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CONTROL OF GRAY MOLD ROT DISEASE OF TOMATO CAUSED BY Botrytis cinerea WITH EMULSION FORMULATED FROM Moringa oleifer Lam. CRUDE EXTRACT

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By

TIJJANI AHMADU

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

December 2017

DEDICATION

The work in this thesis is dedicated to the memory of my late father Haji Ahmad T. Yusuf (30/01/1928-15/10/1969), to my beloved Mom Juwairiyya Haji Idris and my entire family.

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

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Chairman : Assoc. Prof. Khairulmazmi Ahmad, PhD
Faculty : Agriculture

Series of laboratory experiments were conducted with emphasis on the preparation of nano-emulsion formulations from active compounds in *Moringa oleifera* crude extracts and *in vitro* and *in vivo* evaluation of their bio-efficacy against *Botrytis cinerea* causing gray mold rot disease on tomato. In total, nine isolates were successfully isolated from infected tomato sampled from Cameron Highlands, Pahang, Malaysia in 2015. The isolates were identified based on morpho-cultural characteristics such as mycelial texture, conidia shapes and sizes, and sclerotia forms. Conidia (n=40) measured 9.7-17.1 × 6.6-10.5 µm, were smooth one-celled, ellipsoidal, globose or ovate and borne in clusters on branching tree-like conidiophores. To confirm the morphological identification, the primer pair ITS4/ITS5 (ITS region) of rDNA and part of Glyceraldehyde-3-phosphate dehydrogenase gene (G3PDH) were used for amplification and sequencing of the isolates (BCH01 to BCH09). The sequences obtained (GenBank Accession No. KU992692-KU992700 for ITS and KY201456-KY201464 for G3PDH) showed 99-100% homology with *B. cinerea* isolates in the GenBank nucleotides database. Based on the pathogenicity assay, the isolates showed variations in their levels of severity. In ripen tomato fruits, the per cent disease severity ranged from 36 to 97%, with maximum per cent observed in BCH07 (97%) and minimum in BCH04 (36%). Among the organic solvents used in the extraction of the bioactive compounds, methanol gave the highest (14.16%) percentage extraction yield compared to ethanol (10.23%), ethyl acetate (5.24%), hexane (2.3%) and distilled water (1.71%). SEM and TEM micrographs on the effect of the crude extracts of *M. oleifera* on the conidia and mycelium of *B. cinerea* treated with MIC concentration revealed irreversible surface and ultra-structural changes that include lysis, shrinkage, pore formation, aggregation and vacuolation compared to the controls. The results of GC-MS led to the identification of 67 volatile chemical compounds with n-Hexadecanoic acid, Malonic acid, 6-decanoic acid, (Z)-, .beta.-l-Rhamnofuranoside,5-O-acetyl-thio-octyl-, 2-Dimethyl (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran, and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- as the predominant in the extract. Further chemical elucidation of the crude extracts

performed with LC-MS/MS showed the presence of phenylvaleric acid, caffeic acid derivative, quinic acid, apigenin-6,8-di-C-β-D-glucopyranoside (Vicenin II) , Apigenin 6 C glucoside, quercetin-3-O-glucoside dimer and kaempferol 3-o glucoside dimer as the major non-volatile chemical compounds, which are mostly flavones, flavonois and phenolic acids. The results on the characterization of the formulations showed that all formulations were stable at 25°C for two months and 54°C for one month. All formulations have particle size of less than 100 nm, PDI from 0.108-0.415, surface tension below 30 mN/m, viscosity less than 60 mPas, zeta potentials less than 40 mV and pH less than 6.00. TEM micrographs of the formulations confirmed the spherical shape of nano-emulsions and the particle size of less than 100 nm as measured with Malvern Zsizer instrument. The results of SEM and TEM micrographs on the effect of the formulations on the conidia and mycelium of *B. cinerea* confirmed the irreversible surface and ultra-structural alterations such aggregation, abnormal growth, lysis, reduced hyphal diameter and length, destruction of the organelles and irregular cell wall shape compared to the controls. *In vivo* evaluation was conducted with healthy tomato fruits via preventive and curative treatments. The results on *in vivo* indicated 93.3% to 100% disease reduction in treated fruits under preventive method compared with 0% in the negative controls. Results on the effects of the formulations on the post-harvest quality of the fruits showed that % weight loss was as low as 0.72%, loss in firmness (3.09%) and CIE values (L*=37.72, a*=29.15, b*=19.98) for colour, on tomatoes treated with F13 the formulations under preventive method. Similarly, chemical analysis of the F13 formulations treated fruits showed that the final percent TSS was 4.18%, AA (20.95 mg/ml), TA (0.34%) and pH (4.05). Mineral content analysis revealed that tomatoes treated with F13 formulations gave the best results on mineral contents with K (201.96 mg/ml), Ca (28.29 mg/ml), Mg (20.56 mg/ml) and Zn (0.42 mg/ml).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAWALAN PENYAKIT REPUT KULAT PERANG TOMATO YANG DISEBABKAN OLEH *Botrytis cinerea* DENGAN FORMULASI EMULSI DARI EKSTRAK MENTAH *Moringa oleifera*

