Journal of Tropical Agricultural Science

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Pertanika is an international peer-reviewed journal devoted to the publication of original papers, and it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields. Pertanika Journal of Tropical Agricultural Science which began publication in 1978 is a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Pertanika Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other Pertanika series include Pertanika Journal of Science and Technology (JST) and Pertanika Journal of Social Sciences and Humanities (JSSH).

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Pertanika Editorial Office

Office of the Deputy Vice Chancellor (R&I), 1st Floor, IDEA Tower II, UPM-MTDC Technology Centre
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
Tel: +603 8947 1622
E-mail: ndeeps@admin.upm.edu.my
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Editorial[i]

More Science, Better Integration to Meet the Food Challenge

Maintaining adequate global food supplies at a time of rapidly rising population, significant economic growth, increasing food and stockfeed demand, changing climate, declining natural resources, trade liberalisation and regional disturbances is a critical issue for mankind.

To meet this life threatening challenge, we must adopt scientifically sound and sustainable agricultural practices.

Science plays a major role in feeding the world, as clearly demonstrated by the green revolution post 2nd World War. However, future food security challenges will increasingly require a multi-disciplinary approach, involving environmental, economic, social and political solutions.

World leaders increasingly realise that feeding the world with diminishing resources is a massive task and hence, greater co-operation between countries, governments and scientific disciplines is required.

Interestingly, while the need to have food on their plate is shared by all consumers, the more affluent are now demanding their food should also be clean, green and ethically and sustainably produced.

Alarmingly, this is happening as the stockpile of wheat and other major cereal grains has dropped recently to its lowest level since 1980. Food prices are soaring worldwide, while crude oil prices have doubled shipping and fertiliser costs. The UN's Food and Agriculture Organisation (FAO) estimates that by 2050, grain output has to rise 50% and meat output has to double.

Population growth, rising incomes, the declining rate of agricultural productivity trends, climate change, and the increased uses of grain and sugar cane for biofuel production are leading to a competitive surge in food commodity demand. This is occurring in an environment where land and water constraints will limit agricultural production growth. Total urban population will double, changing diets as well as overall demand, because urbanites tend to eat more meat products.

As every human is a net consumer of food, balancing the needs and merits of nutrition, bio-energy, the environment and livelihoods are global concerns. For these reasons, integrating whole aspects of agriculture and the food industry is important in the future.

Five Trends

I see five major trends in the global agriculture and food industry.

1. *Food production must be increased substantially* by the mid 21st century to feed a world population projected to increase from 7 to 9 billion. The challenge is to double world food production output by 2050 using less land and far less water and fewer nutrients, while watching the 'hovering cloud' of climate variability and change.

2. *Economic development is increasing faster than expected* in most countries. With economic growth comes a rapidly changing food preference, increasing purchasing power and greater demand for high standards of food quality. About 40% of the increase in the world's grain production comes from the increase in yields and 60% comes from allocating more land under cultivation. However, increased future food production must come from shrinking land, water and other natural resources, i.e. with increased productivity per unit of land.

3. *Impact* of *agriculture on the environment and our natural resources.* An example is the emerging global shortage of water for urban consumption, industrial use and agricultural purposes.

   The world's 1.5 billion farmers, as guardians of much of what is left of the natural landscape, hold the fate of thousands of threatened species and the world's remaining forests in their hands. Today, agriculture uses 75% of the world's fresh water and its runoff has degraded the earth's major rivers, estuaries and even seas.

4. *Escalating fossil fuel price and the growing popularity of biofuels* are driving demands for grain crops (corn and oil seeds) and sugar cane. Increasing fossil fuel prices further pose major risk to agriculture production and transportation costs, leading to increased price volatility. This presents a serious issue since it takes over arable land and diverts resources from food production. By 2020, we are likely to burn 400 million tonnes of grain a year just to keep our cars on the road – equal to the world's current rice crop.

   Meanwhile, billions of subsidy dollars have been poured into developing sugar and grain-based ethanol and biodiesel to help wean rich economies from their addiction to carbon-blenching fossil fuels, the overwhelming source of human-made global warming. As soaring prices for staples bring more of the planet's most vulnerable people face-to-face with starvation, the image of first generation biofuels has changed from climate saviour to misguided 'experiment'.

5. The fifth trend is *climate change and its impact on agriculture.* Potential changes in climate may reduce productivity and output in agricultural industries in major producing countries, in the medium to long terms. Several analyses indicate that future climate changes and associated declines in agricultural productivity and global economic activity may affect global production of key commodities. For example, global wheat, rice, beef, dairy and sugar productions could decline by 2 - 6% by 2030 and 5 – 11% by 2050.

   The agricultural sector must maintain strong productivity growth to cope with the pressures emerging from climate change and variability. Agriculture occupies 40% of the world's free land surface and is responsible for 30% of global greenhouse emissions.

More world-class scientists must be trained in agronomy/farming systems, environmental science, genetics, biotechnology and plant breeding. By instituting international agricultural training initiatives, we can positively address the global food crisis.

According to ACIAR CEO, Dr Nick Austin, “agricultural science can be a catalyst for lifting many of the world’s estimated 1.4 billion poor people from poverty”.

Addressing the annual Australian Bureau of Agricultural Resource Economics (ABARE) Conference, he said in the past 50 years, agricultural R&D had been pivotal in lifting gross world’s food production by 138%, from 1.84 billion tonnes to 4.38 billion tonnes.

At the moment, it is closer to a nightmare for those going to sleep at night with an empty stomach and this is something that is unpalatable to caring, thinking human beings with the capacity to make the changes necessary for everyone to be adequately fed and cared for.

Thus, we simply can’t claim global food security when one in seven people today still does not have access to sufficient food, while an equal number is over-fed.

Many of our global problems, such as food, water and energy shortages and climate change, are related and it is clear we can no longer take a linear path to a solution. I believe appropriately funded and strategic R&D has the capacity to drive agriculture and, in turn, global food production, to the point where food security can be more a reality than a dream.

Strong political leadership and social planning are also equally necessary to achieve these desired outcomes.

Winthrop Professor Dr Kadambot Siddique, AM FTSE, BSc. (Ag) Hons., MSc., PhD.
Chair in Agriculture and Director
The UWA Institute of Agriculture
The University of Western Australia
and
Associate Dean Research
Faculty of Natural and Agricultural Sciences
The University of Western Australia
Perth

kadambot.siddique@uwa.edu.au
http://www.uwa.edu.au/people/kadambot.siddique

February, 2012

Professor Siddique has 26 years of experience in agricultural research, teaching and management in both Australia and overseas. He has developed a national and international reputation in the fields of farming systems, production agronomy, crop physiology, genetic resources, breeding and market research in cereal, grain and pasture legumes and oilseed crops. Professor Siddique's publications are considered as key papers in the above fields and are widely cited. His pioneering research on chickpea has contributed enormously to the Australian chickpea industry, which is currently valued at more than $250 million per annum.

In 2011, Professor Siddique was made Member of the Order of Australia (AM) in Queen's Birthday Honours List. The citation recognised his lifetime's work in advancing agricultural science as an academic and a researcher in the area of crop improvement and agronomy and through his contributions to professional associations. In 2005 he was elected as a Fellow of the Australian Academy of Technological Sciences and Engineering (FTSE).

Professor Siddique has published more than 200 scientific papers, review articles and book chapters. Professor Siddique is also on the Editorial Board of a number of international scientific journals. He has also trained numerous MSc and PhD students.

He has developed an extensive network of scientists within Australia and also established a diverse range of overseas (China, India, Turkey, Syria, Iraq, Iran, Saudi Arabia, Oman, Malaysia, East Timor, Nepal, Bangladesh, Pakistan, Europe, Canada, USA) collaborative research and educational projects. He holds a number of national and international committee positions, which mark an acknowledgment by the scientific community and industry of the contributions he has made.

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The views expressed in this article are those of the author and do not necessarily represent the views of, and should not be attributed to, the Pertanika Journal or the Pertanika Editorial Board.

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Review Article

Applicability of Virtopsy in Veterinary Practice: A Short Review

Abdulazeez O. Ibrahim, Abu Bakar M. Zuki and Mustapha M. Noordin*
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia
**E-mail: noordin@vet.upm.edu.my*

ABSTRACT

Virtopsy is a new and rapidly evolving non-invasive autopsy procedure involving the use of modern imaging modalities. It basically consists of three-dimensional body surface scanning by photogrammetry, multi-slice computed tomography (MSCT) and magnetic resonance imaging (MRI) for analysis and recording of autopsy lesions to ascertain cause and manner of death. This technique has been shown to be as effective and accurate as a conventional autopsy in the recent forensic studies. The MSCT is the most frequently used image modality for fractures, pathological gas formation and trauma, while the MRI is a good tool for soft tissue pathology. It is hoped that virtopsy will replace or serve as an indispensable adjunct to conventional autopsies in the future. Nonetheless, there is currently a paucity of information on the use of virtopsy in veterinary necropsy practice in which it could be used as a research tool and also possibly replace common procedures. The term 'virtopsy' in this article refers to the use of high throughput imaging techniques in human or animals as it may warrant. This review would look at the history, applications, prospects and limitations of virtopsy in veterinary necropsy.

Keywords: Computed tomography, magnetic resonance imaging, veterinary, virtopsy and 3D-surface scanning

INTRODUCTION

The term virtopsy was derived from virtual autopsy. Virtual was coined from the Latin word *virtus* which mean "useful, efficient and good". Autopsy derived its meaning from the Greek terminologies, *auto* meaning "self" and *opsomie,* "I will see". The team literarily removed the human subjectivity of auto to coin out "virtopsy" (Dirnhofer *et al*., 2006; Bolliger *et al*., 2008). Virtopsy currently means the application of non-invasive 3D-scanning techniques for documentation of body surface and internal organs of dead beings to determine the cause and manner of death. Virtopsy involves the use of modern imaging modalities, including photogrammetry, computed tomography and magnetic resonance imaging. The need to reduce individual subjectivity to post-mortem examinations and diagnoses, as well as reproducibility of lesions over time has been a worrying issue in the forensic arena for quite some time. It was first actualized in Bern University, Switzerland, with the sole objective of documenting radiograph and body surface scans with conventional autopsies. It was a very useful tool in the investigation of a high-profile homicide in Switzerland, where a gunshot bullet trajectory pattern to the skull was reconstructed for evidence in court (Dirnhofer *et al*., 2006). However, the idea of use of imaging tools in

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*Corresponding Author

forensic investigation came to bare about four decades ago when Wullenweber (1977) reported the use of computed tomography (CT) to describe the pattern of gunshot wound to the head. This was followed by the concept of objective non-invasive documentation of body surface for forensic purposes in the nineties with the advent of three-dimensional photogrammetry.

Currently, numerous medical institutes employ radiological imaging, such as whole body CT or MRI scans, to all routine autopsies as a complimentary regime prior to conventional autopsy. Meanwhile, computed tomography has been shown to be more specific and sensitive in identifying pathological gas formation post-mortem, trauma and fractures (Dirnhofer *et al*., 2006; Thali *et al*., 2007; Bolliger *et al*., 2008). This helps the coroner to focus more on lesions of interest as observed from the CT or magnetic resonance imaging (MRI) scan. It is hoped that one day, virtopsy would totally replace invasive autopsies or become an invaluable adjunct for all autopsies.

Autopsy is usually accepted in medico-legal cases as an important procedure in determining the cause and manner of death in man and animals (Shkrum & Ramsay, 2007). However, the century-old scalpel-based technique is still used especially in veterinary necropsy (Cabana, 2008). Results obtained from such necropsy can be tendered in a court of law or used as clinical data for population/herd medicine in the prevention and control of diseases, especially those of zoonotic and economic importance.

Virtopsy of animals has been largely limited to research and rarely to confirmation of clinical diagnoses (Thali *et al*., 2004; Dirnhofer *et al*., 2006; Toklu *et al*., 2006; Aghayev *et al*., 2007; Heng *et al*., 2008). Post-mortem angiography, using imaging techniques and contrast agents, has been broadly studied using dogs as one of the models. This has been proven to be effective in the diagnosis of hemorrhages, vascular rupture and ischemia (Amundsen *et al*., 1997; Jackowski *et al*., 2005; Grabherr *et al*., 2006). Lesions of death by drowning were also studied in rat models by Toklu *et al*. (2006), revealing the ability of imaging techniques to diagnose such at post-mortem, even in small-sized animals. The X-radiographic studies of the abdomen and thorax in cats and dogs revealed an increase in postmortem gas formation over time with remarkable details and appreciation of the gas pockets (Heng *et al*., 2008; Heng *et al*., 2010a, 2010b). A study on the renal adenoma in the pet fish, Red Oscar using CT, revealed the efficacy of CT in identifying the lesion as compared to necropsy (Gumpenberger *et al*., 2004).

Of recent, some universities have adopted digital photography into necropsy routines for the sole purpose of teaching and record keeping. It has become an invaluable method in the digital representation of actual cases and lesions to students. This aside there is no available literature on the use of 2D- or 3D-imaging techniques as an aid to post-mortem examinations in animals. This is a great drawback in this era of sophisticated technology, emerging and re-emerging diseases.

VIRTOPSY: TOOLS

Virtopsy basically consists of two principles revolving around three basic tools (Dirnhofer *et al*., 2006; Bolliger *et al*., 2008), namely:

a. Body volume documentation and analysis with the aid of computed tomography (CT), magnetic resonance imaging (MRI) and microradiology.
b. 3D body surface documentation using photogrammetry and optical scanning.

The Bern virtopsy team have compared post-mortem imaging diagnoses using these tools to invasive autopsy in over 200 cases and found it as effective as, or even better than conventional methods (Farkash *et al*., 2000; Dirnhofer *et al*., 2006; Thali *et al*., 2007; Bolliger *et al*., 2008). These imaging tools carry great prospects for the way veterinary necropsy is practiced today, especially on small animals. Meanwhile, time, efforts and resources can be saved with a rapid CT or MRI scan of carcasses to detect common gross lesions, such as hematoma, abscess, organ size changes, gas formation (especially

in parenchymatous organs), as well as fractures and traumatic injuries. Nevertheless, in view of the current demand, the usage of such technique will ensure an "intact" acceptable carcass prior to last respect before burial or cremation.

The most important record of the use of virtopsy in veterinary forensics was reported by Thali *et al*. (2008), where MRI and CT scans were used to remodel the bullet injury pattern in a lynx helping to determine the type of weapon used and narrowing investigations to such weapon owners.

3D Photogrammetry-based Optical Scanning

This entails the compensation for the 2D resolution of classical photography with the use two digital cameras to obtain photographs of body surface from different angles from a central projection unit. The generated pictures are then fed into a computer and with the aid of the TRITOP/ATOS III (GOM, Braunsweig, Germany), software are converted to appreciable 3D images. Meanwhile, TRITOP gives colour to picture while ATOS calculates the 3D coordinates (Dirnhofer *et al*., 2006; Thali *et al*., 2007; Bolliger *et al*., 2008). It is thus valuable in the assessment of whole body surfaces to document injuries and gross lesions with dimensional precisions. In fact, it has been widely used in computer remodelling of automobile accidents by relating surface injuries with damages on the automobile. Data generated can be analyzed by a third party and even tendered in a legal proceeding. The use of this particular tool in veterinary necropsy may not be that relevant save for requests for detailed forensic investigations on cases of animal cruelty or poaching of endangered species. Here, photographic reconstruction of lesions on body surface may give a clue to the nature of handling or capturing.

Multi-slice Computed Tomography (MSCT)

The 2D- and 3D-documentations of fractures, pathological gas formation and gross tissue trauma are better analyzed with MSCT (Dirnhofer *et al*., 2006). Most medical institutions now use it for routine pre-invasive autopsy whole body scans of cadavers (Thali *et al*., 2007; Bolliger *et al*., 2008). Computed tomography operates with the same principle of photon energy attenuation as X-ray, except that in CT, the photons are emitted in a rotating manner to give sectional slice images of tissues. It creates a gray-scale attenuation map according to the rate of absorption of photons by the body tissues. This is fed into a computer to reconstruct 3D images using mathematical algorithms (Donchin, 1994; Oliver *et al*., 1995; Douglas, 2002; Hayakawa *et al*., 2006; Thrall, 2007). Moreover, it is fast to operate and allows a quick whole body scan in 10-20 minutes. Osseous lesions, foreign bodies, pathological gas formation and organ trauma have been diagnosed with MSCT revealing more precise and accurate results over conventional autopsies (Farkash *et al*., 2000; Dirnhofer *et al*., 2006; Thali *et al*., 2007; Bolliger *et al*., 2008). Such lesions can also be easily diagnosed in veterinary necropsies with the MSCT, thereby stressing its value in this field for whole body scans. The adoption of the MSCT in veterinary post-mortem examinations, however, requires a variety of adjustable machines to accommodate the range of animal sizes and shapes since those used for imaging of cats and dogs may not be suitable for cattle and horses.

Magnetic Resonance Imaging

Body tissues and fluid contain chemicals made up of atoms. Each atom has a positively charged proton which has magnetic properties. MRI harnesses this magnetic property of protons and generates images by introducing high-powered magnetic field to the tissues to cause excitation and misalignment of protons, the magnetic field is then put off to receive signals from the tissues as the protons begin to realign to their former state. This process is called precession and electrical signals generated from this are sent via coils to computers to generate images (Oliver *et al*., 1995; Douglas, 2002; Hayakawa *et al*., 2006; Thrall, 2007). As soft tissues and body fluid contain more chemicals, and hence protons, the

MRI offers an excellent choice for soft tissue imaging ante- and post-mortem. In fact, MR was rated higher than CT in demonstrating soft tissue injury, neurological and non-neurological organ trauma and non-traumatic pathology (Thali *et al*., 2007). Soft tissue pathologies such as trauma, organ size changes, abscesses, edema, and hemorrhages may be diagnosed in veterinary necropsy using MRI.

Current Trends in Virtopsy

Limitations of the MSCT and MRI in virtopsy have been recognized and they have been studied in the effort to improve their efficiency. In particular, the lack of colour can now be tackled by using colour-encoded software to analyze images from the instruments. Meanwhile, size resolution can be improved using micro-radiology (CT and MR) to as small as micro-millimeters (Aghayev *et al*., 2006). The combination of micro-spectroscopy and micro-MR has been suggested to assess ante-mortem and post-mortem metabolite concentrations in tissues (Jackowski *et al*., 2005). This could help differentiate post-mortem from ante-mortem lesions and possibly establish time since death. Rapid advances in imaging technology may play an important role in reducing the cost of imaging instruments and procedures in the future, thereby translating into more clients of veterinarians requesting necropsy. The virtopsy teams plan to develop a robotic version for ease, called "virtobot", which entails every procedure of autopsy in a non-invasive hi-tech approach. A mobile virtobot is also proposed to cater for outstation on-the-scene investigations called "virtomobile" (Dirnhofer *et al*., 2006).

The MSCT and MRI are practically the backbone of virtopsy; it has been suggested that micro-radiology using these two techniques (micro-CT and micro-MRI) would usher in a new perspective of virtual histology, where coroners would be saved the time-consuming histological procedures. In addition, CT guided tissue sampling has also been reported to be accurate (Aghayev *et al*., 2007) and sensitive. Its use in veterinary medicine should be weighed against ultrasound guided tissue sampling for cost, handling and logistics.

Applications of Virtopsy to Veterinary Necropsy Practice

Currently, there is a gap in information on non-invasive necropsies in veterinary medical practice, though animals have been used for research in virtopsy in the areas of post-mortem angiography, post-mortem abdominal and thoracic gas formation, drowning, pulmonary embolism and other related areas (Amundsen *et al*., 1997; Thali *et al*., 2004; Jackowski *et al*., 2005; Grabherr *et al*., 2006; Aghayev *et al*., 2007; Thali *et al*., 2007; Heng *et al*, 2008; Heng *et al*., 2010a, 2010b). Literature on comparative studies, between 2D and/or 3D postmortem imaging procedure and conventional necropsies, are very rare, and most literature deal with correlating clinical radiological findings with necropsy results (Thali *et al*., 2004; Aghayev *et al*., 2007) or directly interpreting post-mortem imaging results (Heng *et al*., 2008; Heng *et al*., 2010a, 2010b). This signifies the relative slow pace of technological development in veterinary necropsy/forensics when compared with medical forensics.

Despite the expensive nature of the MRI and CT machines, some veterinary hospitals especially in the developed countries acquire them for routine clinical procedures (Marta *et al*., 2007); none however has employed them in necropsy. On the contrary, veterinary practice is unique, as a clinician requires a sound judgment for the need and use of certain procedures on his patients. A balance is usually struck between procedures to be done, clients' ability to pay and the salvage value of the animals. This factor, as well as awareness and willingness of owners, has largely limited advanced diagnostic procedures to small animals and pets. Necropsies are carried out on every case ending with death in veterinary establishments that are capable, usually on the request and/or approval of owners. Requests for large animal, reptilian and amphibian necropsies are often prompted by disease control, insurance claim or forensics.

Zoonoses are very important in veterinary practice, where all preventive measures must be rigorously followed. There is still, however, a high risk of veterinarians (specifically pathologists) contracting the zoonoses from carcasses they handle. The principal techniques of necropsy involve visual inspection, palpation and tissue incision (Cabana, 2008) that entail potential risks of disease contraction by veterinary pathologists and students. The problems posed by emerging diseases, as well as the re-emergence of those formally eradicated only stresses the dire need for a review of the ways veterinarians handle animals and dead tissues so as to reduce hazards in veterinary and medical practices. The much desired non-invasive approach presented by virtopsy as well as its superb appreciation of lesions provides a much better method for performance of necropsies. It ensures minimal contact with carcasses and its tissue fluids and blood, thereby reducing risk of infection.

The prospects of virtopsy being applied to veterinary post-mortem investigation are very high taking into cognizance the ease of the procedure, reduction in disease contraction risk, reproducibility, and possibility of third opinion and the convenient storage of data for future reference. Customs and beliefs (Mittleman, *et al*., 1992) would not be limiting factors in the advancement of virtopsy as they are to autopsy. It will also be a vibrant research tool for advancement of veterinary pathologists in an attempt to reduce the arduous nature of conventional necropsies, especially of large animals. Virtopsy is already an important compliment to autopsy and in the authors' opinion, it would be a matter of time for it to be adopted by veterinary pathologists for necropsies.

Appreciation of lesions and acceptance of results from virtopsy would definitely take some time since there is that urgent need for an in-depth research into its use and system of reporting. It is because the available information from virtopsy can be extrapolated for use in veterinary necropsy and may serve as a guide to veterinary pathologists. Ante-mortem clinical finding, imaging and necropsy results of a wide range of diseases and lesions need be compared to generate appreciable information to advance the use of these imaging technologies in veterinary necropsy.

The 3D-photogrammetry may not be readily used in veterinary virtopsy, except if strongly indicated for legal forensic cases requiring surface scanning and reconstruction of events. This may be an avenue for further research and possible application to necropsy.

Most necropsies in veterinary practice are done far and away from established institutions with standard facilities. The use of imaging tools in this scene would demand their handiness and mobility. Proposals by the Bern Virtopsy team are on the way for mobile virtopsy units (Dirnhofer *et al*., 2006).

ADVANTAGES AND DISADVANTAGES OF VIRTOPSY

Advantages

Time saving: A veterinary necropsy procedure takes a time range of thirty minutes to two hours, depending on the size of the animal, and periods are shorter for small-sized animals. This means valuable time is bound to be saved if a rapid MRI, MSCT or even 2D-Xray scan is carried out on all carcasses to pinpoint areas of pathological interests observable through these means, as discussed earlier. For example, a rapid X-ray scan of the thoracic area revealing leafing of the right lung as well as radiolucency in the thoracic cavity would suggest pneumothorax (gas not observable in conventional necropsy). The pathologists' attention would now be focused on this till further lesions provide contradicting evidence.

Non-invasiveness: Tissue destruction is an inevitable part of conventional scalpel-based necropsies which renders the old technique irreproducible and complete destruction of evidence in forensic cases. Virtopsy offers minimal or no tissue destruction as well as preservation of hard forensic evidence for future referrals such as in cases of poaching endangered species (Bolliger *et al*., 2008; Cabana, 2008).

Lesser risk of disease contraction: Risk of disease contraction is the most important drawback to conventional necropsy methods due to its invasiveness and a higher probability of accidental body injuries and contracting the examiners body parts and even mucos membranes (through spills or splashes) with contaminated fluids and secretions. Such zoonotic diseases contraction could be avoided during animal necropsies by the use of imaging techniques. This is a very vital area of concern to veterinary academic institutions for the safety of students during necropsy rounds.

Excellent storage and retrieval system: Electronic and digital storage of lesions and necropsy results is one of the main objectives of virtopsy. Such storage methods are not possible by conventional scalpel-based necropsy and therefore, data transferability is quite difficult. Results of virtopsy can be transferred electronically to another party for multiple opinions with appropriate digital representation of lesions to inform such opinions (Dirnhofer *et al*., 2006). Necropsy cases of diseases of global interests, such as emerging and re-emerging disease, may be stored and transferred internationally amongst veterinarians and policy makers to evolve epidemiological measures, particularly in incubatory stages of such diseases.

Disadvantages

Major limitations to the adoption of virtopsy to veterinary necropsy practice involve cost and technical matters.

Cost

Magnetic resonance imaging and CT machines are very expensive even for veterinary clinical use, where the justifications for their purchase would normally be well thought of based on economics, service quality and research output. This lone factor is the most important limitation to their use of in veterinary necropsy since necropsies do not contribute much to the income of most veterinary institutions. If put in place for necropsies, the next question is how much does a client need to pay?

Technical Limitations

Despite the overwhelming positive values of non-invasive autopsy, there are still technical areas regarding appreciation and interpretation of lesions that need to be researched upon and answered even in the human field. Lesions obtained from such image results may be ambiguous, specifically whenno database is available to support such. Monochromatism (Patowary, 2005) is a single technical hitch that may render the objectives of virtopsy unobtainable as MSCT and MRI records images in the shade of grey, though research is on the way in the areas of colour rendering of images for better appreciation.

Furthermore, minute lesions, such as sporadic or multiple petechial hemorrhages on adipose tissues as well as pustules or minute necrotic foci on organs, such as the liver or kidney, may not be appreciated by either MSCT or MRI. However, more research into virtual histology may alleviate such trouble, particularly if it is buttressed with the colour encoding technique.

The hazard of personnel and environmental exposure to radiation (Thrall, 2007) is there, specifically for veterinary establishments that own and employ X-radiography in necropsy. This is so true, owing to the fact that all necropsy rooms are practically not built with radiation protection in mind.

CONCLUSION

Virtopsy is becoming an indispensable adjunct to autopsy/necropsy with the potentials of replacing the invasive procedure in the future. It holds a bright prospect for applications in veterinary pathology. Though it is expensive to run and faced with technical limitations of colour appreciation, lesion timing and radiological hazards, it reduces the risk of disease contraction by veterinary pathologists and students. Moreover, it is easy to use, preserves valuable organ lesions since it is non-invasive, saves valuable time and efforts and gives the possibility of obtaining a second and third opinion on cases. It is also easily archived

and retrieved. In order to prove its usefulness in veterinary practice, intensive research into different case scenarios for a wide range of species and lesions are therefore required. Only time and research hinder its complete adoption in veterinary necropsy.

REFERENCES

Aghayev, E., Sonnenschein, M., Jackowski, C., Thali, M., Buck, U., Yen, K., Bolliger, S., Dirnhofer, R., & Vock, P. (2006). Postmortem Radiology of Fatal Hemorrhage: Measurements of Cross-Sectional Areas of Major Blood Vessels and Volumes of Aorta and Spleen on MDCT and Volumes of Heart Chambers on MRI. *American Journal of Roentgenology, 187*(1), 209-215.

Aghayev, E., Thali, M. J., Sonnenschein, M., Jackowski, C., Dirnhofer, R., & Vock, P. (2007). Post-mortem tissue sampling using computed tomography guidance. *Forensic Science International, 166*, 199-203.

Albert, L. B. (2008). *Encyclopedia of diagnostic imaging*. New York: Springer.

Amundsen, T., Kvaerness, J., Jones, R. A., Waage, A., Bjermer, L., Nilsen, G., & Haraldseth, O. (1997). Pulmonary embolism: detection with MR perfusion imaging of lung--a feasibility study. *Radiology, 203*(1), 181-185.

Bolliger, S. A., Thali, M. J., Ross, S., Buck, U., Naether, S., & Vock, P. (2008). Virtual autopsy using imaging: bridging radiologic and forensic sciences. A review of the virtopsy and similar projects. *European Radiology, 18*, 273-282.

Brogdon B. G. (1998). *Forensic radiology*. United States of America: CRC Press.

Cabana, J. E., & Cooper, M. E. (2008). *Veterinary necropsy procedures* (p. 22-25). Philippines: CLSU Alumni Association Inc.

Cooper, J. E., & Cooper, M. E. (2008). Forensic veterinary medicine: a rapidly evolving discipline. *Forensic Science Medical Pathology, 4*, 75-82.

Dirnhofer, R., Jackowski, C., Vock, P., Potter, K., & Thali, M. J. (2006). VIRTOPSY: Minimally invasive, imaging-guided virtual autopsy. *Radiographics, 26*, 1305-1333.

Donchin, Y., Rivkind, A. I., Bar-Ziv, J., Hiss, J., Almog, J., & Drescher, M. (1994). Utility of Postmortem Computed Tomography in Trauma Victims. *The Journal of Trauma, 37*(4), 552-556.

Douglas, P. B. (2002). *Radiology source book*. New Jersey: Humana Press.

Farkash, U., Scope, A., Lynn, M., Kugel, C., Maor, R., Abargel, A., & Eldad, A. (2000). Preliminary Experience with Postmortem Computed Tomography in Military Penetrating Trauma. *The Journal of Trauma, 48*(2), 303-309.

Grabherr, S., Djonov, V., Friess, A., Thali, M. J., Ranner, G., Vock, P., & Dirnhofer, R. (2006). Postmortem Angiography After Vascular Perfusion with Diesel Oil and a Lipophilic Contrast Agent. *American Journal of Roentgenology, 187*(5), W515-W523.

Gumpenberger, M., Hochwartner, O., & Loupal, G. (2004). Diagnostic imaging of a renal adenoma in a red oscar (*Astronotus ocellatus* Cuvier, 1829). *Veterinary Radiology and Ultrasound, 45*, 139-142.

Hayakawa, M., Yamamoto, S., Motani, H., Yajima, D., Sato, Y., & Iwase, H. (2006). Does imaging technology overcome problems of conventional postmortem examination? A trial of computed tomography imaging for postmortem examination. *International Journal of Legal Medicine*, *120*, 24-26.

Heng, H. G., Teoh, W. T., & Sheikh-Omar, A. R. (2008). Postmortem abdominal radiographic findings in feline cadavers. *Veterinary Radiology and Ultrasound, 49*, 26-29.

Heng, H. G., Selvarajah, G. T., Lim, H. T., Ong, J.S., Lim, J., & Ooi, J. T. (2009a). Serial postmortem thoracic radiographic findings in canine cadavers. *Forensic Science International, 188*, 119-124.

Heng, H. G., Selvarajah, G. T., Lim, H. T., Ong, J. S., Lim, J., & Ooi, J. T. (2009b). Serial postmortem abdominal radiographic findings in canine cadavers. *Forensic Science International, 192,* 43-47.

Jackowski, C., Sonnenschein, M., Thali, M. J., Aghayev, E., von Allmen, G., Yen, K., Dirnhofer, R., & Vock, P. (2005). Virtopsy: postmortem minimally invasive angiography using cross section techniques-implementation and

preliminary results. *Journal of Forensic Science, 50*, 1175-1186.

Kleinman, P. K. (1990). *Diagnostic imaging of child abuse*. London, England: Mosby.

Marta, S., Jose, M., Rafael, L., Eliseo, B., Maria, J. R., & Amalia, A. (2007). Ultrasonographic, computed tomographic and magnetic resonance imaging anatomy of the normal canine stifle joint. *The Veterinary Journal*, *174*, 351-361.

Mittleman, R. E., Davis, J. H., Kaszil, W., & Graves, W. M. (1992). Practical approach to investigative ethics and religious objections to the autopsy. Journal of Forensic Science, 37(3), 824-829.

Oliver, W. R., Chancellor, A. S., Soltys, M., Symon, J., Cullip, T., Rosenman, J., Hellman, R., Boxwala, A., Gormley, W. (1995). Three-dimensional reconstruction of a bullet path: validation by computed radiography. *Journal of Forensic Science, 40*, 321-324.

Patowary, A. J. (2005). Virtopsy: One step forward in the field of forensic medicine- A review. *Journal of Indian Forensic Medicine, 30,* 32-36.

Pinheiro, J. (2006). *Introduction to Forensic Medicine and Pathology*. In Schmitt, A., Cunha, E. and Pinheiro, J. (Eds.), Forensic Anthropology and Medicine (p. 13-37). New Jersey: Humana Press.

Schumacher, M., Oehmichen, M., König, H. G., & Einighammer, H. (1983). Intravital and postmortal CT examinations in cerebral gunshot injuries [in German]. *Fortschr Röntgenstr, 139*(07), 58,62.

Shkrum, M. J., & Ramsay, D.A. (2007). *Forensic Pathology of Trauma: Common Problems for the Pathologist*. New Jersey: Humana Press.

Swift, B., & Rutty, G. N. (2004). Recent advances in postmortem forensic radiology. *Forensic Pathology Reviews, 4*, 355-404.

Thali, M. J., Dirnhofer, R., Becker, R., Oliver, W., & Potter, K. (2004). Is ‘virtual histology’ the next step after ‘virtual autopsy’? Magnetic resonance microscopy in forensic medicine. *Magnetic Resonance Imaging, 22*, 1131-1138.

Thali, M. J., Jackowski, C., Oesterhelweg, L., Ross, S. G., & Dirnhofer, R. (2007). VIRTOPSY - The Swiss virtual autopsy approach. *Legal Medicine, 9*, 100-104.

Thali, M. J., Kneubuehl, B. P., Bolliger, S. A., Christe, A., Koenigsdorfer, U., Ozdoba, C., Spielvogel, E., & Dirnhofer, R. (2007). Forensic veterinary radiology: Ballistic-radiological 3D computertomographic reconstruction of an illegal lynx shooting in Switzerland. *Forensic Science International, 171*(1), 63-66.

Thrall, D. E. (2007). *Textbook of Veterinary Diagnostic Radiology*. United States of America: Saunders Elsevier.

Toklu, A. S., Alkan, N., Gürel, A., Cimsit, M., Haktanır, D., Körpınar, S., & Purisa, S. (2006). Comparison of pulmonary autopsy findings of the rats drowned at surface and 50 ft depth. *Forensic Science International, 164*(2-3), 122-125.

Wullenweber, R., Schneider, V., & Grumme, T. (1977). A computed-tomographical examination of cranial bullet wounds (in German). *Zeitschrift fur Rechtsmedizin, 80*, 227-246.

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Short Communications

Comparison of Anthocyanin and Phenolic Contents between Tuber and Callus of *Ipomoea batatas* (L.)

Seow Ying Chen[1], Wai Sum Yap[1] and Wilson Thau Lym Yong[2*]
[1]*Biotechnology Department,*
Faculty of Applied Sciences, UCSI University,
No. 1, Jalan Menara Gading, UCSI Heights,
56000 Kuala Lumpur, Malaysia
[2]*Biotechnology Research Institute, Universiti Malaysia Sabah,*
Locked Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia
**E-mail: wilsonyg@ums.edu.my*

ABSTRACT

This study was aimed to investigate and compare the anthocyanin and phenolic contents between the tuber and callus of *Ipomoea batatas* (L.). Callus induction was performed *in vitro* using the Murashige and Skoog media supplemented with 0.5 mg/L 2,4-D and 0.1 mg/L kinetin. Meanwhile, the anthocyanin content was measured by pH differential method using cyanidin-3-glucoside as a standard. The total phenolic content was measured using the Folin-Ciocalteu method with gallic acid as a standard. Based on the results obtained, the anthocyanin content of the tuber *I. batatas* was 1.04 ± 0.12 mg cyanidin-3-glucoside/g fresh weight, while the anthocyanin content of the callus was 0.50 ± 0.07 mg cyanidin-3-glucoside/g fresh weight. The total phenolic content of the tuber *I. batatas* was 0.46 ± 0.01 mg gallic acid/g fresh weight and the phenolic content of the callus was 0.20 ± 0.01 mg gallic acid/g fresh weight. Both the anthocyanin and phenolic contents of the tuber were found to be higher than those of the callus of *I. batatas*.

Keywords: ***Ipomoea batatas* (L.), tuber culture, callus, determination of anthocyanin, phenolic contents**

LIST OF ABBREVIATIONS

MS : Murashige and Skoog
2,4-D : 2,4-dichlorophenoxy acetic acid

INTRODUCTION

Plant tissue culture is a process where cells, tissues or organs of a plant species are isolated, surface sterilized and cultured in an aseptic environment (Rout *et al*., 2006). Tissue culture techniques are capable to be used in minimizing the time needed for propagation of new plantlets and to increase the availability of plants with improved horticultural characteristics. Meanwhile, plant tissue cultures can be used for mutant selection, gene transfer, artificial seed and secondary metabolite production. Callus is undifferentiated cell that emerges from structural tissues in response to the wounding effect. Besides, callus induction and physical disorganization of cells happen when there is a breakdown of intercellular physical and chemical communication. Therefore, callus can be induced by changing the composition

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*Corresponding Author

of media and the concentration of plant growth regulators (Dennis & Sreejesh, 2004).

Ipomoea batatas (L.) belongs to the family *Convolvulaceae* and it is widely grown in the tropical, sub-tropical and warm temperature regions. Jayasinghe *et al*. (2003) reported that *I. batatas* was able to sustain the populations during time of crisis even after the Second World War in Japan, the earthquake in Northern Luzon and the civil disturbances in Rwanda. *I. batatas* is also rich in dietary fibre, minerals, vitamins, and antioxidants, such as phenolic acids, anthocyanin, tocopherol and β-carotene. In addition, *I. batatas* contains β-carotene and vitamin C and these two components are able to eliminate free radicals from the human body. The tuber of this plant is also recognized as an anti-inflammatory food because it can reduce the severity of asthma, rheumatoid arthritis and osteoarthritis. Besides, anthocyanin, calcium and dietary fibre of sweet potatoes can reduce cardiovascular diseases (Huang *et al*., 2007).

Secondary metabolites are compounds produced by organisms which are not directly needed for the cell's survival but required for the plant's survival in the ecosystem. Plants produce secondary metabolites for protection against bacteria, fungi and viruses (Kliebenstein *et al*., 2005). Anthocyanin was known to be non-toxic and non-mutagenic. Previous research revealed that anthocyanin exhibited anti-inflammatory, anti-proliferative, vasoprotective and hepatoprotective activities (Wang *et al*., 2000; Lazzé *et al*., 2003). Phenolic compounds are able to protect cells, food and organs from oxidative degeneration. In human diets, phenolic compounds can prevent diseases, such as cancer, cardiovascular and neurodegenerative diseases (Manach *et al*., 2004). This study was aimed at comparing the anthoycanin and phenolic contents in both the tuber and the callus of *I. batatas*, in order to determine if *in vitro* culture would result in a reduction of the total phenolics and anthocyanins.

MATERIALS AND METHODS

In vitro Callus Induction

Purple sweet potato, *Ipomoea batatas* was obtained from the central market of Kepong, Selangor. The tuber of *I. batatas* was cut into 1 cm × 1 cm × 2 mm size and used as explant materials. The surface-sterilized explants were cultured on the MS medium (Murashige & Skoog, 1962) supplemented with 0.5 mg/L 2,4-D and 0.1 mg/L kinetin for callus induction.

Anthocyanin Content

For the extraction of anthocyanin, approximately 2 g of homogenized tuber *I. batatas* and 2 g of callus were separately put into 20 mL of methanol, 1% (v/v) hydrochloric acid for 1 hour in dark condition under room temperature. The extracts formed were filtered through Whatman No.1 filter paper. About 1 mL of the extract was used for anthocyanin determination. In addition, the pH differential method was used to measure the anthocyanin content in which the extract was diluted in buffers at pH 1.0 (0.025 M potassium chloride buffer) and at pH 4.5 (0.4 M sodium acetate buffer). The absorbance of the extract was then measured at 510 nm and 700 nm. The absorbance of the extract was calculated using the formula stated below:

$$A = (A510 - A700)_{pH\ 1.0} - (A510 - A700)_{pH\ 4.5}$$

The total anthocyanin content (TA) was calculated as follows:

$$TA = (A \times MW \times DF \times 1000)/(\varepsilon \times 1)$$

The result was calculated as milligram of cyanidin-3-glucoside per gram of fresh weight using a molar absorptivity (ε) of 26,900 and a molecular weight (MW) of 449.2, where DF is the dilution factor.

