UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND DEVELOPMENT OF PHENAZINE-BASED COMPOUNDS FROM PSEUDOMONAS AERUGINOSA FOR SUPPRESSION OF GANODERMA BONINENSE

LEE KAI WEI

IPTSM 2016 5

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

CHARACTERIZATION AND DEVELOPMENT OF PHENAZINE-BASED COMPOUNDS FROM *PSEUDOMONAS AERUGINOSA* FOR SUPPRESSION OF *GANODERMA BONINENSE*

By

LEE KAI WEI

September 2016

Chairman: Associate Professor Wong Mui Yun, PhD
Institute: Institute of Tropical Agriculture

Basal stem rot is caused by *Ganoderma boninense* and the disease has significantly reduced the productivity of oil palm. *Pseudomonas aeruginosa* UPMP3 is capable of producing phenazine and phenazine-1-carboxylic acid. It is important to study the potency of these compounds to suppress the fungi, and also to be used as active ingredient in biofungicide formulation. Nanoemulsion is widely used in plant protection, the formulation of phenzine-based compounds in emulsion can be a good substitute to chemicals. The objectives of this project were firstly, to extract and characterize the phenazine-based compounds from *P. aeruginosa*. Secondly, to formulate nanoemulsion formulation of phenazine-based compounds. Thirdly, to evaluate the suppression efficacy of the formulation on *G. boninense in vitro* and *in vivo*. The phenazine compounds were extracted by benzene and characterized by HPLC, FTIR and NMR. The spectra of the characterization were useful to determine the authentic of phenazine-based compounds. Bioassay analysis was run to study the efficacy of the phenazines treatment to suppress *G. boninense in vitro* and *in vivo*. The HPLC results showed that phenazine-1-carboxylic acid and phenazine were successfully extracted using benzene and detected at wavelengths 252 and 237 nm at retention time of 51.32 and 52.13 min, respectively. The quantification analysis indicated 11.81 mg of phenazine-1-carboxylic acid and 679.5 mg of phenazine were obtained per one litre of crude extract sample. The FTIR analysis revealed that spectrum had aromatic nitro compound and aromatic rings were indicative the presence of phenazine compound whereas, spectra with carboxylic acid and aromatic rings were indicative of phenazine-1-carboxylic acid. The compounds structure were confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy with the molecular formula $C_{12}H_8N_2$ (Phenazine) and $C_{13}H_8N_2O_2$ (Phenazine-1-carboxylic acid). The bioassay result showed that 5000 ppm crude extract inhibited mycelial growth of *G. boninense* at 89 %, with LC_{50} of 2232 ppm whereas purified phenazine treatment gave the best efficacy at 1000 ppm for 100% inhibition, with LC_{50} of 234 ppm. For nanoemulsion formulation, a phase diagram was constructed with 30% phenazines crude extract as active ingredient through low-energy method. Nanoemulsion F1 with 174.43 nm size was obtained at a ratio 5:5:90 (Tween80: oil carrier: water), and it was found to be stable in terms of polydispersity index (0.6), zeta potential (-16.0 mV) and surface tension (30.88 mN/m), and effective in controlling *G. boninense* at 70.74% *in vitro*. In glasshouse trial,

Fomulation F1 indicated suppression efficacy identical to positive control (Hexaconazole).The results obtained definitely corroborate to the application of nanoemulsion of phenazine crude extracts as potential candidate for controlling *G. boninense*. The phenazine nanoemulsion is an innovation alternative to chemical fungicide to control basal stem rot. It shows good potential in the antifungal activity of crude phenazine compounds in nanoemulsion formulation against *G. boninense in vitro* and suppresses basal stem rot under glasshouse.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENCIRIAN DAN PEMBENTUKAN KOMPAUN BERDASARKAN PHENAZIN DARI PSEUDOMONAS AERUGINOSA UNTUK PERENCATAN GANODERMA BONINENSE

Oleh

LEE KAI WEI

September 2016

Pengerusi: Profesor Madya Wong Mui Yun, PhD
Institut: Institut Pertanian Tropika

Reput Pangkal Batang (BSR) adalah disebabkan oleh *Ganoderma boninese* dan penyakit ini telah mengurangkan produktiviti kelapa sawit. *Pseudomonas aeruginosa* UPMP3 menghasilkan antibiotic phenazin dan phenazin-1-karbosilik asid. Pengetahuan berkaitan dengan kompaun tersebut adalah penting dalam merencatkan fungi and juga digunakan sebagai bahan aktif dalam penggubalan racun kulat. Nanoemulsi banyak digunakan dalam bidang perlindungan tumbuhan, penggubalan kompaun phenazin dalam emulsi boleh dijadikan sebagai pengganti kepada racun kimia. Objektif pertama projek ini adalah mengektrak dan menyifatkan kompaun phenazin dari *P. aeruginosa*. Kedua, untuk merumuskan nanoemulsi phenazin yang sesuai. Ketiga, untuk menilai keberkesanan perencatan nanoemulsi phenazin pada *G. boninense*. Kompaun phenazin diekstrak dengan benzene dan disifatkan dengan HPLC, FTIR dan NMR. Spektra penyifatan penting dalam menentu ketulenan kompaun phenazin. Keputusan HPLC menunjukkan bahawa phenazin-1-karbosilik asid dan phenazin telah berjaya diekstrak dengan menggunakan benzen dan dikesan pada jarak gelombang 252 dan 237 nm pada masa pengekalan 51.32 dan 52.13 min, masing-masing. Analisis kuantifikasi menunjukkan 11.81 mg phenazin-karbosilik asid dan 679.5 mg phenzin diperolehi setiap satu liter sampel ekstrak mentah. Analisis FTIR menunjukkan spektrum yang mempunyai nitro aromatik dan cincin aromatik, ini menunjukkan kehadiran phenzin sedangkan, spektrum asid karboksilik dan cincin aromatik adalah menunjukkan kehadiran phenazin-1-karbosilik asid. Struktur kompaun telah disahkan dengan ^{1}H NMR dan ^{13}C NMR spektroskopi formula $C_{12}H_8N_2$ molekul (Phenazin) dan $C_{13}H_8N_2O_2$ (Phenazine-1-karboksilik asid). Hasil bioesei menunjukkan bahawa 5000 ppm ekstrak mentah mengawal pertumbuhan mycelial *G. boninense* pada 89%, dengan LC_{50} bernilai 2232 ppm manakala rawatan phenazin extraks memberikan keberkesanan yang terbaik pada 1000 ppm untuk 100% perencatan, dengan LC_{50} bernilai 234 ppm. Untuk penggubalan nanoemulsi, gambar rajah fasa (PD) telah dibina berdasarkan 30% ekstrak phenazine mentah sebagai bahan aktif melalui kaedah-tenaga yang rendah. Nanoemulsi F1 bersaiz 174.43 nm telah diperolehi pada nisbah 5: 5: 90 (w/w/w; surfaktan: pembawa minyak: air), dan ia didapati stabil dari segi indeks polidispersiti (0.6), potensi zeta (-16.0 mV) dan ketegangan permukaan (30.88 mN / m), dan berkesan dalam mengawal *G. boninense* pada 70.74% *in vitro*. Dalam percubaan rumah kaca, Formulasi F1 menunjukkan perencatan berkesan sama dengan kawalan positif