Oleh

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Disember 2017

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Eksperimen makmal telah dilakukan berterusan dengan memberi fokus terhadap langkah penyediaan formulasi nano-emulsi sebatian aktif daripada ekstrak mentah *Moringa oleifera* dan penilaian terhadap keberkesanan produk terhadap *Botrytis cinerea* yang menyebabkan penyakit reput kulat kelabu pada tomato secara *in vitro* dan *in vivo*. Kesemua sembilan pencilan berjaya disisihkan daripada sampel buah tomato terjangkit dari Cameron Highland, Pahang, Malaysia pada tahun 2015. Pencilan telah dikenalpasti berdasarkan ciri-ciri morfo-kultur seperti tekstur miselia, bentuk dan saiz konidia, serta bentuk sklerotia. Ukuran konidia (n=40) adalah 9.7-17.1 × 6.6-10.5 µm, licin dan mempunyai satu sel, elipsoid, globos atau ovat dan terhasil secara berkelompok pada konidiofor yang bercabang seperti pokok. Untuk mengesahkan pengecaman morfologi, sepasang primer ITS4/ITS5 (kawasan ITS) daripada rDNA dan sebahagian gen Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) digunakan untuk tujuan amplifikasi dan penjujukan pencilan tersebut (BCH01 - BCH09). Jujukan yang diperolehi (Number kemasukan GenBank KU992692-KU992700 untuk ITS dan KY201456-KY201464 untuk G3PDH) menunjukkan 99-100% homologi dengan pencilan *B. cinerea* dalam pangkalan data nukleotida GenBank. Berdasarkan ujian patogenisiti, pencilan-pencilan tersebut menunjukkan variasi tahap keparahan penyakit. Peratus keparahan penyakit adalah di antara 36-97% pada buah tomato yang masak, dengan peratus maksimum dicatatkan oleh BCH07 (97%) dan minimum oleh BCH04 (36%). Di kalangan pelarut organik yang diuji dalam pengekstrakan sebatian bioaktif, metanol menunjukkan peratus hasil pengekstrakan tertinggi (14.16%) berbanding pelarut yang lain. Mikrograf SEM dan TEM oleh ekstrak mentah *M. oleifera* terhadap konidia dan miselia *B. cinerea* yang dirawat pada kepekatan MIC menunjukkan kerosakan kekal permukaan dan stuktur ultra seperti lisis, pengecutan, pembentukan liang, penggumpalan dan pemvakulan berbanding dengan rawatan kawalan. Dapatan dari GC-MS telah membawa kepada pengenalpastian 67 sebatian kimia meruap dengan asid n-Hexadecanoik, asid Malonik, asid 6-decanoik, (Z) -, .beta.-l-Rhamnofuranoside, 5-O-asetil-thio- oktil, 2-Dimetil (trimethylsilylmethyl) silyloxymethyl tetrahydrofuran, dan 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- sebagai pradominan dalam