Total Phenolic Content

For the total phenolic content, about 10 g of the homogenized tuber *I. batatas* and 10 g of

the callus were separately put into 100 mL of methanol for 1 hour, followed by filtration through Whatman No. 1 filter paper. The Folin-Ciocalteu method (Vasco *et al*., 2008) was used to measure the total phenolic content with a standard curve prepared using gallic acid at the concentrations of 20, 40, 60, 80 and 100 mg/L. Meanwhile, a reagent blank was prepared using distilled water. About 1 mL of the extract and the standard solutions were added to a beaker containing 9 mL of distilled water. Approximately 1 mL of Folin-Ciocalteu reagent was added to the extract and standard solutions. After 5 minutes, 10 mL of 7% Na_2CO_3 solution was added and mixed. The solution was then immediately diluted to 25 mL with distilled water and mixed thoroughly. The mixtures were incubated for 90 minutes at 23ºC and finally the absorbance was measured at 750 nm. The result was expressed as milligram of gallic acid per gram of fresh weight.

RESULTS AND DISCUSSION

Anthocyanin Content

The absorbance readings of the anthocyanin content of tuber and callus of *Ipomoea batatas* were repeated three times, while the average readings were taken at the wavelength of 510 nm and 700 nm (see Table 1). Based on the pH differential method (Hosseinian *et al*., 2008), the anthocyanin content for the tuber of *I. batatas* was 1.04 ± 0.12 mg cyanidin-3-glucoside/g fresh weight and the anthocyanin content for the callus of *I. batatas* was 0.50 ± 0.07 mg cyanidin-3-glucoside/g fresh weight. The anthocyanin content of the tuber was found to be higher than the callus of *I. batatas*. One of the reasons for the decline in the content of anthocyanins could be the limitations of the physical conditions adopted *in vitro*, as reported by Meyer *et al*. (2002) for the culture of oheloberry (*Vaccinium pahalae*). However, the results obtained contradict with the previous findings in terms of the ranges of higher plant species including wild carrot (*Daucus carota*), parsley (*Petroselinum crispum*) and parsnip (*Pastinaca sativa*), as reviewed by Bourgaud *et al*. (2001) and Matkowski (2008). They reported that the application of tissue culture approach had significantly enhanced the metabolites production of the plant.

Meanwhile, anthocyanin contributes to the purple colour of the *I. batatas* (Borbalan *et al*., 2003). The absorbance readings of the tuber and callus of *I. batatas* were measured at 510 nm, based on the molar absorption coefficient of the cyanidin-3-glucoside. A previous report showed that the content of anthocyanin in sweet potato clones ranged from 0.017 to 0.531 mg/g fresh weight (Teow *et al*., 2007). At low pH values, however, anthocyanin is more

TABLE 1
Absorbance reading for the anthocyanin contents of the tuber and callus of *Ipomoea batatas* (L.)

Purple Sweet Potato	pH Value	Wavelength (nm)	Absorbance (nm)
Tuber	pH 1.0	510	0.514 ± 0.004
		700	0.123 ± 0.003
	pH 4.5	510	0.484 ± 0.006
		700	0.118 ± 0.006
Callus	pH 1.0	510	0.128 ± 0.002
		700	0.044 ± 0.004
	pH 4.5	510	0.123 ± 0.012
		700	0.051 ± 0.004

stable and highly coloured while at higher pH values, anthocyanin gradually loses its colour and becomes colourless between pH 4.0 and 5.0 (Ferreira *et al*., 2007). The pH differential method is used based on the structural change of the anthocyanin chromophores between pH 1.0 and 4.5. In this method, the difference in the absorbance between the potassium chloride buffer and sodium acetate buffer is caused by the monomeric anthocyanin pigments. Meanwhile, monomeric anthocyanin has little or no absorbance in pH 4.5 buffer, whereas polymeric anthocyanin does not exhibit colour changes with pH (Lee *et al*., 2005).

Total Phenolic Content

The standard curve for gallic acid was plotted (see *Fig. 1*), while the average absorbance readings of the total phenolic contents of tuber and callus of *I. batatas* were taken at the wavelength of 750 nm (Table 2). Based on the standard curve, the total phenolic content of the tuber of *I. batatas* was 0.46 ± 0.01 mg gallic acid/g fresh weight and the phenolic content for callus was 0.20 ± 0.01 mg gallic acid/g fresh weight. The total phenolic content for the tuber of *I. batatas* was higher than the callus culture and the difference was about 0.26 mg. Once again, the results obtained contradict with the previous findings by Bourgaud *et al*. (2001) and Matkowski (2008) on various plant species, as discussed above.

TABLE 2
Absorbance reading for the total phenolic contents of the tuber and callus of *Ipomoea batatas* (L.)

Purple Sweet Potato	Absorbance (750nm)
Tuber	0.255 ± 0.006
Callus	0.113 ± 0.007

Phenolic compounds are located in different parts of plant's tissues and cells, such as vacuoles, cell walls and cell nuclei. In particular, phenolics also protect plants from biotic stresses, such as fungi and parasitic plant invasion. Genetic factors and growing conditions might play an important role in the formation of secondary metabolites in plants, such as anthocyanin and

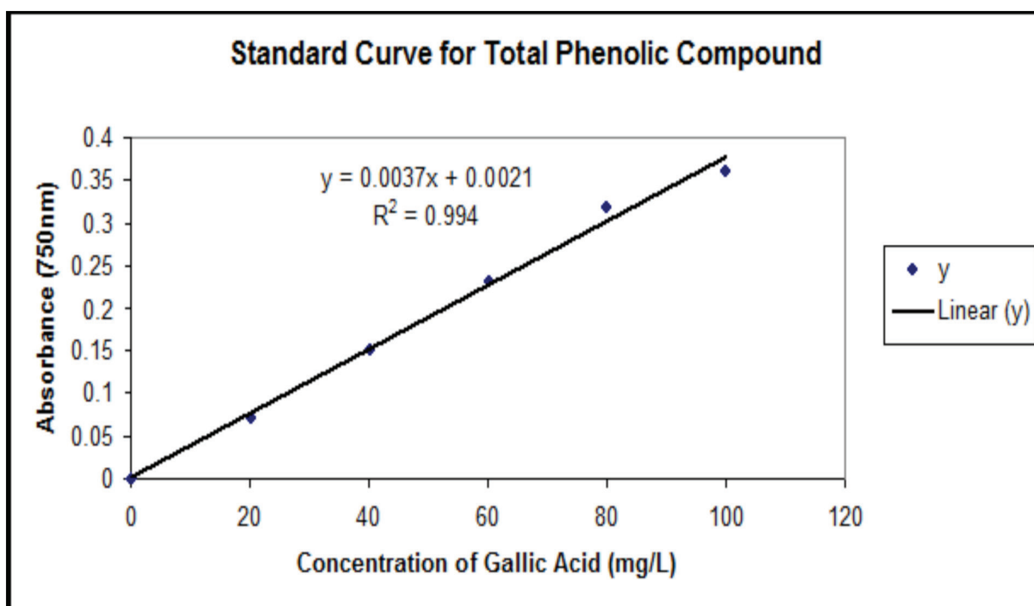

Fig. 1: Standard curve for the total phenolic compound

phenolic compounds. However, the results of the total phenolic content obtained might be affected by other non-phenolic compounds such as ascorbic acid and reducing sugar (Escarpa & Gonzalez, 2001). In the Folin-Ciocalteu method, methanol, acetone, ethanol and boiling water could be used to extract phenolic compounds from plants. However, methanol and acetone were found to be more effective than water for the extraction of phenolic compounds from plants (Yao *et al*., 2004; Zhou & Yu, 2004). Reyes *et al*. (2004) pointed out that the weight of the tuber sweet potato increased with the decrease of its total phenolic content. Meanwhile, fresh cut in the sweet potato would result in an increase in the phenolic compounds and those phenolic compounds would act as a defence against pathogen after the tissue damage (Báidez *et al*., 2006). The total phenolic content varied among different sweet potato cultivars and these differences ranged from 192.7 to 1159.0 mg gallic acid equivalent/100 g dry sample between the cultivars grown in the Philippines (Rumbaoa *et al*., 2009).

CONCLUSION

The anthocyanin content of the tuber of *Ipomoea batatas* was 1.04 ± 0.12 mg/g fresh weight, while the anthocyanin content of the callus was 0.50 ± 0.07 mg/g fresh weight. The total phenolic content of the tuber was 0.46 ± 0.01 mg/g fresh weight and this was 0.20 ± 0.01 mg/g fresh weight for the callus. The results indicated that *in vitro* conditions could have significant impacts on the production of phenolics and anthocyanins. This could be attributed to the interaction of environmental parameters under field conditions, which eventually influenced and induced important metabolic pathways. Although both the anthocyanin and total phenolic contents of the tuber were higher compared to the callus of the *I. batatas*, *in vitro* culture and mass propagation of *I. batatas* continued to serve as an alternative source of secondary metabolite production.

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REFERENCES

Alonso Borbalán, Á. M., Zorro, L., Guillén, D. A., & García Barroso, C. (2003). Study of the polyphenol content of red and white grape varieties by liquid chromatography–mass spectrometry and its relationship to antioxidant power. *Journal of Chromatography A, 1012*(1), 31-38.

Báidez, A. G., Gómez, P., Del Río, J. A., & Ortuño, A. (2006) . Antifungal capacity of major phenolic compounds of Olea europaea L. against Phytophthora megasperma Drechsler and Cylindrocarpon destructans (Zinssm.) Scholten. *Physiological and Molecular Plant Pathology, 69*(4-6), 224-229.

Bourgaud, F., Gravot, A., Milesi, S., & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Science, 161*(5), 839-851.

Dennis Thomas, T., & Sreejesh, K. R. (2004). Callus induction and plant regeneration from cotyledonary explants of ash gourd (Benincasa hispida L.). *Scientia Horticulturae, 100*(1-4), 359-367.

Escarpa, A., & González, M. C. (2001). Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Analytica Chimica Acta, 427*(1), 119-127.

Ferreira Ozela, E., Stringheta, P. C., & Cano Chauca, M. (2007). Stability of anthocyanin in spinach vine (Basella rubra) fruits. *Ciencia e investigación agraria, 34*, 115-120.

Hosseinian, F. S., Li, W., & Beta, T. (2008). Measurement of anthocyanins and other phytochemicals in purple wheat. *Food Chemistry, 109*(4), 916-924.

Huang, Z., Wang, B., Eaves, D. H., Shikany, J. M., & Pace, R. D. (2007). Phenolic compound profile of selected vegetables frequently consumed by African Americans in the southeast United States. *Food Chemistry, 103*(4), 1395-1402.

Jayasinghe, U., Setiawan, A., Kapukha, P., Piggin, C., & Palmer, B. (2003). Performance of some CIP sweet potato clones under East Timorese conditions. *In:* da Costa, H., Piggin, C., da Cruz, C.J., Fox, J.J. (Eds.), *Agriculture: New directions for a new nation East Timor (Timor-Leste)*. ACIAR Proceedings No.113, pp 84-89.

Kliebenstein, D. J., Rowe, H. C., Denby, K. J. (2005). Secondary metabolites influence *Arabidopsis*/*Botrytis* interactions: variation in host production and pathogen sensitivity. *Plant J., 44*, 25-36.

Lazzé, M. C., Pizzala, R., Savio, M., Stivala, L. A., Prosperi, E., & Bianchi, L. (2003). Anthocyanins protect against DNA damage induced by tert-butyl-hydroperoxide in rat smooth muscle and hepatoma cells. *Mutation Research*, *535*(1), 103-115.

Lee, J., Durst, R. W., & Wrolstad, R. E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *Journal of AOAC International*, *88*(5), 1269-1278.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: food sources and bioavailability. *Am J Clin Nutr, 79*(5), 727-747.

Matkowski, A. (2008). Plant *in vitro* culture for the production of antioxidants – a review. *Biotechnol. Adv*. *26*, 548-560.

Meyer, J. E., Pépin, M. F., & Smith, M. A. L. (2002). Anthocyanin production from Vaccinium pahalae: limitations of the physical microenvironment. *Journal of Biotechnology, 93*(1), 45-57.

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum, 15*(3), 473-497.

Reyes, L., Miller, J., & Cisneros-Zevallos, L. (2004). Environmental conditions influence the content and yield of anthocyanins and total phenolics in purple- and red-flesh potatoes during tuber development. *American Journal of Potato Research, 81*(3), 187-193.

Rout, G. R., Mohapatra, A., & Jain, S. M. Tissue culture of ornamental pot plant: A critical review on present scenario and future prospects. *Biotechnology Advances, 24*(6), 531-560.

Rumbaoa, R. G. O., Cornago, D. F., & Geronimo, I. M. (2009). Phenolic content and antioxidant capacity of Philippine potato (Solanum tuberosum) tubers. *Journal of Food Composition and Analysis, 22*(6), 546-550.

Teow, C. C., Truong, V.-D., McFeeters, R. F., Thompson, R. L., Pecota, K. V., & Yencho, G. C. (2007). Antioxidant activities, phenolic and β-carotene contents of sweet potato genotypes with varying flesh colours. *Food Chemistry, 103*(3), 829-838.

Vasco, C., Ruales, J., & Kamal-Eldin, A. (2008). Total phenolic compounds and antioxidant capacities of major fruits from Ecuador. *Food Chemistry, 111*(4), 816-823.

Wang, C.-J., Wang, J.-M., Lin, W.-L., Chu, C.-Y., Chou, F.-P., & Tseng, T.-H. (2000). Protective effect of Hibiscus anthocyanins against tert-butyl hydroperoxide-induced hepatic toxicity in rats. *Food and Chemical Toxicology, 38*(5), 411-416.

Yao, L., Jiang, Y., Datta, N., Singanusong, R., Liu, X., Duan, J., Raymont, K., Lisle, A., & Xu, Y. (2004). HPLC analyses of flavonols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. *Food Chemistry, 84*(2), 253-263.

Zhou, K., & Yu, L. (2004). Effects of extraction solvent on wheat bran antioxidant activity estimation. *LWT - Food Science and Technology, 37*(7), 717-721.

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Short Communications

Fish Biodiversity Survey (2009) of Streams in the Ayer Hitam Forest Reserve, Puchong, Selangor

Yu Abit, L., I. S. Kamaruddin*, Z. Mohd-Rozhan, M. Y. Ina-Salwany and A. S. Mustafa- Kamal.
Department of Aquaculture,
Faculty of Agriculture, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia
**E-mail: hmelintang@hotmail.com*

ABSTRACT

A study was carried out to determine the different fish species inhabiting the streams of Ayer Hitam Forest Reserve (AHFR) in Puchong, Selangor. The study was carried for a period of days (from 13 to 16 April 2009) during the Scientific Expedition of AHFR, Puchong, organized by the Faculty of Forestry, Universiti Putra Malaysia (UPM). The samples were collected using a variety of methods at three stations that were designated as Station A, Station B and Station C within the AHFR riverine system. These samples were then preserved in 90% ethanol solution and sent to the laboratory for identification. From the sampling, eleven indigenous fish species (namely, *Puntius binotatus, Luciocephalus pulcher, Clupeithys sp., Rasbora einthoveni, Hemiramphodon pogognathus, Rasbora heteromorpha, Sphaerichtys osphronemoides, Rasbora sumatrana, Beta pugnax, Glossogobous giuris and Clarias macrocephalus*) were identified to inhabit the AHFR riverine system.

Keywords: Fish biodiversity, Stream, Ayer Hitam Forest Reserve, Puchong

INTRODUCTION

The freshwaters of Peninsular Malaysia can be broadly categorised into 2 groups of environment (Mohsin & Ambak, 1983). The two environments are lentic water bodies which are essentially standing water bodies, such as lakes and reservoirs, whereas the second freshwater environment comprises of lotic water bodies which consist of flowing water bodies such as rivers and streams. Both rivers and streams can subsequently be subdivided into upper, middle and lower stream sections.

The river system found in the Ayer Hitam Forest Reserve (henceforth designated as AHFR), Puchong, can be classified as an upstream section, where the gradients are very steep, with fast flowing water and it consists of a network of interconnecting rapids and waterfalls. These characteristics of the aquatic environment often result in a limited species diversity of fish and only species that can adept sufficiently to these river conditions can survive. In large tropical rivers, sections of rapids often support specialized fishes, such as tiny blind catfishes in the Amazon River (Lundberg, 2001). In high-gradient headwater streams, among typical fishes are streamlined cyprinids (Africa and Asia), highly specialized hillstream fishes (Balitoridae; Asia), and specialized catfishes (South America) (Moyle & Cech, 2004). Fish communities

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*Corresponding Author

in small rivers are actually a reflection of the environment they inhabit, while suitable environments influence the distribution and abundance of different species. Thus, the species is considered as the indicator of the habitat or ecosystem (Hellawel, 1986). The gradient and order of streams factors are usually and strongly interrelated in their effects on fish distribution, and they seem to have the same general effects on the tropical as they do on temperate fishes (Moyle & Cech, 2004).

In the recent years, the areas comprising AHFR have been shrinking, and this is mainly due to regazetting of land for development purposes. This forest reserve has become an island in a sea of urban development. According to Awang Noor *et al*. (2007), the Forestry Department has recorded that the area comprising AHFR has decreased by some 70.4 % from 1965 to 1997, i.e. from 4266.23 ha to 1262.33 ha. Currently, only 1248 ha remain in this forest reserve, comprising six compartments, compartments 1, 2, 12, 13, 14 and 15. In terms of its river system, the AHFR is dissected by two major rivers, i.e. Rasau River on the southern half and Bohol River on the northern side (Ahmad *et al*., 2007). Among the development projects that have been completed in the vicinity include an agriculture project, a world-class sports complex, a multi-million dollar housing project, an incineration plant and waste disposal area, and an equine park (Awang *et al*., 2007). These situations are likely to have affected the fish stock and the fish population inhabiting the AHFR. In addition, over-exploitation and habitat degradation not only deplete wildstock, but also reduce the replacement rate in the population (Khan *et al*., 1996).

Detailed studies on fish species inhabiting the lotic system of this area are still severely lacking. Thus, this study was conducted to determine and identify the different fish species constituting the fish populations within the riverine system of the AHFR. The findings of this study are expected to benefit the planning and management of sustainable fisheries and conservation of the natural resources in this area. As such, the objective of the study was to determine as many fish species and the number of species of fish inhabiting the riverine system in the AHFR Puchong, Selangor within the study period.

MATERIALS AND METHODS

This study site is located in the AHFR Puchong, Selangor, which is a secondary disturbed forest, as it has been logged a few times since the 1930s. The altitudes of the AHFR range between 15 and 233 m above sea level, while the slopes of the forest range between 10 to 20 %. The forest is a production forest categorized as a Lowland Dipterocarp Forest (Awang *et al*., 2007). The AHFR is under the management of the Central Selangor District Forest Office and the Selangor state government leased the forest to Universiti Putra Malaysia (UPM) for 80 years through a memorandum of understanding (MoU) signed in 1996, with the purpose of being a research and educational forest. With an area of 1248 ha, the forest also provides recreation and eco-tourism activities for urban dwellers.

The main data for this study consist of the type of fish species and the number of fish species inhabiting streams in the AHFR. The study was conducted for four days, i.e. from 13 to 16 April 2009, during the Scientific Expedition of the AHFR Puchong, organized by the Faculty of Forestry, UPM.

The sampling stations were located in a section of the AHFR stream/river in compartments 13, 14 and 15. Fish were sampled at three stations that were designated as Station A, Station B and Station C. Station A is located in the rapids in compartment 13, whereas Station B is in compartment 14, i.e. at the pools near a waterfall and Station C is at a small tributary of the river in compartment 15. The sampling gear used included gill nets, cast nets and baited line with hooks. The mesh size of the nets ranged between 0.5 to 2.0 inches. The gill nets were set at dusk, maintained in the position of the stream water and hauled in the next morning. Meanwhile, the cast nets and baited line with hooks were used at each station during the day time. Fish caught were photographed and

preserved in 90% ethanol for further observation. All the fish caught were identified for their species data using standard taxonomic keys, according to Mohsin and Ambak (1983) and Hua (2002).

RESULTS AND DISCUSSION

The AHFR in Puchong is rich in flora and fauna, and it is also home to 10 species of reptiles, 18 species of amphibians and 10 species of fish (Ahmad *et al*., 2007). Norini and Ahmad (2007) listed the fish species of economic importance in the AHFR in 2004, as given in Table 1.

TABLE 1
The fish species that are valuable to the Temuan Ethnic group in AHFR

Type of fish	Species
Ikan tengalan	*Puntius Bulu*
Hampala barb	*Hampala macrolepidota*
Giant snakehead	*Ophicephalus laevis*
Ikan belisik	*Rasbora sumatrana*
Broadhead catfish	*Clarias macrocephalus*
Spotted barb	*Puntius binotatus*
Black snakehead	*Channa melasoma*

Although some previous studies have attempted to list the indigenous fish species found in AHFR, some of the species that were found in those studies differ from the ones found in this study. In this study, eleven species of fish from seven families were recorded during the sampling period. All the eleven species that were recorded are indigenous to Malaysia. The species that were caught in the present study are as shown in Table 2.

In their study, Mohsin and Ambak (1983) caught specimens of *Sphaerichthys osphronemoides* (biji durian) from a clear and flowing stream in Puchong. This chocolate brown to faint red brown colour fish is a beautiful aquarium fish and very attractive to aquarists. This study found that it was difficult to detect this particular species due to its exceptional camouflage ability which closely resembles its surroundings of fallen leaves. Many fish that live close to the substrate, or among floating or rooted plants, to some degree, are camouflaged by the similarity in the colour between them and their background (Keenleyside, 1979).

It is interesting to note that Mohsin and Ambak (1983) mentioned that *Glossogobius giurisis* is not a common fish species in Malaysia, whereby in their study, they managed to capture just one specimen of this particular species from a small stream in Puchong. In this study, the researchers managed to capture one specimen of *G. giurisis* under a rock in the rapids of Station A which is located in compartment 13. Keenleyside (1979) mentioned that the most common hiding species are small, benthic forms such as many of the gobies (Gobiidae). For instance, some gobies occupy burrows excavated by shrimps (Karplus *et al*., 1972). The capture of this particular specimen is indicative that this species is still available in the streams of the AHFR although its population density is unknown.

The fish species in the streams also specialize in their feeding habits and depend on the food availability in the streams of which they inhabit. Mohsin and Ambak (1983) noted that the stomach contents for cyprinids, such as *Rasbora einthoveni, Rasbora heteromorpha* and *Rasbora sumatrana,* consist mostly insects or parts of insects. Most of the time, the food source in upstream section is from allocthonous sources. In the headwater streams, most fishes feed on either terrestrial invertebrates or detritus (Moyle & Cech, 2004).

Meanwhile, *Hemiramphodon pogonognathus* (ikan jolong) are found in a large number in the stream of AHFR and they always remain on the surface of the water unless disturbed. These fish tend to approach nearby objects that fall into the water. The main diet of this species consists of insects, especially ants that fall from the terrestrial canopy cover. Major stomach contents consisted of red ants and other hemipteran and dipteran larvae (Mohsin & Ambak, 1983).

Dorichthys martensii (ikan paip) was mentioned by Mohsin and Ambak (1983) as one of the most abundant fish collected from Sungai Rasau in the AHFR in Pucong. As per this study,

TABLE 2
Fish species inhabiting the streams of AHFR in Puchong, Selangor

No	Species	Common name	Family
1	*Puntius binotatus*	Putih, Tebal Sisek	Cyprinidae
2	*Luciocephalus pulcher*	Pikehead, Ikan Jumo	Luciocephalidae
3	*Clupeichthys sp.*	Bilis	Clupeidae
4	*Rasbora einthoveni*	Bada, Seluang, Susur Batang	Cyprinidae
5	*Hemiramphodon pogonognathus*	Jolong	Hemiramphidae
6	*Rasbora heteromorpha*	Seluang, Bada	Cyprinidae
7	*Sphaerichthys osphronemoides*	Biji Durian	Anabantoidei
8	*Rasbora sumatrana*	Seluang	Cyprinidae
9	*Betta pugnax*	Sepilai	Anabantoidei
10	*Glossogobius giuris*	Ubi	Gobiidae
11	*Clarias macrocephalus*	Keli Kayu	Clarias

however, there were no samples caught or sight observations during the study period. This might be due to the species extinction within the streams of the AHFR or a great reduction in the population size. The results of this study in the AHFR, however, were found to vary from the previous fish biodiversity studies carried out at the same area. Data comparisons by Norini and Ahmad (2007) with the work carried out by Rusli *et al*. (1997) in AHFR revealed that the number of species collected had declined.

The importance of the AHFR, including the forest fish production for the Temuan Ethnic group (subgroup of Orang Asli), was studied by Norini and Ahmad (2007). Based on the findings of their study, it concluded that fishing was not as economically viable as hunting animals (Norini & Ahmad, 2007). As of 2007, the yearly income from fishing in this area was approximately only RM1,200.00, with a total production 51.9 kg of fish sold. Considering the species composition in the AHFR, the disturbed forest is categorized as a commercially poor forest (Isaiah & Ahmad, 2007), but with a rich biodiversity of fish species (Mohsin & Ambak, 1983).

CONCLUSION

The AHFR in Puchong is extremely susceptible to environmental degradation due to decreased water catchment size, which in turn affects the abundance and diversity of the fish species. As it stands, AHFR is still relevant in terms of acting as a shelter for indigenous fish species living within its confines but a coordinated plan between authorities is required in developing and managing AHFR in sustainable ways, particularly in terms of its river systems to negate any further degradation of the fish species. Any further succession of land from the AHFR for commercial use will surely have dire effects on the native population of fish. Degradation can clearly be seen in the downstream portion of the rivers that flow out of the AHFR and are not within the AHFR reserve, while the main fish species found within these areas are introduced species especially tilapia (*Oreochromis spp.*), African catfish (*Clarias gariepinus*) and mosquito fish (*Lebistes reticulates*), and water quality is also poor due to the many drains adjoining the river that pump sewage into its waters; all these have contributed to the demise of the local fish species. Although only eleven indigenous fish species were found

during the study period, it is important to note that the study was only carried out within a short period of time and with limited resources. Further studies with a longer period should be carried out to identify all the indigenous species within the AHFR and to outline suitable steps to ensure that these fish populations are maintained for future generations.

REFERENCES

Ahmad Ainuddin Nuruddin, C. N. Hjortso, Norini Haron, Khamurudin Mohd Nor, Awang Noor Abdul Ghani, & Ismariah Ahmad (2007). Introducing Stakeholder Analysis in Malaysian Forestry – The Case of Ayer Hitam Forest Reserve. *Pertanika J. Trop. Agric. Sci., 30*(2), 131-139.

Awang Noor, A. G., Norini, H., Khamurudin, M. N., Ahmad Ainuddin, N., & Thorsen, B.J. (2007). Economic Valuation of Timber Resources in Ayer Hitam Forest Reserve, Puchong, Selangor. *Pertanika J. Trop. Agric. Sci.*, 30(2), 83-96.

Hellawel, J. M. (1986). *Biological indicators of Freshwater Pollution and Environmental Management*. London: Elsevier Applied Science.

Hua, S. C. (2002). *A Field Guide to the Fish of Tasek Bera*. Malaysia: Wetland International Asia Pacific Publication.

Ismariah, A., & Ahmad Fadli, S. (2007). Valuation of Carbon Stock Sequestration in Ayer Hitam Forest Reserve, Puchong. *Pertanika J. Trop. Agric. Sci.*, *30*(2), 109-116.

Karplus, I., Szlep, R., & Tsurnamal, M. (1972). Associative behavior of the fish Cryptocentrus cryptocentrus (Gobiidae) and the pistol shrimp Alpheus djiboutensis (Alpheidae) in artificial burrows. *Marine Biology*, *15*(2), 95-104.

Keenleyside, M. H. A. (1979). *Diversity and Adaptation in Fish Behaviour*. New York: Springer-Verlag, Berlin Heidelberg.

Khan, M. S., Lee, Patrick K. Y., Cramphorn, J., & Zakaria Ismail, Mohd. (1996) *Freshwater Fishes of the Pahang River Basin, Malaysia.* Malaysia: Wetland International Asia Pacific Publication No: 112.

Lowe-McConnell, R. H. (1975). *Fish communities in tropical freshwaters*. London: Longman.

Lundberg, J. G. (2001). Freshwater riches of the Amazon. *Natural History*, *110*(7), 36-43.

Mohsin, A. K. M., & M. A. Ambak (1983). *Freshwater Fishes of Peninsular Malaysia*. Malaysia: Penerbit Universiti Pertanian Malaysia.

Moyle, P. B., & Cech, J. J. Jr. (2004). *An Introduction to Ichthyology*. Upper Saddle River, NJ: Prentice-Hall.

Norini, H., & S. Ahmad Fadli (2007) The Importance of Ayer Hitam Forest Reserve (AHFR), Puchong, Selangor, to the Temuan Ethnic Subgroup. *Pertanika J. Trop. Agric. Sci.*, 30(2), 97-107.

Rusli, M., Awang Noor, A. G., & Abdul Rahim, O. (1997, October 14-17). *Indigenous people Dependence on non-wood forest produced: A case study*. Paper presented at International Workshop on Non-wood Forest Products, Universiti Putra Malaysia, Serdang, Selangor, Malaysia.

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Short Communications

Detection of Koi Herpesvirus (KHV) in *Cyprinius carpio* (Koi) Stocks using Enzyme-Linked Immunosorbent Assay (ELISA)

Azila, A.[1], Way, K.[2], Wood, G.[2], Ainol, Y. M. Y.[3], Kamisa, A.[1], Norfauzana, M. A.[1], Jafrizah, A. R.[3] and Sabri, M. Y.[4]*

[1] *National Fish Health Research Centre (NaFisH), Batu Maung, Penang, Malaysia*

[2] *Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Weymouth Laboratory, Weymouth, Dorset, UK*

[3] *Biosecurity Division, Department of Fisheries Malaysia, Malaysia*

[4] *Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

* *E-mail: sabri@vet.upm.edu.my*

ABSTRACT

Koi herpesvirus (KHV), which is also known as Cyprinid herpesvirus 3 (CyHV-3) infection, is an OIE (international des epizootis) listed disease that caused high losses in common and koi carp in Indonesia and Japan in 2002 and 2003. Since the mid of 2006, the polymerase chain reaction (PCR) has been used in Malaysia for surveillance of koi fingerlings to detect virus nucleic acid, but it has been found to produce unreliable results. Following this, an alternative enzyme-link immunosorbent assay (ELISA) technique for the detection of antibody against KHV was used to find evidence of KHV infection in koi carp stocks on farms that had been sampled for the PCR. For this purpose, a total of 245 serum samples from koi carp stocks were collected and tested for the antibody to KHV by the ELISA at the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) laboratory, Weymouth, UK. Two hundred and eight samples were found to be negative but 37 others were either definitely positive or close to borderline positive and all were retested. The final results showed that 222 (90%) samples were confirmed as negative and 19 (8%) others were definitely positive. Meanwhile, four samples (1.6%) were positive at dilutions of 1:400 or 1:200, but cross reactions with CyHV-1 (causing herpesviral epidermal hyperplasia) could have occurred at those dilutions. Three of the samples were the only positive fish at two sites, but the fourth sample came from a site at which there were 4 definite positive samples (from 20 fish sampled). Thus this study confirmed that Malaysian koi stocks have previously been exposed to KHV. With the lack of bio-security measures and awareness, there was a high probability that the koi carp had been exposed to KHV, leading to subclinical infections and some fish might possibly have become carriers of the virus. Hence, further surveillance needs to be conducted to determine the true situation of the KHV infection in Malaysia.

Keywords: Koi herpesvirus, *Cyprinius carpio*, ELISA

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*Corresponding Author

INTRODUCTION

Koi herpesvirus disease (KHVD) (Hedrick *et al*., 2004), cyprinid herpesvirus-3 (CyHV-3) (Thomas *et al*., 2005), which is also known as carp interstitial nephritis gill necrosis virus (CNGV) (Ronen *et al*., 2003; Arnon *et al*., 2005), is a unique virus which can cause rapid and widespread disease of koi and carps (Ilouze *et al*., 2006). The disease was first detected in Israel and USA in 1998. This disease was then transferred to other countries such as Poland, England, Germany and the Netherlands (Antychowicz *et al*., 2005). In Israel, for instance, the annual lost was estimated to be $3 million. In Asia, the first KHVD outbreak was detected in Indonesia in April 2002, with an estimated loss of about US$5.5 million (Melba, 2004). The virus then hit Thailand in 2004 during a Koi competition. In Malaysia, KHV was first detected in imported koi broodstocks in 2005 and in juvenile koi at the beginning of 2006 (Nor-Mahya & Azila, 2006). Following this incidence, besides the regular detection of carrier status reported from time to time by AVA Singapore (Azila, pers. comm), NaFisH in collaboration with Perak State Fisheries (PPN), Perak Aquaculture Association, Aquaculture Division (DOF, Putrajaya) and KLIA Fisheries Diagnostic Lab started the monitoring and screening programmes for KHV from July 2006 until December 2008. The technique used for this purpose was polymerase chain reaction (PCR). During this survey, no mass mortality of koi or carps was reported by farmers; however, some of the samples have shown positive detection by commercial kit. As a precaution, positive samples were then retested and confirmed by other labs, whereby the results were usually contradictory.

The PCR is known to have high sensitivity-specificity and able to detect a minimum amount of viral DNA. However, it has some limitation factors, such as the number of fish sampled from the population, the number of DNA copies or virus particles present in the fish, the amount of tissues sampled from the fish and the distribution of virus/DNA in the fish (St-Hillaire *et al*., 2009). Due to the unreliable PCR results, together with the lack of standard protocols for sampling, the EU commission suggested that further screening of broodstocks should be done using enzyme-linked immunosorbent assay (ELISA) to explore whether or not the koi stocks had been exposed to this particular virus. This is in accordance with the EU audit in April 2008 which found big floss in the detection and containment of KHV in Malaysia. Thus, screening of broodstocks might reveal a clearer picture of the occurrence of KHV in Malaysian koi farms.

ELISA is a technique used to detect the presence of KHV antibody in the serum of koi or carps (Adkison *et al*., 2005; Arnon *et al*., 2005; St-Hilaire *et al*., 2009). This immunological based technique is useful in identifying fish that have prior exposure to KHV or fish that have persistent or latent infection, whereby herpesvirus is known to have caused this latent infection (St-Hilaire *et al.*, 2005). ELISA is routinely used in animal disease detection but its usage in aquaculture diseases is still new and thus needs a comprehensive assessment. The advantage of the ELISA is its characteristic of non-lethal sampling procedure, besides its ability to identify positive serum after a long period of exposure. This was demonstrated in the detection of channel catfish virus (CCV) after 6 months of post-exposures (Hedrick *et al*., 1987). Currently, this technique is still under evaluation by the OIE panel and has not been widely used in screening or monitoring the programme of KHV (OIE, 2009). The objective of this surveillance was to provide the information related to the occurrence of KHVD in Malaysia through ELISA, as well as to suggest the control measures of this particular disease in the near future.

MATERIAL AND METHODS

Fish Samples

A total of 245 fish were selected from 15 ponds at koi fish farms in Perak, mainly in the districts of Tronoh and Gopeng. The samples were from the fish aged over 6 months old (14 – 20 cm length),

including the broodstocks. Meanwhile, the source of water for the pond was from the nearby ex-mining pond, with the water temperature normally reached 30°C (between 28 - 2°C) during mid day.

Blood Collection

Fish were anesthetized with clove oil and blood was collected using a 3 cc syringe with a 24 gauge needle. The blood was then transferred into 1.5 mL centrifuge tubes and stored in ice. The blood was allowed to clot for 4 h before centrifuging at 2000 rpm for 20 min to collect the serum. These samples were then kept in -20°C until it was ready for transportation to CEFAS for the ELISA analysis.

Serology

The ELISA plates (Costar®) were coated triplicate with KHV antigens at the concentration of 10 µg/50 µL per well in carbonate buffer, at pH 9.6. The plates were incubated overnight at 20-22°C for 16 h. After washing three times with PBS-Tween 20 (PBST), 50 µL per well koi serum, diluted at 1: 600, was added into each well and it was incubated further for 1 h. The plates were washed three times with PBST and incubated again for 1 h after adding 50 µL mouse anti-carp monoclonal antibody (MAb: Stirling). After three more rounds of washing, 50 µL rabbit anti-mouse conjugate was added into each well, and incubated at 37°C for another hour. After the last washing, the colour reaction was initiated by adding 100 µL of 3,3'5,5'-tetramethyl benzidine (TMB: Sigma), prepared in citrate-buffer and incubated in the dark for 10 min at room temperature. The reaction was stopped by adding 50 µL 1 M H_2SO_4, and the plate was read at 450 nm on iMark microplate reader (BIO-RAD, USA).

RESULTS AND DISCUSSIONS

Out of 245 samples, 37 were either definitely positive or close to borderline positive and all were retested. The final result showed that 222 (90%) samples were confirmed as negative while 19 (8%) others were definitely positive at antibody titre of 1:800 or greater. Meanwhile, four samples (1.6%) were positive at the dilutions of 1:400 or 1:200 but as stated earlier, cross reactions with CyHV-1 (causing herpesviral epidermal hyperplasia) could occur at those dilutions. Three of the samples were the only positive fish at two sites, but the fourth sample came from the site at which there were 4 definite positive samples from 20 fish sampled (*Fig. 1*).

This surveillance finally confirmed that Malaysian koi stocks have previously been exposed to KHV, even though the concerned farmers claimed that they were using local stocks without new introduction or importation of broodstocks in the last 30 years (Azila, pers. comm.). A few data on the import of koi broodstocks between the years of 2000 – 2007 are available. Malaysia took an advanced step to ban the import of koi from Indonesia in 2002, the KHV outbreaks in that country. However, some importation from Japan and the other countries were still continued (Nor-Mahya & Azila, 2006) during these years, and this could have highly contributed to this circumstance.

These imported fish had not been tested for KHV until 2006 with the availability of IQ2000 KHV detection kit in Malaysia. The use of PCR, which is well known for its high specificity and sensitivity, has made this technique the most useful tool for disease diagnosis; however, it does not indicate the state of the disease when dealing with herpesvirus (Morishima, 1999). Herpesvirus is very good in establishing the latent state or carrier status, without any clinical evidence as what has been observed from epidemiological study of KHV conducted by NaFisH in Malaysia (Azila, unpublished data).

The cross reaction between CyHv-1 and KHV, that was observed in 4 of the samples tested at the low serum dilutions of either 1:200 or 1:400, might be due to the high similarity between the 2 viruses. CyHV-1 and KHV are herpesviridae and they infect both koi and common carps but the clinical signs are different. CyHv-1 is usually known as carp pox, the

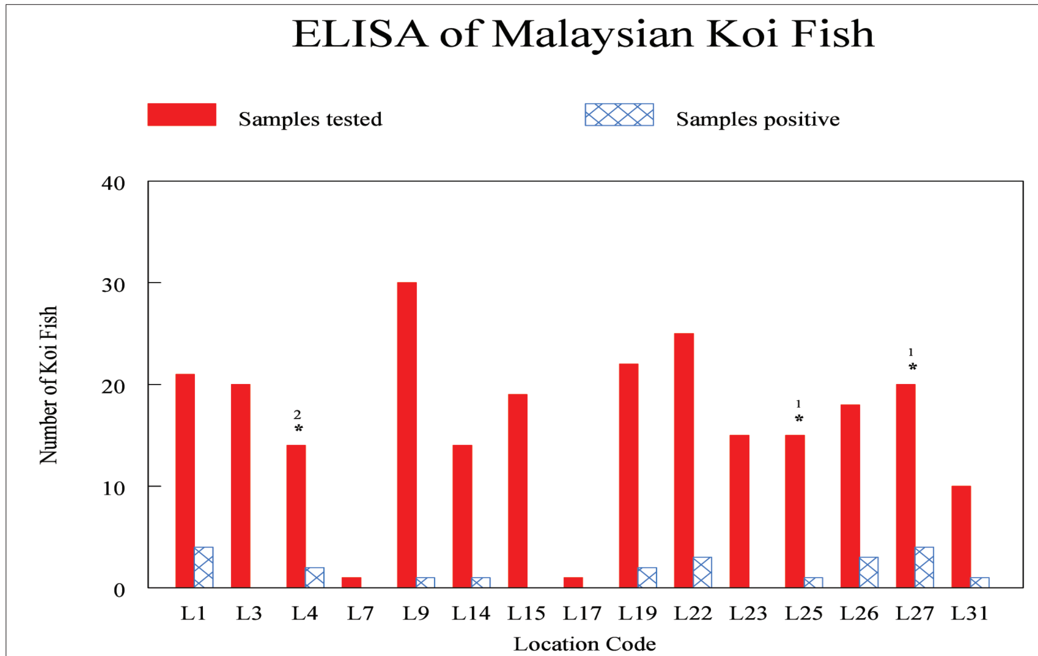

Fig. 1: The number of Malaysian koi fish serum samples retested for ELISA at different dilutions of 1:200, 1:400, 1:800 and 1:1600. At lower dilutions (1:200 & 1:400) cross-reactions with Cyprinid Herpesvirus-1 (CyHV-1) were shown in 4 of the samples (*).

formation of waxy lumps on the skin, fins and lips of fish. However, it is a non-lethal disease, except in small fish and clinical signs usually disappear with the increasing water temperature but the virus can become latent in fish, as what happened in the case of KHV. To some extent, these similarities may complicate the immune system of fish. Following this, Adkison *et al.* (2005) found that cross-reactions between these 2 viruses occurred at 1:50 to 1:500 dilutions. Hence, the researchers suggested higher serum dilutions (i.e. at 1:2500 or greater) should be used to avoid this cross-contamination. In other cases, this cross-reaction was undetectable (St-Hilaire *et al*., 2009), and further testing has to be conducted to clarify this status.