(Hexaconazole). keputusan yang diperolehi menyokong dengan penggunaan ekstrak phenazin nanoemulsi sebagai calon yang berpotensi untuk mengawal *G. boninense*. Phenazin nanoemulsi adalah inovasi yang alternatif untuk menggantikan racun kulat kimia dalam mengawal BSR. Ia menunjukkan potensi yang baik dalam aktiviti antikulat phenazin mentah dalam formula nanoemulsi terhadap *G. boninense in vitro* dan merencat BSR dalam rumah kaca.

ACKNOWLEDGEMENTS

'In the name of God, the most gracious, the most compassionate'

This project would not have been possible without the blessing from God, The Almighty for good health and wellbeing that were necessary to complete this thesis. I wish to thank several beloved people for the strength to complete my Master degree study.

Foremost, I would like to express my sincere gratitude to my supersivor Dr. Wong Mui Yun for the continuous support in research, for her patience, motivation, enthusiasm, and immense knowledge.

Besides my supervisor, I would like to thank the rest of my supervisory committee: Prof. Dr. Dzolkhlifli Omar and Dr. Khalina Abdan, for their encouragement and insightful comments.

.

In particular, I am grateful to Prof. Dr. Gwendoline Chang for her valuable advice in reviewing chemistry parts of my thesis.

I thanks staff of Department of Plant Protection and Institute of Tropical Agriculture, Universiti Putra Malaysia for all their help in laboratory and glasshouse work and not to forget the staff in Institute of Bioscience, Faculty of Engineering and Faculty of Biotechnology for their kind assistance during HPLC, SEM, FTIR and NMR analysis.

Finally, I would like to extend my appreciation to my beloved parents, for their fully support and love. The support, advice and help given by my dearest friends also highly valued.

I certify that a Thesis Examination Committee has met on 9 September 2016 to conduct the final examination of Lee Kai Wei on his thesis entitle "Characterization and development of phenazine-based compounds from *Pseudomonas aeruginosa* for suppression of *Ganoderma boninense*" in accordance with 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 Master of Science.

Members of the Thesis Examination Committee were as follows:

Ganesan a/l Vadamalai, Ph.D
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Radziah bt Othman, Ph.D
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Thong Kwai Lin, Ph.D
Professor
Faculty of Science
Universiti Malaya
(External Examiner)

_____
ZULKARNIAN ZAINAL, PhD
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the supervisory committee were as follows:

Wong Mui Yun, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Dzolkhifli Omar, PhD
Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

Khalina Abdan, PhD
Associate Professor
Faculty of Engineering
Universiti Putra Malaysia
(Member)

_____

BUJANG KIM HUAT, PHD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

Declaration by Graduate Student

I hereby confirm that:

- This thesis is my original work;
- Quotation, illustration and citations have been duly referenced;
- This thesis has not been submitted previously or concurrently for any other degree at any other institutions;
- Intellectual property from the thesis and copyright of thesis are fully-owned by Universiti Putra Malaysia, as according to the Universiti Putra Malaysia (Research) Rules 2012;
- Written permission must be obtained from supervisor and the office of Deputy Vice-Chancellor (Research and Innovation) before thesis is published (in the form of written, printed or in electronic form) including books, journals, modules, proceedings, popular writing, seminar papers, manuscripts, poster, reports, lecture notes, learning modules or any other materials as stated in the Universiti Putra Malaysia (Research) Rules 2012;
- There is no plagiarism or data falsification/fabrication in the thesis, and scholarly integrity is upheld as according to the Universiti Putra Malaysia (Research) Rules 2003 (Revision 2012-2013) and the Universiti Putra Malaysia (Research) Rules 2012. The thesis has undergone plagiarism detection software.

Signature:…….…….. Date: …….....

Name and Matric No.: …….....

Declaration by Members of Supervisory Committee

This is to confirm that:

- The research conducted and the writing of this thesis was under our supervision;
- Supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to

Signature: ……
Name of
Chairman of
Supervisory
Committee: Assoc. Prof. Dr. Wong Mui Yun

Signature: ……
Name of
Member of
Supervisory
Committee: Prof. Dr. Dzolkhifli Omar

Signature: ……
Name of
Member of
Supervisory
Committee: Assoc. Prof. Dr. Khalina Abdan

