Pythium* spp. among the most devastating diseases (American Society for Horticultural Science, 2011; Anusuya and Sathiyabama, 2015).

Many viruses have been associated with important diseases in vegetables but one of the most common and widely distributed viruses is the *Cucumber mosaic virus* (CMV). CMV has been found in Malaysia on a variety of economic, vegetable, and ornamental crops, as well as weed hosts, indicating the pathogen's long presence in the country's agroecosystem (Mazidah et al. 2012), but it has not been reported in either ginger or turmeric. Among the viruses reported to infect ginger were *Ginger mosaic virus*, belonging to CMV group (So, 1980). CMV, a member of the genus *Cucumovirus* with single stranded RNA (ssRNA) and tripartite genome, may reduce crop yield by up to 30% (Zitter and Murphy, 2009; de Breuil et al., 2012). The second virus that has been reported to infect ginger was *Ginger chlorotic fleck virus* (GCFV), which belongs to the genus *Sobemovirus* tentatively, with ssRNA and icosahedral particle of 30 nm in diameter (Thomas, 1986). However, there has been no previous report of any attempt to investigate both these viruses in question in Malaysia. Nevertheless, it is important to identify viruses that might have infected ginger and turmeric for proper management strategy to be deployed and in order to avoid the loss that might be incurred.

A rapid and sensitive identification of the causal agent is imperative in management of plant viral diseases. Serological techniques, such as enzyme-linked immunosorbent assay (ELISA) in its various formats have been used in the diagnosis of CMV and GCFV (Thomas, 1986; El-Borollosy and Waziri, 2013; Eni et al., 2013; Bald-Blume et al., 2017). Nucleic acid-based techniques, using RT-PCR have been widely utilized in CMV and GCFV detection and identification; followed by nucleotide sequencing, phylogenetic analysis and restriction digestion of PCR products (Wylie et al., 1993; Sérémé et al., 2008; Kim et al., 2014; Nouri et al., 2014). However, nucleic acid based, RT-PCR, cloning and sequencing had not been applied for the identification of CMV in ginger and turmeric in the past.

Pathogenicity and host range studies remain fundamental in the diagnostic of plant viruses. CMV is known as the plant virus with the widest host range in contrast with GCFV, which only has a single host. CMV infects wild, domestic, vegetables, monocots and dicots, ornamentals as well as tree crops (Carrère et al., 1999; Paradies et al., 2000; Bald-Blume et al., 2017) whereas, GCFV was only found to infect ginger (Thomas, 1986). However, CMV and GCFV infections were not investigated in ginger and turmeric in Malaysia despite the conspicuous viral symptoms observed on the crops.

To control plant viral diseases, various approaches, ranging from cultural, biological and chemical methods have been applied due the fact that there is no unswerving method to control plant viruses (Lecoq and Katis, 2014). Recently, nanotechnology has evolved with remarkable results so far in the field of plant pathology, against plant pathogenic bacteria, fungi as well as viruses (Kim et al., 2008; Ocsoy et al., 2013; Elbeshehy et al., 2015; Pirtarighat et al., 2019). Therefore, the use of silver nanoparticles was conceived in this study, to evaluate its suppression on CMV in ginger and turmeric plants since it has never been conducted before.

1.1 Statement of problem

Many plant viruses were reported on vegetable crops. The viruses have reduced the crop yield or lower the crop quality, thereby decreasing its marketability. Viral symptoms were observed on ginger and turmeric plants in Peninsular Malaysia. The symptoms observed were mosaic, striping, reduced leaf and plant sizes. However, the causal agent has not been identified and characterized. CMV was reported to cause a yield loss ranging from 10 % to 30 % in various crop plants (Zitter and Murphy, 2009; de Breuil et al., 2012). It was then deemed necessary to screen the infected ginger and turmeric for CMV infection, as early and timely identification of the causal organism is the most important aspect of any disease management.

1.2 Justification of study

Since viruses may devastate ginger and turmeric crops, which may lead to a significant loss in the yield either in quantity or quality, and no research was carried out with respect to the identification of viruses reported to be associated with ginger and turmeric in Malaysia, it is of paramount importance to determine the causal organism. This will aid the quarantine services, virus resistance breeding and production of virus-free planting materials and also to meet the demand of Malaysian government in its intended expansion of fruits and vegetables production that are of higher quality, according to the food safety standards and in order to access premium markets in the developed world.

1.3 Objectives of the study

The study aims at generating information needed, which will help in designing eco-friendly and sustainable viral diseases management in ginger and turmeric crops in Peninsular Malaysia as its overall objective.

The specific objectives were:

1. To identify and characterize viruses on ginger and turmeric in Peninsular Malaysia using electron microscopy, ELISA, RT-PCR, cloning and sequencing.
2. To determine the pathogenicity and host range of the identified virus isolates.
3. To evaluate the efficacy of silver nanoparticles to control viral diseases in ginger and turmeric plants.

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BIODATA OF STUDENT

Buhari Muhammad was born on the 27th August 1983 into the family of Mr. Muhammad Wada Ibrahim and Mrs. Mariyah Abubakar. He attended LEA primary school Tudun Wada, Zaria and Government Secondary School, Zaria, Kaduna State-Nigeria, for his primary and secondary schools' education, respectively. He proceeded to Ahmadu Bello University, Zaria (ABU) where he obtained Bachelor of Agriculture and M.Sc. in Plant Protection certificates in 2009 and 2014, respectively. He is currently a Ph. D. candidate in the Department of Plant Protection, University Putra Malaysia. Buhari is happily married and blessed with children.

LIST OF PUBLICATIONS

International Conference

"Detection and characterization of *Cucumber mosaic virus* (CMV) infecting ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.) in Peninsular Malaysia". **Buhari Muhammad,** Ganesan Vadamalai, Lau Wei Hong and Kong Lih Ling. *Joint Symposium of the 8th International Agriculture Congress and 6th International Symposium for Food and Agriculture 2018* (8TH IAC – 6TH ISFA 2018). 13 – 15 November 2018.

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