Almost all the farms involved in this study are located in the same area and sharing the same water source and to some extent, the broodstocks. This situation was worsened by the lack of bio-security measures, as well as public awareness towards this disease in the earlier studies. Hence, there was a high probability that these koi carp had been exposed to KHV through sharing of the water source, broodstocks and unsecured trade movement between farms and premises. This KHV, however, did not cause any clinical infections and some fish might possibly just became carriers due to the water temperature that was not at the optimum range for virus to grow and caused problem to the fish (Iida & Sano, 2005). Nonetheless, it is still questionable whether the positive ELISA result is due to latent or persistent infection because as far as author is concerned, no outbreak or clinical signs have been reported in this area yet. Furthermore, a one-time sampling is not conclusive enough to confirm this situation. A series of sampling should therefore be done to determine whether the result is consistent, and PCR specific should be done to KHV as well.

As a consequence, suggestion was made to increase the number of the serum samples to be tested using ELISA to determine the cut-off value that could fit the Malaysian KHV scenario. The studies by Adkison *et al.* (2005) and St.

Hilaire *et al.* (2009) described that the use of koi fish which had been exposed and/or infected by KHV revealed that the detection of the antibody was still detectable at the highest dilution (> 1:1600). In the cases of Malaysia, the status of the samples was unknown and there were assumptions made that the antibody could only be detected at certain value to be optimized later. Therefore, the data gathered may be useful for controlling and prevention of KHV in the future without sacrificing the industry in Malaysia.

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REFERENCES

Adkison, M. A., Gilad, O., & Hedrick, R. P. (2005). An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of Koi Cyprinus carpio. *Fish Pathol.*, *40*, 53-62

Antychowicz, J., Matras, M., Bergmann, S. M., & Haenen, O. (2005). Epidemiology, pathogenicity and molecular biology of koi herpesvirus isolated in Poland. *Veterinary Research*, *49*(4), 367-373.

Dishon, A., Perelberg, A., Bishara-Shieban, J., Ilouze, M., Davidovich, M., Werker, S., & Kotler, M. (2005). Detection of Carp Interstitial Nephritis and Gill Necrosis Virus in Fish Droppings. *Appl. Environ. Microbiol., 71*(11), 7285-7291.

Hedrick, R. P., Gilad, O., Yun, S. C., McDowell, T. S., Waltzek, T. B., Kelley, G. O., & Adkison, M.A. (2004, March 13). *Initial isolation and characterization of herpes-like virus (KHV) from koi and common carp*. Paper presented at the International Symposium on Koi Herpesvirus Disease-Strategy for Koi Herpesvirus Disease Control, Yokohama, Japan.

Hedrick, R. P., Groff, J. M., McDowell, T., & Wingfield, W. H. (1987). Response of adult channel catfish to waterborne exposure to channel catfish virus. *Prog. Fish-Cult*, *49*, 181-187.

Iida T., & Sano, M. (2005). Koi herpesvirus disease. *Uirusu, 55*, 145-151.

Ilouze, M., Dishon, A., Kahan, T., & Kotler, M. (2006). Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. *FEBS Letters, 580*(18), 4473-4478.

Melba, G. B. R. (2004). Trans-boundary aquatic animal diseases: Focus on Koi Herpes virus (KHV). *Aquaculture Asia,* 9, 24-28.

Morishima, T. (1999). Progress in diagnosing herpesvirus infections. *Nagoya J. Med. Sci.,* 62, 83-97.

Nor-Mahya, Y., & Azila, A. (2006, June). *Observation of Koi Herpesvirus Disease (KHVD) in ornamental koi carp.* Paper presented at the 4th National Fisheries Symposium, Kuching, Sarawak, Malaysia.

OIE. (2009). Koi herpesvirus disease. Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.3.6. Retrieved from http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/2.3.06_KHVD.pdf

Ronen A., Perelberg, A., Abramowitz, J., Hutoran, M, Tinman, S., Bejerano, I, Steinitz, M., & Kotler, M. (2003). Efficient vaccine against the virus causing a lethal disease in cultured Cyprinus carpio. *Vaccine, 21*(32), 4677-4684.

St-Hilaire, S., Beevers, N., Joiner, C., Hedrick, R. P., & Way, K. (2009). Antibody response of two populations of common carp, Cyprinus carpio L., exposed to koi herpesvirus. *Fish Dis.,* 32, 311-320.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R-M., Martin, P., & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp (*cyprinus carpio*). *Dis. Aquatic Organisms,* 67, 15-23.

Thomas, B. W., Garry, O. K., David, M. S., Keith, W., Larry, H., Hideo, F., Ikuo, H., Takashi, A., Andrew J. D., & Ronald P.H. (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae. Gen. Virology*, 86, 1659-1667.

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Urban Trees Diversity in Kuching North City and UNIMAS, Kota Samarahan, Sarawak

Zainudin, S. R.*, Mustafa, K. A., Austin, D., Helmy, J. and Lingkeu, D. A.
Department of Plant Science and Environmental Ecology,
Faculty of Resource Science and Technology,
Universiti Malaysia Sarawak,
94300 Kota Samarahan, Sarawak, Malaysia
**E-mail: zsrubiah@frst.unimas.my*

ABSTRACT

Tree species composition often varies widely amongst cities, depending to their geographical locations, urban history, land area or population. The objective of the study was to identify the species diversity of urban trees planted along the roadsides of Kuching North city and Universiti Malaysia Sarawak (UNIMAS), Kota Samarahan. A total of 31,181 trees representing 186 species were sampled. The roadside trees of Kuching North city were more diverse with 176 species of trees while 28 species were recorded from UNIMAS. Inverse of Simpson Index of diversity of the roadside trees in Kuching North city and UNIMAS was 21.0 and 10.7, respectively. In particular, five common species dominated the whole study area with indigenous species dominating UNIMAS, while exotic species exceed indigenous species at roadsides in Kuching North city. Five popular species accounted for one third of the total trees planted with *Mimusops elengi* as the dominant species planted at both sites. All the species recorded from both the study areas were less than 10 % and they complied with the urban forest health status guideline, whereby a diverse tree population might slow or prevent the spread of insects or diseases, and in the event that such pests should become established, the impact on a diverse tree population may be less severe. Data on species floristic composition will assist the local authorities in the planting, maintaining and planning for future replanting activities.

Key words: Urban environment, urban trees, species diversity, *Mimusops elengi*

INTRODUCTION

Landscape planting has been widely regarded as an indispensable urban infrastructure in the attempts to ameliorate stresses arising from artificial covers and to furnish a broad range of environmental benefits (Grey & Deneke, 1986; Miller, 1997). Trees in urban settings play an important role in improving urban life by reducing runoff, air pollution and energy use, and improving human health and emotional well being (Schroeder & Cannon, 1983; Ulrich, 1985; Heisler, 1986; Dwyer *et al*., 1992; Nowak & Crane, 2000; Nowak & Crane, 2002; Xiao & McPherson, 2002). The increasing size and proportion of the human population living in towns and cities has also resulted in greater emphasis on the maintenance and improvement of trees within these settings. An understanding of urban floristic composition can help the municipal in managing their resources sustainably. Biological diversity within populations is important in order to minimize plant maintenance needs and disease tolerance of urban tree populations (Richards, 1993; Graves, 1998). Low species diversity may leave the tree population more vulnerable to the new stress environments; both abiotic and

*Corresponding Author

biotic (Graves, 1998). Urban forestry literature generally recommends that not more than 10% of the trees be from any one species and the concern underlying this recommendation is with the possibility of a species-specific pest or disease sweeping through the area damaging or destroying a large segment of the trees (Graves, 1998).

The diversity of the tree population at the level of the genus, species or genotype is an important consideration in terms of the ability of the urban forest to withstand stress (Wen, 1992). A diverse tree population may slow or prevent the spread of insects or diseases, and in the event that such pests should become established, the impact on a diverse tree population may be less severe. Perhaps, one of the most obvious examples is that of the American Elm (*Ulmus americana*), whereby its attractive form and suitability for urban growing conditions resulted in the widespread planting throughout North American cities (Wen, 1992). These cases, and also others like them, have shown that the most serious pests or problems are specific to certain families, genera, or species of plants. Pauleit *et al*. (2002) showed that throughout Europe, there existed a poor diversity of tree genera and species planted in urban areas, especially as street trees. In Finland, for example, there is a trend to use smaller trees as the building density increases. However, in many places, large tree species are still planted, in spite of the complaints that they have too little space for both roots and crown. In Denmark, different cities listed different species as “the most used species”. This may reflect adverse growing conditions, but the choice may also be the “footprint” and personal preferences of the city planners. The species choice for street trees (Pauleit *et al*., 2002) shows that the number of species used increases towards the south of the European region and reflects the more amenable climatic conditions. However, it is rare to find places with such poor species choice as within the two largest Norwegian cities, where 70% of the street trees planted were of one clone, i.e., *Tilia x euopaea* ‘Pallida’. The limited number of species planted in the urban areas is often a result of the use of well-tested cultivars that have been proven to be the most resistant and aesthetically-pleasing, and are also easily propagated and cultivated. Although planners in the Central and North-Western European countries use a relatively broad range of species, only three to four genera predominate in the urban areas, namely *Platanus, Aesculus, Acer* and *Tilia* (Pauleit *et al*., 2002).

Kuching North city and Kota Samarahan have transformed enormously over the past ten years with many developments, such as office buildings, universities, schools, houses and roads to cater the needs of the ever-growing population. In spite of the fast phase of development, greening of the city is not forgotten. Like any other city in the world, benefits of trees in the urban environment have been greatly emphasized and recognized. This study evaluated the species diversity of the trees in Kuching North city and UNIMAS, Kota Samarahan, Sarawak. The data collected would provide information on the diversity of trees planted and assist the local authorities in their planting, maintaining and future replanting activities.

Study Area

The study area, i.e. Kuching North city, is located in the north of the equator line of South East Asia and Kota Samarahan (*Fig. 1*). Kuching North city, which is also the main capital of Sarawak, accommodates a population of over 500 000 in 370 km^2 of land. The climate of Kuching North city and Kota Samarahan is equatorial, with the daytime temperature between 28 to 34°C. The average rainfall is between 330 cm and 460 cm, and the average humidity is 70%. The two study areas represent a range of development history and land use patterns; aggressive tree planting was carried out around residential areas and roadsides in the recent years in line with the recognition of Kuching as a garden city. Issues of landscaping are very important as one of the factors that could promote the city as liveable and environmentally healthy. Kota Samarahan is considered as a university city as two main universities in Sarawak, namely Universiti

Malaysia Sarawak (UNIMAS) and Universiti Technologi MARA (UiTM), are located here. In this study, only UNIMAS was selected as the study site.

MATERIALS AND METHODS

In order to evaluate the patterns and combinations of the planted trees, one or more roads in each of the study areas was/were selected and the trees were measured and analyzed according to the procedures described by Jaenson *et al.* (1992). An accurate street map, with a scale ranging between 1" = 400' and 1"= 900', was taken and the cities were stratified by relatively dividing them into homogeneous zone segments of three zone types known as Rectilinear Residential (RR), Curvilinear Residential (CR), and Downtown (DT). In each RR and DT zone segment, every block is given a number. Stratified random sampling was done within each zone segment to ensure that every block has an equal probability of being selected. For each zone segment, the tree density or the number of trees per street unit was counted. A list of the roads surveyed in Kuching North city and UNIMAS is given in Table 1. The trees were identified up to species level and their diameters at breast height (dbh) were measured and recorded. Data analysis was aided by Microsoft Excel 2007 and SPSS/PC 9.0. The Shannon-Weiner diversity, Inverse of Simpson's Index of diversity, Maximum equitability and Equitability species indices were calculated using the standard formulae (Greig-Smith, 1983; Mueller-Dombois, 1974) listed in the footnote of Table 2. Shannon-Wiener's and Simpson's inverse diversity indices are derived from the aggregation of relative proportions of individual species, and they provide a synoptic summary of the diversity of species in a given flora. Maximum equitability is derived directly from species richness. Equitability is a ratio between Shannon-Wiener and Maximum equitability indices depicting the relationship between species diversity and richness, and a high value denotes that the constituent species are more evenly represented.

RESULTS AND DISCUSSION

A total of 31,181 trees, represented by 204 species, from both the study sites were sampled (Table 2). The roadside trees of Kuching North city were more diverse with 176 species of trees, while 28 species were recorded from UNIMAS. For an area of 14,578 ha, the number of species recorded at the roadside in Kuching North city was 176 and this is considered as high compared with temperate-latitude cities (Kunick, 1987; Richards, 1983; Whitney, 1985). Similar results were reported by Jim (2002) for the city of Guangzhou in China, whereby for an area of 5519 ha, species richness was 246, and

TABLE 1
Name of roads surveyed and their distance in Kuching North city and UNIMAS

No	Name of road	Distance (km)
1	Tun Abang Haji Openg	5
2	Tunku Abdul Rahman Yaakub	10
3	Tun Zaidi Adruce	5.5
4	Sultan Tengah	5
5	Nanas	2
6	Bako	25
7	Rubber	2.5
8	Kampung Malaysia	3.0
9	Kampung Tunku	2.5
10	Santubong Damai	57.5
11	UNIMAS	9.0

TABLE 2
Basic and derived quantitative attributes of the two forest types in Kuching North city (roadsides) and UNIMAS, Kota Samarahan, Sarawak.

Quantitative attributes	Roadsides	UNIMAS	Whole Study Area
Basic statistics			
Tree Frequency, N	25819	5362	31181
No. of Species	176	28	204
Area, A(ha)	14,578	110	14,688
Road length, L (km)	118 km	9 km	127 km
Tree statistics			
Indigenous tree,%	44.4%	71.4 %	63.2%
Tree density (area),N/A (tree/ha)	1.8	48.7	50.5
Tree density (road length), N/L(tree/km)	218.8	595.8	
Species indices			
Shannon-Weiner diversity, H	20.3	1.07	21.1
Inverse of Simpson diversity index, D	21.0	10.7	23.1
Maximum equitability, H_{max}	7.46	4.81	7.53
Equitability, E	2.72	0.22	2.90

Shannon-Wiener diversity, $H = -\sum pi \log_2 pi$
Inverse of Simpson diversity index, $D = \sum p^2 i$
Maximum equitability, $H = \log_2 S$
Equitability, $E = H/H\text{max}$.

Hong Kong with 149 species at roadside (Jim, 2000). Inverse of Simpson Index of diversity of roadside trees in Kuching North city and UNIMAS was 21.0 and 10.7, respectively, and these are also considered as high compared to other Asian countries, such as Hong Kong and Fujian, China, with the values of 12.7 and 6.0, respectively (Jim, 1992, 1999). Similarly, the Shannon-Weiner diversity index of roadsides trees in Kuching North city and UNIMAS was 20.3 and 1.07, respectively. Meanwhile, equitability value (E) was 2.72 and 0.22 for Kuching North city and UNIMAS, respectively. A high equitability value denotes that the constituent species represented roadsides trees in Kuching North city more evenly as compared to those in UNIMAS. The length of the roads in Kuching North city is 10 times longer than those in UNIMAS and they can account for the high values of the calculated parameters. Another contributing factor could be the fact that UNIMAS new campus was established in 2003 compared to the aggressive tree planting in Kuching North city. Data on species diversity and composition would serve as a guide for the local authorities and UNIMAS in planting, maintaining and planning for future replanting activities.

For the whole study area, a small number of popular species dominate the tree population, with the remainder making limited contributions (Table 3). The top five popular species planted along the roadsides accounted for one third of the total trees planted, while 16.4% of the said roadsides were dominated by the family Leguminoseae. The five most popular species were *Mimusops elengi, Cinnamomum iners, Tabebuia pentaphylla, Samanea saman* and *Andira surinamensis.* For UNIMAS, the top five species accounted for 32.1% of the trees planted. Leguminoseae is the main family dominating the two planting zones (Table 3). Meanwhile, five common species dominated the whole study area, with indigenous species dominating the area in UNIMAS, while exotic species exceed the natives at roadsides in Kuching North city**.** Ten species were found to be unique to UNIMAS as they have not been planted along the roadsides of Kuching North city, and these included *Araucaria excelsa, Casuaurina nobile,*

TABLE 3
Five most common tree species planted in Kuching North city and UNIMAS

Kuching North city			UNIMAS		
Species Name	Family	% of trees planted	Species Name	Family	% of trees planted
Mimusops elengi	Sapotaceae	8.8	*Filicium decipiens*	Leguminoseae	9.2
Cinnamomum iners	Lauraceae	8.5	*Syzygium polyantha*	Myrtaceae	6.8
Tabebuia pentaphylla	Leguminoseae	6.6	*Roystonea regia*	Palmae	6.2
Samanea saman	Leguminoseae	5.2	*Cinnamomum iners*	Lauraceae	5.1
Andira surinamensis	Leguminoseae	4.6	*Mimusops elengi*	Sapotaceae	4.8

Hopea odorata, Salix babylonica, Dacrydium araucarioides, Dyera costulata, Gmnostema nobille, Plumeria rubra, Plumeria obtuse and Parkia angulatum. Roadside trees of Kuching North city have similar frequency distributions that are pronouncedly skewed towards the popular species. Similar results were also reported by Jim (2002) for Guanghzou China whereby the popular species included *Ficus virens*, *Bauhinia purpurea*, *Aleurites moluccana*, *Bauhinia variegata* and *Ficus microcarpa*. The selection of the species planted in Kuching North city was made by the officials in the municipals; however, due to restrictive habitat conditions, the selection tended to favour heavy planting of popular species. Roadside amenity strips and associated sites have been heavily filled by trees. On the basis of species selection in both study sites, Leguminoseae dominate the planting stocks. This is probably due to the fact that most of the legume trees have been proven to be successful in urban habitats, and also the existence of the nitrogen–fixing bacteria, *Rhizobium*, in nodules associated with the roots (Wee, 1989). *Mimusops elengi* and *Filicium decipiens* were the most popular species planted in Kuching North city and UNIMAS. *Cinnamomum iners* from the family Lauraceae has striking pinkish leaves and its attractive shape contributes significantly to the aesthetic values of urban trees. *Samanea saman* is among the most common tree species planted in Kuching North city and this is probably due to its dense and wide-spreading foliage suitable for shading.

CONCLUSIONS

As an artificial plant community, roadside trees in Kuching North city and UNIMAS have been planted to meet human's demands such as beautifying the area and for environmental benefits. A total of 31,181 trees were sampled from both the study sites. The roadside trees of Kuching North city were found to be more diverse with 176 species of trees, while 28 species were recorded from UNIMAS. Inverse of Simpson Index of diversity of roadside trees in Kuching North city and UNIMAS were 21.0 and 10.7, respectively. The five common species dominated the whole study area with indigenous species dominating UNIMAS, while exotic exceeds indigenous species at roadsides in Kuching North city. As indicated earlier on, five popular species accounted for one third of the total trees planted, with *Mimusops elengi* and *Filicium decipiens* as the most popular species planted in Kuching North city and UNIMAS.

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REFERENCES

Dwyer, J. F., McPherson, E. G., Schroeder, H.W., & Rowntree, R. A. (1992). Assessing the benefits and costs of the urban forest. *J. Aboric., 18*(5), 227–234.

Grey,G. W. and Deneke, F. J. (1986). *Urban Forestry* (2nd Ed.). New York: Wiley.

Graves, W. R. (1998). Drought resistance on trees: An overview of mechanisms and new research with Iowa Black Maple. *Metropolitan Tree Improvement All*iance *(METRIA*), *5,* 1-5.

Greig-Smith, P. (1983). *Quantitative Plant Ecology* (3rd Ed.). Oxford: Blackwell.

Heisler, G. M. (1986). Energy savings with trees. *J. Aboric., 12*(5), 113–125.

Jaeson, R., Bassuk, N., Schawer, S., & Headley, D. (1992). A statistical method for the accurate and rapid sampling of urban tree populations. *J. Arboric*., *18*(4), 171-183.

Jim, C. Y. (1992). Provenance of amenity-tree species in Hong Kong. *J. Aboric., 16*, 11-23.

Jim, C. Y. (1999). A planning strategy to augment the diversity and biomass of roadside trees in urban Hong Kong. *Landscape Urban Planning, 44*, 13-32.

Jim, C. Y. (2000). The urban forestry programme in the heavily built-up milieu of Hong Kong, Cities. *J. Aboric., 4*, 271–283.

Jim, C. Y. (2002). Heterogeneity and differentiation of the tree flora in three major land uses in Guangzhou City, China. *Ann. For. Sci*., *59*, 107-118.

Krebs, C. J. (1994). Ecology: The Experimental Analysis of Distribution and Abundance (4th ed.). New York: Harper Collins.

Kunick, W. (1987). Woody vegetation in settlements. *Landscape Urban Planning*, *14*, 57–78.

Miller, R.W. (1997). *Urban Forestry-Planning and Managing Urban Greenspaces (2nd Ed.)*. New York: Prentice-Hall.

Mueller-Dombois, D., & Ellenberg, H. (1985). *Aims and Methods of Vegetation Ecology.* New York: John Wiley & Sons.

Nowak, D. J., & Dwyer, J. F. (2000). Understanding the Benefits and Costs of Urban Forest Ecosystems. In J. E. Kuser (Ed.), *Handbook of Urban and Community Forestry in the Ecosystems.*

Nowak, D. J., Civerolo, K. L., Rao, S.T., Sistla, G., Luley, C. J., & Crane, D. E. (2000). A modeling study of the impact of urban trees on ozone. *Atmospheric Environment, 34*, 1601–1613.

Nowak, D. J. & Crane, D. E. (2002). Carbon storage and sequestration by urban trees in the USA. *Environmental Pollution, 116*, 381–389.

Pauleit, S., Jones, N., Garcia-Martin, G., Garcia-Valdecantos, J. L., Riviere, L. M., Vidal-Beaudet, L., Bodson, M., & Randrup, T.B. (2002). Tree establishment practise in towns and cities -Results from a European survey. *Urban For. & Urban Greening, 1*(2), 83–96.

Richards, N. A. (1993). Optimum stocking of urban trees. *J. Aboric*., *18*, 64-68.

Richards, N. A. (1983). Diversity and stability in a street tree population. *Urban Ecology, 7*, 159–171.

Schroeder, H. W., & Cannon, W. N. (1983). The esthetic contribution of trees to residential streets in Ohio towns. *J. Aboric., 9*(9), 237–243.

Ulrich, R. S. (1985). Human responses to vegetation and landscapes. *Landscape and Urban Planning, 13*, 29–44.

Wee, Y. C. (1989). *A Guide to the Wayside Trees of Singapore. Singapore Science Center, Singapore*. 160 pp.

Wen, Q. S. (1992). Quantifying species diversity of streetside trees in our cities. *J. Aboric., 18*(2), 91-93.

Whitney, G. G. (1985). A quantitative analysis of the flora and plant communities of a representative midwestern U.S. town. *Urban Ecology, 9*, 143–160.

Xiao, Q., & McPherson, E. G. (2002). Rainfall interception by Santa Monica's municipal urban forest. *Urban Ecosystems, 6,* 291–302.

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Characterization and Quantification of Dragon Fruit (*Hylocereus polyrhizus*) Betacyanin Pigments Extracted by Two Procedures

Nassim Naderi[1], Hasanah M. Ghazali[2], Anis Shobirin Meor Hussin[1], Mehrnoush Amid[1] and Mohd Yazid Abd. Manap[1*]

[1]Food Technology Department,
[2]Food Science Department,
Faculty of Food Science and Technology
Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia
**E-mail: myazid@putra.upm.edu.my*

ABSTRACT

A method for the extraction of betacyanins pigments of dragon fruit (*Hylocereus polyrhizus*) grown in Malaysia was studied. A processing scheme consisting of solvent system selection (ethanolic and aqueous ethanolic) was proposed to study the effect of water in enhancing betacyanin recovery from the pulp of H. polyrhizus fruit. Betacyanins, in concentrated extracts from the dragon fruit (*H. polyrhizus*), were identified as betanin, phyllocactin, hylocerenin and their respective C-15 isoforms using High-performance liquid chromatographic (HPLC) analysis. Structural alteration was monitored by using selected solvent systems. As for the relative peak area ratios, some betacyanins showed a higher stability than others. Betanin, one of the main betacyanin in selected Malaysian *H. polyrhizus* cultivars, displayed the most stable structure. Comparing the peak area ratios of individual betacyanins, it was noticed that ethanolic assay might induce co-occuring of the C-15 isoforms.

Keywords: Dragon fruit, *Hylocereus polyrhizus*, Betacyanins, Betanin, Phyllocactin, Hylocerenin

INTRODUCTION

Exempt (natural) colours are recognized as organic products which have gained a growing interest from health-conscious consumers and researchers (Griffiths, 2005). Food and Drug Administration (FDA) assigned "exempt colour additives" label to specify that colourings are free from the certification process (Meggos, 1995). These colorants are obtained from natural sources by solvent extractions (Sajilata & Singhal, 2006). Despite the numerous number of anthocyanin containing food colorant extracts, there is only one single betacyanin source, i.e. red beet (*Beta vulgaris L. ssp. vulgaris*), which has been approved in the market (Castellar *et al*., 2003). Nevertheless, a high molar absorbency index of betacyanins and their potential as colorant are equivalent for synthetic colorants (Strack *et al*., 2003). As betacyanins and anthocyanins are chemically related, the methods of anthocyanin extractions can therefore be applied for betacyanin (Sajilata & Singhal, 2006). Compared to polar anthocyanins, betacyanins are more hydrophilic. In more specific, they can dissolve in three common polar solvents (namely, water, methanol and ethanol) and their mixtures; to certain extent, water and organic acids are miscible (Schoefs, 2004). Thus, finding a good separation system is rather challenging. Based on their chemical structure, Betacyanins belong to alkaloids.

[*]Corresponding Author

These pigments are also water-soluble and localized in vacuole as bis-anions (Stintzing & Carle, 2004). They are more soluble in water than in non-polar solvents and this characteristic helps extraction and separation processes (Strack *et al*., 2003). Methanol has generally been used to extract betacyanins (Moreno *et al*., 2007). Since methanol has toxic characteristics, food scientists prefer other extraction systems (Xavier *et al.*, *2008*). In this study, a processing scheme of solvent system selection (ethanolic and aqueous ethanolic) was proposed to study the effect of water in enhancing betacyanin recovery from the pulp of *Hylocereus polyrhizus* fruit in the final concentrated colour extract.

MATERIALS AND METHODS

Plant Material and Chemicals

The fruit of two-year-old plants of the dragon fruit *(Hylocereus polyrhizus),* grown on trellis system in a modern agriculture farm in Kluang, Johor, Malaysia, were used in this investigation. The clones were originally introduced as cuttings from Vietnam several years ago and the plantation has been done through a modern technology directed by the Ministry of Agriculture and Agro-Based Industry (Kluang, Johor, Malaysia). The fruit were harvested for analysis when reaching the full ripening stage, i.e. 30-35 days after pollination. The skin was separated from the pulp using a stainless steel knife. Vacuum packaging was used for a complete removal of oxygen. Subsequently, the fruit were stored at -38˚C for further analysis.

Solvents and Reference Substances

Trifluoroacetic Acid 98% (TFA), Acetonitril and MeOH were purchased from Sigma-Aldrich (Selangor D.E., Malaysia) and they were of analytical or HPLC grade. Food grade EtOH (98%) was retrieved from Merck (Germany). For reference, authentic standard containing betanin and isobetanin was donated by Scott M. Engel (D.D. Williamson Co., WA, USA).

Preparation of the Concentrated Betacyanin Extract

Two extraction methods were applied to monitor the concentration of betacyanin and pigment retention, as well as their composition in concentrated colorants. This was followed by a direct extraction of the pigments by homogenization using a 1/1 (w/v) ratio of fruit/solvent. Typically, 100g of the peeled fruit (pH =4.5; TSS=10%) of watery consistency was shaken and macerated with 100 mL solvents (EtOH, aqueous ethanol 50:50) for 15 minutes under ice cooling condition. The aqueous mixture was centrifuged at 18000 rpm and 4˚C for 20 min, followed by a fast filtration on nylon mesh. The extract obtained was concentrated in a vacuum at 35°C, using a rotary evaporator, to 3-4 mL. The ethanol was completely removed after the concentration process and the samples were then kept in a dark vessel. Once again, 100 gm of the peeled fruit was pressed and filtered to obtain purified juice which was immediately stored at 4°C in a dark vessel as a control. Each sample was analyzed for ^{o}Brix (which is a percentage by weight of sugar in a solution at room temperature), pH, titrable Acidity and betacyanins.

Photometric Quantification of the Total Betacyanins

The betacyanin content was measured in triplicate in deionised water. The extracted samples were diluted by 100-fold with deionised water to obtain the absorption values. After 20 min of equilibration, the quantification of betacyanins was carried out by applying the following equation (Cai & Corke, 1999):

$$\text{BC (Betacyanin Concentration)} = \frac{A \times DF \times MW \times 1000}{\varepsilon \times L} \quad (1)$$

Where BC is the betacyanin concentration in milligrams per litre, A is the absorption value at the absorption maximum (λ_{max} = 540 nm), F is the dilution factor, MW is the molecular weight of betanin (550 g/mol), ε is the molar extinction

coefficient of betanin (ε = 60,000 L mol^{-1} cm^{-1} in H2O) and L is the pathlength of the cuvette.

High-Performance Liquid Chromatography (HPLC)-UV Visible Detector Analysis

A modified method from Wybraniec and Mizrahi (2002) and Esquivel *et al*. (2007) was utilized to determine the pigment patterns of betacyanins in the concentrated extracts. The HPLC analysis for identification of betacyanins was carried out with a liquid chromatographic apparatus (Waters, Ca, USA), equipped with a pump Waters 600 Controller, and a UV-Vis detector (Waters™ 486 Tunable Absorbance Detector). An analytical Lichrocart® 250×4.6 mm i.d. Purospher ®Star RP18-column, with a particle size of 5 µm (Merck, Darmstadt, Germany) was used for the pigment analyses. The separation was performed isocratically using a mixture of 90% solvent A (0.5% aqueous TFA) with 10% solvent B (Acetonitril) for 40 min at a flow rate of 1 mL/min (injection volume: 10µL); detection was carried out at a wavelength of 540 nm, while the betanin and isobetanin were identified by comparing their retention times with those of the standards. The concentrations of betacyanins were calculated from the standard curves of betanin and isobetanin, at four concentrations (namely, 0.02, 0.04, 0.06, 0.08 mg/L) using a linear regression analysis ($r > 0.99$). Later, individual betacyanin composition (%) was measured by analytical HPLC and was expressed as the percentage of the peak area. All determinations were performed in duplicate.

Other Analytical Measurements

Titrable acidity was calculated as the percentage of citric acid by titrating 10mL of the concentrated betacyanin extract with a solution of NaOH (0.1N) to get constant pH 8.2. The pH was measured using a pH meter. The soluble solid content was measured as °Brix using a manual refractometer (ATAGO, Japan). All the analytical measurements were repeated three times.

Statistical Analysis

Statistical analyses were carried out utilizing ANOVA by using Minitab (Minitab 13.1 Inc., USA). Significant differences between the values are at $P<0.05$ levels using Tukey's test.

RESULTS AND DISCUSSIONS

Isolation of the Concentrated Betacyanin Extracts by Solvent System Selection

The extraction method for the production of concentrated betacyanin consists of three general steps, namely, extraction, centrifugation and concentration. The fruit of *Hylocereus polyrhizus* were harvested and transported to the laboratory. These fruit were subsequently peeled and placed in vacuumed plastic bags exempt of air prior to freeze (-38°C) storage. As the pulp of fruit consists of thousands small soft seeds that were distributed homogeneously throughout the flesh, defrosted fruit flesh was pre-treated. Freeze-thawing of the fruit pulps in an air tight condition was found to damage tissues and consequently cause the pigments to leak out. Thus, the freeze-thawing method was chosen for tissue disruption instead of the mechanical method. This method was applied to prevent disruption to the seeds during the procedure. Meanwhile, it is important to note that broken seeds may contain components that degrade betacyanins or increase viscosity.

Defrosted fruit pulp was macerated with selected solvents (ethanol and ethanol:water) in an ice cooling condition. This solvent system assay was selected to reduce the concentration of mucilages that was present in *H. polyrhizus* which could increase the viscosity of the extracts, and this characteristic is certainly not desirable in concentrated extract. Although pectin could be extracted in water and afterward precipitated with organic solvents but, with such a procedure, pigments could also be precipitated, along with pectin (Castellar *et al*., 2006). Thus, a system consisting of a mixture of ethanol and water was selected to extract pigments from the fruit. The next step was centrifugation to discard the residue and the concentration of extracted

betacyanin by evaporation under vacuum at low temperature (35°C). Each extract obtained from the assays was concentrated by 3-4 folds and analyzed. Table 1 shows the analysis of the concentrated betacyanin extracts obtained from each assay.

The pH value was rather low and similar for all the concentrated extracts. This pH value was slightly higher from 4.6 to 4.67 for the sample that was obtained from the ethanolic assay. This pH value is considered as favourable because it has also been reported to be the optimum pH of betacyanins in several studies (Prudencio *et al.,* 2007; Stintzing *et al*., 2006; Stintzing *et al*., 2003; Cai & Corke, 1999). The soluble solid (°Brix) also was not different in the concentrated extract from each assay. °Brix was increased by applying both assays, and therefore, sugar was extracted using both the solvent system assays. The significant difference was observed in the concentration of betacyanin and titrable acidity. Meanwhile, the concentration of betacyanin ($811 mgL^{-1}$) in *H. polyrhizus* fruit was found to be higher than those shown for the red beet (Stintzing *et al*., 2006) and *Opuntia stricta* (Moreno *et al*., 2008) fruit. The highest pigment concentration (655.5 mg/L) was obtained for the aqueous ethanolic assay. The acidity expressed as citric acid (0.004%) was higher in the ethanolic assay than that obtained for the aqueous ethanolic.

Pigment Characterization and Betacyanin Retention in Concentrated Extracts

The analysis of betacyanins can be deduced from their chromatographic behaviour, and corroborative data may be retrieved from the analysis of their absorption spectra. *Fig. 1* shows the chromatographic pattern of betacyanins of the pulp of *H. polyrhizus* fruit. Peaks 1 and 1' were readily identified by UV-vis chromatography with authentic standards (*Fig.1-D*). Based on the HPLC elusion order (Esquivel *et al*., 2007; Wybranieca *et al*., 2002), compounds 2/2' and 3/3' were inferred as phyllocactin/isophyllocactin and hylocerenin/isohylocerenin. Phyllocactin was considered as a major betacyanin in the pigment pattern of *H. polyrhizus* extracts, followed by betanin. To get an insight into the impacts of selected solvent systems on structural alteration, the development of betanin and phyllocactin was monitored. To this extent, betanin isomerisation (i.e. isomerisation index and phyllocactin deacylation) was evaluated by comparing their respective peak area and peak area ratios. In the purified juice (control), five betacyanin structures were found to exist in the equilibrium, namely betanin, isobetanin, phyllocactin, isophyllocactin and hylocerenin. As a rule, it was confirmed that betacyanins were accompanied by their respective isobetacyanins (Herbach *et al*., 2006a).

TABLE 1
Characteristic of liquid concentrated extracts using solvent assays

Extraction solvent	pH	Titratable acids[a]	Soluble solid Contents (°Brix)	Betacyanin concentration mgL^{-1}
EtOH	4.67±0.01a	0.004 ± 0.0a	23.2 ± 0.00a	449 ± 13.58a
EtOH:Water (50:50,v/v)	4.6±0.05a	0.002 ± 0.0b	23 ± 0.00a	655.5 ± 4.38b
Control [b]	4.2± 0.02b	0.01 ± 0.0c	9.5 ± 0.7b	811 ± 6.48c

[a] % of citric acid
[b] purified juice
Means in the same column are significantly different at $p<0.05$, using Tukey's HSD test.

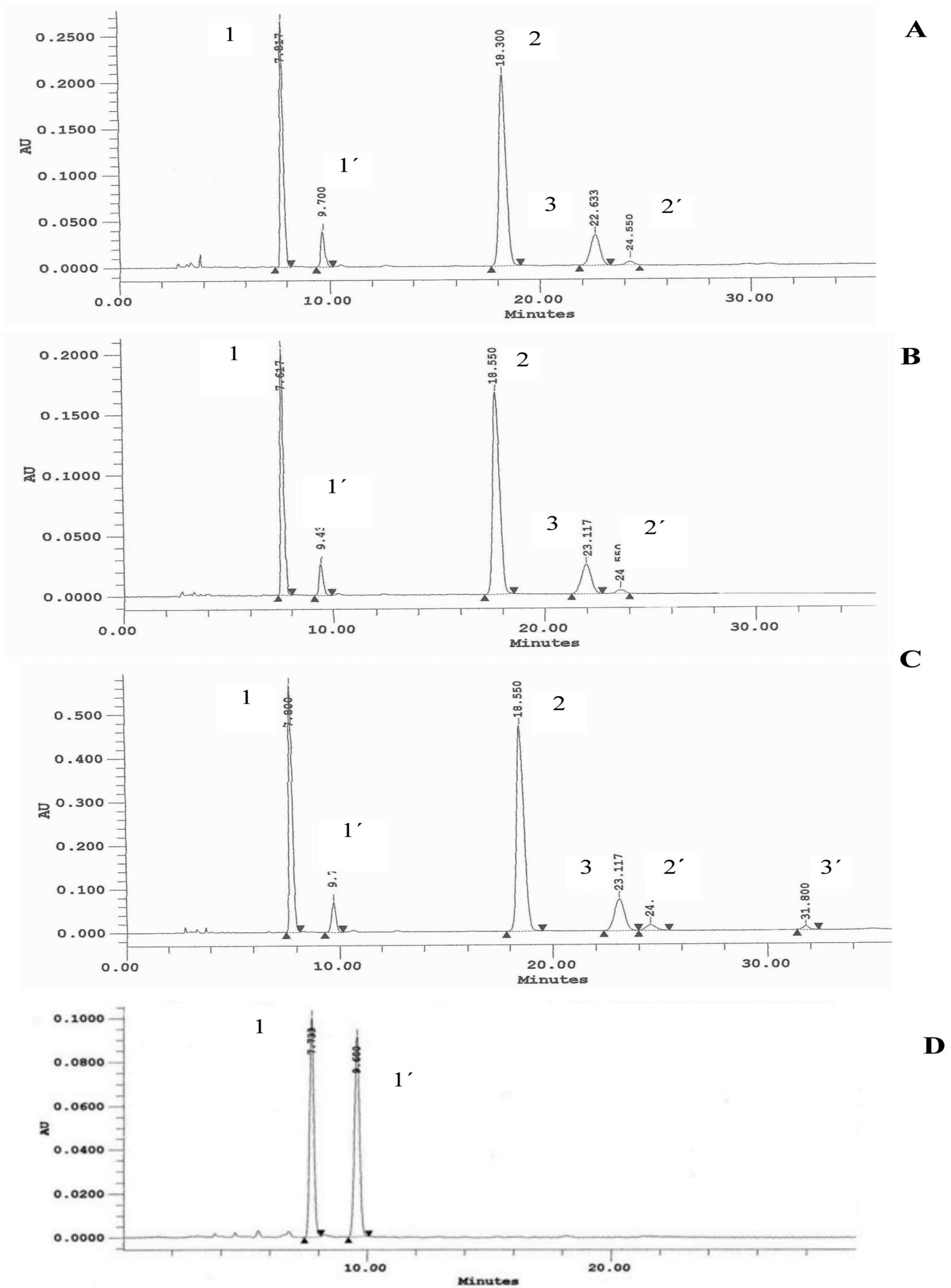

Fig. 1: HPLC pattern of extracted betacyanins from fruit-pulp of H. Polyrhizus. (A) Purified juice (B) aqueous ethanolic assay (C) ethanolic assay (D) Standard: (1)betanin, (1´)isobetanin. (1) Betanin, (1´) Isobetanin, (2) Phyllocactin, (2´) Isophyllocactin, (3) Hylocerenin, (3´) Isohylocerenin

Meanwhile, the relative amounts of these structures at equilibrium varied with the application of two solvent system assays. As for the relative peak area ratios, some betacyanins have showed higher stability than others. Betanin, one of the main betacyanins in selected Malaysian *H. polyrhizus* cultivars, displayed the most stable structure. As shown in Table 2, selected solvent system assays in the pigment extraction did not result in betanin isomerisation but rather in the betanin/isobetanin peak area ratio. The highest peak area ratio of betanin/isobetanin was observed in the concentrated colorant which had been extracted by ethanol, whereas, phyllocactin peak area ratio was found to have declined. In all the samples, however, phyllocactin represented the predominant betacyanin. The peak area in the samples obtained from the aqueous ethanolic assay was almost twice than that of the betanin, as reflected by the betanin/phyllocactin peak area ratio presented in Table 2. As reported previously, phyllocactin is more prone to deacylation, but this reaction was only tentatively observed in the thermally treated samples (Herbach *et al*., 2006b; 2005). The findings of the present study revealed that the ethanolic system might also provoke this reaction, revealing that phyllocactin is less stable than betanin. Isomerization was observed in hylocerenin, where isohylocerenin ratios were found to have increased in the ethanolic assay. Nevertheless, this compound was not detectable in the samples from the aqueous ethanolic assay as well as the purified juice. On the basis of the previous studies on purified betacyanins, as well as considering the retention time and elution order, the presence of isobetacyanins could not be degradation products of their parent betacyanins (2, 3) (Wybraniec & Mizrahi, 2005). Conclusion could be drawn by the observation of a more acidic condition resulted from the ethanolic assay that might induce the co-occurring of C-15 isoforms. Further analysis was carried out by calculating the content of betanin and isobetanin using calibration curves. Interestingly, the highest concentration of betanin was obtained in the ethanolic extraction method, whereby it comprised 50% of the total betacyanins in the concentrated pigments yielded.