TABLE OF CONTENTS

LISTS OF TABLES

LISTS OF FIGURES

LIST OF ABBREVIATIONS

2-OH-PCA	2-hydroxyphenazine-1-carboxylic acid
2-OH-PHZ	2-hydroxyphenazine
A.I.	Active ingredient
AUDPC	Area under Disease Progress Curve
BSR	Basal Stem Rot
CV	Coefficient of Variation
DAD	Diode Array Detector
DI	Disease Index
DR	Disease Reduction Rate
DS	Disease Severity Index
E2126	Emereen 2126
ER	Epidemic rate
FTIR	Fourier Transform Infra-red
HPLC	High Performance Liquid Chromatography
IR	Infrared
LC	Lethal Concentration
LCMS	Liquid Chromatography Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MPOB	Malaysian Palm Oil Board
NMR	Nuclear Magnetic Resonance
O/W	Oil in Water emulsion
PCA	Phenazine-1-carboxylic acid
PCN	phenazine-1-carboxamide
PD	Phase Diagram
PDA	Potato Dextrose Agar
Pdi	Polydispersion index
PHZ	Phenazine
Plt	Pyoluteorin
PYO	Pyocyanin
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RSD	Relative Standard Deviation
RWB	Rubber Wood Block
SEM	Scanning Electron Microscopy
TLC	Thin Liquid Chromatography

CHAPTER 1

INTRODUCTION

Oil palm tree (*Elaeis guineensis Jacq*) is a monocotyledon in the family Arecaceae (formerly Palmae) within the subfamily Cocosoideae (Corley and Tinker, 2003). It originates from West Africa and introduced to South East Asia by the British in Early 1870s. Oil palm trees start bearing fruits after 30 months of planting and remain productive for 20-30 years. With the planting density of 136 stem per hectare, needing just 0.17 ha of land to produce one tone of oil per year, by far it is the highest yielding monocotyledon plants in producing low cholesterol oil, biofuels, and has multiple uses of all its plant parts (Hushiarian *et al.,* 2013).

The Malaysian palm oil industry is significant contributor to Malaysia's overall economy, providing both employment and income from exports. In of 2011, the total planted area was 4.917 million hectares, the sector was the fourth largest contributor to Malaysia's economy, accounting for RM53 billions of Malaysia's Gross National Income (MPOB, 2014). However, oil palm plantations in Malaysia are facing problems associated with *Ganoderma* disease infections-Basal Stem Rot (BSR) that reduce quantity and quality of oil-palm products (Paterson *et al.,* 2013). The situation deteriorates with the climate changes that ingress fungal disease and more importantly it have resulted in economic losses. The disease can cause losses up to 50% of palm trees and 45% yield from fresh fruit bunch in oil palm plantations (Naher *et al.,* 2013).The seriousness of this disease in Malaysia has attracted attention where 3.5% of plantation areas infected render lost in revenue of RM 1 billion/year (Ishaq *et al.,* 2014).

Basal stem rot (BSR) caused by a white rot fungus, *Ganoderma boninense* is the most destructive disease of oil palm, notably in Malaysia and Indonesia. *G. boninense* is extraordinarily capable of degrading lignin and converting it to carbon dioxide and water where cellulose as a by-product of lignin degradation is used by the fungus as a nutrient source. Prevention steps have been taken by identification of diseased plants with rapid diagnostic tests at the nursery stage, but the increasing BSR incidence can be associated with post-planting processes under poor growth conditions of palms. Curative methods such as isolation pits, curative surgery and chemical control, have been applied but adequate control of this disease has never been achieved (Hushiarian *et al*., 2013).

In term of biological control, incorporation of beneficial microbes into planting materials were implemented but were only effective in delaying the disease incidence in the early stages and were reported to be ineffective at later stages due to inability of these beneficial microbes to survive in variable soil conditions of planting sites (Paterson, 2007). *Pseudomonas aeruginosa* produces phenazine-based compounds through secondary metabolism (Kanner *et al.,* 1978). The phenazine-based compounds are antibiotics that fluorescent under ultra-violet emission. These antibiotics have been discovered as key determinants for *P. aeruginosa* to perform as a biocontrol agent (Audenaert *et al*., 2002; Rane *et al*., 2007; Shanmugaiah *et al*., 2010; Raio *et al*., 2011).

Phenazine is a nitrogen-containing heterocylic antibiotic agent. More than 6,000 phenazine derivatives have been identified and described during the last two centuries.

Phenazine antibiotics are well-known in demonstrating toxicity towards various pathogens by having potent anti-fungal activity for control of economically important plant pathogens. A previous finding indicated that the isolated phenazine-based compounds exhibited antifungal activity on *G. boninense*.

Phenazines can be produced in two ways, namely biosynthesis and chemosynthesis (Cheluvappa, 2014). Biosynthesis, the natural way of this compound production is achieved via phenazine-producing bacteria such as *P. aeruginosa* when the bacteria is incubated in a suitable media and regulated by nutrient depletion. The high cell density converts the bacterium to biofilm form. Chemosynthesis of this compound is through serial oxidation of phenazine core (Nansathit *et al.,* 2011). In this present study, phenazine compounds were produced via biosynthesis by antagonistic bacterial *P. aeruginosa* UPMP3.

Phenazine-based compounds have diverse effects on eukaryotic hosts and host tissues. The mechanism of phenazines to manifest antibiotics properties are attributed to their ability to generate reactive oxygen species in other organism. In basal stem rot case, phenazines give good effect to oil palm, by inhibition of *G. boninense*. Phenazine can disturb the electron flow in pathogen cells by accept or donate electron, this redox reaction control the oxidative phosphorylation and shunting the endogenous pathway of *G. boninense* (Pierson and Pierson, 2010). Phenazines are not just inhibiting *G. boninense*, but to control wide range of plant pathogenic fungi with this mechanism (Mavrodi *et al.,* 2006).

Phenazines have been shown to induce plant defence system by activate induced systemic resistance in plants. Plant's induced system resistance is activated by the presence of bacterial secondary metabolites, outer membrane components, lipopolysaccharide, siderophores and volatile compounds. Phenazines are bacterial compounds and these foreign entities modulate the plant jasmonic acid pathway, leading to the rapid systemic expression of broad spectrum resistance against numerous pathogen (Pierson and Pierson, 2010).