ekstrak. Penjelasan kimia selanjutnya mengenai ekstrak mentah yang dijalankan oleh LC-MS/MS menunjukkan kehadiran asid fenilvalerik, derivatif asid caffeic, asid quinik, apigenin-6,8-di-C-β-D-glucopyranoside (Vicenin II), Apigenin 6 C glucoside, dimensi quercetin-3-O-glucoside dan kaempferol 3-o glucoside dimer sebagai sebatian kimia utama yang tidak meruap, yang kebanyakannya flavon, flavonois dan asid fenolik. Keputusan pencirian formulasi menunjukkan semua formulasi adalah stabil pada 25°C selama dua bulan dan 54°C selama satu bulan. Semua formulasi mempunyai saiz zarah kurang daripada 100 nm, PDI dari 0.108-0.415, ketegangan permukaan di bawah 30 mN/m, kelikatan kurang daripada 60 mPas, potensi zeta kurang daripada 40 mV dan pH kurang daripada 6.00. Mikrograf TEM dari formulasi tersebut mengesahkan nano-emulsi berbentuk sfera dan saiz zarah kurang dari 100 nm telah diukur menggunakan alat Malvern Zsizer. Dapatan mikrograf SEM dan TEM ke atas kesan formulasi terhadap konidia dan miselia *B. cinerea* mengesahkan terdapat kesan kerosakan kekal terhadap permukaan dan struktur ultra seperti penggumpalan, pertumbuhan tidak normal, lisis, pengecilan diameter dan panjang hifa, kerosakan pada organel, bentuk dinding sel yang tidak sekata berbanding dengan rawatan kawalan. Penilaian *in vitro* telah dilakukan pada buah tomato yang sihat melalui kaedah pencegahan dan pemulihan. Keputusan *in vivo* menunjukkan pengurangan penyakit 93.3-100% pada buah-buahan yang dirawat dengan kaedah pencegahan berbanding 0% pada rawatan kawalan negatif. Keputusan kesan formulasi F13 menggunakan kaedah pencegahan terhadap kualiti selepas tuai tomato menunjukkan peratusan kehilangan berat adalah serendah 0.72%, kehilangan ketegangan (3.09%), nilai CIE warna buah tomato terawat (L*=37.72, a*=29.15, b*19.98). Begitu juga dengan analisis kimia terhadap formulasi F13, buah yang dirawat menunjukkan bahawa peratusan akhir TSS adalah 4.18%, AA (20.95 mg/ml), TA (0.34%) dan pH (4.05). Analisa kandungan mineral mendedahkan bahawa formulasi tersebut mampu meningkatkan kandungan mineral K (201.96 mg/ml), Ca (28.29 mg/ml), Mg (20.56 mg/ml), dan Zn (0.42 mg/ml).

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I certify that a Thesis Examination Committee has met on 15 December 2017 to conduct the final examination of Tijjani Ahmadu on his thesis entitled "Control of Gray Mold Rot Disease of Tomato Caused by *Botrytis cinerea* with Emulsion Formulated from *Moringa oleifera* Lam. Crude Extract" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

±SD	Standard deviation of means
a.i	active ingredient
AA	Ascorbic Acid
ANOVA	Analysis of variance
AUDPC	Area Under Disease Progress Curve
Bp	Base Pair
Ca	Calcium
CIE	Commission Internationale de l'E- clairage
CMA	Corn Meal Agar
cm	Centimeter
CRD	Complete Randomize Design
DAI	Days after Inoculation
DNA	Deoxyribonucleic acid
DS	Disease Severity
DSI	Disease Severity Index
ED	Edonol
EDTA	Ethylene Diamine Tetraacetic Acid
FAO	Food and Agricultural
GC-MS/MS	Gas Chromatography-Mass Spectrophotometry/ Mass Spectrophotometry
g	grams
ISSR	Inter Simple Sequence Repeats
Kb	Kilo base
LBC	lactophenol-cotton blue
LCB	Lactophenol-cotton blue
LC-MS/MS	Liquid Chromatography- Mass Spectrophotometry/Mass Spectrophotometry
MEA	Malt Extract Agar
Mg	Magnesium
Min	Minutes
MLDW	*Moringa* Leaves Distilled Water extracts
MLE	*Moringa* Leaves Ethanol extracts
MLEA	*Moringa* Leaves Ethyl Acetate extracts
MLH	*Moringa* Leaves Hexane extracts
ML	Millilitres
MLM	*Moringa* Leaves Methanol extracts
Mm	millimetre
mN/m	millinewtons per meter
mPas	Millipascal-second
NICB	National Centre for Biotechnology Information
Nm	Nanometer
No.	Number
PBD	Potato Dextrose Broth
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDI	Polydispersity Index
pH	Logarithm of hydrogen ion activity
PIRG	Percentage Inhibition Radial Growth