TABLE 2
Pigment characteristics of the concentrated extract using organic and aqueous organic extraction assay and Betanin/isobetanin, betanin/phyllocactin peak area ratios at 540 nm in the preparation of *H. polyrhizus* pigment

Extraction solvent	BC (%)[a] (±SD)	Relative concentration (%) for peaks[c]			Betanin/ Isobetanin	Betanin/ Phyllocactin
		1 1´	2 2´	3 3´		
EtOH	50.39 (±9.39)	33.9(±5.1) 5.02(±0.8)	47.8(±6.1) 1.8(±0.2)	11.2(±0.1) 0.22(±0.3)	6.7	0.71
EtOH:Water (50:50,v/v)	24.3 (±3.16)	29.7(±1.5) 4.8(±0.1)	52.3(±0.1) 1.89(±1.0)	11.1(±0.01) -[d]	6.19	0.57
Control [b]	19.28 (±1.0)	32.3(±0.9) 5.4(±0.1)	51.0(±0.5) 0.59(±1.0)	10.6(±1.0) -[d]	6.0	0.6

[a] Betacyanin content expressed as betanin, isobetanin
[b] purified juice
[c] 1:Betanin, 1´:Isobetanin, 2: phyllocactin, 2´: Isophyllocactin, 3:Hylocerenin, 3´:Isohylocerenin
[d] not detected
Mean values of duplicate measurements; (±SD)=standard deviation

CONCLUSION

As commercial production of betacyanins involves solvent extraction and that these pigments are water soluble, therefore in this work, a system based on the admixture of water with organic solvent (ethanol) was chosen for pigment extraction. Through HPLC analysis, betacyanins in the concentrated extracts from *H. polyrhizus* were identified as the known betanin, phyllocactin, hylocerenin and their respective C-15 isoforms. Meanwhile, an addition of water to the organic solvent (ethanol), which were partly miscible together, resulted in a better solubility of betacyanins in the organic phase. Although water extraction is simple, a highly efficient and low cost method for crude betacyanin extraction led to a difficult separation of betacyanin and water-soluble protein components (Cai *et al*., 1998). In such procedure, mucilage and pectines could be extracted in water and later precipitated with organic solvent like ethanol, but pigments might be precipitated with such a procedure as well (Castellar *et al*., 2006). The quantification of betacyanin has shown that the extraction of the pigments with aqueous ethanolic assay resulted in a higher yield. In addition, a closer inspection for the impact of selected solvent systems on structural alteration was carried out by comparing individual peak area ratio using the HPLC analysis. Since the absence of isohylocerenin was the characteristic of betacyanins in the purified juice and the aqueous ethanolic extracts, the co-occurring of hylocerenin C-15 isoform in the ethanolic assay was considered as structural modification (Herbach *et al*., 2006a) due to the more acidic condition, as indicated by a comparatively high pH value.

REFERENCES

Cai, Y., & Corke, H. (1999). Amaranthus Betacyanin Pigments Applied in Model Food Systems. *Journal of Food Science, 64*(5), 869-873.

Castellar, R., Obón, J. M., Alacid, M., & Fernández-López, J. A. (2003). Color Properties and Stability of Betacyanins from Opuntia Fruits. *Journal of Agricultural and Food Chemistry, 51*(9), 2772-2776.

Esquivel, P., Stintzing, F. C., & Carle, R. (2007). Pigment pattern and expression of colour in fruits from different Hylocereus sp. genotypes. *Innovative Food Science & amp; Emerging Technologies, 8*(3), 451-457.

Griffiths, J. C. (2005). Coloring Foods & Beverages. *Food Technology, 59*, 38-44.

Herbach, K. M., Stintzing, F. C., & Carle, R. (2006a). Betalain Stability and Degradation Structural and Chromatic Aspects. *Journal of Food Science, 71*(4), 41-50.

Herbach, K. M., Stintzing, F. C., & Carle, R. (2006b). Stability and Color Changes of Thermally Treated Betanin, Phyllocactin, and Hylocerenin Solutions. *Journal of Agricultural and Food Chemistry, 54*(2), 390-398.

Herbach, K. M., Stintzing, F. C., & Carle, R. (2005). Identification of heat-induced degradation products from purified betanin, phyllocactin and hylocerenin by high-performance liquid chromatography/electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry, 19*(18), 2603-2616.

Meggos, H. (1995). Food colors-An international perspective, *In Manuf. Confect. 75*(2), 59-65.

Moreno, D. A., Garcia-Viguera, C., Gil, J. I., & Gil-Izquierdo, A. (2008). Betalains in the era of global agri-food science, technology and nutritional health. *Phytochemistry Reviews, 7*, 261-280.

Prudencio, I. D., Prudêncio, E. S., Gris, E. F., Tomazi, T., & Bordignon-Luiz, M. T. (2008). Petit suisse manufactured with cheese whey retentate and application of betalains and anthocyanins. *LWT - Food Science and Technology, 41*(5), 905-910.

Sajilata, M. G., & Singhal, R. S. (2006). Isolation and stabilisation of natural pigments for food applications. *Stewart Post-harvest Solutions*, *5*(11), 1-29.

Schoefs, B. (2004). Determination of pigments in vegetables. *Journal of Chromatography A*, *1054*, 217–226.

Stintzing, F. C., & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants,food, and in human nutrition. *Trends in Food Science & Technology*, *15*, 19-38.

Stintzing, F. C., Schieber, A., & Carle, R. (2003). Evaluation of colour properties and chemical quality parameters of cactus juices. *European Food Research and Technology, 216*(4), 303-311.

Stintzing, F. C., Trichterborn, J., & Carle R. (2006). Characterisation of anthocyanin–betalain mixtures for food coloring by chromatic and HPLC-DAD-MS analyses, *Food Chemistry, 94*, 296–309.

Strack, D., Vogt, T., & Schliemann, W. (2003). Recent advances in betalain research. *Phytochemistry*, *62*, 247–269.

Wybraniec, S., & Mizrahi, Y. (2002). Fruit Flesh Betacyanin Pigments in Hylocereus Cacti. *Journal of Agricultural and Food Chemistry, 50*(21), 6086-6089.

Xavier, M. F., Lopes, T. J, Quadri, M. G. N., & Quadri, M. B. (2008). Extraction of Red Cabbage Anthocyanins: Optimization of the Operation Conditions of the Column Process. *Brazilian Archives of Biology and Technology., 51*, 143-152.

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Characterization of Cucumber Mosaic Virus (CMV) Causing Mosaic Symptom on *Catharanthus roseus* (L.) G. Don in Malaysia

Mazidah, M.[1], Yusoff, K.[2], Habibuddin, H.[3], Tan, Y. H.[1] and Lau, W. H.[1*]

[1]Department of Plant Protection, Faculty of Agriculture,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Ministry of Science, Technology and Innovation (MOSTI),
Putrajaya, Malaysia
[3]Rice and Industrial Crops Research Centre,
MARDI HQ, PO Box 12301, General Post Office,
50774 Kuala Lumpur, Malaysia
[*]E-mail: lauweih@putra.upm.edu.my

ABSTRACT

A cucumber mosaic virus (CMV) isolate, causing leaf mosaic and distortion, malformed flowers or colour-breaking on the petals of *Catharanthus roseus* in Serdang, Selangor, Malaysia, was identified and designated as Malaysian periwinkle isolate (CMV-MP). The virus was spherical in shape with the size of 28.6 ± 0.48 nm in diameter with a central core. It was mechanically transmitted to various test plants which produced typical symptoms of CMV infection. The coat protein (CP) gene of the virus was amplified using reverse transcriptase-polymerase chain reaction (RT-PCR) and cloned in *Escherichia coli* using TOPO-TA vector. A single open reading frame of 657 nucleotides, potentially encoding for 218 amino acids was sequenced. A comparison with the CP genes of other CMV isolates indicated that CMV-MP shared 100% sequence homology to the CP gene sequence of *C. roseus* isolate of CMV in India. This is the first aetiology report on *C. roseus* in Malaysia showing natural mosaic disease symptoms supported with the nucleotide sequence analysis of the causal virus.

Keywords: *Catharanthus roseus*, cucumber mosaic virus, mosaic disease, nucleotide sequence analysis, coat protein gene

INTRODUCTION

Catharanthus roseus (L.) G. Don or periwinkle, which is also known as 'kemunting cina' in Malaysia is widely used as an ornamental plant to decorate gardens and landscapes. The plant is also famous for its medicinal properties, particularly as anti-cancer (Manganey *et al*., 1979; Svoboda, 1983; Cragg & Newman, 2005), anti-diabetic (Ghosh & Gupta, 1980; Chattopadhyay *et al*., 1991; Singh *et al*., 2001; Wiart, 2002) and antihypertensive remedies (Van de Heijden *et al*., 2004). Two important *Catharanthus* alkaloids, namely vinblastine and vincristine, have been developed into cancer chemotheraphy agents since 1960s and also marketed as vinblastine sulphate (Velbe®) and vincristine sulphate (Oncovin®) (Van de Heijden *et al*., 2004). In Malaysia, *C. roseus* has long been used in traditional medicine and one of the popular and potential medicinal plants for both cultivation and conservation (Loh, 2008; Musa *et al*., 2009).

As a medicinal plant, tremendous research efforts have been given to study the bioactive compounds of *C. roseus* compared to its phytopathological aspect. Due to very little

[*]Corresponding Author

emphasis on the diseases of the plant, very limited records of virus infections are available. Among other, Espinha and Gaspar (1997) reported cucumber mosaic virus (CMV) infection in *C. roseus,* showing mild mosaic, chlorosis and plant distortion. Meanwhile, tomato spotted wilt virus (TSWV) has also been reported in *C. roseus* with black spots, systemic mosaic, leaf deformation and browning of larger leafs at the bottom part of the plant (Chatzivassiliou & Livieratos, 2000). Samad *et al*. (2008) recently reported the natural infection of *C. roseus* with an isolate of CMV in India.

In Malaysia, CMV has been reported to be present in many important economic crops (Mohamad Roff & Anang, 1989; Sidek & Sako, 1996; El-Sanousi, 1997) and weeds (Sidek *et al*., 1999). Although CMV infection on *C. roseus* has been mentioned elsewhere (Ong & Ting, 1977; Inon *et al*., 1999), information associated with the viral disease and its characterization has not reported. This paper describes the morphology, symptom and molecular characterization of CMV as the causal agent of mosaic disease on *C. roseus*.

MATERIALS AND METHODS

DAS-ELISA

Leaf extracts from the diseased and symptomless *C. roseus* plants growing under natural conditions in Serdang, Selangor were tested by DAS-ELISA as described by Clark and Adams (1977), following the procedure recommended in the diagnostic kit by the manufacturer (Bioreba, Switzerland). Antisera against cucumber mosaic virus (CMV), tobacco mosaic virus (TMV), tomato spotted wilt virus (TSWV) and impatien necrotic spot virus (INSV) were used in the assays to determine the presence of CMV, TMV, TSWV and INV. Briefly, each well was initially coated with 200 µl anti-virus IgG diluted in coating buffer (20 µl anti-virus IgG in 20 ml coating buffer). Plates were covered tightly and placed in humid boxes and incubated at 30ºC for 4 h. The contents of the wells were discarded and washed 3-4 times with washing buffer. The plates were blotted on paper towels to remove any liquid residue. The leafs were homogenized in an extraction buffer (0.05 g tissue in 1 ml buffer). Two hundred µl of the crude sap was loaded into each well and the plates were incubated at 4ºC overnight. The plates werethen subjected to washing before the addition of 200 µl enzyme conjugates (20 µl enzyme conjugate in 20 ml buffer) to each well. After incubation at 30ºC for 5 h, the plates were washed and loaded with substrate (p-nitrophenyl phosphate at 1 mg/ml in substrate buffer). The plates were incubated at room temperature in the dark. The ELISA reactions were read at 405 nm by using an ELISA reader (Thermolab System, USA) after 30-120 min incubation. All the samples were tested in duplicates and the average A_{405} values of more than twice compared to that of the healthy controls were considered as positive for virus detection.

Virus Isolate and Maintenance

C. roseus var. rosea plant, which exhibited mosaic symptoms and showed positive CMV detection in DAS-ELISA, was used as the source of virus isolate. The symptomatic leafs were ground in chilled phosphate buffer (0.01 M phosphate, pH 7.0, containing 0.25% DIECA) and carborundum (600 mesh) and the extract was rubbed on the leafs of healthy *Chenopodium amaranticolor* to obtain pure virus culture through three serial single-lesion transfers and the inoculum was maintained on *C. roseus*, *Nicotiana tabacum* and *N. glutinosa* for subsequent studies.

Virus Purification

The virus was isolated and purified from the primary leafs of the inoculated *N. tabacum* cv. Coker 254 harvested 15-20 days post-inoculation and kept at -80ºC prior to purification. The procedures of Scott (1963) were used with major modifications. Briefly, 100 g of infected leaf tissues were homogenized in 0.5 M sodium citrate buffer (pH 7.5) containing 0.005 M EDTA and 0.5% thioglycolic acid and filtered with 2

layers of muslin cloth. The filtrate was mixed with an equal volume of chloroform, stirred for 40 min before it was centrifuged at 9500x *g* for 10 min at 4ºC. The aqueous phase was collected and mixed with 10 % polyethylene glycol (PEG 6000). The mixture was then centrifuged at 8000x *g* for 20 min and the pellet was resuspended in 10 ml of 0.005 M sodium borate buffer (pH 9.0). The suspension was centrifuged at 9500x *g* to collect supernatant, followed by centrifugation at 139,000x *g* for 3 h at 4ºC to obtain the pellet. Suspension of the pellet in 2 ml of borate buffer was centrifuged at 9500x *g* for 15 min and the supernatant was layered onto a 10-50% sucrose density gradient in 0.5 M sodium citrate buffer prior to centrifugation at 185,000x *g* for 3 h. The virus band was collected and pelleted through high speed centrifugation at 139,000x *g* for 2 h and resuspended in 2 ml of borate buffer. The virus preparation was analyzed spectrophotometrically and the UV spectra values of $A_{260}:A_{280}$ and $A_{max}:A_{min}$ were determined. The virus yield per 100 g leaf tissues was calculated by assuming the extinction coefficient at 260 nm for CMV to be 5 (Francki *et al*., 1979). The purified virions were used for symptomatological studies of the test plants, morphological determination, virion RNA extraction and RT-PCR.

Morphological Determination of the Virions

A formvar-carbon coated copper grid was floated on a drop of purified virus for 5 min and subsequently stained with 2% uranyl acetate adjusted to pH4.2. The grids were examined under a transmission electron microscope (Phillips HMG 4000). The mean virion diameter was determined from the measurements of 138 virus particles at a magnification of 100,000x.

Symptomatological Studies

Four different plant families, grown in pots under insect proof condition, were mechanically inoculated with the purified virus at 1.2-1.5 mg/ml: *Solanaceae* (*N. tabacum* cv. White Burley, *N. glutinosa*, *N. benthamiana*, *Datura metel*, *Capsicum annuum* cv. MC11, *Lycopersicon esculentum* cv. MT1), *Chenopodiaceae* (*C. amaranticolor*), *Leguminosae* (*Vigna sesquipedalis* cv. MKP5, *Phaseolus vulgaris* cv. MKB1) and *Cucurbitaceae* (*Cucumis sativus* cv. local). Five plants were inoculated for each species and kept under observation for 2 months. Both the symptomatic and symptomless plants were recorded and checked for the presence of virus by back inoculation onto *C. amaranticolor* and *C. roseus*. The CMV infection on the test plants were confirmed by DAS-ELISA using the CMV antiserum.

Viral RNA Extraction and RT-PCR

Viral RNA was isolated from the purified virus using proteinase K and phenol-SDS procedures, as described by Sambrook *et al.* (1989). The virus was incubated at 50ºC for 20 min with an equal volume of RNA extraction buffer (0.02M Tris-Cl, pH 7.4, 0.03M KCl, 3 mM $MgCl_2$, 0.01M SDS) and 50 µl of proteinase K (2 mg/ml). After the addition of 80 µl of 1 M NaCl, the mixture was subjected to 2 times phenol extractions (50ºC) and 3 times chloroform: isoamylalcohol (24:1) extractions, and this was followed by the precipitation of the RNA with 3 M NaAc (pH 5.2) and cold 100% ethanol. The RNA was air-dried prior to resuspension in TE buffer (pH 8). Meanwhile, RT-PCR was performed using degenerate primers which were designed based on the conserved regions of CMV coat protein (CP) genes available in the GenBank. The upstream primer CMVF1 (5'-TAGACAT/ACTGTGACGCGA-3') and the downstream primer CMVR2 (5'-GTAAGCTGGATGGACAAC-3') were designed to amplify a region of about 1000bp in length covering complete CP region of CMV. The synthesis of cDNA was carried out with Reverase™ M-MuLV reverse transcriptase (BIORON, Germany), following the protocol recommended by the manufacturer. One µg of the viral RNA or 2.0 µg virions and 10 pmole of the downstream primer CMVR2 were used for reverse transcription reaction at 42ºC for 90 min. For PCR, 5 µl of the cDNA was used

as a template and this was proceeded to PCR using the following conditions: one cycle of denaturation at 94ºC for 3 min, 35 cycles of 94ºC for 1 min; 60ºC for 1 min; 72ºC for 1 min followed by one cycle of elongation at 72ºC for 10 min. The PCR product was electrophoresed on a 2.0% agarose gel in TAE buffer, stained with 0.5 µg/ml ethidium bromide, and viewed on a UV-transilluminator. One hundred bp extended DNA blue ladder (BIORON) was used as a standard marker.

Cloning and Sequencing of the Amplified PCR Product

The amplified product was gel-purified, ligated into T&A cloning vector and transformed into competent *E. coli* cells using TOPO TA Cloning kit (Invitrogen). The recombinant clones were identified using PCR and three clones were selected for sequencing. The obtained sequences were compared with the sequences from the GenBank through the BLAST programme of the National Centre for Biotechnology Information (NCBI) (Altchul *et al*., 1990).

RESULTS AND DISCUSSION

DAS-ELISA

All *C. roseus* plant samples used in the assays at flowering stage. Two kinds of viruses were detected throughout the assays (Table 1). Amongst 100 plant samples of *C. roseus* tested, 25 symptomless plant samples were determined to be positive for TMV, one plant sample with mild mosaic symptoms was CMV positive and one plant sample with severe leaf mosaic and deformed flowers were found positive for both TMV and CMV infections. 73 other samples reacted negatively against all antisera tested. For TSWV and INSV, the absorbance readings of the tested samples were as similar as the healthy controls, suggesting no occurrence of the viruses in the assays.

Table 2 shows the results of rescreening for CMV and TMV in the original diseased *C. roseus* and the inoculated plants. Rescreening on 5 original *C. roseus* plants which had initially detected positive TMV failed to detect the virus. Forty *C. roseus plants* inoculated with the crude leaf extract from the TMV positive plants also exhibited negative reaction against TMV antibody, whereas CMV was consistently detected in the original diseased *C. roseus* as well

TABLE 1
DAS-ELISA detection (absorbance at 405 nm) of viruses in *C. roseus* grown wild or cultivated in pots in Serdang, Selangor.

Total number of sample	Visual observation	Antisera[1]			
		CMV	TMV	TSWV	INSV
25	SL	0.131-0.151	0.339-0.523*	0.137-0.160	0.135-0.156
65	SL	0.114-0.151	0.138-0.310	0.132-0.232	0.132-0.157
1	MM, CB	0.276*	0.150	0.149	0.131
1	SM, DF	0.635*	0.538*	0.123	0.140
8	MM	0.114-0.216	0.170-0.309	0.139-0.175	0.084-0.144
PC	-	3.510	3.131	2.172	3.56
NC	-	0.114	0.165	0.134	0.177
BC	-	0.115	0.165	0.130	0.136
HS	-	0.114	0.156	0.141	0.150

[1]Asterisk (*) indicates positive reactions (greater than 2x negative mean). SL: symptomless; MM: mild mosaic; CB: colour breaking on petals; SM: severe mosaic; DF: deformed flowers; PC: positive control; NC: negative control; BC: buffer control; HS: healthy sap.

TABLE 2
Rescreening assay (absorbance at 405 nm) of CMV and TMV using DAS-ELISA in original diseased *C. roseus* grown under natural conditions and the inoculated plants grown in a glasshouse in Serdang, Selangor.

Total number of sample	Visual observation	Antisera[1]	
		CMV	TMV
7	SM, DF, CB	2.447-3.328*	0.195-0.396
1[a]	MM, CB	3.039*	NT
17[b]	SM, DF	2.447-3.483*	0.173-0.396
5[c]	SL	NT	0.310-0.374
40[d]	SL	NT	0.252-0.422
1[e]	SM, DF	3.102*	0.198
10[f]	SM, DF	2.233-3.324*	0.190-0.235
Positive control		3.076	3.158
Negative control		0.549	0.263
Buffer control		0.202	0.206
Healthy sap		0.222	0.212

[1]Asterisk (*) indicates positive reactions (greater than 2x negative mean). NT: not tested; SL: symptomless; MM: mild mosaic; CB: colour breaking on petals; SM: severe mosaic; DF: deformed flowers.
[a] Original diseased plant detected CMV positive in the first assay.
[b] Plants inoculated with leaf extract from original CMV positive *C. roseus* in the first assay.
[c] Original diseased plants detected TMV positive in the first assay.
[d] Plants inoculated with leaf extract from original TMV positive *C. roseus* in the first assay.
[e] Original diseased plant detected positive of TMV and CMV infection in the first assay.
[f] Plants inoculated with leaf extract from original TMV and CMV positive *C. roseus* in the first assay.

as in the inoculated *C. roseus* plants. For the *C. roseus* plant with mix infection, only CMV was consistently detected during the rescreening of the original plant and the inoculated plants. Very high absorbance readings of CMV detections were observed (>2.0) throughout rescreening DAS-ELISA, and this revealed the occurrence of the virus in very high concentration in the plants. The results of rescreening also confirmed the prevalence of CMV in diseased *C. roseus,* showing mosaic symptoms with malformed flowers or slight colour breaking on the petals. Failure to detect CMV in the plants with mosaic symptoms, as indicated in the first screening, was probably due to very low concentration of the virus, which was below the detection limit of the assays used. Based on the DAS-ELISA results, aetiology studies only focused on CMV. The naturally diseased *C. roseus* exhibited leaf mosaic and distortion, malformed flowers or colour-breaking on the petals (see *Fig. 1*).

Purification of the Virus

The virus was banded as single light-scattering zone at 2.4 cm depth from meniscus in a sucrose density gradient. The purified virus preparation exhibited a typical nucleoprotein absorption spectrum with a maximum and a minimum absorption at 258-260 nm and 240-245 nm, respectively. Meanwhile, the $A_{260}:A_{280}$ and $A_{max}:A_{min}$ ratios were calculated as 1.5 and 1.2, respectively. The values of $A_{260}:A_{280}$ and $A_{max} : A_{min}$ ratios for the purified virus were close to the values reported for other CMV isolates (Noordam, 1973; Srivastava *et al*., 1992; Sarma *et al.*, 2001). The differences in the values may be due to the impurities which present in the purified preparations. The virus concentration as calculated spectrophotometrically using an Extinction coefficient ($E^{0.1\%}$ 1 cm*)* at 260 nm = 5 (Francki *et al.*, 1979) varied from 1.2 mg/ml to 5.0 mg/ml per 100 g leaf sample. The purified virus preparation at 1.3-1.5 mg/

ml was found infectious when tested on *N. tabacum* and *C. sativus,* as well as on *C. roseus*. The symptoms developed on *C. roseus* were identical to the natural diseased *C. roseus*. The reproduction of the disease by inoculating healthy plants with plant sap and purified virus preparations confirmed the pathogenicity of the virus according to Koch's postulates (Rivers, 1937).

Morphological Determination of Virions

Plenty isometric particles with a central core were observed in negatively stained purified preparations diluted to 0.2 mg/ml (*Fig. 2*). No other virus particles were observed in the preparation. For size determination, the histogram represents the diameter distribution of the virus particles in the purified preparation

Fig. 1: Mosaic symptoms on leafs (A and B), flowers of the deformed shape (C) and slight colour breaking on the petals (D) of naturally-infected C. roseus were detected positive for CMV infection.

TABLE 3
Host range and symptomatology of cucumber mosaic virus isolated from *C. roseus*.

Test plants	Reactions	Days required for for symptom expression
Chenopodiaceae		
Chenopodium amaranticolor	LL	5-7
Cucurbitaceae		
Cucumis sativus	SM	4
Leguminosae		
Phaseolus vulgaris cv. MKB1	Neg.	Neg.
Vigna sesquipedalis cv. MKP5	NLL	3-5
Solanaceae		
Capsicum annuum cv. MC11	Neg.	Neg.
Datura metel	Neg.	Neg.
Lycopersicon esculentum cv MT1	Neg.	Neg.
Nicotiana benthamiana	SM, M, LD	10-13
Nicotiana glutinosa	C, SM	7-10
Nicotiana tabacum cv. White Burley	SM	10-15

SM: systemic mosaic; M: mottling; LD: leaf deformation; C: chlorosis; NLL: necrotic local lesion; LL: local lesions; SL: symptomless; Neg: negative reaction.

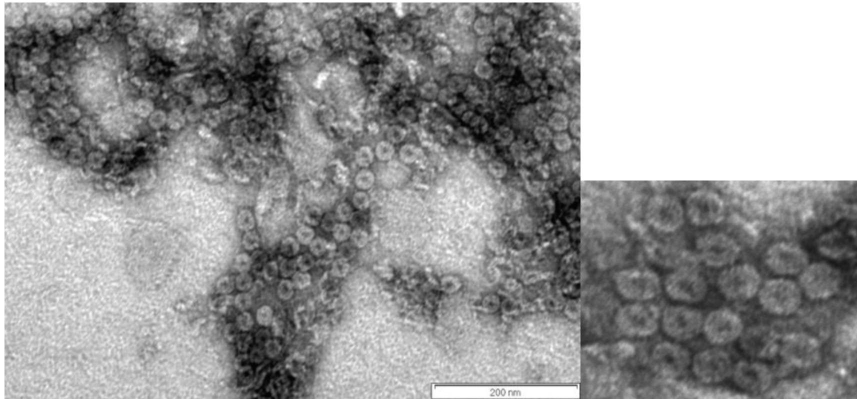

Fig. 2: Electron micrograph of negatively stained virus particles with 2% uranyl acetate. The inserted picture shows the particles. Bar = 200 nm.

TABLE 4
Percentage identity of nucleotide (nt) and predicted amino acid (aa) sequence between Malaysian CMV isolate (GenBank: EU726631) extracted from *C. roseus* and other published CMV isolates.

GenBank Accession number	Natural host	Sub-group	Location	% identities at the level of	
				nt	aa
EU310928	*Catharanthus roseus*	IB	India	100	100
EF593025	*Rauvolfia serpentine*	IB	India	98	99
EF593023	*Amaranthus tricolor*	IB	India	97	96
EF153733	*Chrysanthemum morifolium*	IB	India	97	95
AY545924	*Piper nigrum*	IB	India	95	99
AY965892	*Capsicum* sp	IB	China	93	97
AJ810264	*Cucumis sativus*	IB	Thailand	93	96
DQ070746	*Beta vulgaris*	IB	China	93	95
AM183119	*Lycopersicon esculentum*	IB	Spain	92	95
AY380533	*Chrysanthemum* sp	IA	Brazil	92	95
DQ295914	*Gladiolus* sp	IA	India	91	95
AJ810258	*Cucurbita* sp	IA	USA	91	94
AB109908	*Capsicum annuum*	II	Korea	76	78
EF202597	*Lycopersicon esculentum*	II	China	76	78
AJ242585	*Nicotiana* sp	II	China	76	78
EU642567	*Daucus carota*	II	India	76	76
EF424777	*Catharanthus roseus*	Not known	China	92	97
EF424778	*Catharanthus roseus*	Not known	China	92	97
AY376840	*Catharanthus roseus*	Not known	Brazil	92	95

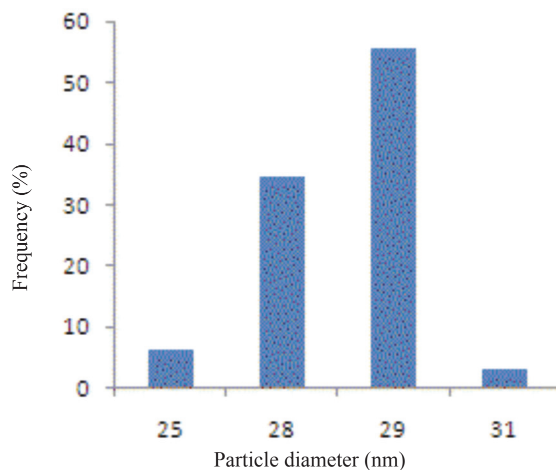

Fig. 3: Histogram showing the particle diameter distribution of C. roseus CMV in purified preparation observed under EM.

(*Fig. 3*) which shows the maximum number of particles with a modal diameter between 28 to 29 nm. The mean diameter of virions, which was determined from the measurements of 138 particles, was found to be 28.6 ± 0.48 nm. Meanwhile, the shape and size of the virions resembled CMV as described by Francki *et al.* (1979).

Symptomatology of the Test Plants

The virus was easily transmitted by mechanical means to selected test plants, while the symptoms induced varied according to plant species (*Fig. 4*). Details of the host range and symptomatology of the virus are given in Table 3. Inoculated *N. tabacum* cv. White Burley showed a systemic mosaic within 10-15 days post-inoculation. The top leafs of the inoculated *N. glutinosa* exhibited chlorosis within 7-10 days of post-inoculation, and this was frequently followed by mosaic symptoms. Systemic mosaic, mottling and leaf deformation were noted on *N. benthamiana* within 10-13 days post-inoculation. Severe leaf mosaic was induced by the virus four days after inoculation on *C. sativus* plants. Local lesions were observed on the inoculated leafs of *V. sesquipedalis* cv. MKP5 and *C. amaranticolor* within 3-5 days and 5-7 days post-inoculation, respectively, suggesting the plants as local lesion hosts for the virus. On the contrary, no symptom was observed on *C. annum* cv. MC11, *L. esculentum* cv. MT1, *D. metel* and *P. vulgaris* cv. MKB1. When back inoculated to the healthy seedlings of *C. roseus,* the leaf extracts of the symptomatic test plants produced similar mosaic symptoms as the natural diseased host. Back inoculation to *C. amaranticolor* from the test plants showed only susceptible hosts reproduced the symptoms on *C. amaranticolor.* Positive reactions with CMV antiserum in DAS-ELISA confirmed the CMV infection in the symptomatic test plants. The symptoms induced by the virus on the susceptible test plants were identical to those induced by a number of CMV isolates (El-Sanousi *et al*., 1997; Madhubala *et al*., 2005), even though no symptom was exhibited on *D. metel, L. esculentum* cv. MT1*, C. annuum* cv. MC11 and *P. vulgaris* cv. MKB1. Factors such as temperature, age of the test plants and the source of the virus inoculum may also greatly influence the symptomatology of the test plants studied.

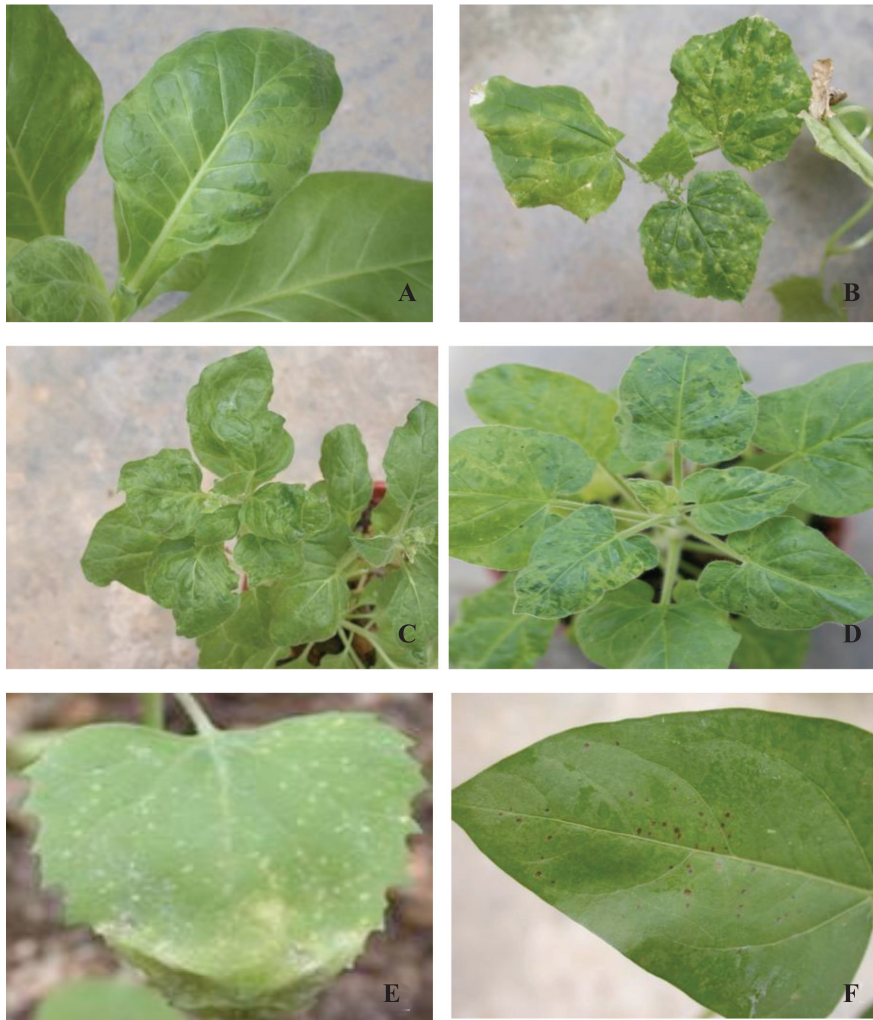

Fig. 4: The response of test plants following mechanical inoculation with the virus isolate. A) N. tabacum cv. White Burley; B) C. sativus; C) N. benthamiana; D) N. glutinosa; E) C. amaranticolor; F) V. sesquipedalis.

Sequence Analysis of CP Gene

The amplification of the CP gene of the virus isolate was successfully performed using RT-PCR on the viral particles and its RNA. A DNA fragment of 1000bp was amplified using the primers, CMVF1 and CMVR2 (*Fig. 5*). No amplicon was obtained in water control. The sequenced region was analyzed and confirmed to have a single open reading frame which comprised of 657 nucleotides potentially coding for 218 amino acids. The sequence obtained showed 92-100% sequence homology to the CP sequences of CMV isolates in the Genbank, confirming the identity of the virus. The local CMV CP gene sequence data was submitted to the GenBank (Accession number EU726631) and the database search was also performed. The sequence data revealed 100% nucleotide and amino acid identity to a CP gene of the CMV isolated from *C. roseus* in India (GenBank

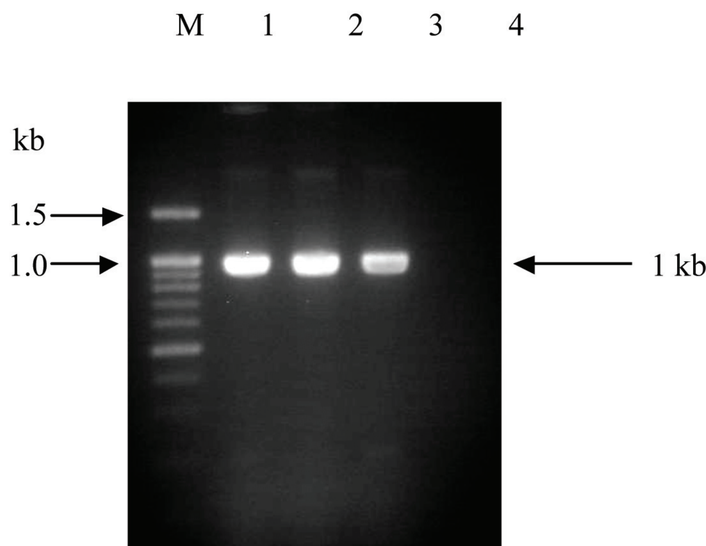

Fig. 5: Gel photograph of RT-PCR amplicons of 1000 bp using CMV RNA and its particle as a template. Lane 1, CMV RNA; lane 2, CMV partial purified particles; Lane 3, CMV purified particles; lane 4, water control; M, I00 bp blue extended DNA ladder (Bioron).

accession EU310928) which is a member of the subgroup 1B (Samad *et al*., 2008).

Meanwhile, the sequence analysis clearly indicated that the Malaysian CMV isolated from *C. roseus* belonged to sub-group 1 (with >90% sequence identity at nucleotide and >93% at amino acid levels) compared to sub-group II (<79% at both nucleotide and amino acid levels) (Table 4). A further analysis revealed that the Malaysian CMV isolate possesses higher sequence identity with subgroup IB strains, with nucleotide percent identity ranging between 92 and 100%. It has only 91-92% sequence identity with subgroup IA. At the amino acid level, the percentage of identity of the local isolate with IB members was higher (95-100%) as compared to those of IA members (95%).

Alignment of the deduced amino acids of the CP for this isolate with four other CMV of *C. roseus* from abroad showed unique differences at five positions (*Fig. 6*). The CP of this isolate and an Indian isolate (EU310928) are unique as they have threonine, arginine, lysine, valine and threonine residues at position 31, 76, 82, 172 and 193, respectively, compared to asparagine, lysine, arginine, alanine and alanine residues which were conserved in all other sequences. The existence of CMV isolates that are genetically related but occur in geographically distinct areas, as noted in this work, suggests that they may move together with infected plant materials between the countries.

CONCLUSIONS

The causal agent of the *C. roseus* mosaic symptom consists of virions (28.6 ± 0.48 nm in diameter) which are spherical in shape with a central core. It induces typical symptoms of CMV infection on various test plants and shows a positive reaction to CMV antiserum in DAS-ELISA. The coat protein (CP) gene sequence analysis revealed 100% sequence identity to the CP gene of *C. roseus* CMV isolated from India. The results of this study have revealed that the causal agent that induces mosaic symptoms on local *C. roseus* was an isolate of CMV. Meanwhile, the highest homology

AY376840 Brazil	MDKSESTSAG RNRRRRPRRG SRSAPSSADA NFRVLSQQLS RLNKTLAAGR PTINHPTFVG
E F424778 China	. . . . S . . . . . N . . . .
E F424777 China	. . . . S . . . . . N . . . .
EU726631 Malaysia	. . . . S . . . . . **T** . . . .
EU310928 India	. . . . S . . . . . **T** . . . .
	61
AY376840 Brazil	S E RCRPGYTF TS I TLKPP KI DRGSYYGKRL LLPDSITEYD KKLV SRI Q IR VNPLPKFDST
E F424778 China	. . . . K. . . . . K. . . . . R . . . . .V. . F . . . .
E F424777 China	. . . . K. . . . . K. . . . . R . . . . .V. . F . . . .
EU726631 Malaysia	. . . . K. . . . . **R**. . . . . **K** . . . . V. . F. . . . .
EU310928 India	. . . . K . . . . **R**. . . . . **K** . . . . V. . F. . . . .
	121
AY376840 Brazil	VWVTVRKVPA SS DLSVTAI S AMFADGASPV LVYQYAASGV QANNKLLYDI SAMRADIGDM
E F424778 China	. . . . T . . . . A . . . .
EF 424777 China	. . . . A . . . . A . . . .
EU726631 Malaysia	. . . . S . . . . **V** . . . .
EU310928 India	. . . . S . . . . **V** . . . .
	181 218
AY376840 Brazil	RKYAVLVYSK DDALETDELV LHVDIEHQRI PTSGVLPV
E F424778 China	. . . . A . . . .
EF 424777 China	. . . . A . . . .
EU726631 Malaysia	. . . . **T** . . . .
EU310928 India	. . . . **T** . . . .