Formulation of crop protection products in agrochemical industries are developed, general types of formulation are emulsified concentrates, wettable powder, solution, granules, water dispersible granules, microcapsule, emulsion and microemulsion (Lim *et al.,* 2012). Nanoemulsion are submicron emulsion with a nanometric-scaled droplet, enhance solubility and dissolution properties of poorly water soluble substrates, they are referred as miniemulsion of ultrafine emulsion and have small droplet size ranged from 20 to 200 nm (Fernandes *et al.,* 2014; Forgiarini *et al.,* 2001). It was identified as promising delivery systems, good alternatives for toxic and non-renewable chemicals such as petroleum and oil adjuvant.

In past decades, nanoemulsions have gained immense interest in water-soluble pesticide of various kinds of active ingredient (Wang *et al.,* 2007), research has highlighted tremendous improvement on physical characteristics and biological performance. Yet, biofungicide nanoemulsion formulation with phenazine crude extracts, characteristics and its functionality in suppression on fungal pathogen have not been reported.

Currently, there is a need to formulate nanoemulsion that effectively controls BSR. Thus, objectives of the present study were as followed:

1. To extract and to characterize the phenazine-based compounds from *Pseudomonas aeruginosa*.
2. To formulate the nanoemulsion formulation of the phenazine-based compounds from *Pseudomonas aeruginosa*.
3. To evaluate the suppression efficacy of the formulation on *Ganoderma boninense*

REFERENCES

Abdullah, F, Ilias, G.N.M., Nelson, M., Izzati, N.A.M.Z. and Yusuf, U.K. (2003). Disease assessment and the efficacy of *Trichoderma* as a biocontrol agent of basal stem rot of oil palms. *Sci. Putra*, 12:31-33.

Abdullah, F., Ilias, G.N.M., Vijaya, S.K., and Leong, T.T. (1999). Diversity of *Trichoderma* and its *in vivo* efficacy against *Ganoderma boninense*. In *Sustainable Crop Protection Practices in the Next Millenium. MCBMAPPS Plant Protection Conference* (Vol. 99, pp. 2-3).

Abraham, A., Philip, S., Jacob, M.K., Narayanan, S.P., Jacob, C.K., and Kochupurackal, J. (2015). Phenazine-1-carboxylic acid mediated anti-oomycete activity of the endophytic *Alcaligenes sp*. EIL-2 against *Phytophthora meadii*. *Microbiological research*, *170*, 229-234.

Ahmad, S.N., Islam, M.T., Abdullah, D.K., and Omar, D. (2012). Evaluation of physicochemical characteristics of microemulsion formulation of rotenone and its insecticidal efficacy against *Plutella xylostella* L. (Lepidoptera: Plutellidae). *Journal of Food, Agriculture and Environment*, *10*(3and4), 384-388.

Anton, N., and Vandamme, T. F. (2009). The universality of low-energy nano-emulsification. *International Journal of Pharmaceutics*, *377*(1), 142-147.

Ariffin, D., Idris, A.S. and Abdul Halim, H. (1989). Significance of the black line within oil palm tissue decay by *Ganoderma boninense*. *Journal of Elaeis*, 1:11-16.

Ariffin, D. and Idris, A.S. (1991). A selective medium for the isolation of *Ganoderma* from diseased tissues. In: Yusof, B. *et al.* (eds), Proceeding of the 1991 PORIM International Palm Oil Conference-Progress, Prospects and Challenges Towards the 21st Century-Module 1, Agriculture. Palm Oil Research Institute of Malaysia, Bangi, Selangor, Malaysia, pp. 517-519.

Ariffin, D., Idris, A.S. and Singh, G. (2000). Status of *Ganoderma* in oil palm. In: Flood, J. *et al.*, (eds), *Ganoderma* Diseases of Perennial Crops. CABI International Publishing, Wallingford, UK. pp 249-666.

Assis, K., Chong, K. P., Idris, A. S., & Ho, C. M. (2016). Economic Loss due to *Ganoderma* Disease in Oil Palm. World Academy of Science, Engineering and Technology, International Journal of Social, Behavioral, Educational, Economic, Business and Industrial Engineering, 10(2), 631-635.

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. (2002). Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. *Molecular Plant-Microbe Interactions*, *15*(11), 1147-1156.

Azadeh, B. F., and Meon, S. (2009). Molecular characterization of *Pseudomonas aeruginosa* UPMP3 from oil palm rhizosphere. *American Journal of Applied Sciences*, *6*(11), 1915.

Azam, A., Ahmed, A.S., Oves, M., Khan, M.S. and Memic, A. (2012). Size-dependent antimicrobial properties of CuO nanoparticles against Gram-positive and-negative bacterial strains. *International journal of nanomedicine*, *7*, 3527.

Aziz, L.M., Hamza, S.J. and Abdul-Rahman, I.A. (2012). Isolation and characterization of phenazine produced from mutant *Pseudomonas aeruginosa*. *Al-Anbar J Vet Sci*, *5*, 42-53.

Aziz, L. M., حمزة صـ بحي, and إبـ راهيم عـ بد الـ رحمن. (2011). Isolation and characterization of phenazine produced by SP9 strain of *Pseudomonas aeruginosa*. *International Journal for Sciences and Technology*, *6*(4), 75-84.

Babchin, A.J. and Schramm, L. L. (2012). Osmotic repulsion force due to adsorbed surfactants. *Colloids and Surfaces B: Biointerfaces*, *91*, 137-143.

Backman, P.A. (1978). Fungicide formulation: relationship to biological activity. *Annual Review of Phytopathology*, *16*(1), 211-237.

Barradas, T.N., de Campos, V.E.B., Senna, J.P., Coutinho, C.D.S.C., Tebaldi, B.S., e Silva, K.G.D.H. and Mansur, C.R.E. (2014). Engineering Aspects.

Bentzmann, S. and Plésiat, P. (2011). The *Pseudomonas aeruginosa* opportunistic pathogen and human infections. *Environmental microbiology*,*13*(7), 1655-1665.

Bloemberg, G.V. and Lugtenberg, B.J. (2003). Phenazines and their role in biocontrol by *Pseudomonas* bacteria. *New phytologist*, *157*(3), 503-523.

Bouchemal, K., Briançon, S., Perrier, E. and Fessi, H. (2004). Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. *International journal of pharmaceutics*, *280*(1), 241-251.