PSGI	Percentage Spore Germination Inhibition
RAPD	Random Amplified Polymerase DNA
RNA	Ribonucleic acid
Rpm	revolution per minute
SEM	Scanning Electron Microscopy
spp	species
SSR	Simple Sequence Repeats
TA	Titrable Acidity
TEM	Transmission Electron Microscopy
Ter.	Termul
TSS	Total Soluble Solids
Tw	Tween 20
UPM	Universiti Putra Malaysia
Uv	Ultraviolet
Ver.	Version
Viz	*videlicet*, that is, namely
V	Volt

CHAPTER 1

INTRODUCTION

Tomato (*Solanum lycopersicum Syn Lycopersicon esculentum Mill.*) is an herbaceous plant that belongs to the family *Solanaceae*, a nightshade family that originated from South America (Agrios, 2005). The fruits are rich in minerals, vitamins and lycopene (Etebu *et al*., 2013; Borghesi *et al*., 2016). Farmers at all levels of production including small scale, medium and large commercial farmers were attracted by tomato because of its quick maturity and high yielding potentials. Moreover, the crop has some peculiar economic importance that includes domestic trades, exports and development of local food and agro-allied industries (Borghesi *et al*., 2016).

Highlands of Peru with good climatic conditions especially the temperature of below 25°C, is believed to be the centre of diversity for tomato (Smith, 1994) and this explains the reason why tomato production is highly successful in Cameron Highlands of Pahang in Malaysia. Currently production of tomato in Malaysia is increasing due to demand of continuous supply of the crop. In Malaysia, fresh tomato fruits were produced in open-fields or under greenhouse conditions. The yield in an open-field cultivation is about 100-120 ton/ha while the greenhouse yield is about 300-500 tons/ha (FAO, 2012; Ismail *et al*., 2015). The yield of the crop is generally far below its potentials due to the activities of large number of pests and diseases (Ana *et al*., 2003). About 200 known diseases affecting tomato were reported by Agrios (2005), out of which 70 % has been caused by bacterial and fungal pathogens (Janisiewiez *et al*., 2001; Wani, 2011). In Malaysia postharvest rot diseases caused by fungal pathogens are perhaps the most common and most widely distributed disease of tomato and this can cause a significant reduction in postharvest yield potential of the crop. One of such diseases is gray mold rot caused by *B. cinerea*. The occurrence of the disease is worldwide, particularly widespread in Malaysia, around areas of Cameron Highlands in Pahang, northern part of Malaysia. Enyiukwu *et al*. (2014) and Kader (2005) reported that the estimated postharvest losses of tomato due to postharvest diseases worldwide range between 30-40%. In Malaysia, occurrence of *B. cinerea* caused gray mold rot disease on tomato in the greenhouses particularly in the Cameron Highlands area of Pahang. This resulted in severe damages at both pre- and post-harvest periods with subsequent yield losses and economic downfall. At present, the pathogen (*B. cinerea*) causing gray mold rot have not been extensively studied in Malaysia despite its ranking as second most destructive or aggressive fungal pathogen in the top 10 devastating fungal plant pathogens worldwide (Dean *et al*., 2012; Kim *et al*., 2016).

For sustainable tomato production there is need to search for remedies to this incessant problem threatening tomato production in Malaysia and worldwide. In a bid to control these fungal pathogens causing losses to valuable crops at present, several phytosanitary measures such as chemical control, physical, cultural and use of resistant varieties have been adopted to reduce the menace of diseases on the crop but each method was found to be associated with some drawbacks. For instance, chemical fungicides has been recognized as the most common, popular, potent and primary method for managing postharvest fungal decay of fruits and vegetables but their use

has been regulated or limited due to their teratogenicity, carcinogenicity, acute and high residual toxicity, long period of degradation, environmental pollution and possible side effects or outcomes on human health through food chain (Ai-ying *et al*., 2011; Enyiukwu *et al*., 2014). In line with the public opinions and concerns related to the environmental and human safety, development of resistance by plant pathogens due to progressive use of chemicals and increasing regulations on chemical pesticides use (Elliott *et al*., 2009), it is imperative to explore new alternatives that will reduce the use of synthetic chemicals.