Fig. 6: Amino acid sequence alignment of the coat protein gene of five CMV C. roseus isolates. The Malaysian CMV isolate from C. roseus is in bold. Identical residues are denoted as a dot. Five positions of amino acid sequence unique to Malaysian and Indian isolates are in bold and highlighted.

scored for both the nucleotide and predicted amino acid sequences of the CP region of the local and Indian CMV isolates of *C. roseus* suggest a similar virus origin. To the best of the researchers' knowledge, this is the first aetiology report of a natural mosaic disease symptom of *C. roseus* in Malaysia, which is supported with the nucleotide sequence analysis of the causal virus.

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REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol, 215*(3), 403-410.

Chattopadhyay, R. R., Sarkar, S. K., Ganguly, S., Banerjee, R. N., & Basu, T. K. (1991). Hypoglycemic and antihyperglycemic effect of leaves of Vinca rosea linn. *Indian J Physiol Pharmacol, 35*(3), 145-151

Chatzivassiliou, E., Livieratos, I., Jenser, G., & Katis, N. (2000). Ornamental plants and thrips populations associated with tomato spotted wilt virus in Greece. *Phytoparasitica, 28*(3), 257-264.

Clark, M. F., & Adams, A. N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology, 34*, 475-483.

Cragg, G. M., & Newman, D. J. (2005). Plants as source of anticancer agents. *Journal of Ethnopharmalogy, 100*, 72-79.

El-Sanousi, O. M. (1997). *Differentiation and genetic studies of several isolates of cucumber mosaic virus*. Universiti Putra Malaysia: PhD Thesis.

Espinha, L. M., & Gaspar, J. O. (1997). Partial characterization of CMV isolated from *Catharanthus roseus*. *Fitopatologia Brasileira 22*(2), 209-212.

Francki, R. I. B., Milne, R. G., & Hatta, T. (1979). *Cucumber mosaic virus. CMI/AAB Descriptions of Plant Viruses. no. 213*.

Ghosh, R. K., & Gupta, I. (1980). Effect of *Vinca rosea* and *Ficus racemososus* on hyperglycemia in rats. *Indian Journal of Animal Health 19*, 145-148.

Inon, N, S., Hamizah, I., Ramani, P., Yaakob, D., & Zakaria, S. (1999). Reactions of several *Capsicum* spp to two severe cucumber mosaic virus isolates. *5th International Conference on Plant Protection in the Tropics*. 15-18 March. Kuala Lumpur, Malaysia.

Loh, K. Y. (2008). Know the medicinal herb: *Catharanthus roseus* (*Vinca rosea*). *Malaysian Family Physician 3*(2), 123.

Madhubala, R., Bhadramurthy, V., Bhat, A. I., Hareesh, P. S., Retheesh, S. T., & Bhai, R. S. (2005). Occurrence of Cucumber mosaic virus on vanilla (Vanilla planifolia Andrews) in India. *Journal of Biosciences*, *30*(3), 339-350.

Manganey, P., Andriamialisoa, R. Z., Langlois, Y., Langlois, N., & Pottier, P. (1979). Preparation of vinblastine, vincristine and leurosidine: antitumor alkaloids from *Catharanthus* spp (Apocyanaceae). *Journal of American Chemistry Society, 101,* 2243-2245.

Mohamad Roff, M. N, & Anang, S. H. (1989). Virus diseases in Malaysian vegetables. *Teknologi Sayur-sayuran, 5*, 1-6.

Musa, Y., Azimah, A. K., & Zaharah, H. (2009). Kemunting cina. *In Tumbuhan Ubatan Popular Malaysia* (1st edn.), Kuala Lumpur: MARDI, 57.

Noordam, D. (1973). Spectrophotometry. In *Identification of Plant Viruses: Methods and Experiments,* 88-102.

Ong, A. C., & Ting, W. P. (1977). A review of plant virus disease in Peninsular Malaysia. *Tropical Agriculture Research Series no. 10*.

Rivers, T. M. (1937). Viruses and Koch's postulates. *Journal of Bacteriology 33,* 1-12.

Samad, A., Ajayakumar, V., Gupta, M. K., Shukla, A. K., Darokar, M. P., & Alam, M. (2008). Natural infection of periwinkle (*Catharanthus roseus*) with CMV subgroup 1B. *Australasian Plant Disease Notes 3*, 30-34.

Sarma, Y. R., Kiranmai, G., Sreenivasulu, P., Anandaraj, M., Hema, M., Venkatramana, M., Murthy, A. K., & Reddy, V. R. (2001). Partial characterization and identification of a virus associated with stunt disease of black pepper (*Piper nigrum*) in South India. *Current Science 80*(3), 459-462.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual,* 2nd edn. New York: Cold Spring Harbor Laboratory.

Scott, H. (1963). Purification of cucumber mosaic virus. *Virology 20,* 103-106.

Sidek, Z., Barin, J., & Sulaiman, I. (1999). Weed hosts of cucurbit viruses. *Agro- Search Research Bulletin 6(1)*, 1-3.

Sidek, Z., & Sako, N. (1996). Isolation of five viruses naturally infecting cucurbit plants in Malaysia. *Journal of Bioscience 7*, 114-121.

Singh, S. N., Vats, P., & Suri, S. (2001). Effect of an antidiabetic extract of *Catharanthus roseus* on enzymic activities in streptozotocin induced diabetic rats. *Journal of Ethnopharmacology 76*, 269-277.

Srivastava, K. M., Raj, S. K., & Singh, B. P. (1992). Properties of a cucumber mosaic virus strain naturally infecting chrysanthemum in India. *Plant Disease, 76*, 474-477.

Svoboda, G. H. (1983). The role of the alkaloids of *Catharanthus roseus* (L.) G. Don (*Vinca rosea*) and their derivatives in cancer chemotherapy. *Workshop Proceedings Plants: The Potentials for Extracting Protein, Medicines, and Other Useful Chemicals*. Congress of the United states, Office of Technology Assessment. Washington D.C. pp 154-169.

Van De Heijden, R., Jacobs, D. I., Snoejer, W., Hallard, D., & Verpoorte, R. (2004). The *Catharanthus* alkaloids: pharmacognosy and biotechnology. *Current Medicinal Chemistry 11,* 607-628.

Wiart, C. (2002). *Catharanthus roseus G. Don. In Medicinal Plants of Southeast Asia,* 2nd edn. Kuala Lumpur: Prentice Hall, 224-225.

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Evaluation of the Use of Farmyard Manure on a Guinea Grass (*Panicum maximum*) - Stylo (*Stylosanthes guianensis*) Mixed Pasture

Ahmed S. A., Halim, R. A.* and Ramlan, M. F.
Department of Crop Science,
Faculty of Agriculture, Universiti Putra Malaysia,
43400 UPM Serdang, Selangor, Malaysia
**E-mail: ridzwan@agri.upm.edu.my*

ABSTRACT

The effects of different levels of farmyard manure (FYM) and inorganic phosphorus (P) and potassium (K) fertilizer application on the physiology, forage yield and quality of Stylo (*Stylosanthes guianensis* cv. Pauciflora) – guinea grass (*Panicum maximum* cv. Green panic) pasture grown on an acid soil in Malaysia were evaluated in this study. The treatments were six rates of FYM application (0, 10, 20, 30, 40, 50 t FYM/ha and a combined application of 50 kg P with 50 kg K/ha) as triple-superphosphate and muriate of potash, respectively. Four consecutive cuttings were taken at 6-week intervals. Dry matter (DM) yield of guinea increased linearly with increasing levels of FYM with rates of increase from 239 to 457 kg/ha per tonne increase of the FYM applied. On the other hand, the DM yield of Stylo was found to decline with the increasing rates of FYM. Crude protein (CP) concentration of guinea increased linearly from 9.27% to 11.93% from 0 to 50 t FYM applications, while the CP concentration of Stylo increased from 17% to 20%. The photosynthetic rate, leaf area index and stomatal conductance of guinea significantly increased with the increasing rates of the FYM applied. On the contrary, acid detergent fibre and neutral detergent fibre concentrations of guinea declined with the increasing rates of the FYM used. Meanwhile, the use of inorganic P and K fertilizers gave a higher yield and higher quality of both the species compared to the control at the first cut but they were not significantly different from the control at the fourth cut.

Keywords: Forage, guinea, manure, organic, Stylo, quality, physiology, yield

INTRODUCTION

Shortage of feeds is one of the major problems that the ruminant industry is facing in Malaysia. In 2008 alone, the country spent more than RM2.14 billion (USD 621 million) to import animal feeds (Ministry of Agriculture and Agro-Industry, Malaysia, 2009). This method of ruminant production that is based on formulated concentrates is expensive, indicating that the production of ruminants has to rely on conventional green feeds or pasture and some substitutes. However, the productivity of pasture is low mainly due to the low soil fertility (Coulter, 1972). In Malaysia, Ultisols and Oxisols occupy about 72% of Peninsular Malaysia (IBSRAM, 1985). In these soil types, the efficiency of applied fertilizer is relatively low because the root uptake of nutrients is inhibited by the acidic pH of the soil (Baligar & Bennett, 1985).

Research on plant nutrition carried out on representative soil has shown that phosphorus deficiency is an important factor limiting legume establishment in sedentary soil (Oxisols and

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*Corresponding Author

Ultisols). Widespread deficiencies of nitrogen, potassium, and molybdenum were also recorded, while calcium, magnesium and sulphur are also deficient in some soil series (Tham, 1976; Tham & Kerridge, 1979). A grazing trial was conducted in Serdang, in which guinea and other grass species were evaluated in the soil fertilized at 150 and 300 kg N/ha/year. All the grasses produced had higher live weight gain when the fertilizer rate was increased to 300 kg N/ha/year (Chen *et al*., 1982). This finding indicated that the application of fertilizer is needed to improve the fertility of the soil and consequently, the productivity and quality of the pasture. Thus, the general recommendation is to apply inorganic fertilizers, including urea, to meet the nutrient requirements of the pastures. An alternative method is to make use of organic fertilizer, such as farmyard manure (FYM), because it is available locally and it can avoid pollution of water resources from leaching and runoff of inorganic fertilizer.

Since animal performance is directly related to forage quality, information is therefore needed on the effects of the FYM on forage quality. However, little information is available on the effects of the FYM application on forage quality, particularly on the fibre content. Studdy *et al.* (1995) reported that the total N of reed canarygrass was increased with the increasing rate of dairy manure. On the other hand, the applications of 112 and 336 kg N/ha from dairy manure and 112 and 336 kg/ha from ammonium nitrate fertilizer immediately after the first cutting of alfalfa were found to have no effect on the herbage N concentrations (Daliparthy *et al.,* 1994). The common viewpoint has been that manure should be applied to row crops or grasses rather than legumes since the latter can fix their own nitrogen. Klausner (1995) reported that when manure has to be applied, grasses should have the first priority because they are nitrogen accumulators.

Organic fertilizers, such as green manure, have been investigated for their effects on forage yield and they have shown promising results (Tham & Kerridge, 1982; Eng, 1983). Comparative studies on the effects of the rates and frequencies of animal manure application on forage quality have not previously been conducted. Therefore, the objective of this study was to evaluate the effects of various rates of FYM application on forage growth and quality of a Stylo-guinea mixed pasture.

MATERIALS AND METHODS

The experiment was conducted on acidic (pH5.5) ex-tin mine soil at the Faculty of Agriculture, Universiti Putra Malaysia. Each of the plots has an area of 4m^2. Seven fertilizer treatments were applied, namely control (zero fertilizer), 50 kg P and 50 kg K/ha, and 10, 20, 30, 40, 50 t FYM/ha, in four randomized complete blocks. The FYM application was done only once, i.e. before planting. Dairy cattle bedded pack manure from a covered barn was broadcast on the soil surface of each plot and immediately incorporated manually on 20 July 2002. The manure contained 10.5, 2.3 and 10.2 kg/t (wet basis) of the total N, P and K, respectively. *Stylosanthes guianensis* cv. Pauciflora and *Panicum maximum* cv. green panic were planted in 0.5 x 0.5 m spacing using rooted tillers, while Stylo was sown in between planting rows of guinea using seeds hand sown at the rate of 2 kg/ha.

Yield and Physiology

Photosynthetic rate (Pn) of the plants leaf was monitored using a LCA-3 portable infrared gas analyzer between 10 and 11 am. It was an open system used with Parkinson broad leaf cuvette with a leaf area of 6.2 cm^2. The leaf area index (LAI) was measured using LAI-2000 plant canopy analyzer for both Stylo and guinea during different stages of growth. The reading was taken separately for each species. The plots were harvested manually using a sickle, followed by fresh weight measurement for both the species. The samples were cut in 0.5 x 1.0 m quadrate, and the fresh weight of the shoot components was determined using an electronic balance. The plants were immediately enclosed in paper bags after they had been cut to prevent dehydration.

After weighing and determining their fresh weights, the plant parts were enclosed in paper bags and placed in an oven at 85°C for 72 hours. After that, the dried samples were re-weighed for dry matter determination.

Chemical Composition

The dried samples were ground and sieved (0.2 mm sieve) for the NDF and ADF analysis (Goering & Van Soest, 1970). The NDF is a measure of the cell wall constituent of the plant cell and is inversely correlated to the voluntary intake of feed by ruminants, while the ADF is the cell wall after removal of hemicellulose component and is inversely correlated with digestibility of forages (Van Soest & Robertson, 1980). Crude protein was determined by Kjeldahl digestion (AOAC, 1990) with concentrated sulphuric acid, followed by steam distillation and acid titration.

Statistical Analysis

The results obtained were subjected to an analysis of variance for a randomized complete block design, with the aid of SAS software (SAS, 2001). The differences between inorganic and organic fertilizer treatments were tested using the LSD, while a linear regression was fitted for the different levels of organic fertilizer used.

RESULTS

Dry Matter Yield

There was a significant linear increase in the dry matter yield of guinea, with an increasing rate of the FYM application at all harvests (*Fig. 1*). The highest rate of FYM (i.e. at 50 t/ha) produced the highest mean DM yield (10.05 t/ha). The treatment receiving inorganic fertilizer showed a yield that was equivalent to an application of 20 t/ha of FYM. The lowest DM yield of guinea was obtained from the control, where no fertilizer was applied (7.43 t/ha). During the second cut, the DM yield of guinea showed the same trend of a linear increase in the DM yield with the increasing rate of FYM applied (P<0.001). At the third and the fourth cut, the DM yield obtained at 50 t FYM/ha (7.94 and 7.04 t/ha/harvest, respectively) was significantly

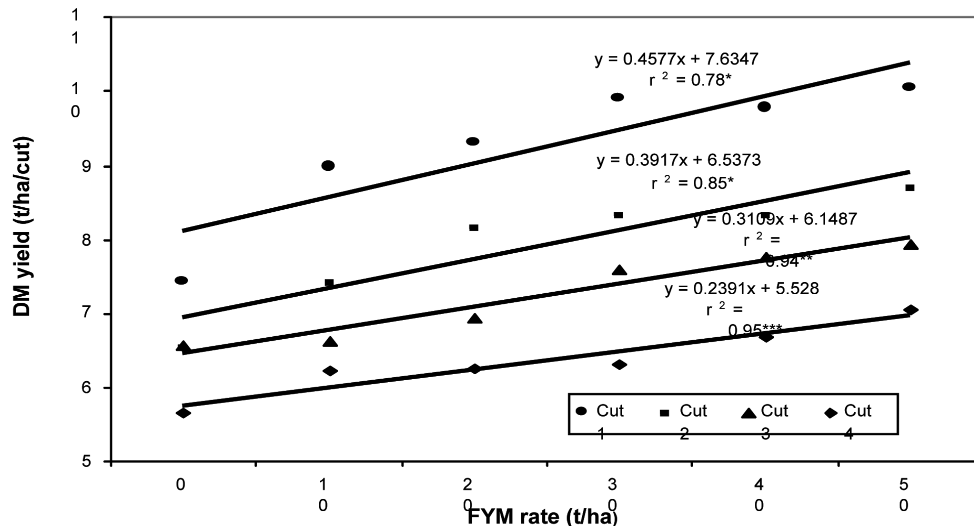

Fig. 1: Regressions of dry matter yield of guinea on the rates of farmyard manure (FYM) applications at four consecutive cuttings

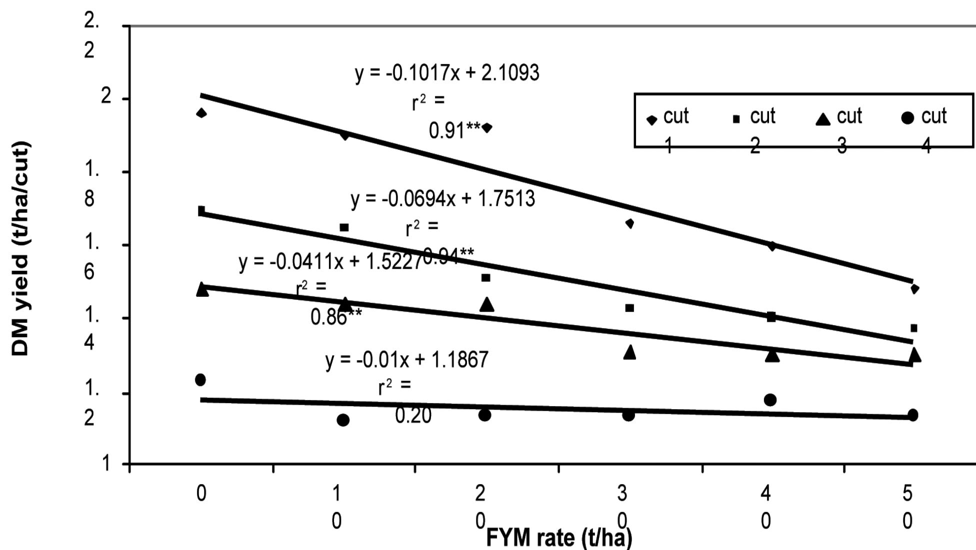

Fig. 2: Regressions of dry matter yield of Stylo on the rates of FYM application at four consecutive cuttings

higher (P<0.01) than those of the control and inorganic fertilizers.

As compared to guinea, Stylo showed a linear decrease in the DM yield with the increasing rate of FYM applied at the first three harvests (*Fig. 2*). However, at the fourth cut, Stylo yield was shown to be not affected by the FYM rates.

The total dry matter yield (sum of guinea and Stylo) showed a significant linear increase with the increasing rate of the FYM applied. This finding indicated that the decline in the Stylo yield was more than offset by increasing guinea yield with the increase in the FYM rates. Meanwhile, the use of inorganic fertilizer was shown to be equivalent to the application of the FYM at 10 t/ha in all the harvest (Table 1).

PHYSIOLOGY

Photosynthesis Rate

There was a significant linear increase in Pn of guinea with the increasing rate of FYM used (P<0.01) at both the first and the fourth cut. At the first cut, Pn increased from 24.7 to 32.0 µmol $m^2 s^{-1}$ from 0 to 50 t/ha FYM (*Fig. 3*). The Pn of guinea at 40 and 50 t FYM/ha was significantly higher than that of the inorganic fertilizer. The Pn of guinea was greater in cut one than in cut four. In contrast, there was a significant linear decrease in Pn of Stylo from 21 to 15 µmol $m^2 s^{-1}$, with the increasing rate of FYM applied (P<0.001) (*Fig. 4*).

Leaf Area Index

There was a significant linear increase in the LAI of guinea with the increasing rate of FYM application (p<0.001) (*Fig. 5*). Correspondingly, the LAI of Stylo declined with the increasing rates of the FYM used (*Fig. 6*).

Crude Protein (CP)

There was a significant linear increase in the CP concentration of guinea with the increasing rate of FYM application (P<0.01) (Table 3) and the value ranged from 9.27% (control) to 11.93% (50 t FYM/ha).

TABLE 1
Dry matter yield of total herbage at four cuttings

Treatment	Cut 1	Cut 2	Cut 3	Cut 4
	t ha^{-1} cut^{-1}			
50 kg/ha P, 50 kg/ha K	10.58	8.69	8.01	7.12
Control (0 FYM)	9.39	8.22	8.06	6.89
10 t/ha FYM	10.88	9.06	8.06	7.35
20 /ha FYM	11.22	9.67	8.37	7.37
30 t/ha FYM	11.56	9.77	8.90	7.46
40 t/ha FYM	11.37	9.72	9.06	7.86
50 t/ha FYM	11.52	10.06	9.24	8.17
$LSD_{0.05}$	0.65	0.58	0.34	0.22
R	0.81^{*}	0.90^{**}	0.97^{**}	0.96^{**}
Slope	0.36	0.32	0.27	0.23
SE	0.756	0.835	0.694	0.934

FYM= Farmyard Manure; P=phosphorus; K=potassium; LSD0.05=least significant difference at P<0.05; SE=standard error of mean; r=correlation coefficient (for linear regression of dry matter yield on FYM application rate *, ** regression significant at P< 0.05, 0.01, respectively)

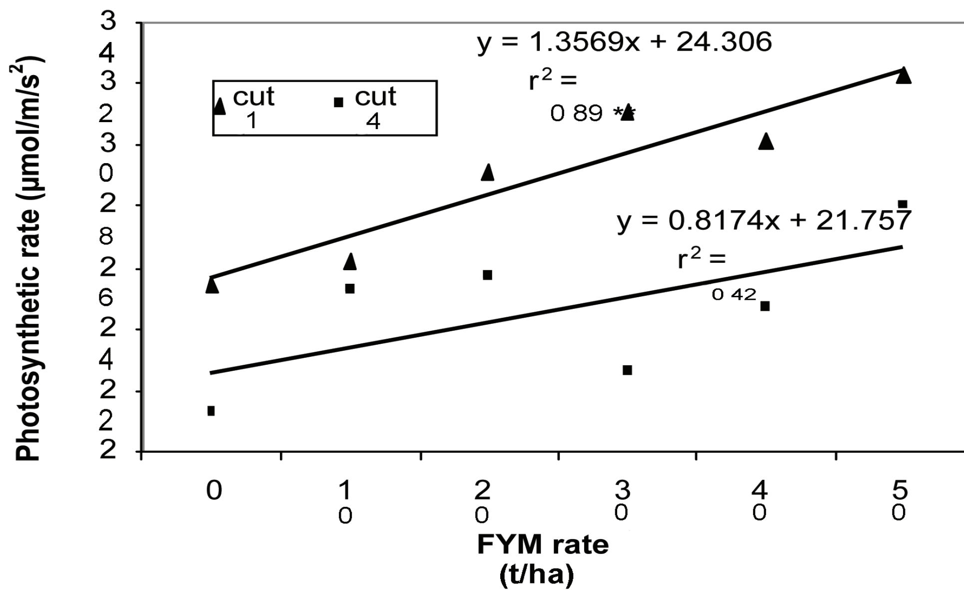

Fig. 3: Regressions of the photosynthetic rate of guinea on the rate of FYM application at the first and fourth cycles of vegetative growth

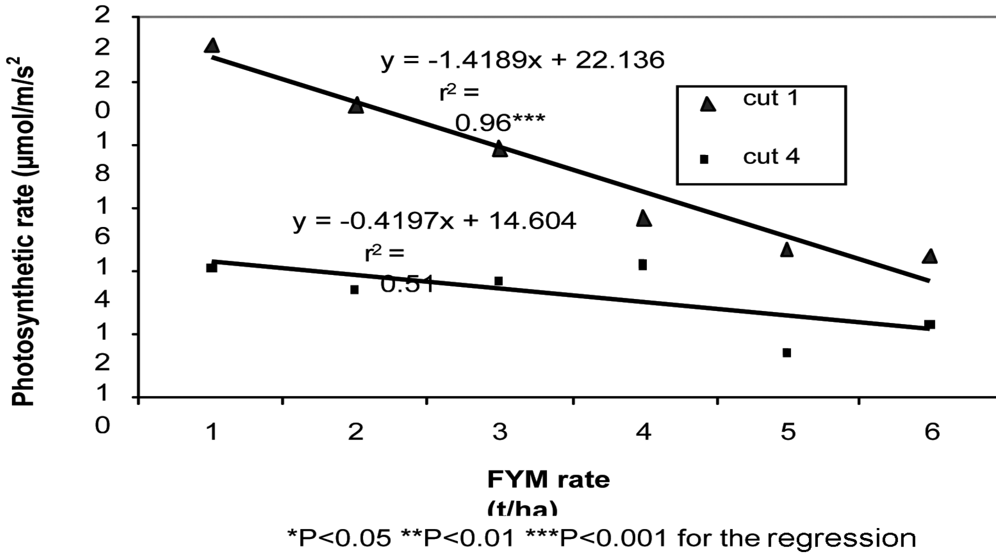

Fig. 4: Regressions of the photosynthetic rate of Stylo on the rate of the FYM application at the first and fourth cycles of vegetative growth

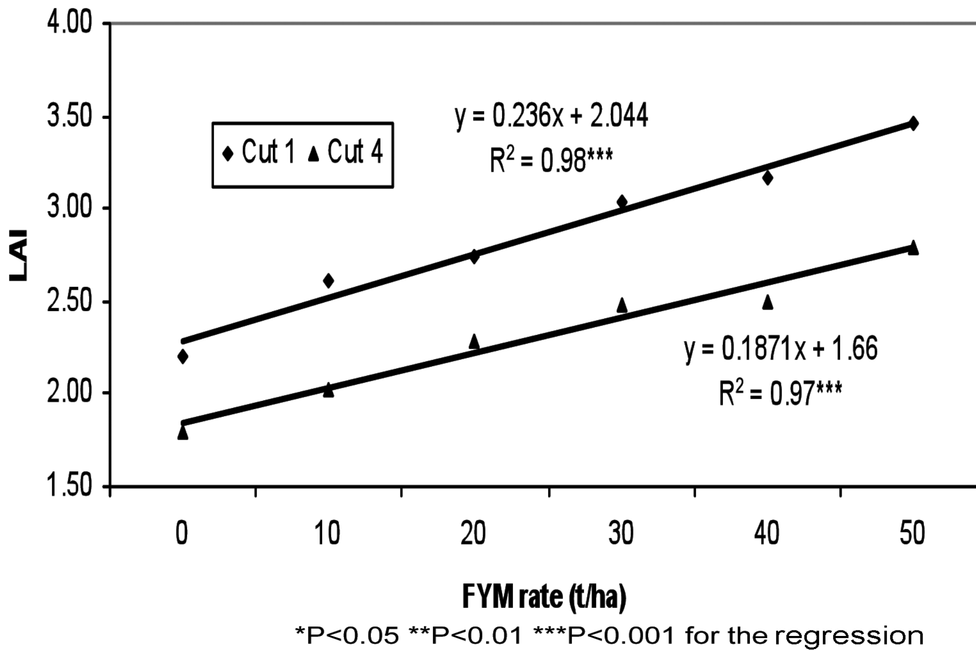

Fig. 5: Regressions of the leaf area index (LAI) of guinea on the rate of application of FYM at the first and fourth cycles of vegetative growth

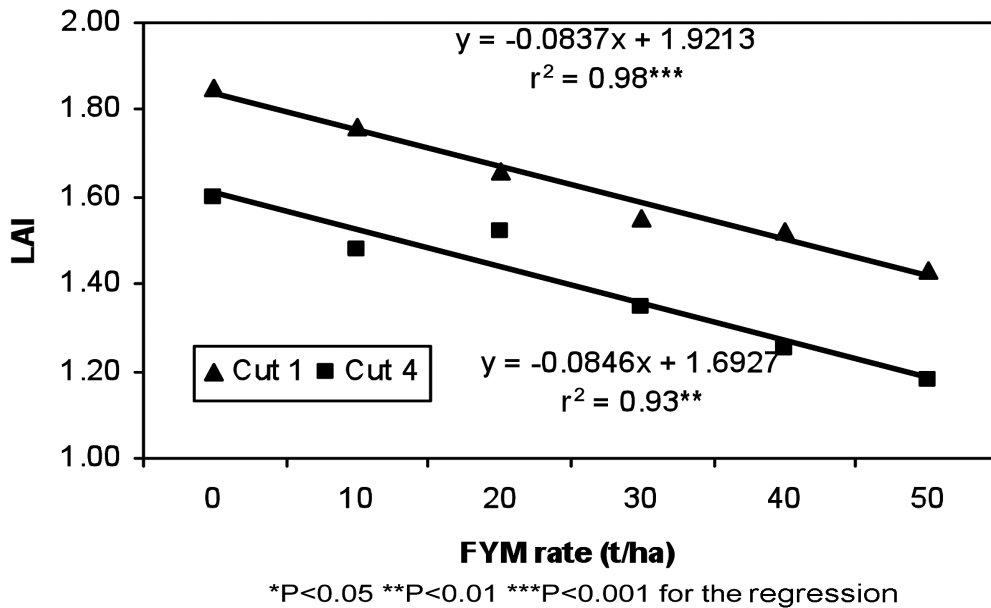

Fig. 6: Regressions of the leaf area index (LAI) of Stylo on the rate of application of FYM at the first and fourth cycles of vegetative growth

In Stylo, the CP concentration increased with the increasing rates of the FYM application for both the cuttings (Table 4). At the first cut, the CP increased from 17 to 21% and this was from 16 to 17% in the fourth cut.

Neutral Detergent Fibre (NDF)

There was a significant effect of the FYM on the fibre concentration of the pasture whereas the NDF concentration declined significantly (from 69.78 to 61.89 % for guinea, and this was 52.56 to 51.31% for Stylo) with the increasing rate of FYM application (Tables 3 and 4).

Acid Detergent Fibre (ADF)

The ADF concentration of guinea declined with the increasing rate of the FYM used (i.e. from 43.87 to 40.20 %). The results showed an increase in the ADF concentration from the first to the fourth cut (Table 3). The ADF concentration of Stylo, however, did not show any significant variation with the increasing rate of the FYM applied (Table 4).

DISCUSSION

The composition of guinea was found to have been affected by the input of plant nutrients which were available in the manure. This input might be substantial to maintain plant growth by improving leaf growth and photosynthesis. A similar result was also reported by Jarvis (1993) as a pasture land in the UK cycled and recycled N input through animal excreta alone.

The results obtained in this study showed the contribution of FYM on the growth of guinea. The fodder yield of guinea increased linearly with the increasing rates of the FYM applications for all the cuttings. The nutrient from FYM, particularly N, plays a major role in leaf growth via its involvement in cell division and as a primary component of enzymes for all the living systems and processes (Duru *et al.,* 1997). In addition, the FYM also increased

TABLE 2
Crude protein, NDF and ADF concentrations of guinea at cut 1 and cut 4 under different fertilizer treatments

Treatment	*Crude protein (CP) %*		*Neutral Detergent Fibre (NDF) %*		*Acid Detergent Fibre (ADF) %*	
	Cut 1	Cut 4	Cut 1	Cut 4	Cut 1	Cut 4
50 kg/ha P, 50 kg/ha K	11.60	8.76	67.99	72.00	44.22	46.31
Control (0 FYM)	9.27	8.74	70.93	74.03	45.62	46.58
10 t/ha FYM	9.96	8.89	69.78	71.41	43.87	45.93
20 /ha FYM	10.20	8.97	67.34	71.37	41.85	45.63
30 t/ha FYM	11.19	9.10	66.40	69.13	41.74	44.77
40 t/ha FYM	11.70	10.20	63.89	69.10	40.19	43.71
50 t/ha FYM	11.93	10.49	61.89	65.44	40.20	43.50
$LSD_{0.05}$	0.95	1.12	3.28	3.24	0.99	1.17
R	0.93^{**}	0.90^{**}	-0.99^{***}	-0.95^{***}	-0.95^{***}	-0.98^{***}
Slope	0.59	0.19	-1.82	-1.49	-1.09	-0.66
SE	0.402	0.377	0.888	1.478	0.551	0.461

FYM= Farmyard Manure; LSD0.05=least significant difference at P<0.05; SE=standard error of mean; r=correlation coefficient (for linear regression of quality variables on FYM application rate *, **, *** regression significant at P< 0.05, 0.01, and 0.001, respectively)

TABLE 3
Crude Protein, NDF and ADF concentrations of Stylo at cut 1 and cut 4 under different fertilizer treatments

Treatment	*Crude protein (CP) %*		*Neutral Detergent Fibre (NDF) %*		*Acid Detergent Fibre (ADF) %*	
	Cut 1	Cut 4	Cut 1	Cut 4	Cut 1	Cut 4
50 kg/ha P, 50 kg/ha K	19.02	16.34	52.48	55.84	37.36	39.09
Control (0 FYM)	17.44	16.16	52.62	56.32	38.74	39.62
10 t/ha FYM	17.50	16.30	52.55	56.42	37.07	38.45
20 /ha FYM	17.55	16.20	51.95	55.22	37.37	37.97
30 t/ha FYM	18.67	16.58	52.24	55.17	36.44	35.42
40 t/ha FYM	19.97	16.60	51.24	54.08	35.64	34.71
50 t/ha FYM	19.87	17.15	51.31	52.70	35.04	34.12
$LSD_{0.05}$	1.24	1.28	1.28	1.68	2.66	1.59
r	0.98^{***}	0.92^{**}	-0.89^{**}	-0.95^{**}	-0.69	-0.95^{***}
Slope	0.56	0.37	-0.52	-0.71	-0.38	-1.20
SE	0.65	0.514	0.75	0.561	2.93	0.536

FYM= Farmyard Manure; LSD0.05=least significant difference at P<0.05; SE=standard error of mean; r=correlation coefficient (for linear regression of quality variables on the FYM application rate *, **, *** regression significant at P< 0.05, 0.01, and 0.001, respectively)

both plant growth and photosynthesis directly. The increased rate of extension of leaves on the existing tillers in turn stimulates greater light capture and photosynthesis (Lemaire & Chapman, 1996; Gastal *et al.,* 1992). Nitrogen has also been shown to have the same positive effect on the tillering of tropical tufted species at the beginning of the growth stage (Coris, 1984;

Gastal *et al.,* 1992). The fact that the treatment using inorganic P and K fertilizers gave yields similar to the use of only 20 t/ha FYM indicated that N obtained from the legume fixation was less than that obtained from the higher rates of FYM application.

Plots which had a higher LAI were faster in sward growth. The increasing rates of FYM also increased with the rates of photosynthesis and a similar result was also reported by Bisoondat *et al.* (2002) who stated that the total annual forage DM yield increased from 21.0 t/ha at the low dairy manure level to 23.1 t/ha at the high manure level during Year 1, and similarly from 22.3 to 25.5 t/ha during Year 2.

Meanwhile, the use of inorganic fertilizer gave the CP concentration in grass of 11.6%, which was similar to that of 40 t FYM/ha at the first cut. However, the CP concentration of grass treated with inorganic fertilizer (8.76%) was equivalent only to the control at the fourth cut. This finding showed that the effects of inorganic fertilizer did not last long in terms of the CP concentration. The reason for the decline in the CP could probably be due to early availability of inorganic fertilizer to the plant, followed by rapid losses due to leaching or fixation in the later harvests.

In contrast to the response of guinea, high rates of the FYM application decreased the yield of Stylo. The shade effect of the grasses might have depressed the growth of legume through reduction in photosynthesis. Even during short periods of low light intensity, photosynthesis and the supply of materials for growth were also reduced (Volenec & Nelson, 1994). Stylo leaves developed in low light, under higher canopy of the grass, had a poorer photosynthetic capacity (*Fig. 4*). The same results have been reported by many researchers (see for example, Woledge, 1971; Prioul *et al.,* 1975). Leaves expanded in low light were thinner (Woledge, 1971), and they might have fewer stomata (Wilson & Cooper, 1969) and fewer mesophyll cells per unit leaf area, fewer and smaller chloroplasts, and reduced activities of RuBP carboxylase (Prioul *et al.,* 1980).

There was an overall decline of the DM yield from cut one to cut four and this might be due to the mineralization of FYM, the reduction in vegetative growth of the grass and the slow growth of the legume after cutting. Guinea dry matter yield was highly and positively correlated with the rates of FYM (*Fig. 1*). The applications of the manures at higher rates could therefore immensely improve fertility of slightly acidic soil and others with similar properties.

However, there have been contradictory reports on the effects of inorganic and organic fertilizer on fibre content of forage crops. Min *et al. (*2002) reported that the application of inorganic and organic fertilizers did not decrease the fibre content of grasses. In the present study, on the other hand, both the NDF and ADF of guinea were found to have decreased with the increasing rates of FYM applied. Similar results were also reported by Getnet and Inger (2001), as NDF and ADF contents of oats and vetches pasture slightly decreased with the increasing fertilizer rate. Valk *et al.* (1996) also reported that the undegradable NDF fractions decreased by using more N fertilizer. In addition, Cox and Cherney (2001) reported that the NDF and ADF contents of corn showed a negative linear-plus-plateau response to increased N fertilizer. Other researchers (Cox *et al.,* 1998) also reported a negative linear relationship of the fibre content with high rate of fertilizer application.

The effect of FYM on the fibre content of grass might depend on the rate of organic fertilizer application, soil type, climatic condition and plant species. Meanwhile, the effect of FYM on the soil properties, particularly on water retention characteristics, might cause less fibre accumulation by grass. On top of that, at a high rate of FYM application, vegetative growth of guinea was denser and less exposed to direct sunlight, and this in turn might be one of the reasons for the difference in the rates of lignification. The plants at higher FYM rate were in active growth as compared to other treatments.

CONCLUSION

The application of FYM resulted in the improvement of both the productivity and quality of guinea. The dry matter yield was significantly higher at high FYM rates. Unlike inorganic fertilizer, which showed a high CP concentration only in the first cut, the FYM consistently gave a high concentration of CP even at the fourth cut. This result demonstrates that the effect of FYM lasts longer than that of inorganic fertilizer. In addition, the FYM also gave a significant improvement on the mixed pasture by reducing the fibre content (NDF). The lowest values of NDF were obtained at a higher rate of application (50 t FYM/ha). This findings reveals that recycling nutrient in cattle-mixed pasture system could help maintain the quality of the feed. Meanwhile, the utilization of animal wastes as organic fertilizer would have a great potential to recycle the nutrients in cattle-pasture production system and this in turn could avoid dependence on chemical fertilizer. As shown in the present study, at a rate of 50 t/ha and above, FYM could be applied to improve grass productivity and quality.

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REFERENCES

AOAC (Association of Official Analytical Chemists) (1990). *Protein (crude) determination in animal feed: semi-automated method (976.06) Official methods of analysis.*

Baligar, V. C., & O. L. Bennett. (1985). Outlooks on fertilizer efficiency in the tropics. *Fertilizer Research 10*(1), 83-96

Bisoondat M., R. W. Kenneth, E. S. Lynn, C. F. Edwin, P. M. Kenneth, A. G. Donald., M. P. Gordon, & H. J. Harold. (2002). Dairy effluent effects on herbage yield and nutritive value of forage cropping systems. *Agronomy Journal,* 94, 1043-1049.

Chen, C. P., S. S. Ajit, & T. R. Evans. (1982). Beef production from three nitrogen fertilized grasses in Peninsular Malaysia. *MARDI Resource Bulletin*, 9, 207-224.

Coulter, J. K. (1972). Soils of Malaysia. A review of investigation on their fertility and management. *Soils and Fertilizer, 35*, 475-498.

Coris, M. (1984). Effects of nitrogen rates and harvesting intervals on dry matter production, tillering and quality of the tropical grass *Panicum maximum* Jacq. Dissertation Abstracts *International Sciences and Engineering*, *45*, 1-24.

Cox, W. J., & Cherney, D. J. R. (2001). Row spacing, plant density and nitrogen effects on corn forage. *Journal of Agronomy*, *93*, 597-602.

Cox, W. J., D. J. R., Cherney, & J. J. Hancher. 1998. Row spacing, hybrid, and plant density effects on corn silage yields and quality. *Journal of Production Agriculture. 11*, 128-134

Daliparthy, J., Herbert, S. J., & Veneman, L. M. (1994). Dairy Manure Applications to Alfalfa: Crop Response, Soil Nitrate, and Nitrate in Soil Water. *Agronomy Journal*, *86*(6), 927-933.