Breitmaier, E., & Hollstein, U. (1976). Carbon-13 nuclear magnetic resonance chemical shifts of substituted phenazines. The Journal of Organic Chemistry,41(12), 2104-2108.

Breton, F., Hasan, Y., Hariadi, S., Lubis, Z., De Franqueville, H. (2006). Characterization of parameters for the development of an early screening test for basal stem rot tolerance in oil palm progenies. *Journal of Oil palm research* (Special Issue, April 2006), 24-36.

Bruxel, F., Laux, M., Wild, L.B., Fraga, M., Koester, L.S. and Teixeira, H.F. (2012). Nanoemulsões como sistemas de liberação parenteral de fármacos. *Quim Nova*, *35*, 1827-1840.

Campbell, C.L. and Madden, L.V. (1990). *Introduction to plant disease epidemiology*. John Wiley and Sons.

Cheluvappa, R. (2014). Standardized chemical synthesis of *Pseudomonas aeruginosa* pyocyanin. *MethodsX*, *1*, 67-73.

Cheluvappa, R., Shimmon, R., Dawson, M., Hilmer, S. N., and Le Couteur, D. G. (2008). Reactions of *Pseudomonas aeruginosa* pyocyanin with reduced glutathione.

Chen, F., Wang, Y., Zheng, F., Wu, Y. and Liang, W. (2000). Studies on cloud point of agrochemical microemulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *175*(1), 257-262.

Chen, Y., Shen, X., Peng, H., Hu, H., Wang, W. and Zhang, X. (2015). Comparative genomic analysis and phenazine production of *Pseudomonas chlororaphis*, a plant growth-promoting rhizobacterium. *Genomics Data*, *4*, 33-42.

Cheong, J.N., Tan, C.P., Man, Y.B.C., and Misran, M. (2008). α-Tocopherol nanodispersions: preparation, characterization and stability evaluation. *Journal of Food Engineering*, *89*(2), 204-209.

Chong, K.P (2010). *The role of phenolics in the interaction between oil palm and Ganoderma boninense the causal agent of basal stem rot* (Doctoral Dissertation, University of Nottingham).

Chung, G.F. (1991). Preliminary results on trunk injection of fungicides against *Ganoderma* basal stem rot in oil palm. In: Ariffin, D. and Sukaimi, J. (Eds), Proceedings of *Ganoderma* workshop, Bangi, Selangor, Malaysia. Palm Oil Research Institute of Malaysia, pp 81-97.

Coates, J. (2000). Interpretation of infrared spectra, a practical approach. *Encyclopedia of analytical chemistry*.

Cooper, R. M., Flood, J. and Rees, R. W. (2011). *Ganoderma boninense* in oil palm plantations: current thinking on epidemiology, resistance and pathology. *Planter*, *87*(1024), 515-526.

Corley, R.H.V. and Tinker, P.B. (2003). The oil palm. 4th edition. *Oxford, UK: Blackwell Publishing*.

De Vleesschauwer, D., Cornelis, P. and Höfte, M. (2006). Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances Rhizoctonia solani susceptibility in rice. *Molecular Plant-Microbe Interactions*, *19*(12), 1406-1419.

Dias, D.D.O., Colombo, M., Kelmann, R.G., Kaiser, S., Lucca, L.G., Teixeira, H. F. and Koester, L. S. (2014). Optimization of Copaiba oil-based nanoemulsions obtained by different preparation methods. *Industrial Crops and Products*, *59*, 154-162.

Durand-Gasselin, T., Asmady, H., Flori, A., Jacquemard, J.C., Hayun, Z., Breton, F. and De Franqueville, H. (2005). Possible sources of genetic resistance in oil palm (*Elaeis guineensis* Jacq.) to basal stem rot caused by *Ganoderma boninense* – prospects for future breeding. *Mycopathologia*, 159: 93-100.

El-Fouly, M. Z., Sharaf, A. M., Shahin, A. A. M., El-Bialy, H. A. and Omara, A. M. A. (2015). Biosynthesis of pyocyanin pigment by *Pseudomonas aeruginosa*. *Journal of Radiation Research and Applied Sciences*, *8*(1), 36-48.

El-Sayed, W., El-Megeed, M. A., El-Razik, A. A., Soliman, K. H. and Ibrahim, S. A. (2008). Isolation and identification of phenazine-1-carboxylic acid from different *Pesudomonas* isolates and its biological activity against *Alternaria solani*. *Res J Agric Biol Sci*, *4*(6), 892-901.

Feklistova, I. N. and Maksimova, N. P. (2008). Obtaining *Pseudomonas aurantiaca* strains capable of overproduction of phenazine antibiotics. *Microbiology*, *77*(2), 176-180.

Fernandes, C.P., de Almeida, F.B., Silveira, A.N., Gonzalez, M.S., Mello, C.B., Feder, D. and Falcão, D.Q. (2014). Development of an insecticidal nanoemulsion with *Manilkara subsericea* (Sapotaceae) extract. *J. Nanobiotechnology*, *12*, 22.

Fernández, R.O. and Pizarro, R.A. (1997). High-performance liquid chromatographic analysis of *Pseudomonas aeruginosa* phenazines. *Journal of Chromatography A*, *771*(1), 99-104.

Flores, F.C., Ribeiro, R.F., Ourique, A.F., Rolim, C.M.B., Silva, C.D.B.D., Pohlmann, A.R. and Guterres, S. S. (2011). Nanostructured systems containing an essential oil: protection against volatilization. *Química Nova*,*34*(6), 968-972.

Forgiarini, A., Esquena, J., Gonzalez, C. and Solans, C. (2001). Formation of nano-emulsions by low-energy emulsification methods at constant temperature. *Langmuir*, *17*(7), 2076-2083.

Gao, B., Peng, H., Wang, W., Xu, Y., and Zhang, X. (2010). Determination of a novel fungicide phenazine-1-carboxylic acid in soil samples using sample stacking capillary electrophoresis combined with solid phase extraction. *Analytical Letters*, *43*(12), 1823-1833.