In recent time, plant-based pesticides as a form of biological control that are enabling and instrumental to manage postharvest decay of fruits and extend their shelf life without pollution to the environment and risk to the public health were investigated as an alternative to chemical pesticides for controlling diseases in fruits and vegetable incited by plant pathogenic fungi. The plants contain natural phytochemicals which could be exploited for use as biopesticides (Satish *et al*., 2007). For example medicinal plants like *Moringa oleifera* (Pereira *et al*., 2015) extracts containing a quantum of crucial phytochemicals rich in phenolic compounds, flavonoids, tannins, saponins and alkaloids are now emerging as secure, safer and more compatible way to manage plant pathogens. *Moringa oleifera* also known as a miracle plant, all plant parts having diverse range of medicinal uses and other purposes such as water purification, fertilizer, biogas and biopesticides (Stohs and Hartman, 2015). The flowers, roots, bark, leaves, stem, and seeds of *M. oleifera* have antimicrobial qualities and/or properties (Dwivedi and Enespa, 2012; Arora *et al*., 2013), antifungal (Kadhim and AL-Shamma, 2014; Batista *et al*., 2014), antioxidant (Verma *et al*., 2009), antibacterial, antiulcer (Arora and Onsare, 2014; Belay and Sisay, 2014), anti-inflamatory, diuretic (Caceres *et al*., 1992; Krishnamurthy *et al*., 2015) and anticancer (Pinto *et al*., 2015).

The active ingredients in these valuable medicinal plants need to be upgraded or enhanced into plant-based pesticides in order to compete with the commercial synthetic pesticides in the market. This therefore, necessitate the need for the inclusion of pesticide formulation which will ease the handling of the active ingredients, extend their shelf life, assist in application and increase their effectiveness on the target organisms (Asib *et al*., 2015; Ribeiro *et al*., 2015). Emulsion is the most common commercial formulation and currently, nano-emulsion has been introduced. Nano-emulsion is an important delivery technology for chemical compounds that comprises a small droplet size ranging from 5 nm to 100 nm (Ranjan *et al*., 2014; Asib *et al*., 2015), low surface tension, low viscosity and good appearance (Ribeiro *et al*., 2015). It composes of a surfactant, an oil phase and an aqueous phase in appropriate proportions or ratios (Changez and Varshney, 2000). The nano-emulsion has a very good stability at storage over a wide range of temperatures (-10°C to 55°C). It has an excellent long term dilution characteristics in water since it is water based and highly capable of solubilizing lipophilic and hydrophilic compounds leading to the use of minimal inert ingredients. Additionally, nano-emulsions can carry active compound to the desired site of action, protect it against adverse environmental conditions and enhance efficacy (Asib *et al*., 2015; Ribeiro *et al*., 2015).

Despite the efficacy and environmental compatibility of nano-emulsion delivery technology and the quantum of crucial and diverse phytochemicals in *M. oleifera*, there

is no report regarding its formulation as nanomulsion biopesticide against plant pathogens. Hence, this research aimed at the formulation of *M. oleifera* crude extracts as nano-emulsion for controlling *B. cinerea* causing gray mold rot on tomato. Thus, it is hoped that this work will provide new information that could be used as a phytosanitary measure for the control of gray mold disease globally especially in Malaysia.

The objectives of this study were:

1. To identify *B. cinerea* associated with gray mold disease of tomato based on their morpho-cultural and molecular properties.
2. To extract and profile phytochemical compounds from *M. oleifera* crude extracts and determine their MIC, MFC and antifungal activities against *B. cinerea* isolates.
3. To formulate *M. oleifera* crude extracts as nanoemulsions and evaluate their physical characteristics.
4. To validate the effectiveness of the formulations on selected isolate of *B. cinerea in vitro* and *in vivo* and their effects on the postharvest qualities of the tomato.

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