Duru, M., G. Lemaire, & P. Cruz (1997). The nitrogen requirement of Major agricultural crops: and grasslands. In Lemaire, G. (Eds.), *Diagnosis of N Status in Crops. Advanced Series in Agricultural Sciences* (pp. 59-72). Berlin, Springer-Verlag.

Eng, P. K. (1983). *Effect of phosphorus on pasture yields, botanical composition and live weight from guinea grass-mixture.* Paper presented at the 7th Malaysian Society of Animal Production Conference, Port Dickson, Malaysia

Gastal, F., C. J. Nelson, & J. M. Coutts, (1992). *Role of N on leaf growth of grasses: assessment of a root signal hypothesis*. Paper presented at the Annual Meeting of the American Society of Agronomy, Minneapolis, USA.

Goering, H. K., & Van Soest P. J. (1970). Forage fiber analysis (Apparatus, reagent, procedures and some applications). In *Superintendent of*

Document, Agriculture Handbook No. 378. US Department of Agriculture, Washington, DC, USA.

Getnet, A., & L. Inger (2001). Effect of variety, soil type and fertilizer on the establishment, growth, forage yield, quality and VI by cattle of oats and vetches cultivated in pure stands and mixture. *Journal Animal Feed Science and Technology. 92* (1-2): 95-111.

IBSRAM (1985). *Report of Inaugural Workshop and Proposal for Implementation of the Acid Tropical Soils Management Network*. Bangkok : International Board of Soil Research and Management, Bangkok.

Jarvis, S. C. (1993). Nitrogen cycling and losses from dairy farms. *Soil Use and Mangement 9,* 99-105

Klausner, S. D. (1995). *Nutrient Management: Crop Production and Water Quality*. Department of Soil, Crop, and Atmospheric Sciences, Cornell University, 95CUWFP1

Lemaire, G., & D. Chapman (1996). Tissue flows in grazed plant communities. In: Hodgson, J., & A.W. Illius, (eds.). *The Ecology and Management of Grazing Systems* (pp. 3-36). CAB International, Wallingford

Min, D. H., L. R. Voughb, & J. B. Reeves. (2002). Dairy slurry effects on forage quality of orchardgrass, reed canarygrass and alfalfa-grass mixtures. *Journal of Animal Feed Science and Technology, 95*(3-4): 143-157.

Ministry of Agriculture and Agro-Industry, Malaysia (2009). *Agriculture Statistical Handbook, 2008*. Malaysia, MARDI.

Prioul, J. L., A. Reyss, & P. Chartier (1975). *Relationship between carbon dioxide transfer resistances and some physiological and anatomical featyres*. In: R. Marcelle (Ed.). *Environmental and Biological Control of Photosynthesis*, 17-28. Junk. The Hague

Prioul, J. L., J. Brangeon, & A. Reyss, (1980). Interaction between external and internal conditions in the development of photosynthetic features in a grass leaf. I. Regional response along a leaf during and after low-light or high-light acclimation. *Plant Physiology, 66*, 762-769.

Tham, K. C. (1976). Nutrient response by pasture legumes on older rubber land soil of the Serdang series. *Malaysian Agriculture Journal 50*, 452-461.

Tham, K. C., & P. C. Kerridge (1979). Nutrient responded by *Stylosanthes guianensis* on coastal marine sands (Bris) of Peninsular Malaysia. *MARDI Res. Bull. 7*, 43-54.

Tham, K. C., & Kerridge, P. C. (1982). Response to lime, K, Mo, and Cu by grass-legume pasture on some Ultisols and Oxisols of Peninsular Malaysia. *MARDI Res. Bull., 10*, 350-369

Studdy, C. D., Morris, R. M., & Ridge, I. (1995). The effects of separated cow slurry liquor on soil and herbage nitrogen in *Phalaris arundinacea* and *Lolium perenne*. *Grass Forage Sci. 50*, 106–111.

Wilson, D., & J. P. Cooper, (1969). Effect of light intensity during growth on leaf anatomy and subsequent light-saturated photosynthesis among contrasting. Lolium genotypes. *New Physiologist, 68*, 1125-1135.

Woledge, J. (1971). The effect of light intensity during growth on the subsequent rate of photosynthesis in leaves of tall fescue (*Festuca arundinacea* Schreb). *Annals of Botany 35*, 311-312.

Valk, H., L. E., Kappers, & S. Tamminga (1996). In Sacco degradation characteristics organic matter and nutrient detergent fiber on fresh grass fertilized with different amount of N. *Journal of Feed Science and Technology, 63*(1-4), 63-67.

Van Soest, P. J., & J. B. Robertson (1980). Systems of analysis for evaluating fibrous feeds. pp. 49-60 In Pigden *et al*. (Eds.) *Standardization of Analytical Methodology for Feeds*. Proceedings of Workshop in Ottawa, Canada, 12-14 March 1979. International Development Research Center and International Union of Nutritional Sciences.

Volenec, J. J., & C. J. Nelson (1984). Carbohydrate metabolism in leaf meristems of tall fescue.II. Relationship to leaf elongation rates modified by nitrogen fertilization. *Plant Physiology, 74*, 595-600.

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Honey Hydrogel Dressing to Treat Burn Wound in Rats - A Preliminary Report

Rozaini Mohd. Zohdi[1], Md. Zuki Abu Bakar@Zakaria[1,3*], Norimah Yusof[4], Noordin Mohamed Mustapha[1], Muhammad Nazrul Hakim Somchit[2] and Asnah Hasan[3]

[1]Faculty of Veterinary Medicine,
[2]Faculty of Medicine and Health Sciences,
[3]Laboratory of Molecular Biomedicine, Institute of Bioscience,
Universiti Putra Malaysia, 43400 Serdang,
Selangor Darul Ehsan, Malaysia
[4]Division of Agrotechnology & Bioscience,
Malaysian Nuclear Agency, Bangi, 43000 Kajang,
Selangor Darul Ehsan, Malaysia
[*]E-mail: zuki@vet.upm.edu.my

ABSTRACT

Various studies have shown that honey is effective in healing burns and wounds. In this study, Malaysian honey was incorporated into hydrogel dressing formulation using electron beam irradiation technique and introduced as Honey Hydrogel dressing. The wound healing efficacy of Honey Hydrogel dressing on deep partial thickness burns was monitored on the basis of gross appearances, rate of wound contraction and histopathological changes. Deep partial thickness burns were created by applying an aluminium template preheated to 85°C to the backs of rats for 5 s and randomly treated with Honey Hydrogel or hydrogel while control group received no treatment. Wound appearance was photographed and the rate of wound contraction was calculated at 7, 14, and 21 days post burn. Rats were euthanized after 21 days of treatment and skin samples were taken for histopathological examination. The wounds treated with Honey Hydrogel dressing showed better gross appearances and significantly ($p<0.05$) enhanced the rate of wound contraction as compared to the control group at 21 days post burn. Faster epithelialization was also seen in the Honey Hydrogel treated group as compared to the other groups, although this was not statistically significant. The results substantiate the potential efficacy of Honey Hydrogel in accelerating burn wound healing.

Keywords: Honey, hydrogel, burn wound healing, wound contraction, rats

INTRODUCTION

Major injuries such as in thermal burns resulting in extensive damage to the skin necessitate immediate coverage to aid repair and regeneration to restore normal skin function (Cuttle *et al.,* 2006). Different therapies that affect burn wound repair have been proposed over the last few decades due to technology and strategic advances in the biomedical field (Branski *et al.,* 2009). Recently, however, there has been a surge of interest in the use of alternative therapies and natural remedy among modern societies, mainly due to its potential to improve healing and reduce the financial burden at the same time (Salmah & Sidik, 2005; Davis & Perez, 2009).

Honey is the nectar and saccharine exudation of plants gathered, modified and

[*]Corresponding Author

stored by the honey bee (Molan, 2000). It is one of the oldest and most enduring materials to be used in managing wound (Molan, 1998). It may be used alone or in combination with other substances (Salmah & Sidik, 2005) and has been administered both topically and systemically (Suguna *et al.,* 1992). Much of the effectiveness of honey in many of its medicinal uses is attributed to the antibacterial activity and antioxidant properties (Molan, 1999).

Topical application of Malaysian honey has also been reported to be effective to treat burn and wound in rats (Rozaini *et al.,* 2005; Aljady *et al.,* 2000). However, rapid clearance from the wound site occurred as honey tends to flow out from the wound area, making it difficult to maintain therapeutic concentration over prolonged period of time. In order to further enhance the use of honey in wound management and for easy handling, local honey was incorporated into hydrogel dressing formulation, cross-linked and sterilized using irradiation technique. The present study reports on the preliminary evaluation of Honey Hydrogel dressing in improving the outcome of burn wound by monitoring the morphological changes of the burn wound tissue healing after the treatment with Honey Hydrogel.

MATERIALS AND METHODS

Honey Sample

Local monofloral *Apis mellifera* honey from the floral source of *Melaleuca spp.* (Gelam) trees was used in this study. The honey was supplied by the Department of Agriculture Malaysia through Malaysian Nuclear Agency (Nuclear Malaysia) and was irradiated with 25 kGy gamma irradiation using radioactive source Cobalt 60, with the dose rate of 2 kGy per hour for sterilization purposes at MINTec-Sinagama, Malaysian Nuclear Agency (Model JS 8900). Dosimetry was performed using ceric/cerous sulphate solution and analyzed by potentiometrics.

Preparation of Honey Hydrogel Dressing

Polyvinyl pyrolidone (PVP) with a molecular weight of 1.2 x 10^6 (Kollidon 90) and Polyethylene glycol (PEG) with molecular weight of 400 were obtained from BASF, Ludwigshafen, Germany. Technical grade agar was supplied by Oxoid. The mixture, containing 15% PVP (Kollidon 90), 1% Protein Free Agar solution, and 1% PEG, was added with 6% honey (Yusof *et al.,* 2007). The mixture was poured into plastic molds (5 cm in diameter; 3-4 mm in thickness), and left to set at room temperature (37°C) before it was covered with polyethylene sheet and individually packed. The gels were cross-linked and sterilized by electron beam at 25 kGy at Alutron Irradiation Facility, Malaysian Nuclear Agency (Model EPS-3000, conveyer speed of 4.4m/minute, beam current of 10mA and energy of 3MeV).

Study Design

A complete randomized design was used to determine the efficacy of Honey Hydrogel dressing to treat deep partial thickness burns in rats. The experimental protocol was approved by the Animal Care and Use Committee (ACUC) at the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) (Reference No: 08R36/July 08-Jun09).

Animals

In this study, a total of 18 male Sprague-Dawley rats (weight 200-300 g) were used and they were randomly divided into three experimental groups of 6 rats each. The sample size was designed to minimize the number of animals required, which was still adequate to generate statistical analysis. The animals were acclimatized to the laboratory conditions for one week prior to the onset of experiment. The rats were individually caged and given commercial pellet and water *ad libitum* throughout the study.

Skin Preparation

Rats were anaesthetized with an intramascular (IM) injection of ketamine (50 mg/kg) and xylazine (5 mg/kg) into the caudal thigh muscle. Under anaesthesia, the back and flank of both sides of the body were shaved. Following this procedure, rats were returned to their cages for 24 hours to allow any oedema caused by the shaving procedure to recede.

Thermal Source

A method described by Kaufman *et al.* (1990) was used with modification. Cylindrical aluminium templates (2.5 cm diameter × 3 cm length, a handle measuring 24 cm, and total weight 400 g) were heated in a water bath at a constant temperature of 85°C for 3 hours prior to inflicting burn areas on the skin of the rats. Five templates were heated simultaneously, used alternately, and then returned to the water bath to ensure maintenance of the desired temperature of the template surface. There was approximately 5 minutes elapsed between each use of a template.

Burn Lesions

Rats were again anaesthetized with an IM injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The anaesthetized rat was positioned in sternal recumbency, restrained and stretched on a metal stage. The location of the burn was marked between the last ribs and the horizontal line of the sacroiliac joints. Deep partial thickness burn was inflicted on the dorsal part of the rat between the last thoracic vertebra and the first sacrum by placing the heated and moistened template at the right angles perpendicular to the dorsum of the rat on the pre-marked location for 5 seconds, using an analogue stopwatch. Minimal and constant pressure was applied to ensure a perfect contact between the template surface and the skin. The shaved skin was smoothened to ensure sufficient contact and uniform pressure over the entire lesion.

Treatment Protocol

Approximately 15 minutes after wound creation, all wounds in the treatment groups were dressed with Honey Hydrogel or hydrogel followed by OpSite® film dressing (Smith and Nephew, Hull, England) as secondary dressing. The dressings were held in place by wrapping the whole trunk with sterile gauze and plastered with Leukoplast® (BSN Medical, Pinetown, SA). Leukoplast® was applied to firmly affix the dressings to the animal's skin. Every 7 days, wounds were redressed with fresh hydrogel or Honey Hydrogel, while the rats were under anaesthesia. The secondary dressing and the hydrogels were removed and the wounds were flushed with sterile saline to remove debris and to clean the wound area. Sterile techniques were utilized when changing the dressings to minimize infection by pathogens to the wound site. Once the wounds have been analyzed, fresh dressings were placed on the wounds. Control group did not receive any treatment.

Assessment of the Wound

The progress of burn wound healing was recorded at 0, 7, 14 and 21 days post-burn. All the wounds were digitally photographed in the presence of a standard reference ruler. The wound area was measured immediately by placing a transparent tracing paper over the wound and tracing it. The tracing paper was placed on a 1 mm^2 graph sheet and traced accordingly. The squares were counted and the area was recorded. The wound area was assessed by the same blinded observer.

Histopathological Analysis

Rats were euthanized at day 21 post-burn by halothane inhalation and the skin samples were taken for histopathological examination. The skin samples were fixed in 10% formalin solution and embedded in paraffin. Tissue sections of 4-5 μm thickness were cut, stained with haematoxylin and eosin (H&E), and examined under light microscope. Digital photomicrographs were

captured at representative locations using a digital camera attached to a Nikon Eclipse FX-35DX microscope.

Statistical Analysis

Data are expressed as mean ± Standard Deviation (S.D). The statistical analysis of data was performed using two-way ANOVA using the SPSS® Statistical package (SPSS, Version 10.0, Chicago, Illinois, USA). The effects with $P<0.05$ were considered statistically significant.

RESULTS

Measurement of the Wound Size

On days 7 and 14 post-burn, there were no significant differences ($p>0.05$) observed in the wound area measurement between all the experimental groups (Table 1). On day 21 post-burn, nevertheless, the wound area measurement showed a significant ($p<0.05$) reduction in the wound size of the treated group as compared to the control with Honey Hydrogel dressing, showing the lowest mean of the wound size (60.8±2.2), followed by the hydrogel treated wound (80.8±2.6). In addition, the wound size of the untreated control group was significantly

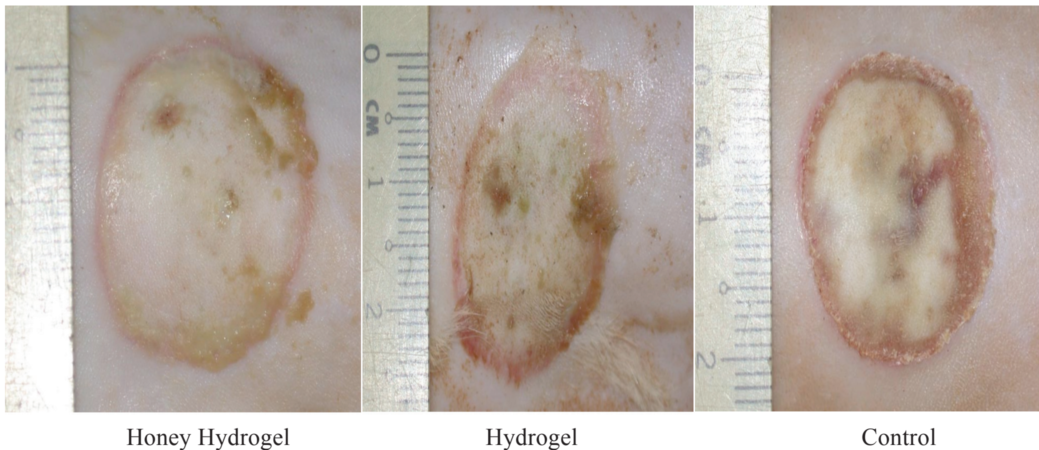

Fig. 1: General appearance of the wound sites in all the experimental groups at day 7 post-burn. Burned skin started to exhibit moist, intact, soft and supple yellow-brown discoloration in the treated groups, while the control group exhibited dry, intact, light-dark brown discolouration.

TABLE 1
Measurement of the wound sizes (mm^2) of the control and the treated groups at 0, 7, 14 and 21 days post-burn. Results are shown in mean ± standard deviation (S.D). *$P<0.05$ compared with the control group.

Measurement of the wound area (mm^2) (mean±S.D)				
Groups	0	7	14	21
Control	430.4±3.26	400.4±3.2	285.6±1.3	149.6±3.6
Honey Hydrogel	427.2±1..03	388±1.33	258.8±5.4	60.8±2.2*
Honey	423.3±2.23	388.0±3.1	272.3±1.7	80.8±2.6*

($p<0.05$) higher at day 21 post-burn with 149.6±3.6 mm². No significant difference was observed in the treated groups at 21 days post-burn, although there was a trend towards improvement in the Honey Hydrogel treated group.

General Appearance of the Wound

Gross changes in the general appearance and the size of burn wounds were monitored at 7, 14 and 21 days post-burn by capturing digital images of each animal. Towards the end of the first week (day 7 post-burn), the wounds in both the treated groups exhibited moist, soft and supple, yellow-brown discolouration, with red rim around the lesion, while the control group was covered with dry scab (*Fig. 2*). After 14 days, the treated groups still exhibited moist appearance with soft, yellow-brown lesions, while the control wounds were covered with dry, intact, dark brown-coloured scab (*Fig. 3*). The best healing was seen in Honey Hydrogel treated wounds after 21 days of post-burn with the wounds getting considerably smaller, while the control wound still exhibited dry appearance with dark brown scab (*Fig. 4*).

Histopathological Study

After 21 days of injury, epidermal regeneration was observed in all experimental wounds. Histopathologic comparisons showed that on day 21, Honey Hydrogel treated wounds resulted in better re-epithelialization as compared to the control and hydrogel treated rats. In addition, the inflammatory cells were absent in both treated wounds. In the untreated control wounds, though new epithelium was noted to regenerate, inflammatory cells particularly neutrophils and macrophages were still present on the upper dermis. Less scab formation was seen in the wounds treated with Honey Hydrogel as compared to the hydrogel and untreated control wounds (*Fig. 5*).

DISCUSSION

Burn wounds lead to a loss of integrity of the skin and have complex healing process. Wound healing proceeds through an overlapping pattern of events consisting of inflammation, proliferation and tissue remodelling. A number of studies have indicated that honey is a potential agent for wound healing, mainly due to its antibacterial, anti-inflammatory and antioxidant

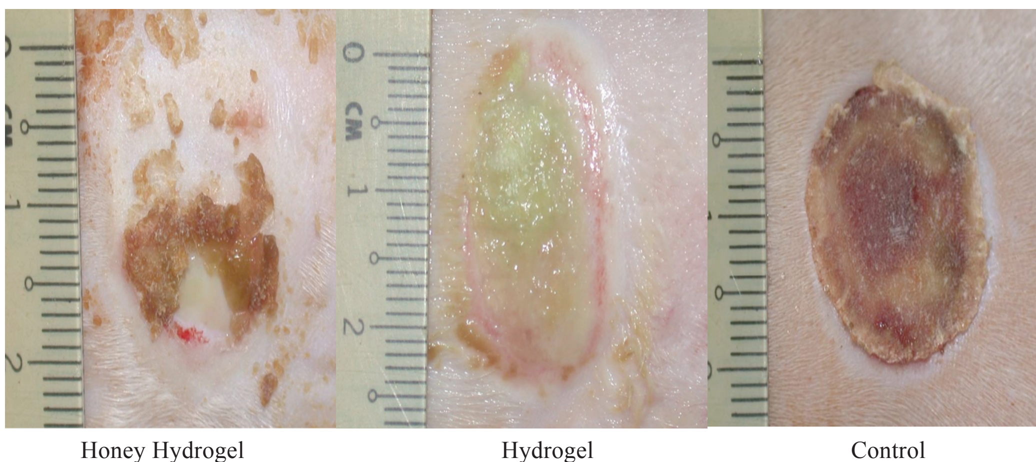

Fig. 2: General appearances of the wound sites in all the experimental groups at day 14 of post-burn. The wound treated with Honey Hydrogel showed better healing with scab started to slough off while hydrogel treated wound exhibited moist appearance with soft, yellow-brown discolouration. However, the untreated control wound was covered with dry, intact, dark brown discoloured scab.

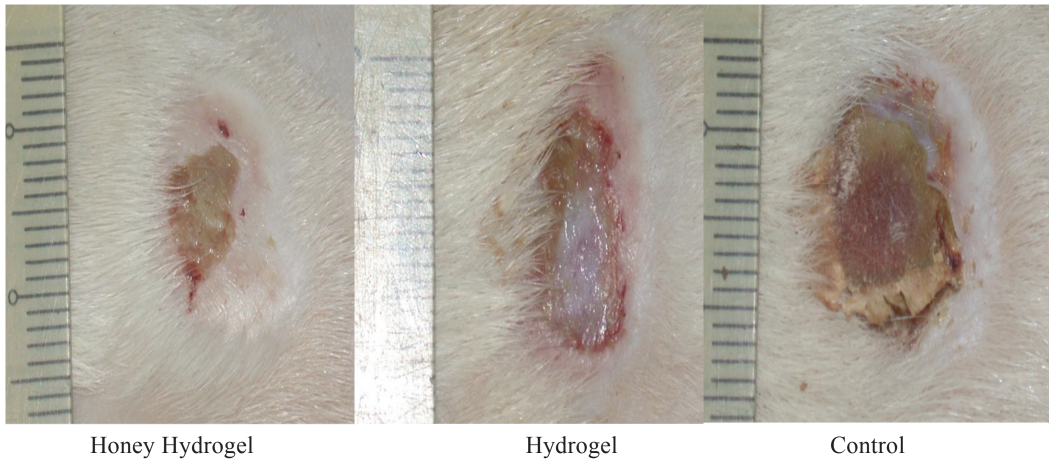

Fig. 3: General appearance of the wound sites in all the experimental groups at day 21 post burn. Better sign of healing in the wound treated with Hydrogel Honey with wound getting considerably smaller. However, the control wound still exhibited dry appearance with dark brown scab.

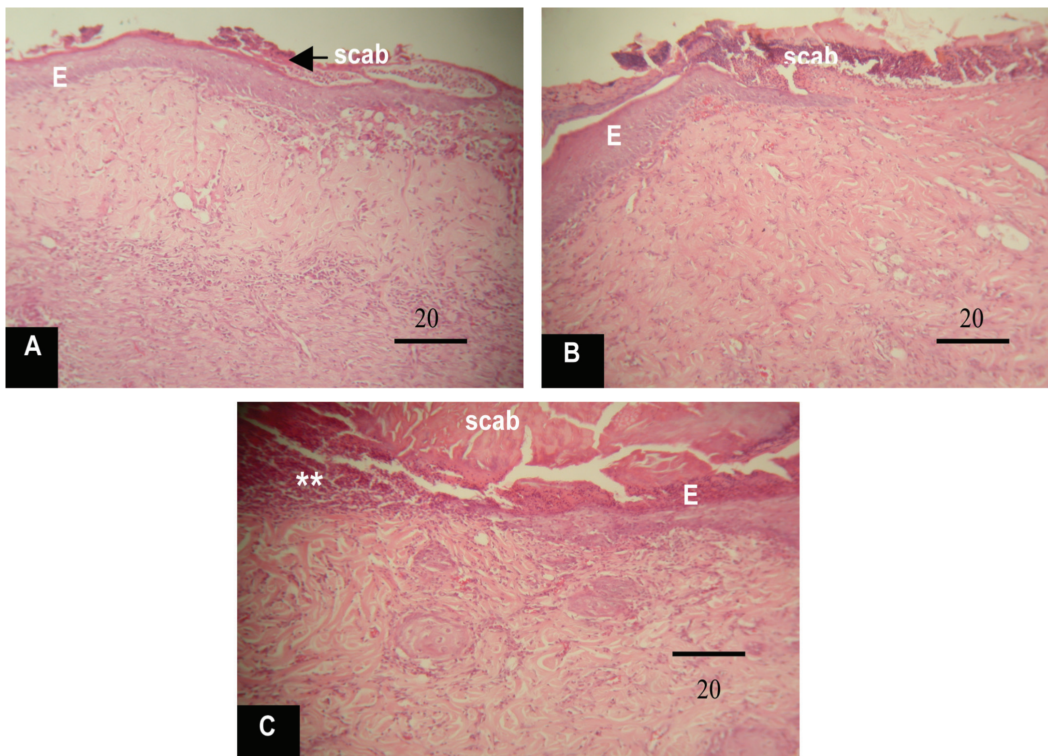

*Fig. 4: Representative micrographs of the histological sections at 21 days of post-burn stained with H&E. Note the advanced epidermal regeneration in the wound treated with (A) Honey Hydrogel. Marked inflammatory response still persists in (B) hydrogel (C) control group. (E=epidermis, **=inflammatory cells; 40x mag.). Bars on the photomicrograph represent 20 µm.*

properties (Molan, 2000; Subrahmanyam, 1998). Recent advances in radiation have resulted in the ability to incorporate honey into the hydrogel matrix for better handling. The present study showed that topical application of Honey Hydrogel accelerated the rate of wound healing as demonstrated by the increased rate of wound closure and better cosmetic appearances. Meanwhile, the histopathological evaluation of the wound site also provided evidence that Honey Hydrogel stimulated the healing process by reducing the inflammatory response and enhancing re-epithelialization.

One of the goals of wound therapy is to reduce excess inflammatory responses (Cho *et al.,* 2003). Honey has been reported to reduce inflammation when it is applied to wounds (Subrahmanyam, 1998); its anti-inflammatory activity may be associated with the antioxidant property of honey which is responsible to scavenge free radicals involved in various aspects of inflammation. Aljadi and Kamaruddin (2004) reported that honey has antioxidative and radical scavenging properties, which are mainly due to its flavonoids and phenolic constituents.

Re-epithelialization was also found to have remarkably advanced in the Honey Hydrogel treated wounds as compared to other groups. Meanwhile, the nutrient contents of honey, such as laevulose and fructose, improved the local substrate supply and helped in promoting epithelialization and tissue growth (Subrahmanyam, 1998). The application of Honey Hydrogel also kept the wounds moist and it has been proven that keratinocytes migrated more easily over a moist wound surface than that underneath a dry scab (Winter and Scales 1963). Furthermore, Honey Hydrogel dressings may be clinically easier to use, maintaining shape and consistency on or within the wound cavity.

CONCLUSION

The current study has shown that Honey Hydrogel is effective in healing burns, although not statistically more effective than hydrogel. Nonetheless, perhaps statistical significance would be reached if the study period was prolonged and the wounds had been followed longer, as there was clearly a trend to greater improvement in the Honey Hydrogel treatment compared to hydrogel. Further studies on the cellular and molecular mechanism of Honey Hydrogel were carried out.

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REFERENCES

Aljadi, A. M., & Kamaruddin M. Y. (2004). Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chemistry, 85*, 513-518.

Aljadi, A. M., Kamaruddin, M. Y., Jamal, A. M., & Mohd.Yassim, M. Y. (2000). Biochemical study on the efficacy of Malaysian Honey on inflicted wounds: an animal model. *Medical Journal of Islamic Academy of Sciences, 13*(3), 125-132.

Branski, L. K., Gauglitz, G. C., Herndon, D. N., & Jeschke, M. G. (2009). A review of gene and stem cell therapy in cutaneous wound healing. *Burns 35*, 171-180.

Cho, M. K., Sung, M. A., Kin, D. S., Park, H. G., Jew, S. S., & Kim, S. G. (2003). 2-Oxo-3,23-isopropylidene-asiatate (AS2006A), a wound-healing - asiatate derivative, exerts anti-inflammatory effect by apoptosis of macrophages *International Immunopharmacology, 3*, 1429-1437.

Corsetti, G., D'Antona, G., Dioguardi, F. S., & Rezzani, R. (2010). Topical application of dressing with amino acids improves cutaneous wound healing in aged rats. *Acta Histochemica, 112*(5), 497-507.

Cuttle, L., Kempf, M., Philips, G. E., mill, J., Hayes, M. T., Fraser, J. F., Wang, X. Q., & Kimble, R. M. (2006). A porcine deep dermal partial thickness burn model with hypertrophic scarring. *Burns, 32*, 806-820.

Davis, S. C., & Perez, R. (2009). Cosmeceuticals and natural products: wound healing. *Clin Dermatol, 27*(5), 502-506

Kaufman, T., Lusthaus, S. N., Sagher, U., & Wexler, M. R. (1990). Deep partial skin thickness burns: a reproducible animal model to study burn wound healing, *Burns, 16*(1), 13-16.

Molan, P. C. (1998). The evidence for honey promoting wound healing. *Primary intention (The Australian Journal of wound management), 6*, 148-158.

Molan, P. C. (1999). The role of honey in the management of wounds. *Journal of Wound Care. 8*, 423-426.

Molan, P. C. (2000). Establishing honey as a recognized medicine. *The Journal of the American Apitherapy Society, 7*, 7-9.

Rozaini, M. Z, Zuki, A. B. Z., Noordin, M. M., Norimah, Y., & Nazrul Hakim, A. (2005). Macroscopic evaluation of burn wounds healing progress treated with different types of honey. *Pakistan Journal of Biological science, 8*(5), 672-678.

Salmah, I., Mahmood, A. A., & Sidik, K. (2005). Synergistic effects of alcoholic extract of sweet basil (*Ocimum basilicum* L.) leaves and honey on cutaneous wound healing in rats. *International Journal of Molecular Medicine and Advance Sciences, 1*, 220-224.

Subrahmanyam, M. (1998). A prospective randomized clinical and histological study of superficial burn wound healing with honey and silver sulphadiazine. *Burns, 24*, 157-161.

Suguna, L., Chandrakasan, G. and Thomas Joseph, K. (1992). Influence of honey on collagen metabolism during wound healing in rats. *Journal of Clinical Biochemical Nutrition, 13*, 7-12.

Yusof, N., Ainul Hafiza, A. H., Zohdi, R. M., & Bakar, M. Z. A. Development of honey hydrogel dressing for enhanced wound healing. *Radiation Physics and Chemistry, 76*(11-12), 1767-1770.

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Heavy Metal Concentrations in Ceiling Fan and Roadside Car park Dust Collected from Residential Colleges in Universiti Putra Malaysia, Serdang, Selangor

C. K. Yap[1]*, Chew, W. Y.[1], and S. G. Tan[2]

[1]Department of Biology, Faculty of Science,
[2]Department of Cell and Molecular Biology,
Faculty of Biotechnology and Biomolecular Science,
Universiti Putra Malaysia, 43400 Serdang,
Selangor, Malaysia
**E-mail: yapckong@hotmail.com*

ABSTRACT

In this study, dust samples were collected from 4 residential colleges (K2, K5, KOSAS and K10) in Universiti Putra Malaysia (UPM) Serdang, Selangor. The samples were collected from ceiling fans and car parks roadside dust. Sand dust on top of the car park cover were collected using polyethylene brush, tray and kept in a polyethylene bag. Dust from ceiling fan on the first floor was collected and put into a polyethylene bag. The dust samples collected were analysed for the concentrations of Cd, Cu, Fe, Ni and Zn. It was found that K5 [Cu (62.94 ± 0.77 µg/g dw), Fe (1802.40 ± 9.81 µg/g dw), and Zn (253.34 ± 22.76 µg/g dw) of car park dust; Zn (997.20 ± 16.10 µg/g dw) (of ceiling fan dust] and K10 [Ni (26.88 ± 1.84 µg/g dw) and Zn (199.77 ± 6.64 µg/g dw) of car park dust; Cu (468.55 ± 3.67 µg/g dw), Ni (83.96 ± 0.75 µg/g dw), and Fe (3131.58 ± 27.01 µg/g dw) of ceiling fan dust] exhibited elevated concentrations of heavy metals that might be related to vehicular activities as compared to K2 and KOSAS. In general, ceiling fan dust had significantly ($P < 0.05$) higher concentrations of heavy metals than the car park dust. In comparison to other reported studies in the literature, the maximum levels of Ni and Cu were comparable or higher than those reported for major cities in the world. Hence, more monitoring studies should be conducted in the future to check for metal contamination in the dust, as this can serve as an atmospheric indicator of heavy metal pollution.

Keywords: Dust, heavy metals, UPM residential colleges

INTRODUCTION

Dust is also known as a particulate matter that can range from 1 to 1000µm in size and it receives inputs from various urban sources (Meza-Figueroa *et al.,* 2007). Heavy metals in dust particulate form will eventually be deposited on the ground and can easily be suspended by wind into the atmosphere (Sharma *et al.,* 2008; Amato *et al.,* 2009). They are potentially being hazardous to human health, especially to children (Lin *et al.,* 2002; Ng *et al.*, 2003; Meza-Figueroa *et al.,* 2007). Ferreira-Baptista and Miguel (2005) define street dust as solid particles that comprise of impervious materials and accumulate outdoors in urban environments. Street or urban roadside dust consist of vehicular exhaust particles, household dust, soil dust, construction dust and aerosols that are carried freely by air and water (Takada *et al.*, 1991; Al–Khashman, 2004; Meza-Figueroa *et al.,* 2007). Atmospheric pollution is one of the major sources of heavy metal contamination

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*Corresponding Author

in soils and street dust in urban areas (Lin *et al.,* 2001). Although studies carried out on ceiling fan dust are rather few, there are more closely related research done on household or indoor dust that have mainly focused on floor or carpet dust (e.g. Kim & Fergusson, 1993; Tong & Lam, 1998; Jabeen *et al.,* 2001). Loading of heavy metals in the indoor environment are actually related to the outdoor environment (Kim & Fergusson, 1993; Tong & Lam, 1998; Jabeen *et al.,* 2001). Despite the abundant literature about dust, only Yap *et al.* (2007) have reported heavy metal levels in indoor ceiling fan dust in Ipoh and Serdang, Malaysia.

Since dust always presents in the urban environment and has the potential to contribute to metal toxicity in humans, there is a growing concern over this threat in the scientific community (Ng *et al*., 2003). The US EPA (1986) reported that populations exposed to Cd, Cu, and Zn pollutants are in risk of developing nervous system alterations which can lead to health hazards. The emissions from roadways can cause respiratory illnesses (Lin *et al.,* 2002). There have been studies done on street dust near places where children play (e.g. Ng *et al*., 2003; Meza-Figueroa *et al.,* 2007) since children are more sensitive to contaminant-bearing dust (Meza-Figueroa *et al.,* 2007).

The environmental impacts of atmospheric deposition have been studied for more than a century, and the first effect that was described on a scientific basis was probably the decline of epiphytic lichens in areas with high levels of atmospheric pollution (Wolterbeek, 2002). Heavy metals such as Cu, Pb, and Zn can be accumulated in soils from atmospheric deposition from sources such as vehicular exhausts, industrial discharges, oil lubricants, automobile parts, and corrosion of building materials (Li *et al.,* 2001). The determination of metal levels in environmental samples in top soils and urban roadside dust is therefore necessary for monitoring environmental pollution since such levels can affect the surrounding ecosystems and heavy metals can accumulate in organisms (Aksoy & Demirezen, 2006). Due to the significant contribution of street dust to pollution in the urban environment, numerous studies on heavy metals of street dust have been carried out in developed countries (Charlesworth *et al.,* 2003; Manno *et al.,* 2006), but such studies are very much lacking in developing countries.

The objectives of this study were to determine the concentrations of metals on ceiling fan and car park dust collected from four different residential colleges in the Serdang campus of Universiti Putra Malaysia (UPM) and to relate their anthropogenic sources to the human activities observed in the surroundings.

MATERIALS AND METHODS

Samples of dust were collected from four residential colleges in UPM, namely K2, K5, KOSAS, K10, in February 2009 (*Fig. 1*). The dust was collected from ceiling fans at each college, and sandy dust at car parks. For the ceiling fan dust, only the one in the rooms located on the first floor was collected. All the samples were collected from non-rusty ceiling fans. For the roadside car park dust, sand dust on top of the car park was collected using a polyethylene brush, and tray. The dust was stored in clean and labelled polyethylene plastic bags. For the preliminary study, three (3) sites at KOSAS, K2, and K5 were considered as the reference sites to K10 which is closest to main north-south highway. However, it was not certain if the metals levels would be the highest at K10, and it was for this reason that the present study was conducted.

After drying at 80ºC in an oven until constant dry weights, the car park sand dust was sieved through a 53µm sieving tool. However, the fan dust samples were not sieved as they were processed immediately after drying. This is because all the airborne particles that can be trapped by fans are potentially inhaled by humans (Yap *et al.,* 2007).

The methodology of the analysis of heavy metals in the dust is similar to those described by Yap *et al.* (2002). In brief, the direct aqua-regia method was used to process the digested sample. About 0.5g of the dust sample was weighed and placed in digestion tube (3 replicates) for ceiling

Fig. 1: Map showing the sampling site of the dust samples collected from the four residential colleges in UPM, Serdang (Insert: Map of Peninsular Malaysia).

fan dust and 1.0g of car park sand dust was used. The dust was digested with HNO_3 and $HClO_3$ in the ratio of 4:1. The digestion tube was placed on the digestion block and heated at 40°C for the 1 hour and 140°C for the next 2-3 hours (Yap *et al.,* 2002). Then, the digested solution was topped-up to 40ml by adding distilled water. The solution was filtered with Whatman No.1 filter paper and stored in an acid-washed polyethylene bottle (Yap *et al.,* 2002, 2007). The filtered samples were determined for their Cd, Cu, Fe, Ni, and Zn concentrations using an air-acetylene flame Atomic Absorption Spectrophotometer (AAS) Perkin-Elmer Model AAnalyst 800. All the data were presented in μg/g dry weight (dw).

All the equipment and glassware were first acid-washed to avoid external contamination. Procedural blanks and quality control samples, made from the standard solution for each metal, were analyzed together with the digested samples. The quality of the method used was checked against the Certified Reference Material (CRM) for Soil (International Atomic Energy Agency, Soil-5, Vienna, Austria). The agreement between the analytical results for the certified reference material and the measured values for each metal was satisfactory with recovery percentages between 80 and 110%. All the software statistical calculations, such as (Mann-Whitney for comparison between ceiling fan dust and car park dust, One-way ANOVA for multiple testing, Pearson's Correlation and Spearman's Correlation), were carried out using the SPSS version 16 for statistical calculation.