George, S.T., Chung, G.F. and Zakaria, K. (1996). Updated result (1990-1005) on trunk injection of fungicides of the control of *Ganoderma* basal stem rot. In: Ariffin, D., *et al.* (eds), Proceedings of the 1996 PORIM International Palm Oil Congress-Agriculture. Palm Oil Research Institute of Malaysia, Selangor, Malaysia, pp 508-515.

Goff, H. D. (1997). Colloidal aspects of ice cream—a review. *International Dairy Journal*, *7*(6), 363-373.

Griesbeck, A. G. (2014). Science of synthesis: Houben-Weyl Methods of Organic Chemistry Vol. E 9b/2, 4th Edition Supplement:Hetarenes III (Six-Membered Rings and Larger Hetero-Rings with Maximum Unsaturation) - Part 2b. Lulu Press, Inc. pp. 267.

Ho, Y.W. and Nawawi, A. (1986). *Ganoderma boninense* from basal stem rot of oil palm (Elaeis guineensis) in Peninsular Malaysia. *Pertanika Journal of Tropical Agricultural Science, 8:* 425-428.

Hoeller, S., Sperger, A., and Valenta, C. (2009). Lecithin based nanoemulsions: A comparative study of the influence of non-ionic surfactants and the cationic phytosphingosine on physicochemical behaviour and skin permeation. *International journal of pharmaceutics*, *370*(1), 181-186.

Hu, L., Song, W., Niu, F., Jiao, K., and Jia, Z. (2013). Preparation, characterization and tableting of cilnidipine solid dispersions. *Pak. J. Pharm. Sci*, *26*(3), 629-636.

Hushiarian, R., Yusof, N. A., and Dutse, S. W. (2013). Detection and control of *Ganoderma boninense*: strategies and perspectives. *SpringerPlus*, *2*(1), 555.

Idris, A.S., Ariffin, D., Swinburne and Watt, T.A. (2000). The identity of *Ganoderma* species responsible for basal stem rot disease of oil palm in Malaysia – Pathogenecity test. MPOB information series NO. 103(77).

Idris, A.S., Kushairi, D., Ariffin, D., and Basri, M. (2006). Technique for inoculation of oil palm germinated seeds with *Ganoderma*. *MPOB Inf Ser*,*314*, 1-4.

Idris, A.S., Khushairi, A., Ismail, S. and Arifin, D. 2002. Selection of partial resistance in oil palm to *Ganoderma* basal stem rot. Seminar: elevating the national oil palm productivity and recent progress in the management of peat and *Ganoderma*, Kuala Lumpur, Malaysia, 6-7 May, 2002, p.11.

Ishaq, I., Alias, M. S., Kadir, J., & Kasawani, I. (2014). Detection of basal stem rot disease at oil palm plantations using sonic tomography. *Journal of Sustainability Science and Management*, *9*(2), 52-57.

Jones, M. and Fleming, S.A. 2009. Organic Chemistry 4th Edtion, Norton, pp14-16.

Kanner, D., Gerber, N. N. and Bartha, R. (1978). Pattern of phenazine pigment production by a strain of *Pseudomonas aeruginosa*. *Journal of bacteriology*,*134*(2), 690-692.

Karthikeyan, M., Bhaskaran, R., Radhika, K., Mathiyazhagan, S., Jayakumar, V., Sandosskumar, R. and Velazhahan, R. (2008). Development and comparison of ELISA and PCR methods for the early detection of *Ganoderma* disease of coconut. *Archives of Phytopathology and Plant Protection*, *41*(6), 396-406.

Kavitha, K., Mathiyazhagan, S., Sendhilvel, V., Nakkeeran, S., Chandrasekar, G.O. P.A.L. and Dilantha Fernando, W.G. (2005). Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. *Archives of Phytopathology and Plant Protection*, *38*(1), 69-76.

Kelmann, R.G., Kuminek, G., Teixeira, H.F. and Koester, L.S. (2007). Carbamazepine parenteral nanoemulsions prepared by spontaneous emulsification process. *International journal of pharmaceutics*, *342*(1), 231-239.

Khairuddin, H. (1990). Basal Stem rot of oil palm: Incidence, etiology and control (Master degree Dissertation, Universiti Pertanian Malaysia).

Kho, K., Cheow, W.S., Lie, R.H. and Hadinoto, K. (2010). Aqueous re-dispersibility of spray-dried antibiotic-loaded polycaprolactone nanoparticle aggregates for inhaled anti-biofilm therapy. *Powder Technology*, *203*(3), 432-439.

Kim, K. J. (2000). Phenazine 1-carboxylic acid resistance in phenazine 1-carboxylic acid producing *Bacillus sp*. B-6. *Journal of Biochemistry and Molecular Biology*, *33*(4), 332-336.

Komaiko, J. and McClements, D. J. (2014). Optimization of isothermal low-energy nanoemulsion formation: Hydrocarbon oil, non-ionic surfactant, and water systems. *Journal of colloid and interface science*, *425*, 59-66.

Lightbown, J.W. (1954). An antagonist of streptomycin and dihydrostreptomycin produced by *Pseudomonas aeruginosa*. *Journal of general microbiology*, *11*(3), 477-492.

Lim, C. J., Basri, M., Omar, D., Rahman, M. B. A., Salleh, A. B., and Rahman, R. N. Z.R.A. (2012). Physicochemical characterization and formation of glyphosate-laden nano-emulsion for herbicide formulation. *Industrial Crops and Products*, *36*(1), 607-613.

Liu, H., Dong, D., Peng, H., Zhang, X., and Xu, Y. (2006). Genetic diversity of phenazine-and pyoluteorin-producing *pseudomonads* isolated from green pepper rhizosphere. *Archives of microbiology*, *185*(2), 91-98.

Liu, H., He, Y., Jiang, H., Peng, H., Huang, X., Zhang, X., and Xu, Y. (2007). Characterization of a phenazine-producing strain *Pseudomonas chlororaphis* GP72 with broad-spectrum antifungal activity from green pepper rhizosphere. *Current microbiology*, 54(4), 302-306.

Mahdi, E.S., Noor, A.M., Sakeena, M. H., Abdullah, G.Z., Abdulkarim, M. F. and Sattar, M. A. (2011). Formulation and *in vitro* release evaluation of newly synthesized palm kernel oil esters-based nanoemulsion delivery system for 30% ethanolic dried extract derived from local *Phyllanthus urinaria* for skin antiaging. *International journal of nanomedicine*, *6*, 2499.

Mavrodi, D. V., Blankenfeldt, W. and Thomashow, L.S. (2006). Phenazine compounds in fluorescent *Pseudomonas spp*. biosynthesis and regulation*. *Annu. Rev. Phytopathol.*, *44*, 417-445.

Mavrodi, O.V., Walter, N., Elateek, S., Taylor, C.G., and Okubara, P.A. (2012). Suppression of *Rhizoctonia* and Pythium root rot of wheat by new strains of *Pseudomonas*. *Biological control*, *62*(2), 93-102.

Mazurek, A. and Jamroz, J. (2015). Precision of dehydroascorbic acid quantitation with the use of the subtraction method–Validation of HPLC–DAD method for determination of total vitamin C in food. *Food chemistry*, *173*, 543-550.

Mehnaz, S., Baig, D.N., Jamil, F., Weselowski, B. and Lazarovits, G. (2009). Characterization of a phenazine and hexanoyl homoserine lactone producing *Pseudomonas aurantiaca* strain PB-St2, isolated from sugarcane stem. *J Microbiol Biotechnol*, *19*(12), 1688-1694.

Meon, S. (1998). Potential for Biological Control of Sclerotium Foot Rot of Chilli by *Trichoderma spp*. *Pertanika Journal of Tropical Agricultural Science*, *21*(1), 1-10.

Miller, R. N. G. (1995). *The characterization of Ganoderma populations in oil palm cropping systems* (Doctoral dissertation, University of Reading).

Mohd Zainudin, N.A.I. and Abdullah, F. (2008). Disease suppression in *Ganoderma*-infected oil palm seedlings treated with *Trichoderma harzianum*. *Plant Protection Science*, *44*(3), 101-107.

MPOB. (2014). Retrieved from http://www.palmoilworld.org/applications.html. Accessed on 11 March 2014.

Mulqueen, P. (2003). Recent advances in agrochemical formulation. *Advances in colloid and interface science*, *106*(1), 83-107.

Naher, L., Yusuf, U.K., Ismail, A., Tan, S.G., and Mondal, M.M.A. (2013). Ecological status of *Ganoderma* and basal stem rot disease of oil palms (*'Elaeis guineensis*' Jacq.).

Nansathit, A., Phaosiri, C., Pongdontri, P., Chanthai, S., and Ruangviriyachai, C. (2011). Synthesis, isolation of phenazine derivatives and their antimicrobial activities. *Walailak Journal of Science and Technology (WJST)*, *6*(1), 79-91.

Nowroozi, J., Sepahi, A. A. and Rashnonejad, A. (2012). Pyocyanin biosynthesis genes in clinical and environmental isolates of *Psedomonas aeruginosa* and detection of pyocyanin's antimicrobial effects with or without colloidal silver nanoparticles. *Cell Journal*, 14(1), 7-18.

Parvin, W., Othman, R., Jaafar, H. Wong, M. Y. (2013). Detection and Optimization of Indole-3 acetic acid in rhizobacterial strains Pseudomonas aeroginosa UPM3 and burkholderia cepacia UPMB3 isolated from Oil Palm Rhizosphere in vitro in agroindustrial Biotechnology for global sustainable prosperity . Proceeding of the 25th annual meeting of the That society for biotechnology and International conference, Bangkok Thailand (Unpublished).

Paterson, R.R.M. (2007). *Ganoderma* disease of oil palm—A white rot perspective necessary for integrated control. *Crop protection*, *26*(9), 1369-1376.

Paterson, R. R. M., Sariah, M., and Lima, N. (2013). How will climate change affect oil palm fungal diseases? *Crop Protection*, *46*, 113-120.

Pierson III, L. S., & Pierson, E. A. (2010). Metabolism and function of phenazines in bacteria: impacts on the behavior of bacteria in the environment and

biotechnological processes. *Applied microbiology and biotechnology*,*86*(6), 1659-1670.

Pilotti, C.A., Sanderson, F.R., and Aitken, E.A. (2002). Sexuality and interactions of monokaryotic and dikaryotic mycelia of Ganoderma boninense. *Mycological Research*, *106*(11), 1315-1322.

Raio, A., Puopolo, G., Cimmino, A., Danti, R., Della Rocca, G., and Evidente, A. (2011). Biocontrol of cypress canker by the phenazine producer Pseudomonas chlororaphis subsp. aureofaciens strain M71. *Biological Control*, *58*(2), 133-138.

Ramsey, R.J.L., Stephenson, G.R., and Hall, J.C. (2005). A review of the effects of humidity, humectants, and surfactant composition on the absorption and efficacy of highly water-soluble herbicides. *Pesticide Biochemistry and Physiology*, *82*(2), 162-175.

Rane, M.R., Sarode, P.D., Chaudhari, B.L. and Chincholkar, S.B. (2007). Detection, isolation and identification of phenazine-1-carboxylic acid produced by biocontrol strains of *Pseudomonas aeruginosa*. *Journal of Scientific and Industrial Research*, *66*(8), 627.

Reyes, E.A., Bale, M.J., Cannon, W.H. and Matsen, J.M. (1981). Identification of *Pseudomonas aeruginosa* by pyocyanin production on Tech agar. *Journal of clinical microbiology*, *13*(3), 456-458.

Rozet, E., Marini, R.D., Ziemons, E., Boulanger, B. and Hubert, P. (2011). Advances in validation, risk and uncertainty assessment of bioanalytical methods. *Journal of pharmaceutical and biomedical analysis*, *55*(4), 848-858.

Sadyś, M., Skjøth, C.A. and Kennedy, R. (2014). Back-trajectories show export of airborne fungal spores (*Ganoderma sp*.) from forests to agricultural and urban areas in England. *Atmospheric Environment*, *84*, 88-99.