RESULTS AND DISCUSSION

Table 1 presents the overall heavy metal concentrations (μg/g dry weight) in the ceiling fan car park dust from the four sampling. Based on the car park sand dust, K5 was found to have the highest metal concentrations of Zn (253.34 ± 22.76), Fe (1802.40 ± 9.81), and Cu (277.29 ± 7.69) as compared to other residential

TABLE 1
Comparison of the mean concentrations (µg/g dry weight) of Cd, Cu, Fe, and Zn between ceiling fan dust and car park sand dust using the Mann-Whitney test

Metal	College	N	Ceiling Fan Dust	N	Car Park Dust	Mann-Whitney (p-value)
Cd	K2	9	4.15 ± 0.84 [A]	3	3.01 ± 0.29 [c]	P=0.518 (P>0.05)
	K5	6	3.84 ± 0.03 [A]	3	2.06 ± 0.04 [ab]	P=0.020 (P<0.05)
	KOSAS	9	3.50 ± 0.25 [A]	3	2.19 ± 0.05 [b]	P=0.013 (P<0.05)
	K10	15	3.58 ± 0.22 [A]	3	1.24 ± 0.08 [a]	P=0.008 (P<0.05)
	Average	39	3.73 ± 0.17	12	2.11 ± 0.12	P< 0.05
	(Min–Max)		(3.15-5.83)		(0.72-3.76)	
Zn	K2	9	800.15 ± 8.44 [A]	3	103.68 ± 16.34 [a]	P=0.013 (P<0.05)
	K5	6	997.20 ± 16.10 [C]	3	253.34 ± 22.76 [c]	P=0.020 (P<0.05)
	KOSAS	9	850.24 ± 12.20 [B]	3	164.13 ± 5.65 [b]	P=0.013 (P<0.05)
	K10	15	897.13 ± 20.74 [B]	3	199.77 ± 6.64 [b]	P=0.008 (P<0.05)
	Average	39	892.11 ± 18.72	12	177.61 ± 9.68	(P< 0.05)
	(Min–Max)		(785.60-1029.08)		(52.27-304.64)	
Fe	K2	9	2631.62 ± 14.83 [A]	3	1687.22 ± 37.88 [a]	P=0.013 (P<0.05)
	K5	6	3008.97 ± 68.11 [BC]	3	1802.40 ± 9.81 [c]	P=0.020 (P<0.05)
	KOSAS	9	2894.41 ± 36.34 [B]	3	1691.61 ± 28.66 [ab]	P=0.013 (P<0.05)
	K10	15	3131.58 ± 27.01 [C]	3	1783.25 ± 5.40 [bc]	P=0.008 (P<0.05)
	Average	39	2976.41 ± 56.36	12	1742.89 ±13.47	(P< 0.05)
	(Min–Max)		(2603.72-3243.56)		(1552.55-1846.71)	
Ni	K2	9	46.64 ± 2.03 [A]	3	15.68 ± 1.21 [a]	P=0.013 (P<0.05)
	K5	6	46.74 ± 0.46 [A]	3	19.66 ± 0.57 [a]	P=0.020 (P<0.05)
	KOSAS	9	58.12 ± 2.23 [B]	3	13.80 ± 0.95 [a]	P=0.013 (P<0.05)
	K10	15	83.96 ± 0.75 [C]	3	26.88 ± 1.84 [b]	P=0.008 (P<0.05)
	Average	39	66.13 ± 5.35	12	20.17 ± 1.19	(P< 0.05)
	(Min–Max)		(42.59-96.35)		(9.85-38.41)	
Cu	K2	9	226.27 ± 1.23 [A]	3	18.95 ± 0.70 [a]	P=0.013 (P<0.05)
	K5	6	277.29 ± 7.69 [B]	3	62.94 ± 0.77 [c]	P=0.013 (P<0.05)
	KOSAS	9	304.22 ± 11.64 [C]	3	30.93 ± 4.08 [ab]	P=0.013 (P<0.05)
	K10	15	468.55 ± 3.67 [D]	3	43.59 ± 3.99 [b]	P=0.008 (P<0.05)
	Average	39	360.51 ± 31.11	12	37.96 ± 2.91	(P< 0.05)
	(Min–Max)		(223.84-536.00)		(16.11-76.46)	

Note: N= numbers of samples analysed

Metal concentrations at the different sites sharing common letters ([a], [b], and [c]) are not significantly different for Gabriel, One-way ANOVA testing. (P<0.05)

Metal concentrations at the different sites sharing common capital letters ([A], [B], and [C]) are not significantly different for Student-Newman-Keuls, One-way ANOVA testing (P<0.05)

colleges, whereas K10 had the highest metal concentration of Ni (26.88 ± 1.84), and K2 had the highest concentration of Cd (3.01 ± 0.29). On the other hand, K2 was found to have the lowest concentrations of Zn (103.68 ± 16.34), Fe (1687.22 ± 37.88), while Cu (18.95 ± 0.70). KOSAS was found to have the lowest concentration of Ni (13.80 ± 0.95) and K10 had the lowest concentration of Cd (1.24 ± 0.08).

As for the ceiling fan dust, K2 was found to have the highest concentration of Cd (4.15 ± 0.84) as compared to other residential colleges. Meanwhile, K5 had the highest concentration of Zn (997.20 ± 16.10). The highest concentrations of Fe (83.96 ± 0.75), Ni (83.96 ± 0.75), and Cu (468.55 ± 3.67) were found in K10. Among all the residential colleges, the lowest concentrations of Cu (226.27 ± 1.23), Ni (46.64 ± 2.03), Fe (2631.62 ± 14.83), and Zn (800.15 ± 8.44) were found in K2, while KOSAS had the lowest concentration of Cd (3.50 ± 0.25).

Looking at the location of K5, the residential college's car park is situated next to a hill slope. Thus, higher amounts of fuel were used for motor vehicles (buses, cars, motor, etc.) in low gear to go up the hill in addition to frequent braking while going downhill. Furthermore, buses also stop frequently at the Putra Food Court which is located near to K5. Hence, higher heavy metal concentrations could be expected at this site, and this corresponded well with the highest concentrations of Cu, Fe and Zn found in the car park sand dust at K5 and the high Zn concentration in the ceiling fan dust of K5. Cu and Zn could be derived from the mechanical abrasion of vehicles as they are used in the production of brass alloy and they could also come from brake linings, oil leak sumps and cylinder head gaskets (Jiries *et al.,* 2001). Tyre to road friction would be higher for up slope and down slope driving and increased braking manouvers which contribute to the higher mechanical abrasion of vehicles and greater stop-start manouvers (Ellis & Revitt, 1982). However, K5 had significantly lower Ni concentration than K10 ($p<0.05$, One-way ANOVA test), the residential college which is located nearest to the highways (Lebuhraya Utara-Selatan and Lebuhraya Sungai Besi). There is no doubt that car fuel combustion is higher (with higher volume of cars on the highway) along the vicinity of the highways. Thus, this could explain the high concentration of Ni in the car park dust and the high concentrations of Cu, Ni, and Zn in the ceiling fan dust. In particular, Cu is a common element that is used in automobile thrust bearing, brake lining, and other parts of the engine (Ng *et al*., 2003). Zn may also originate from lubricant oil and tyres of motor vehicles (Meza-Figueroa *et al.,* 2007). Ni could be involved in the vehicular fuel combustion process (Meza-Figueroa *et al.,* 2007). However, the ceiling fan dust collected in K10 had a significantly ($p<0.05$) higher concentration of Fe as well. Fe is often the major component of brake pads (Amato *et al.,* 2009). This could be due to the outdoor sources of Fe contributing to the room dust, as the residential college is situated next to the highway. The concentrations of heavy metal were reported to be higher on streets where traffic was more likely to undergo stop-start manouvers (Ellis & Revitt, 1982).

In general, the accumulation of metal found in the ceiling fan dust was observed to be resulted from different factors such as indoor pollutant sources, infiltration of outdoor dust, and the absorption of metals due to indoor humidity (Davies *et al.,* 1987). Most of the rooms where the samples were taken lack proper ventilation as the windows were either seldom or never opened by the occupants. This is in line with study by Jabeen *et al.* (2001) who showed that the interior house dust had higher concentrations of Cd and Pb, even for houses with little ventilation. Furthermore, the ceiling fan dust collected in this study had been there for a long time as the fans were not cleaned for several months to years. Therefore, heavy metal concentrations may have increased with time as compared to the outdoor street dust, as the conditions of the interior are much more stable than the outdoors.

In addition, weather is likely to influence the heavy metal distribution, concentration and retention time of the deposited street dust (Charlesworth *et al.,* 2003). A study by

Fergusson *et al.* (1984) showed that metal loadings for Cd, Cu, Pb, and Zn in house dust correlated strongly with the amount of dust in the house and this in turn correlated strongly to the amount of carpet used (Jabeen *et al.,* 2001).

K2 was found to have significantly ($p<0.05$) lower levels of Cu, Fe, Ni, and Zn for its car park dust, while Cu, Ni, Fe, and Zn ceiling fan dust when compared to the other residential colleges. KOSAS had significantly ($p<0.05$) lower concentrations of Cu, Ni, and Zn for the car park dust, and Cu and Zn for ceiling fan dust relative to the other residential colleges. The lower metal concentrations found in these two colleges could be due to the lesser road traffic activities around there as compared to K10 and K5. However, K2 had a significantly ($p<0.05$) higher concentration of Cd for its car park dust, and this was also higher but insignificant for the ceiling fan dust as compared to K10.

The correlations between heavy metal concentrations among all the car park sand dust are presented in Table 2. Based on the data given, significant (at least $p<0.05$) and positive correlations were found between Zn-Fe (0.320), Zn-Ni (0.502), Zn-Cu (0.625), Ni-Fe (0.323), and Cu-Fe (0.580). However, the positive relationships were found to be not convincing, and thus, further investigations are needed. As for the ceiling fan dust (Table 3), significant (at least $p<0.05$) and positive correlations were found between Fe-Ni (0.732), Fe-Cu (0.846), and Cu-Ni (0.918). The strong and positive correlations among Cu, Fe, Ni, and Zn in the car park dust, as well as Cu, Fe, and Ni in the ceiling fan dust might indicate that they could have come from the same sources, such as road traffic, as these metals are contained in motor vehicles. The study by Al-Khashman (2004) revealed a high correlation of the metal concentrations of Cu, Fe, Pb, and Zn which convinced him that anthropogenic activities are the main sources of heavy metal in soils. Table 3 shows that a larger proportion of Cd might have come from different sources, even though it could also be due to road traffic activities, as reported by Meza-Figueroa *et al.* (2007). This could explain why K2 had a significant ($p<0.05$) higher concentration of Cd than K10. Cd might have come from colour pigments, like the ones from carpets (Jabeen *et al.,* 2001). Cd could also come from air-borne emissions from various sources (Barnejee, 2003), as it is used to protect the surface of brass from corrosion (Charlesworth *et al.,* 2003).

TABLE 2
Pearson's Correlations for the car park dust (N=39)

	Cd	Zn	Fe	Ni	Cu
Cd	1	-0.404*	-0.747**	-0.439**	-0.371*
Zn		1	0.320*	0.502**	0.625**
Fe			1	0.323*	0.580**
Ni				1	0.279
Cu					1

TABLE 3
Spearman's correlations for the ceiling fan dust (N=15)

	Cd	Zn	Fe	Ni	Cu
Cd	1	0.064	-0.018	-0.129	0.007
Zn		1	0.664**	0.089	0.407
Fe			1	0.732**	0.846**
Ni				1	0.918**
Cu					1

Note: *= Correlation is significant at the 0.05 level (2-tailed).
**= Correlation is significant at the 0.01 level (2-tailed).

When compared to other related studies in the literature, the mean Zn concentration for the car park dust in the present study (Table 4) was found to be higher than that of the street dust in Bahrain (Akhter & Madany, 1993). However, the concentrations of Cd, Cu, and Ni found in the car park dust in this study were lower as compared to the street dust from other studies (e.g. as reported by Fergusson & Ryan, 1984; Lehame *et al.,* 1992; Akhter & Madany, 1993; Chon *et al.,* 1995; Wang *et al*., 1998).

Table 5 shows that the overall maximum concentration ranges of Cu, Ni, and Zn in the ceiling fan dust were higher than those reported by Yap *et al.* (2007), and these increase the concern over their influence on human health. On the contrary, the overall concentrations of Cd and Fe in the ceiling fan dust were lower than those reported by Yap *et al*. (2007). Fe

TABLE 4
Comparison of the results from the global studies on heavy metal concentrations in street dust (µg/g), as compiled by Charlesworth *et al.* (2003) and in this study

City	Reference	Metal			
		Cd	Cu	Ni	Zn
New York	Fergusson & Ryan (1984)	8	355	-	1811
Seoul	Chon *et al.* (1995)	3	101	-	296
London	Lehame *et al.* (1992)	6250	61-323	32-74	-
Hong Kong	Wang *et al*. (1998)	-	92-392	-	574-2397
Bahrain	Akhter & Madany (1993)	72	-	126	152
Serdang	This study	0.72-3.76	16.11-76.46	9.85-38.41	52.27-304.64

- indicates data are available

TABLE 5
Comparisons for the values (minimum-maximum) of heavy metal concentrations (µg/g dry weight) in the fan dust collected in this study and those reported by Yap *et al.* (2007)

Location	Cd, µg/g	Zn, µg/g	Fe, µg/g	Ni, µg/g	Cu, µg/g	Reference
Serdang, Malaysia	3.15-5.83	785.60-1029.08	2603.72-3243.56	42.59-96.35	223.84-536.00	This study
Ipoh, Malaysia	5.37-16.70	563.20-815.90	3470.70-4455.46	43.97-58.55	159.99-229.32	Yap *et al*. (2007)
Serdang, Malaysia	11.40-13.71	688.39-868.10	3446.50-4440.39	28.51-36.22	163.54-270.05	Yap *et al*. (2007)

is a common element of the earth's crust, so its levels in the car park dust could be related to the mobilization of soil particles (Fung & Wong, 1995).

CONCLUSION

In conclusion, K5 (Zn, Fe, and Cu, car park dust; Zn, ceiling fan dust) and K10 (Zn and Ni, car park dust; Cu, Ni, and Fe, ceiling fan dust) were found to have high concentrations of heavy metals, since they are comparable to other reported data for major cities, and this could be related to the vehicular traffic at these residential colleges. In general, ceiling fan dust had significantly ($P< 0.05$) higher concentrations of heavy metals compared to that in the car park dust. Meanwhile, most of the heavy metals concentrations (Cd, Cu, Ni, and Fe) found in the car park dust were still low than the data reported in the literature. However, the concentrations of Zn in the car park dust and the concentrations of Cu, Ni and Zn in the ceiling fan dust undertaken in this study were found to be comparable to or higher than those reported for other cities in the literature. Thus, further monitoring studies are needed since dust is considered as a potential atmospheric indicator of heavy metal pollution and its relationship to human health should be considered seriously.

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REFERENCES

Akhter, M. S., & Madany, I. M. (1993). Heavy metals in street and house dust in Bahrain. *Water, Air and Soil Pollution, 66*, 111 – 119.

Aksoy, A., & Demirezen, D. (2006). *Fraxinus excelsior* as a biomonitor of heavy metal pollution. *Polish Journal of Environmental Studies*. *15,* 27-33.

Al- Khashman, O. A., (2004). Heavy metal distribution in dust, street dust and soils from the work place in Karak Industrial Estate, Jordan. *Atmospheric Environment, 38*, 6803-6812.

Amato, F., Pandolfi, M., Viana, M., Querol, X., Alastuey, A., & Moreno, T. (2009). Spatial and chemical patterns of PM10 in road dust deposited in urban environment. *Atmospheric Environment*, *43*, 1650-1659.

Banerjee, A. (2003). Heavy metal levels and solid phase speciation in street dust of Delhi, India. *Environmental Pollution*, *123,* 95-105.

Charlesworth, S., Everett, M., Mc Carthy, R., Ordonez, A., & De Minguel, E. (2003). A comparative study of heavy metal concentration and distribution in deposited street dust in large and a small urban area: Birmingham and Coventry, West Midlands, UK. *Environmental International*, *29*, 563-573.

Chon, H-T., Kim, K-W., & Kim, J-Y. (1995). Metal contamination of soils and dust in Seoul metropolitan city, Korea. *Environmental Geochemistry and Health*, *17*, 139–146.

Davies, D. J. A., Watt, J. M., & Thornton, I. (1987). Lead levels in Birmingham dust and soils. *Science of the Total Environment, 67,* 177– 85.

Ellis, J. B., & Revitt, D. M., (1982). Incidence of heavy metals in street surface sediments: solubility and grain size studies. *Water, Air, and Soil Pollution*, *17,* 87-100.

Fergusson, J. E., & Ryan, D. E. (1984). The elemental composition of street dust from large and small urban areas related to city type, source and particle size. *Science of the Total Environmen, 34,* 101– 116.

Ferreira-Baptista, L., & De Miguel, E. (2005). Geochemistry and risk assessment of street dust in Luanda, Angola: A tropical urban environment. *Atmospheric Environment*, *39*, 4501-4512.

Fung, Y. S., & Wong, L. W. Y. (1995). Apportionment of air pollution sources by receptor models in Hong Kong. *Atmosphere Environment*, *29*, 2041-2048.

Jabeen, N. A., Ahmed, S., Hassan, S. T., & Alam, N. M. (2001). Levels and sources of heavy metals in house dust. *Journal of Radioanalytical and Nuclear Chemistry*, *247*, 145-149.

Jiries, A. G., Hussein, H. H., & Halas, Z., (2001). The quality of water and sediments of street runoff in Amman, Jordan. *Hydrology Proceedings*, *15*, 815-824.

Kim, N., & Fergusson, J. (1993). Concentrations and sources of cadmium, copper, lead, and zinc in house dust in Christchurch, New Zealand. *Science of the Total Environment*, *138,* 1-21.

Leharne, S., Charlesworth, D., & Choudhry, B. (1992). A survey of metal levels in street dust in an inner London neighbourhood. *Environment International*, *18*, 263–270.

Li, X., Poon, C. S., & Liu, P. S., (2001). Heavy metal contamination of urban soils and street dust in Hong Kong. *Applied Geochemistry, 16,* 1361-1368.

Lin, S., Munsie, J. P., Hwang, S.-A., Fitzgerald, E., & Cayo, M. R. (2002). Childhood asthma hospitalization and residential exposure to state route traffic. *Environmental Research*, *88*, 73-81.

Manno, E., Varrica, D., & Dongarra, G. (2006). Metal distribution in road dust samples collected in an urban area close to a petrochemical plant at Gela, Sicily, Italy. *Atmospheric Environment*, *40*, 5929 – 5941.

Meza-Figueroa, D., De La O-Villanueva, M., & De La Parra, M. L. (2007). Heavy metal distribution in dust from elementary schools in Hermosillo, Sonora, Mexico. *Atmospheric Environment* , *41,* 276-288.

Ng, S. L., Chan, L. S., Lam, K. C., & Chan, W. K. (2003). Heavy metal contents and magnetic properties of playground dust in Hong Kong. *Environmental Monitoring and Assessment*, *89,* 221–232.

Sezgin, N., Ozcan, H. K., Demir, G., Nemlioglu, S., & Bayat, Cuma. (2003). Determination of heavy metal concentrations in street dust in Istanbul E-5 highway. *Environment International*, *29,* 979-985.

Sharma, R. K., Agrawal, M., & Marshall, F. M. (2008). Atmospheric deposition of heavy metals (Cu, Zn, Cd and Pb) in Varanasi City, India. *Environment Monitoring and Assessment, 142*, 269–278.

Takada, H., Honda, T., Harada, M., & Ogura, N. (1991). Distribution and sources of polycyclic aromatic hydrocarbons (PAHs) in street dust from the Tokyo Metropolitan area. *Science of Total Environment, 107,* 45-69.

Tong S. T. Y. (1998). Indoor and outdoor household dust contamination in Cincinnati, Ohio, USA. *Environmental Geochemistry and Health*, *20,* 123– 133.

Tong, S. T. Y., & Lam, K. C. (1998). Are nursery schools and kindergartens safe for our kids? The Hong Kong study. *Science of Total Environment, 216,* 217-225.

U.S. EPA (US Environment Protection Agency), (1986). *Air Quality Criteria for Mercury and Lead.* EPA. Research Triangle Park, NC.

Wang, W. H., Wong, M. H., Leharne, S., & Fisher, B. (1998). Fractionation and biotoxicity of heavy metals in urban dust collected from Hong Kong and London. *Environmental Geochemistry and Health*, *20,* 195– 198.

Wolterbeek, B. (2002). Biomonitoring of trace element air pollution: principles, possibilities and perspectives. *Environmental Pollution*, *120,* 11-21.

Yap, C. K., Ismail, A., & Tan, S. G. (2007). Heavy metal concentrations in indoor fan dust of residential areas: A preliminary study. *Malaysian Applied Biology, 36,* 47-49.

Yap, C. K., Ismail, A., Tan, S. G., & Omar, H., (2002). Correlations between speciation of Cd, Cu, Pb and Zn in sediment and their concentrations in total soft tissue of green-lipped mussel *Perna viridis* from the west coast of Peninsular Malaysia. *Environment International*, *28*, 117-126.

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The Distribution of Palms and *Pandans* in Teluk Bahang Permanent Forest Reserve, Penang

Asyraf Mansor* **and Rahmad Zakaria**
School of Biological Sciences, Universiti Sains Malaysia,
11800 Penang, Malaysia
**E-mail: asyrafm@usm.my*

ABSTRACT

Woody species of the coastal forest of Penang island have received substantial focus in term of their diversity, composition and spatial distribution. In comparison, comprehensive data on the non-woody plant species component, specifically the palms and *pandans*, are relatively lacking. The objective of this study was to provide baseline data on the non-woody component of Penang coastal forest flora which would become a platform for more extensive research on their population dynamic. In order to provide these baseline data, an assessment of the diversity and distribution of two non-woody families, namely palms and *pandans*, was conducted in Teluk Bahang Permanent Forest Reserve (TBPFR) located in Penang, from February 2005 to January 2008. One ha plot (100 metre x 100 metre) was established in the TBFR and all palms and *pandans* were enumerated and individual positions were recorded. A total of seven species of palms and two species of *pandans* (i.e. *Pandanus ovatus* and *Pandanus penangiensis*) were recorded. The most abundant palm species is *Eugeisona tristis*, with a total count of 551 individuals, whilst a total of 312 *P. ovatus* were also enumerated. Based upon the Morisita Standardized Modified Index of Dispersion, the distribution of the two monocots ranged from random to clumped distribution.

Keywords: Coastal forest, clumped distribution, non-woody species, Penang Island

INTRODUCTION

Arecaceae of palm family is a common sight in Malaysian forest (Turner, 1989; Gong & Ong, 1983; Manokaran, 1992). Studies have shown that *Eugeissona tristis* is one of the common palm species growing wild in most types of Malaysian forests, particularly under the canopy of giant Dipterocarpaceae (e.g. Aiken & Leigh, 1992; Fuller, 2000; Gavin *et al*., 1996). The plant also occupies the spaces immediately after the formation of tree gap (LaFrankie & Saw, 2005). In certain cases, *E. tristis* is able to form monospecific stands, which are at time impenetrable, under tropical forest canopy (Baker *et al*., 1998). Even though this species has no commercial value, a recent study has suggested that this species does play a role in protecting the soil surface from direct contact with rainfall and it also can suppress seedling growth (Montgomery & Chazdon, 2001).

Another common monocots that thrive on the forest floor is the member of Pandanaceae. Early studies have suggested the importance of Pandanaceae in terms of its biodiversity and phylogenetic study (see for example the early work by van Steenis, 1954; Kam, 1971; Stone, 1972). Among the locally known uses of pandan (e.g. *Pandanus odoratisimus* and *P. ovatus*) are such as in the traditional mat making industry, whereas they ecologically form a protective place for small mammals in the tropical forest (Whitmore, 1985). Nonetheless, studies on its

*Corresponding Author

exclusive impacts on the ecosystem and dynamic of forest are rather limited.

The distribution of *Arecaceae* in the tropical forest has been described by many authors (Uhl & Dransfield, 1987; Dransfield, 1992), but a more specific study of the Penang forest reserves is particularly lacking, especially for *pandans*. Many species of these plants occupy a small area and can be found on almost all soil types. Some studies have shown that many populations of animals and plants are aggregated in nature, and a few are spaced out in a regular pattern (e.g. Krebs, 2002). One reason for knowing these patterns is that they affect decisions about what method to use for estimating population density. However, the second reason for determining the pattern is to describe it objectively and explain it biologically (Krebs, 2002).

The objectives of this study were to describe the distribution pattern of these common species and to determine whether the pattern was related to soil condition, availability of light on the forest floor or a combination of these factors.

MATERIAL AND METHODS

Study Site

Teluk Bahang Permanent Forest Reserve (TBPFR) in Pulau Pinang (latitude 100° 12' 55.79" U and longitude 5° 15' 56.88" T) has been designated as an educational and recreational forest. This particular forest is bordered by bukit Laksamana Permanent Forest Reserve on the west side and Bukit Kerajaan Permanent Forest Reserve on the east. A preliminary survey of the TBPFR shows that both palm and *pandan* species are concentrated within the boundary recreational forest of the TBPFR (A. Mansor & R. Zakaria, pers. obs.). Thus, in order to assess palm and *pandan* diversity, only one ha plot was decided to be established. The one ha study plot within the TBPFR was a coastal hill lowland forest (*Fig. 1*) which is dominated by Anacardiaceae, Clusiaceae and Myrtaceae (Zakaria *et al.,* 2009).

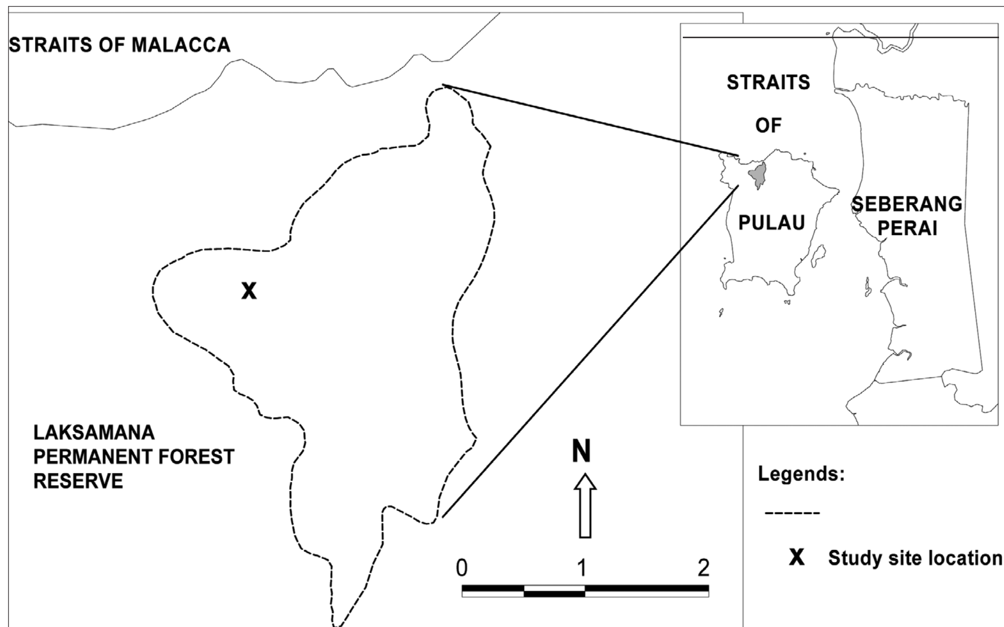

Fig. 1: Site location and the boundry of Teluk Bahang Permanent Forest Reserve, Penang, Malaysia.

Study Plot Design

This study was conducted between February 2005 to January 2008 at a one hectare plot (100 x 100 m) which had been randomly selected. The established study plot was divided into 100 quadrates with individual dimensions of 10 m x 10 m. Light illuminance was recorded using handheld digital Lux meter (Model: LX1010B), soil properties (i.e. soil type and soil pH) were tested using a method suggested by Bouyucos (1962), while temperature and humidity were recorded using HOBO® H8 Pro series Logger (Model: H08-031-08) from Onset Computer Corporation, USA. These parameters were recorded from four (4) randomly selected points within the study plot.

Species Assessment

All the palms with a height of 1 meter tall were recorded and mapped to the nearest 0.1 m in each quadrate. Using the Morisita Standardized Modified Index of Dispersion (MSMID; Krebs, 2002), the distribution pattern of the species was calculated. Meanwhile, the standardized Morisita Index of Dispersion (I_P) ranged from -1.0 to +1.0, with 95% confidence limits at +0.5 and -0.5. The random patterns gave I_P of zero, clumped patterns above zero, and uniform patterns below zero. The standardized Morisita Index is one of the best measures of dispersion because it is independent of population density and sample size. The recommended minimum sample size should be 50 quadrates, and that when the pattern is highly clumped, at least 200 quadrates are required (Krebs, 2002). The identification of the species was done based upon Whitmore (1972,1973) and Ng (1978, 1989).

RESULTS AND DISCUSSIONS

Limitation

There are two limitations on data interpretation and final outcome from this study. First, the study was designed to address the lack of data especially on non-woody component of TBPFR. Thus, the plot size was deliberately established as one ha plots with smaller size subplots and should be sufficient (see Putz & Chai, 1987; Appanah *et al.*, 1993). Second, the plot location was located within a hilly section of TBPFR and may contribute to low number of palm and *pandan* species.

Species Diversity and Composition

Seven species of palms and two species of *pandans* were recorded within the study plot (Table 1). Highest individual count was recorded for *Eugeissona tristis* (551 individuals) and only one individual plant was recorded for *Oncosperma tigillarium*. In terms of individual occurrences for *pandan*, *Pandanus ovatus* is the most abundant (312 clump count) compared to *Pandanus penangensis* (62 clump count). Statistical analysis shows that *E. tristis* and *P. ovatus* are the two most common palm and *pandan* found in this study site (Table 1). The distribution pattern calculated for palm and *pandan* shows that, at 95 % confident level, both families are distributed randomly toward clumping with the index reading of between 0 to 1 (Table 2). From the observation of the surrounding habitat, *O. tigillarium* adapt well in damp and wet habitats but less likely to establish on a higher ground. In addition, Putz & Chai (1987) stated that climbing palm (rattan) were more abundant on ridges than valleys. This could also explain the low number of palm species recorded in this study especially *Calamus exilis*.

Distribution

The finding in MSID shows that, all major species are distributed randomly to clump (Table 2; *Fig. 2*). According to Baker *et al*. (1998) and Tomlison (1979), the distribution of this type of plant is influenced by light availability. Monocotyledons are random-clumply distributed whereas large main dicotyledon are randomly distributed (Index near to 0). This type of dispersion is largely light oriented which reflected by the *pandans* and palms composition. These two families are commonly thrived under low light intensity (Baker *et al*., 1998; Tomlison, 1979).

TABLE 1
The statistical information of all Arecaceae and Pandanaceae found in the Teluk Bahang Forest Reserve study site.

Family	Species	Count	Mean	Std. Dev
Arecaceae	*Calamus exilis*	9	0.36	0.86
	Eugeissona tristis	551	22.04	13.15
	Licuala longipes	55	2.2	2.43
	Myrialepis scortechinii	58	2.32	2.72
	Oncosperma tigillarium	1	0.04	0.20
	Pinanga malaiana	67	2.68	2.69
	Plectocomia griffithii	36	1.44	1.85
Pandanaceae	*Pandanus ovatus*	312	12.48	1.40
	Pandanus penangensis	62	2.48	2.45

TABLE 2
Arecaceae and *Pandan*aceae dispersion in TBFR based on Morisita's Modified Index.

Parameters	*E. tritis*	*L. longipes*	*P. penangensis*	*M. scortechinii*	*P. ovatus*
Sample size (number of plots)	100	100	100	100	100
Frequency	545	59	62	58	309
Mean	5.45	0.59	0.62	0.58	3.09
Variance	21.08	1.13	1.35	1.30	20.16
Ratio of variance/mean	3.87	1.92	2.18	2.24	6.52
Chi-square	382.89	190.15	215.42	221.31	646.02
Morisita's index	1.52	2.57	2.91	3.15	2.78
Modified Morisita's Index	0.50	0.51	0.51	0.51	0.51
Type of distribution	*	*	*	*	*

Notes: * = clumping dispersion. Other Arecaceae found are not included in this analysis due to their low number of frequency detected in TBFR, thus their dispersion pattern need wider study plots.

Other possible explanation is palm is probably clumped together as water surface flowing on the forest floor is likely to wash away the seeds. However, we cannot confirm this particular event and suggested for a more in-depth study to be conducted in future.

Forest Ecology

The forest canopy in most part of the study site are continuous with only a few small gap (Mansor & Zakaria, pers. obs.), therefore the light illuminance recorded for 12 month on the forest floor never exceeded 2000 Lux at 1200 H as most of the light are captured by the upper forest canopy and less than 5 % of light penetrate to the ground (Montgomery & Chazdon, 2001; Table 3). This phenomenon has been described by Dorothy *et al*., (2001) which stated that light is very unlikely to penetrate to the forest floor during early morning and late evening. In addition, at times only 1% of sunlight ever reached the forest floor while mean temperature ranged from 24.9°C to 29.5°C and humidity from 85% to 95% (MSTEM, 1997).

Importance and implication for conservation

Palm family can be a primary target for conservation, due to their richness and abundance of taxa, their occurrences in all strata of forest as well as their importance as source of food

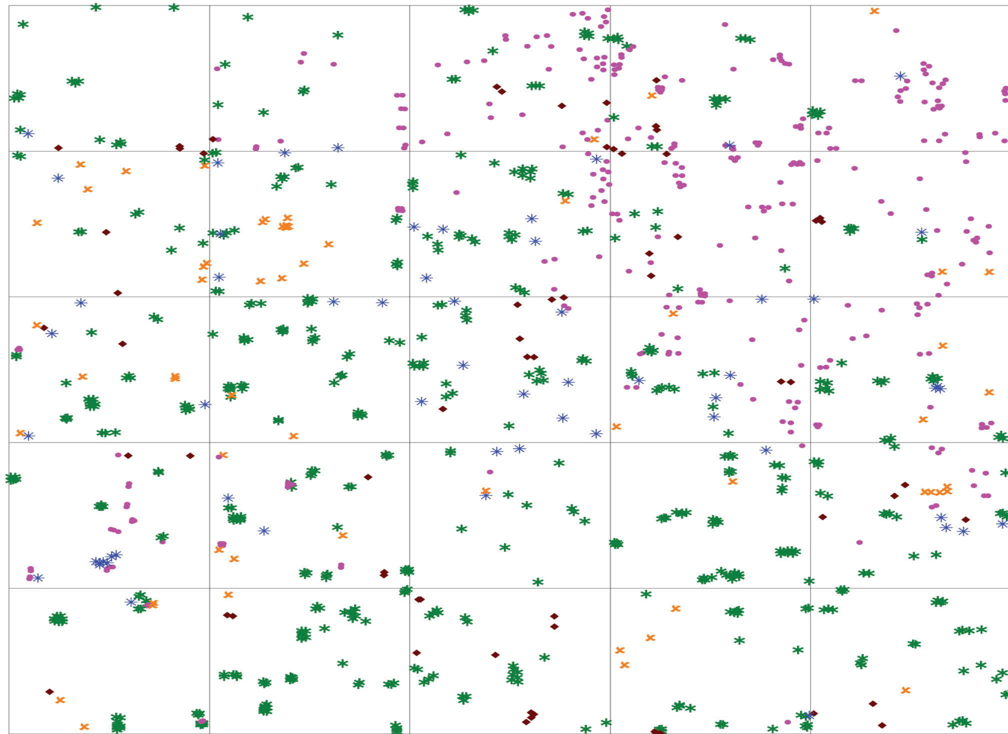

Fig. 2: Distribution of Arecaceae and Pandanaceae within the one ha study plot. Other Arecaceae found are not included in this because their number detected in TBFR is very low, thus their dispersion pattern is non-detectable.

for wild life (Terborgh, 1986). This may also applicable to the *pandan* family. According to a recent finding, *pandans* can also play an important part in providing shelter for small gecko (*Phelsuma cepediana*), which in turn act as a pollinator of another rare endemic plant, *Trochetia blackburniana* of Mauritius island growing nearby (see Hansen *et al.,* 2007). It is important to note that this particular information on plant-animal interaction is still lacking especially for coastal forest ecosystem (e.g. Penang Island), thus more research on this aspect is highly recommended. In general for non-woody species exclusively in Penang, the information on their population dynamics and how the biotic and abiotic components regulate their propagule distribution is still a big gap and would necessitate further investigation.

CONCLUSION

The composition and distribution of these two non-woody group of plant, namely palm (Arecaceae) and *pandan* (Pandanaceae) are regulated by light hence the regulated distribution pattern. In addition, the runoff water on the forest

TABLE 3
Average values for soil pH, air temperature, relative humidity, light illumination and soil type, recorded *in-situ*.

Parameter	Value (mean ± sd)
Soil pH	4.42 ± 0.16
Daily temperature at 1200H	28.167 ± 0.363 (n=12 reading)
Relative Humidity at 1200H	85.392 ± 2.849 (n=12 reading)
Light at 1200H	1341.7 ± 155.00 Lux (Highest around 1200 H to 1300 H, n=12 reading)
Soil type	Sandy loam

floor in addition to the location of study plot (i.e. hilly) are a probable cause for the clumped distribution observed for some palms and *pandans*, as seeds (or propagule) were washed and deposited at certain location. However, more experimental data is needed to further confirm this claim and future research is on its way.

REFERENCES

Appanah, S., Gentry, A. H., & LaFrankie, J. V. (1993) Liana diversity and species richness of Malaysian Rain Forests. *Journal of Tropical Forest Science*, *6*(2), 116-123

Baker, W.J., J. E., Coode, J., Dransfield, S., Dransfield, M. M., Harley, P., Hoffmann, & R. J., Johns (1998). Patterns of distribution of Malesian vascular plants. In Hall, R. and J.D., Holloway (Eds.) *Biogeogarpahy and Geological Evolution of SE Asia* (p. 243-258). Backbuys Publishers, Leiden, the Netherlands.

Brandani, A., Hartshorn, G. S., & Orians, G. H. (1988). Internal heterogeneity of gaps and tropical tree species richness. *Journal of Tropical Ecology*, *4*, 99-119.

Brokaw, N. V. L. (1985). Tree falls, regrowth and community structure in tropical forests. In S.T.A. Picket and P.S. White (Eds.) *The Ecology of natural disturbance and patch dynamic*. (p. 53-69). Orlando, Florida: Academic Press.

Clark, D. B., & Clark, D. A. (2000). Landscape-scale variation in forest structure and biomass in a tropical rain forest. *Forest Ecology and Management*, *137*, 185-198.

Dransfield, J. (1992). *The rattans of Sarawak*. Kew, UK: Royal Botanic Gardens and Sarawak Forest Department.

Fuller, R. J. (2000). Influence of treefall gaps on distributions of breeding birds within interior old growth stands in Bialowieza Forest, Poland. *The Condor, 102*, 267-274.

Gavin, D. G., Peart, D. R., & Leighton, M. (1996). Canopy gaps in the peat swamp forest in West Kalimantan, Indonesia. *Tropical Biodiversity*, *3*(3), 243-249.

Gong, W. K., & Ong, J. E. (1983). Litter production and decomposition in a coastal hill dipterocarp forest. In *Tropical Rain Forest: Ecology dan Management. Special Publication no. 2 of The British Ecological Society* (Eds: Sutton, S.L., Whitmore, T.C., & Chadwick, A.C.). Blackwell Scientific Publications.

Hansen, D. M., Kiesbüy, H. C., Jones, C. G., & Müller, C. B. (2007) Positive indirect interactions between neighboring plant species via a lizard pollinator. *The American Naturalis*, *169*(4), 534-542.

Kam, Y. K. (1971) Morphological studies in *Pandanaceae* III: Comparative systematic foliar anatomy of Malayan *Pandanus*. *Bot. J. Linn. Soc., 64*(4), 315-351.

Krebs, C. J. (2002). *Program for Ecological Methodology*. Version 6.1. *Ecological Methodology (2nd Edition)*. , New York: Benjamin-Cummings.

Krebs, C. J. (1999). *Ecological Methodology*, second Edition. Menlo Park, California: Addison Wesley Longman Inc.

LaFrankie, J. V., & Saw, L. G. (2005). The understorey palm *Licuala* (Arecaceae) suppresses tree regeneration in a lowland forest in Asia. *Journal of Tropical Ecology*, *21*, 703-706.

Manokaran, N., (1992). An overview of biodiversity in Malaysia. *Journal of Tropical Forest Science*, *5*(2), 271-290.

Montgomery, R. A., & Chazdon, R. L. (2001). Forest structure, canopy architecture and light transmittance in tropical wet forests. *Ecology*, *82*(10), 2707-2718.

MSTEM (Ministry of Science, Technology and Environment, Malaysia), (1997). *Assessment of Biological Diversity in Malaysia (Malaysia: Country study on Biological Diversity)*. Ministry of Science, Technology and Environment, Malaysia.

Ng, F. S. P. (Ed.) (1978) *Tree flora of Malaya*. Vol 3. Malaysia: Longman.

Ng, F. S. P. (Ed.) (1989) *Tree flora of Malaya*. Vol 4. Malaysia: Longman.

Putz, F. E. & Chai, P. (1987) Ecological studies of lianas in Lambir National Park, Sarawak, Malaysia. *Journal of Ecology, 75*, 523-531.

Shaharudin, A. (1990). An investigation of forest microclimate: The Pasoh Forest Reserve, Negeri Sembilan, Malaysia. *Singapore Journal of Tropical Geography*, *11*(1), 68-75.

Stone, B. C. (1972). A Reconsideration of the evolutionary status of the family *Pandan*aceae and its significance in monocotyledon phylogeny. *The Quarterly Review of Biology*, *47*(1), 34-45.

Terborgh, J. (1986) Keystone plant resources in the tropical forest. *Conservation Biology: The Science of Scarcity and Diversity* (ed. M.E. Soule), pp. 330-344. New York, NY: Sinauer.

Turner, I. (1989). An enumeration of one hectare of Pantai Aceh Forest Reserve, Penang. *Garden Bulletin of Singapore*, *42*(1), 29-44.

Tomlinson, P. B. (1979). Systematic and ecology of the palmae. *Annual Review of Ecology Systematic*, *10*, 85-107.

Uhl, N., & Dransfield, J. (1987). *Genera Palmarum*. Allen Press: Lawrence, Kansas, USA.

Van Steenis, C. G. G. J. (1954). *Pandanus* in Malaysian vegetation types. *Flora Malesiana, I, 4*(1), 3-12.