Sanderson, F.R., Pilotti, C.A. and Bridge, P.D. (2000). Basidiospores: Their influence on our thinking regarding a control strategy for Basal Stem Rot of Oil palm. In: Flood, J. *et al.*, (eds), *Ganoderma* Diseases of Perennial Crops. CAB International Publishing, Wallingford, UK. pp. 113-121.

Sapak, Z., Meon, S., Ahmad, M. and Abidin, Z. (2008). Effect of Endophytic bacteria on growth and suppression of ganoderma infection in oil palm.*International Journal of Agriculture and Biology*, *10*(2), 127-132.

Shanmugaiah, V., Mathivanan, N., and Varghese, B. (2010). Purification, crystal structure and antimicrobial activity of phenazine-1-carboxamide produced by a growth- promoting biocontrol bacterium, *Pseudomonas aeruginosa* MML2212.*Journal of applied microbiology*, *108*(2), 703-711.

Singh, G. (1991). *Ganoderma*-the scourge of oil palms in the coastal areas. *Planter 67*:421-444.

Spínola, V., Llorent-Martínez, E. J. and Castilho, P.C. (2014). Determination of vitamin C in foods: Current state of method validation. *Journal of Chromatography A*, *1369*, 2-17.

Steyaert, R.L. (1967). Les *Ganoderma palmicoles*. Bulletin du Jardin Botanique Nationale Belgique 37, 465-492.

Strobel, G., Daisy, B., Castillo, U. and Harper, J. (2004). Natural products from endophytic microorganisms. *Journal of Natural products*, *67*(2), 257-268.

Sundram, S. (2013). First report: isolation of endophytic *Trichoderma* from oil palm (*Elaeis guineensis* Jacq.) and their in vitro antagonistic assessment on *Ganoderma boninense*. *Journal of Oil Palm Research*, *25*(3), 368-372.

Sundram, S. (2013). The effects of *Trichoderma* in surface mulches supplemented with conidial drenches in the disease development of *Ganoderma* basal stem rot in oil palm. *Journal of Oil Palm Research*, *25*(3), 314-325.

Sundram, S., Meon, S., Seman, I.A. and Othman, R. (2011). Symbiotic interaction of endophytic bacteria with arbuscular mycorrhizal fungi and its antagonistic effect on *Ganoderma boninense*. *The Journal of Microbiology*,*49*(4), 551-557.

Susanto, A., Sudharto, P.S. and Purba, R. Y. (2005). Enhancing biological control of basal stem rot disease (*Ganoderma boninense*) in oil palm plantations. *Mycopathologia*, *159*(1), 153-157.

Tadros, T. F. (Ed.). (2013). *Emulsion formation and stability*. John Wiley and Sons.

Tang, S.Y., Manickam, S., Wei, T.K. and Nashiru, B. (2012). Formulation development and optimization of a novel Cremophore EL-based nanoemulsion using ultrasound cavitation. *Ultrasonics sonochemistry*, *19*(2), 330-345.

Teh, K.S. and Sariah, M. (1999). Improved inoculation technique for testing pathogenicity of *Ganoderma boninense* on oil palm seedlings. In: Plant protection in the information Age. Fourth MAAPS International Conference on Plant Protection in the Tropics 142-145.

Thompson, A. (1931). Stem rot of oil palm in Malaya. Bulletin Department of Agriculture, Straits Settlements and F.M.S., *Science Series* 6:23 pp.

Turner, P.D. (1981). Oil palm disease and Disorders. *Oxford University Press*.

Wagner, H., Kostka, K.H., Lehr, C.M. and Schaefer, U.F. (2003). pH profiles in human skin: influence of two in vitro test systems for drug delivery testing. *European journal of pharmaceutics and biopharmaceutics*, *55*(1), 57-65.

Wang, L., Li, X., Zhang, G., Dong, J. and Eastoe, J. (2007). Oil-in-water nanoemulsions for pesticide formulations. *Journal of colloid and interface science*, *314*(1), 230-235.

Warisnoicharoen, W., Lansley, A.B. and Lawrence, M.J. (2000). Nonionic oil-in-water microemulsions: the effect of oil type on phase behaviour. *International Journal of Pharmaceutics*, *198*(1), 7-27.

Wilson, R., Pitt, T., Taylor, G., Watson, D., MacDermot, J., Sykes, D. and Cole, P. (1987). Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia *in vitro*. *Journal of Clinical Investigation*, *79*(1), 221.

Yuan, L.L., Li, Y.Q., Wang, Y., Zhang, X. H. and Xu, Y.Q. (2008). Optimization of critical medium components using response surface methodology for phenazine-1-carboxylic acid production by *Pseudomonas sp*. M-18Q. *Journal of bioscience and bioengineering*, *105*(3), 232-237.

Yuan, Y., Gao, Y., Mao, L. and Zhao, J. (2008). Optimisation of conditions for the preparation of β-carotene nanoemulsions using response surface methodology. *Food chemistry*, *107*(3), 1300-1306.

Zaiton, S. (2006). Bactrial endophytes from oil palm (*Elaeis guineensis*) and their antagonistic activity against *Ganoderma boninense* (Master degree dissertation, Universiti Putra Malaysia).

BIODATA OF STUDENT

Kai Wei was born in Kempas, Johor Bahru on 2nd OCT 1988. He received his primary education from Sekolah Jenis Kebangsaan (Cina) Kempas Baru and completed his SPM and STPM from Sekolah Menengah Kebangsaan Kempas in 2006 and Sekolah Menengah Kebangsaan Tasek Utara in 2008, respectively. Later, he attended Universiti Malaysia Sabah and received his bachelor's degree with First Class honour in Agricultural Science Major in Horticulture and Landscaping in 2013.

After he graduated in DEC 2013, he joined J.C.Chang group as a Junior Assistant Manager in Segamat Estate. To fulfil his ambition as Plant Doctor, he enrolled as a full time Master student in Institute of Tropical Agriculture. He completed his Master's degree under the supervision of Associate Professor Dr. Wong Mui Yun, a dedicated scientist in the Plant Pathology.