Whitmore, T. C. (1985). *Tropical rain forest of the Far East (2 nd Edition)*. Oxford: Clarendron Press.

Whitmore, T. C. (Ed.) (1972). *Tree Flora of Malaya* Vol. 1. Malaysia: Longman

Whitmore, T. C. (Ed.) (1973). *Tree Flora of Malaya* Vol. 2. Malaysia: Longman

Zakaria, R., Mansor, A., Nik-Rosely, N. F., & Mansor, M. (2009). Comparison of plant communities at six study plots in Penang forest reserves, Malaysia. *Tropical Ecology, 50*(2), 259-265.

Zarr, J. H. (1999). *Biostatistical Analysis (4th Edition)*. London: Prentice Hall International, Inc.

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Mas Cotek *(Ficus deltoidea)*: A Possible Supplement for Type II Diabetes: (A Pilot Study)

S. Draman[1*], M. AM Aris[1], Razman[1], Akter SFU[1], Azlina H.[1], Nor Azlina A. R[2], Muzaffar[2] , Norazlanshah H.[2] and Azian[3]

[1]Kulliyyah of Medicine,
[2]Kulliyyah of Allied Health Sciences,
[3]Kulliyyah of Pharmacy,
International Islamic University Malaysia (IIUM),
25200 Bandar Indera Mahkota,
Kuantan, Pahang, Malaysia
**E-mail: joedah@yahoo.com*

ABSTRACT

The aim of this research was to study the effect of the *Ficus deltoidea* (Mas Cotek) leaves on fasting blood sugar, renal and lipid profile of Type II diabetic patients. This study was carried out at Polyclinic Balok, located in Kuantan, Pahang, Malaysia. Twenty patients participated in the study and they were divided into two (2) groups of ten (10) patients each. The inclusion criteria were registered as diabetic patients in the health centre, diagnosed as type II diabetes mellitus for more than one (1) year, age 18 years and above, HbA1c more than 6.5% and have contactable telephone number. The patients in the intervention group has been given *F. deltoidea* 350 mg twice daily orally and monitored every 20 days for two (2) months. In conclusion, the effects on fasting blood sugar, HbA1C, renal and lipid profiles were not significant. The patients in the intervention group felt energetic and fresh compared to the control.

Keywords: Diabetes mellitus, energetic, fasting blood sugar, feeling fresh, ficus deltoidea, lipid profile, renal profile.

INTRODUCTION

Diabetes mellitus is a syndrome characterized by chronic hyperglycemia which is due to relative insulin deficiency, or resistance, or both (Kumar & Clark, 1998). The primary defect is a tissue insulin receptor disorder resulting in resistance to insulin action. Compensatory hyperplasia of pancreatic beta cells and increased insulin production occur and account for fasting hyperinsulinaemia and an exaggerated insulin response to glucose ingestion. Prolonged exposure over time to fasting hyperglycaemia causes beta cells desensitization and failure of insulin secretion by the beta cells (Weiss Barry, 1999). Treatment consists of oral hypoglycemic agents and insulin (Hanninen *et al.*, 1998).

The prevalence of diabetes mellitus is dramatically rising worldwide according to the International Diabetes Federation (IDF). In 2000, about 171 million people were affected, and this number was expected to increase to 366 million by 2030 (Wild *et al.*, 2004). In Malaysia, the prevalence of diabetes is found to be 8.2% (Rugayah, 1997). Based on the National Health Morbidity Survey conducted in 1986, the prevalence was 6.3%, and this was increased to

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*Corresponding Author

8.3% in 1996, and 14.9% in 2006. The National Health Morbidity Survey III showed that the diabetes prevalence rate had risen drastically and at a much faster rate than expected (Zanariah H. *et al.*, 2006).

Botanical products may improve glucose metabolism, state of well-being, lipid metabolism, antioxidant status and capillary function (Bailey, 1989). Some example of these are bitter melon, Gymnema, Korean Ginseng, onions, garlic, flaxseedmeal, alphalipoicacid, biotin, carnitine, vanadium, chromium, magnesium, zinc and vitamins B_3, E and K (Shapiro *et al.*, 2002). In India, 85% of the traditional medicines used for the primary healthcare are derived from plants (Basu, 1999). It is reported that out of 17,209 different kinds of plants, more than 7,918 plants were reported to have high medicinal value (Indian Botanical Survey Report, 2002). In addition to that, there are 2400 unique species of plants that are fully documented in terms of their biological properties, actions and drug formulations for a range of health conditions (Shanker, 2006).

Although herbs can be found in the wild, it is the individuals that control their usage (Goffman, 2003). The use of herbal medicines in Asia represents a long history of human interactions with the environment. Among other, plants used for traditional medicine contain a wide range of substances that can be used to treat chronic as well as infectious diseases (Duraipandiyan *et al*., 2006). More than 400 traditional plant treatments for diabetes mellitus have been recorded, but only a small number of these have received scientific and medical evaluations to assess their efficacy (Bailey, 1989). In Malaysia, a few plants have been scientifically investigated and reported to have anti-diabetic properties. These plants include *Morinda citrifolia, Averrhoa bilimbi, Tinospora crispa, Parkia speciosa, Andrographis paniculata, Gynura procumbens* and *Orthosiphon stamineus* (Mafauzy, 2004).

However, there are still plants with anti-diabetic properties which have not yet been studied. One of them is Mas Cotek or scientifically known as *Ficus deltoidea*. It is a shrub that reaches a height of six feet, used as an ornamental plant in the tropics or in the home and conservatories. *F. deltoidea* is in the division of Magnoliophyta, class Magnoliopsida, order of Rosales, and in the family of *Moracea* (Starr, 2003). It contains flavonoid, which could result in antioxidant activity of the plant. Flavonoid also gives the yellow pigmentation, and it also helps the plant protect itself from microorganism and insects. Any herbs that contain flavonoid could also have the ability to act as anti-allergy, anti-inflammatory, anti-microbial, and anti-cancer agent (Buhler, 2007). Furthermore, the plant also contains tannins, triterpenoids and phenols. Tannins are astringent, bitter-tasting plant polyphenols that bind and precipitate proteins. Tannins may be employed medicinally in anti-diarrheal, hemostatic, and antihemorrhoidal compounds (Vattem *et al*., 2005).

Research done on *f. deltoidea* also showed that a mixture of the extracts of its fruits and leaves can reduce blood glucose level. Animal study was conducted by Aminudin N. in 2006 using rats. The extract was given as tea and fruit extracts. After two (2) hours, the rats that took the tea had 15.6% glucose reduction, whereas those which had fruit had 14.8% reduction. The researcher conducted another study by comparing *F. deltoidea* to other herbs, namely *Orthosiphon siamineus* (Misai Kucing), *Momordica charantia* (Peria), *Andrographis paniculata* (Hempedu bumi) and *Labisia pumila* (Kacip Fatimah) in terms of glucose reduction. Among the five herbs, *Ficus deltoidea* was found to be have the highest percentage of glucose reduction, which is 18.4%, whereas *Orthosiphon siamineus* (Misai Kucing), *Momordica charantia* (Peria), *Andrographis paniculata* (Hempedu Bumi) and *Labisia pumila* (Kacip Fatimah) had 17.3%, 7.8%, 6.7% and 1.5%, respectively.

Similarly, a study by Zainah A. *et al.* (2007) also showed that the aqueous extract of *Ficus deltoidea* at a concentration of 1000mg/kg had hypoglycaemic activity in post-prandial mild diabetic rats. The extract mechanism of the action was suggested through enhancement of glucose uptake in muscle tissue and reduction

of hepatic gluconeogenesis. This plant extract can also be used by mothers after giving birth because they are believed to aid in strengthening of the uterus, or used to regulate menstrual flow (Yaakob *et al.,* 2005). The leaves of *F. deltoidea* are traditionally boiled and the decoction is taken by women after giving birth. It is believed that *F. deltoidea* helps to contract the uterus and the vaginal muscles. It also improves blood circulation and regains body strength as well as treats disorders related to menstrual cycle (Anon, 2000; Burkhill & Haniff, 1930; Fasihuddin & Din, 1995). In Indonesia, women use the decoction of the whole plants as aphrodisiac tonic (Sri Yuliani, 2001; Sorolangum, 1999). According to the Ministry of Health Malaysia, the plant is supported by evidence based on the raditional and complementary medicine. The Ministry hopes that more research will be conducted to provide this evidence (MMA, 2008).

The objective of this study was to look at the effects of Mas Cotek (*F. deltoidea*) on fasting blood glucose, lipid profile and renal profile of Type II diabetic patients. The authors hope that the study can be used as a reference to find possible supplements for diabetic patients in the future, and thus improve diabetic control.

MATERIALS AND METHOD

This study was carried out at Polyclinic Balok, Kuantan, Pahang. For the purpose of this study, 20 diabetic patients who participated voluntarily were used as subjects. In particular, ten patients took *Ficus deltoidea* 350 mg twice daily orally (Intervention) and the other ten (10) patients were used as control. The patients were followed up at 0, 20, 40 and 60 days. The patients in the control group continued their standard treatment without receiving any *F. deltoidea*.

Recruitment was undertaken from November 2008 until December 2008. The recruitment of the subjects was continuously done until a total of 20 subjects were enrolled. Prior to the recruitment, the patients received a counselling session that lasted between 10 to 30 minutes from the researchers. During these sessions, they also received advice on the complications of diabetes mellitus. The inclusion criteria were:

Inclusion Criteria

- Registered as diabetic patients in the health centre
- Diagnosed as type II DM for more than one year
- Age 18 years and above
- Poor control of DM (HbA_{1c} more than 6.5%)
- Have contactable telephone number

Exclusion criteria

Meanwhile, the exclusion criteria were:

- Age > 75 years old
- Having acute illness which needs hospitalization for a duration of 3 months before and during the study period
- On haemodialysis
- Unable to comprehend and give cooperation
- Unable to continue follow up

This study was conducted according to the ethical principles of clinical trial involving human subjects. Informed consent and protocols were approved by the Ethics Committee of the Kulliyyah of Medicine, International Islamic University Malaysia, Kuantan, Pahang.

After the counseling session, eligible subjects were explained that the study involved the use of *F. deltoidea*. Each subject was given an information sheet and a full explanation of the nature and purpose of the study. The researcher explained the possible adverse effects, risks and benefits, as well as the study protocol.

After the subjects had been fully informed about the study, the informed consent form was given to each of the subjects to sign. Only those who agreed to sign the informed consent form were accepted for enrolment. The informed consent form was also signed and dated by the researcher and one witness and later kept in the

researcher's files. A copy of the information sheet and the signed informed consent was given to the subjects for their own personal record.

Fasting blood sugar (each follow up), renal profile and lipid profile (pre and post study) were investigated during the study period. The results were compared between the intervention and controls to see the effectiveness of *F. deltoidea* in diabetic patients. The medication *F. deltoidea* 350mg, with a registration number **MLMAL 06071300TC,** was sponsored by Delto Medicana Plantation Sdn. Bhd. The patients were monitored every 20 days. The patients were provided with the researcher's handphone number, if any untoward incidents happened during the study period.

The data was analysed using Statistical Package for Social Science (SPSS) version 16. The Mann-Whitney test was used and the statistical significant level was taken at 0.05.

RESULTS

A total of ten (10) diabetic patients for each intervention and the control group were recruited in this study. However, one patient from the intervention group did not complete the study, resulting in the drop-out rate of 5%.

Tables 1 and 2 below show the comparison of the socio-demographic and clinical characteristics of the patient participants in the intervention and control group. Table 1 also presents the comparison based on the categorical characteristics using *Fisher's Exact* test because the assumption on the expected frequency was not met and the *chi-square* test could not be used, whereas Table 2 reveals the comparison of the numerical characteristics using the Mann-Whitney test.

DISCUSSIONS

Besides *Eurycoma longifolia* (Tongkat Ali) and *Labisia pumila* (Kacip Fatimah), *Ficus deltoidea* (Mas Cotek) is another medicinal plant that is gaining popularity among the herbal practitioners (Chang *et al.*, 2004). The name of Mas Cotek is given mainly due to the presence of golden spots at the upper surface of the leaf. It is also known as *sempit-sempit* in Sabah and *tabak barito* in Indonesia (Sri Yuliani, 2001; Sorolangum, 1999). The collectors have classified *Ficus deltoidea* into more than 30 accessions based on the morphology of the leaf, stem and its growth habits. However, these accessions are commonly grouped into two (2), namely female *F. deltoidea* and male *F. deltoidea* (Musa & Wan Zaki, 2004; Musa *et al.*, 2004).

As presented in Table 1, the number of patients was reduced to only 19. One of the patients in the intervention group had default the treatment due to logistic problem. The patient was also unable to be contacted. As can be seen in Table 1, the intervention and control groups were comparable because there was no significant difference found in all the pre-intervention variables, except for their diastolic blood pressure. Similarly in Table 2, it can be observed that there was also no significant

TABLE 1
Socio-demographic factors of the study subjects in the intervention and control groups

Variable	Intervention (n=9) Frequency (%)	Control (n=10) Frequency (%)	*p*-value
Sex			
Male	4 (44.4)	3 (30.0)	0.650
Female	5 (55.6)	7 (70.0)	
Education level			
Primary education	3 (33.3)	7 (70.0)	0.179
Secondary & above	6 (66.7)	3 (30.0)	

TABLE 2
A comparison of the socio-demographic and clinical characteristics of the study subjects in the intervention and control groups

Variable	Intervention (n=9) median (iqr[a])	Control (n = 10) median (iqr)	*p*-value
Age (years)	49 (26)	58 (10)	0.153
Duration of diabetes (years)	4 (2)	5.5 (12)	0.593
HbA_{1c} (%)	7.95[b] (2.261)[c]	8.85[b] (1.831)[c]	0.396[d]
Fasting blood glucose (mmol/L)	9.80 (6.15)	9.35 (6.03)	0.806
Total cholesterol (mmol/L)	5.50 (1.85)	5.35 (2.56)	1.000
Triglycerides (mmol/L)	1.32 (1.28)	1.68 (1.42)	0.775
High-density lipoprotein (mmol/L)	1.49 (0.60)	1.405 (0.42)	0.838
Low-density lipoprotein (mmol/L)	3.4 (1.85)	3.25 (1.78)	0.806
Systolic blood pressure (mmHg)	130 (20)	145 (22.5)	0.066
Diastolic blood pressure (mmHg)	80 (15.0)	90 (12.5)	0.016
Body mass index (kg/m^2)	32.45 (7.590)	28.69 (8.380)	0.221
Uric Acid (mmol/L)	304.0 (79.50)	337.5 (120.50)	0.414
Urea (mmol/L)	3.7 (1.30)	4.05 (2.28)	0.967
Creatinine (mmol/L)	82.0 (25.00)	73.5 (29.00)	0.838
Potassium (mmol/L)	4.5 (0.70)	4.35 (0.63)	0.967
Sodium (mmol/L)	138.0 (3)	137.5 (4)	0.619
Chloride (mmol/L)	102 (5.00)	100.5 (2.50)	0.185

[a] iqr = interquartile range
[b] mean
[c] standard deviation
[d] independent t-test (normally distributed variables)

association between the intervention and control groups with sex and education level.

In order to assess whether there was any change in the fasting blood glucose, fasting lipid profile and renal function tests between the intervention and control group after *F. deltoidea* had been given to the patients in the intervention group, the RM Anova analysis was used. The multivariate test results showed that there was no change in the clinical variables between the two groups. Even though the fasting blood glucose showed a reducing trend, after controlling for possible confounders (such as age, sex, education level, duration of diabetes mellitus and body mass index), no significant changes were found between the two groups (*p*-value = 0.365). Similarly, there was no significant change in the HbA1c level between the intervention and control groups (*p*-value = 0.855). Detailed results are shown in Table 3.

Table 3 reveals that the FBS from both groups were reduced, although it was not significant. Meanwhile, the intervention group was happy with the use of *F. deltoidea* and found that the medications had no side effects. A patient from the intervention group was able to work for 2 hours continuously after taking *F. deltoidea*. Before taking *F. deltoidea*, he had to stop every half an hour. Two (2) patients on insulin noted that their blood sugar controls were getting better after taking *F. deltoidea*.

Fruits and vegetables have the capabilities to deliver health benefits besides fulfilling physiological needs. Thus, a routine consumption of fruits and vegetables confer significant benefits to human health (Steinmetz & Potter, 1996). Epidemiological data as well as *in vitro* studies strongly suggest that food containing phytochemicals with anti-oxidation potential possesses strong protective effects against major disease risks (Knekt *et al.*, 1997; Elliot, 1999; Kaur & Kapoor, 2001). However, according to Weiger *et al.* (2002), no herb or supplement has sufficient evidence to actively recommend or discourage its use among diabetes patients.

Those on oral hypoglycaemic agents also showed improved blood sugar range (5.7-8.4 mmol/L). More importantly, nobody claimed

TABLE 3
Glucose level between the intervention and control groups

	Mean (sd[a])	Estimated Marginal Mean* (95% CI[b])	F-statistic (df^c)	p-value
FBS				
Intervention				
Pre	10.55 (3.177)	9.773 (7.713, 11.833)	1.127	0.365
20 days post	10.60 (3.789)	9.822 (7.262, 12.381)	(3, 18)	
40 days post	9.73 (4.608)	8.051 (5.162, 10.939)		
60 days post	8.10(3.302)	7.070 (4.723, 9.418)		
Control				
Pre	10.21(3.277)	11.05 (8.630, 13.463)		
20 days post	9.36 (3.005)	11.60 (8.600, 14.604)		
40 days post	8.69 (2.402)	9.530 (6.142, 12.918)		
60 days post	8.03 (1.179)	8.382 (5.627, 11.136)		
HbA1c				
Intervention				
Pre	7.95(2.261)	7.237 (4.661, 9.814)	0.037	0.855
Post	9.00 (2.048)	8.219 (5.176, 11.262)	(1,5)	
Control				
Pre	8.85 (1.831)	8.229 (4.814, 11.783)		
Post	8.55 (2.430)	8.088 (3.973, 12.203)		

[a] sd = standard deviation
[b] CI = confidence interval
[c] *df* = degree of freedom
FBS = fasting blood glucose
* Controlled for age, sex, education level, duration of diabetes mellitus and body mass index

any side effects during the study period. One female patient noted improved sexual life. Compliance wise, they were much better, and two (2) other patients were willing to buy the product once the research was finished. These two patients noticed the edema on their legs, secondary to diabetic nephropathy, was reduced and they were less lethargic. Compliance was checked by asking patients, counting pills and HbA1c level. However, HbA_{1c} was also not that significant. A clinical review by R. Nahas in 2009 found that fibre, green tea and fenugreek have other benefits but there is little evidence that they could substantially improve glycemic control HbA_{1c}.

Tables 4 and 5 revealed that lipid profile and renal profile were found to be insignificant. The p-value from the multivariate test for fasting lipid profile after controlling for possible confounders (such as age, sex, body mass index and duration of diabetes mellitus) was 0.405. The results of the univariate analysis for each component of fasting lipid profile are shown in Table 4. The p-value from the multivariate test for renal function test after controlling for possible confounders (such as age, sex, systolic blood pressure, diastolic blood pressure, presence of hypertension, duration of hypertension and duration of diabetes mellitus) was 0.713. The results from the univariate analysis for each component of the renal function tests are shown in Table 5. It is important to check the renal profile because an early detection and an aggressive intervention are needed to retard the progression of diabetic nephropathy to end stage renal failure. Diabetic nephropathy is a spectrum of progressive renal lesions secondary to diabetes mellitus ranging from renal hyperfiltration to end stage kidney disease (CPG, 2004).

However, the researchers found that *F. deltoidea* slightly increased the HDL level, i.e. from 1.41mmol/L to 1.42mmol/L, whereas the control group showed a reduction from

TABLE 4
Fasting lipid profile between the intervention and control groups

	Mean (sd[a])	Estimated Marginal Mean* (95% CIb)	F-statistic (*df*[c])	*p*-value
TC				
Intervention				
Pre	5.51 (1.185)	4.673 (3.229, 6.117)	0.003	0.956
Post	5.54 (2.180)	4.925 (2.981, 6.869)	(1, 10)	
Control				
Pre	5.86 (1.765)	6.391 (5.118, 7.663)		
Post	5.42 (1.283)	5.713 (4.000, 7.426)		
TG				
Intervention				
Pre	2.02 (1.804)	1.300 (-0.062, 2.662)	0.799	0.392
Post	2.06 (1.685)	1.761 (0.707, 2.816)	(1, 10)	
Control				
Pre	2.16 (1.823)	3.061 (1.861, 4.261)		
Post	1.89 (1.000)	2.332 (1.403, 3.262)		
HDL				
Intervention				
Pre	1.41 (0.433)	1.471 (1.151, 1.791)	0.784	0.397
Post	1.42 (0.430)	1.397 (1.089, 1.706)	(1, 10)	
Control				
Pre	1.36 (0.292)	1.251 (0.968, 1.533)		
Post	1.21 (0.217)	1.132 (0.860, 1.404)		
LDL				
Intervention				
Pre	3.33 (1.136)	2.718 (1.520, 3.917)	0.107	0.750
Post	3.21 (1.928)	2.745 (1.107, 4.383)	(1, 10)	
Control				
Pre	3.58 (1.333)	3.859 (2.803, 4.916)		
Post	3.33 (0.920)	3.518 (2.074, 4.962)		

[a] sd = standard deviation [b] CI = Confidence interval [c] *df* = degree of freedom
TC = total cholesterol; TG = triglycerides; HDL = high-density lipoprotein; LDL = low-density lipoprotein
* Controlled for age, sex, body mass index and duration of diabetes mellitus

1.36mmol/L to 1.21mmol/L. The LDL level was also found to have been reduced in both the groups. Nonetheless, the clinical studies of *F. deltoidea* are still scarce as compared to other studies using cinnamon. Khan *et al*. (2003) found that intakes of 1g, 3g, or 6 g of cinnamon per day reduces serum glucose, triglyceride, LDL cholesterol, and total cholesterol in people with type II diabetes and suggested the inclusion of cinnamon in the diet of people with type II diabetes would reduce risk factors associated with diabetes and cardiovascular diseases. However, a meta analysis study by Baker *et al*. in 2008 indentified five prospective randomized controlled trials (n=282), and the use of cinnamon did not significantly improve HbA1c, FBG or lipid parameters in the patients with Type I or Type II diabetes.

However, the researchers only conducted a 60-day study period. The study by Khan *et al.* also took around 60 days using cinnamon, whereas Vanschoonbeek *et al*. (2006) only took 6 weeks to complete their study using cinnamon.

TABLE 5
Renal function test between the intervention and control groups

	Mean (sd[a])	Estimated Marginal Mean* (95% CI[b])	F-statistic (df[c])	p-value
Uric acid				
Intervention				
Pre	320.1 (63.97)	233.7 (130.74, 336.61)	0.321	0.587
Post	342.4 (55.27)	269.0 (177.14, 360.94)	(1, 8)	
Control				
Pre	353.7 (115.86)	457.0 (369.87, 544.23)		
Post	400.8 (114.63)	498.6 (420.76, 576.43)		
Urea				
Intervention				
Pre	4.52 (2.228)	4.997 (1.739, 8.255)	0.445	0.523
Post	4.26 (1.584)	5.991 (4.188, 7.794)	(1, 8)	
Control				
Pre	4.46 (2.387)	4.710 (1.951, 7.470)		
Post	5.15 (2.473)	4.381 (2.854, 5.908)		
Creatinine				
Intervention				
Pre	77.33 (16.016)	63.195 (43.257, 83.133)	4.561	0.065
Post	87.44 (30.582)	85.084 (58.256, 111.912)	(1, 8)	
Control				
Pre	82.40 (32.377)	107.6 (90.67, 124.44)		
Post	87.30 (36.661)	102.0 (79.30, 124.75)		
Sodium				
Intervention	138.89 (3.180)			
Pre	137.11 (3.689)	137.3 (132.29, 142.27)	0.071	0.797
Post		135.7 (130.83, 140.49)	(1, 8)	
Control	138.30 (2.869)			
Pre	137.70 (3.713)	138.4 (134.17, 142.63)		
Post		137.6 (133.50, 141.68)		
Potassium				
Intervention				
Pre	4.44 (0.368)	4.235 (3.798, 4.673)	0.105	0.754
Post	4.61 (0.491)	4.340 (3.819, 4.860)	(1, 8)	
Control				
Pre	4.39 (0.354)	4.672 (4.301, 5.042)		
Post	4.26 (0.372)	4.620 (4.180, 5.061)		
Chloride				
Intervention				
Pre	103.11 (3.689)	105.3 (101.50, 109.02)	3.642	0.093
Post	101.33 (2.872)	100.3 (97.89, 102.62)	(1, 8)	
Control				
Pre	101.10 (2.685)	98.3 (95.14, 101.51)		
Post	101.90 (2.079)	102.0 (100.02, 104.02)		

[a] sd = standard deviation [b] CI = Confidence interval [c] *df* = degree of freedom
* Controlled for age, sex, systolic blood pressure, diastolic blood pressure, presence of hypertension, duration of hypertension and duration of diabetes mellitus

CONCLUSION

The effect of *Ficus deltoidea* on fasting blood sugar, lipid and renal profile showed that the results were not significant. However, a few patients in the intervention group had relatively fresh feeling and became energetic compared to the control group. Nonetheless, more studies are still needed in order to explore the efficacy of *F. deltoides*.

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REFERENCES

Aminuddin, N. (2006). *Keputusan kajian saintifik mas cotek oleh Universiti Malaya*. Retrieved on 29 December 2006 from http://www.delto.com.my/cert%20sah.htm.

Anon (2000). More over viagra. *Daily Express Sabah*, 22nd January.

Bailey, C. J., & Day, C. (1989). Traditional plant medicine as treatments for diabetes. *Diabetes Care,* 12, 553-564.

Basu, S. K. (1999). *Health problems and health care of the tribal population of India. Tribal Economy, Health and Wasteland Development*. New Delhi: Inter-India Publications *34,* 45-49.

Buhler, D., & Cristobal, M. (2000). Antioxidant activities of flavonoids. Retrieved on 7 February 2007 from http://lpi.oregonstate.edu/f-w00/flavonoid.html. and http://www.quackwatch.org/01QuackeryRelatedTopics/herbs.html.

Burkill, I. H., & Haniff, M. (1930). Malay Village Medicine. *Garden's Bulletin*, *6*(2), 167-332

Chang, Y. S., Vimala S., Mazura, M. P., & Ong B. K. (2004). Current trends and perspectives. *Proceedings of the Seminar on Medicinal and Aromatic plants*. FRIM: Malaysia.

Clinical Practice Guidelines for Diabetic Nephropathy (2004). The Malaysian consensus.

Duraipandiyan, V., Muniappan A., & Savarimuthu, I. (2006). Antimicrobial activity of some ethnomedicinal plants used by Paliyar Tribe from Tamil Nadu, India. *BMC Complementary and Alternative Medicine.*

Elliot, J. G. (1999). Application of antioxidant vitamins in foods and beverages. *Food Technology, 53*, 46-48.

Fasihuddin, B. A., & Din, L. B. (1995). *Medicinal plants used by various ethnic groups in Sabah.* The French Malaysian-Symposium on Natural Products. Dept of Chemistry, (p. 85). University of Malaya, Kuala Lumpur.

Goffman, Nicole. (2003). Why should we endeavor to include herbs in our daily diet. Retrieved from http://www.aarongoffman.com/nicole/Pages/writings/Writings/whyUseHerbs.html.

Hanninen J., Takala J., & Keinanen- Kiukaanniemi S. (1998). Quality of life in NIDDM patients assessed with SF-20 questionnaire. *Diabetes Research and Clinical Practice, 42*, 17-27.

International Diabetes Federation (IDF), Facts and figures, prevalence. Retrieved from http://www.idf.org/home/index.cfm?node=264

Kaur, C., & Kapoor, H. C. (2001). Antioxidant in fruits and vegetables in the millennium's health. *International Journal of Food Science and Technology*, *36,* 703-725.

Khan, M. Safdar, Ali Khan, M. M., Khattak, K. N., & Anderson, R.A. (2003). Cinnamon improves glucose and lipids of people with Type 2 Diabetes. *Diabetes Care, 26,* 3215-3218.

Vanschoonbeek, K., Thomassen, B. J. W., Senden, J. M., Wodzig, W. K. W. H., & Van Loon, L. J. C. (2006). Cinnamon supplementation does not improve glycemic control in postmenopausal Type 2 diabetes patients. *Journal of Nutrition April 1, 13,* 977-980.

Knekt, P., Jarvinen R., Seppanen R, Heliovaara M., Teppo L., & Aroma A. (1997). Dietary flavonoids and the risk of lung cancer and other malignant neoplasms. *American Journal of Epidemiology, 146,* 23-230.

Kumar, & Clark (1998), Clinical Medicine Fourth Edition, Elsevier.

Mafauzy, M. (2004). *Research on Malaysian Plants for Diabetes Mellitus*. The Malaysian-Swedish Dialogue in Medical Research. 5-6 February 2004, Legend Hotel, Kuala Lumpur.

Malaysian Medical Association News (2008). *Traditional & Complementary Medicine & hope more research will be conducted to provide this evidence*. 2008, May.

Musa, Y., Yahaya H., Wan Zaki W. M., & Zaharah H. (2004). Mas Cotek - Tumbuhan ubatan baru yang amat berpotensi. *Agromedia*, 2004, Mac.

Musa, Y., Yahaya H., & Wan Zaki W. M. (2004). Mas Cotek - Tumbuhan ubatan untuk kesejahteraan wanita. *Agromedia*, 2004, Mac.

Nahas, R., & Moher, M. (2009). Complementary and alternative medicine for the treatment of type 2 diabetes. *Canadian Family Physician, 55,* 591-596.

Rugayah, B. (1997). *Diabetes Mellitus: Among adults aged 30 years and above*.

Second National Health and Morbidity Survey Report. Volume 9. Retrieved on 10 May 2009 from http://www.diabetes.org.my/DiabMalaysia.htm

Shanker, K. (2006). Ethnomedical plants used by the Valaiyan Community of Malanadu Hills (Reserved Forest), Karnataka India-A Pilot Study. *African Journal of Traditional, Complementary and Alternative Medicines 3,*101-114.

Shapiro, K., & Gong, W. C. (2002). Natural products used for diabetes. *J Am Pharm Assoc (Wash), 42*(2), 217-226.

Sorolangum (1999). Deforestation ruins Jambi's rich medicinal heritage. *Jakarta Post,* 1999, September 22.

Sri Yuliani (2001). *Tabat Barito (Ficus deltoidea Jack). Dalam Tumbuhan obat Indonesia. Penggunaan dan Khasiatnya*. Jakarta: Pustaka Popular.

Starr, F., Kim, S., & Lloyd, L. (2003). *Plants of Hawaii*. Retrieved on, 2009, February 8 from http://www.hear.org/starr/hiplants/reports/html/Ficus_deltoidea.htm.

Steinmetz, K. A., & Potter, J. D. (1996). Vegetable, fruit and cancer prevention. *Journal of American Dietetic Association, 2*, 325-351.

Vattem, D. A., Ghaedian, R., & Shetty, K. (2005). Enhancing health benefits of berries through phenolic antioxidant enrichment: focus on cranberry. *Asia Pac J Clin Nutr, 14*(2), 120-130.

Weiger, W. A., Smith, M., Boon, H., Richardson, M. A., Kaptchuk, T. J., & Eisenberg, D. M. (2002). Advising Patients Who Seek Complementary and Alternative Medical Therapies for Cancer. *Annals of Internal Medicine, 137*(11), 889-903.

Weiss Barry, D. MD. (1999). *20 common problems in Primary Care* (1st Ed.). New York: McGraw-Hill.

Wild, S., Roglic, G., Green A., Sicree, R., & King, H. (2004). Global prevalence of Diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care,* 27, 1047-1053.

Baker, W. L., Gutierrez-Williams, G., White, C. M., Kluger, J., & Coleman, C. I. (2008). Effect of Cinnamon on Glucose Control and Lipid Parameters. *Diabetes Care, 31*(1), 41-43.

Yaakob, M., & Wan Zaki W.M. (2004). Emas Cotek, Tumbuhan Ubatan Untuk Kesejahteraan Wanita. *Agro Media,* 2004, March, 66-68.

Zainah Adam, Muhajir Hamid, Amin Ismail, & Shafii Khamis (2007). Effects of Ficus deltoidea Aqueous Extract on Blood Glucose Level in Normal and Mild Diabetic Rats. *Jurnal Sains Kesihatan Malaysia, 5*(2), 9-16.

Zanariah, H., Chandran, L. R., Wan Mohamad, W. B., Wan Nazaimoon, W. M., Letchuman, G. R., Jamaiyah, H., Fatanah, I., Nurain, M. N., Helen Tee, G. H., & Mohd Rodi, I. (2006). *Prevalence of Diabetes Mellitus in Malaysia in 2006*. 3rd National Health and Morbidity Survey (NHMS III), Ministry of Health, Malaysia.

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Diversity and Morphological Characteristics of *Aspergillus* Species and *Fusarium* Species Isolated from Cornmeal in Malaysia

Nithiyaa, P.[1], Nur Ain Izzati, M.Z.[1*], Umi Kalsom, Y.[1], Salleh, B.[2]

[1]*Department of Biology,*
Faculty of Science, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia
[2]*School of Biological Sciences,*
Universiti Sains Malaysia,
11800 Minden, Penang, Malaysia
**E-mail: izzati@science.upm.edu.my*

ABSTRACT

Corn is a vital food component that serves as a nutritional diet element for human and feedstuff for livestock. Despite its vast importance, corn frequently faces contamination problem caused by a range of microbes especially fungi. For the purpose of this study, cornmeal samples were collected from nine states in Malaysia, and were cultured onto Peptone Pentachloronitrobenzene Agar (PPA) to isolate the fungi. Single spore isolation was done on Potato Dextrose Agar (PDA) to obtain the pure culture. A total of 314 isolates of microscopic fungi were obtained, 284 isolates belonging to the *Aspergillus* species, namely *A. flavus* (241), *A. niger* (24), *A. nidulans* (14) and *A. fumigatus* (5). Another 30 isolates were *Fusarium* species, identified as *F. verticillioides* (14), *F. semitectum* (10) and *F. proliferatum* (6). The diversity of the fungi was determined by using Shannon-Weiner Index. The diversity index indicated that *A. flavus* was the most abundant, recorded as 0.203.

Keywords: ***Aspergillus flavus, A. niger, A. nidulans, A. fumigatus, Fusarium verticillioides*, *F. semitectum, F. proliferatum,* cornmeal**

INTRODUCTION

Corn is categorized as a main staple food and industrial material which serves as an additional food and nutritional supplement for both human and animals. It also serves as an important food component for poultry and swine consumption and is directly used in the farming industry, hence contributing to the high demand of corn worldwide.

Corn plantations are vulnerable to fungal infection caused by *Fusarium* species, especially in cool and humid climates (Lacey, 1989; Sewram *et al.,* 1999; Vanara *et al.,* 2009). *Fusarium* species play a significant role as plant pathogens, causing a broad range of diseases on various host plants, such as vascular wilt, pre and post-appearance blight as well as root and stem rots (Pascale *et al.,* 2002; Schollenberger *et al.,* 2006). *Fusarium* is also considered as a vital genre associated with cereal mycology, where this pre-harvest fungal infection prolongs until post-harvest and storage stage (Hussein *et al.,* 1991; Plattner *et al.,* 1991; Mubatanhema *et al.,* 1999; Ezekiel *et al.,* 2008). According to Ezekiel *et al.* (2008), poor storage condition, storage period, temperature, humidity levels and suitable climate could lead to infection caused by various storage fungi, such as *Aspergillus*

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*Corresponding Author

species, *Penicillium* species, *Curvularia* species *Alternaria alternata*, *Cladosporium cladosporioides* and *Phoma* species (Horn *et al.,* 2000; Reddy *et al.,* 2009).

The fungi that contaminate the feed, produce a wide range of secondary metabolites that give negative effects to animal and human. They are known as mycotoxin. Mycotoxin causes several losses in the quality of the corn. It has been recorded to affect the health of it consumers, human and animal (Mubatanhema *et al.,* 1999; Sewram *et al.,* 1999; Zinedine & Manes, 2009). Livestock fed with contaminated feeds were diagnosed with symptoms of weight decrease, liver or kidney cancer, leukoencephalomalacia and death (Munkvold *et al.,* 1998; Pascale *et al.,* 2002). Since contamination on grain cereal has been proven to be one of the major causes of toxicity affecting animals and human, special attention must therefore be paid during the processes of storage, handling and packaging.

The objectives of this study were to investigate the diversity and distribution of microscopic fungi associated with cornmeal, as well as to identify the fungi based on morphological diagnostics.

MATERIALS AND METHODS

Samples Collection and Isolation of Aspergillus *and* Fusarium *Isolates*

The cornmeal samples were collected from 9 states throughout Malaysia, namely, Pulau Pinang, Selangor, Melaka, Negeri Sembilan, Johor, Pahang, Terengganu, Sabah and Sarawak (Table 1). The samples were surface sterilized with 0.525% Sodium hypochlorite (NaOCl), soaked for 10 seconds and rinsed with sterile distilled water twice. The sterilized samples were cultured onto Peptone Pentachloronitrobenzene Agar (PPA) to isolate the fungi (Papavizas, 1967). Single spore isolation was then carried out onto Potato Dextrose Agar (PDA) to obtain pure cultures. These cultures were incubated for 7 days at room temperature (25 ± 2ºC) and were further proceeded for identification. *Fusarium* was identified according to the *Fusarium* Laboratory Manual by Leslie and Summerell, (2006), whereas *Aspergillus* identified according to Raper and Fennell (1965).

The Macromorphology of Aspergillus *and* Fusarium

The *Fusarium* and *Aspergillus* isolates were cultured on PDA and incubated for 7 days under standard temperature, lighting and period of incubation conditions (Salleh & Sulaiman, 1984). The macromorphological features of *Aspergillus* and *Fusarium* isolates, such as the colony features, pigmentation and presence or absence of sporodochia on PDA (only *Fusarium*), were observed. Meanwhile, the growth rates of the isolates were examined according to Diba *et al.,* (2007), with minor modification by measuring the diameter of the fungal colony after 72 hours of incubation.

The Micromorphology of Aspergillus *and* Fusarium

Fusarium were grown on the Carnation Leaf Agar (CLA) and incubated for 7-10 days. The carnation leaves were obtained from a local florist and washed under running tap water for 12 hours. The clean leaves were cut into 1cm length and dried in the oven (Memmert) at 60ºC until the leaf pieces became dry and brittle. The leaves were then autoclaved at 121ºC for 15 minutes. After that, the water Agar (WA) was prepared by dissolving 20g of straw agar into 1L distilled water and autoclaved. The WA was poured into sterile petri dish and 5 pieces of sterile carnation leaves were placed onto the medium and allowed to dry before culturing the *Fusarium* isolates. After 7-10 days of incubation, an *in situ* observation was done by cutting a block of *Fusarium* colonized agar and placed on a clean slide with no cover slip. The agar cube was observed of light microscope (Carl Zeiss) to examine the arrangement of the conidia. Water mount of CLA cultures was done by scrapping the fungal mycelia and placing on a drop of sterile distilled water on a clean slide. The shape and size of conidia, conidiophores

and presence and the absence of chlamydospores were observed (Burgess *et al.,* 1994; Summerell *et al.,* 2003; Leslie & Summerell, 2006).

Moist chamber cultures were prepared for *Aspergillus* by cutting a block (1cm^2) uninoculated PDA and placed on a clean slide. The *Aspergillus* isolate was cultured on all four sides of the agar block and covered with a cover slip and incubated for 7 days in a sterile petri dish layered with a damp filter paper. The inner surface of the cover slip colonized by the mycelia was placed on a new slide containing a drop of sterile distilled water. The slide culture was observed using light microscope (Carl Zeiss) to examine the size and arrangement of conidia on the conidial heads, conidiophores and vesicle structures (Samson, 1979).

Diversity of Aspergillus *and* Fusarium

The diversity of all the microfungi isolates was calculated by using the Shannon-Weiner Index, based on the following formula (Cuenca & Meneses, 1996).

$$H' = -\sum_{i=1}^{s} p_i (\ln p_i)$$

Where: H'= value of Shannon-Weiner Index
∑= total
p_i= proportion of ith species
ln= natural log

RESULTS AND DISCUSSION

A total of 314 isolates of microscopic fungi consisting of *Fusarium* species and *Aspergillus* species were obtained from the cornmeal samples collected throughout Malaysia (Table 1). Two hundred and eighty four isolates (90.4%) were *Aspergillus* species, identified as *A. flavus*, *A. nidulans, A. niger* and *A. fumigatus*. The remaining 30 isolates (9.6%) belonging to *Fusarium* species were identified as *F. semitectum, F. proliferatum* and *F. verticillioides*.

The colony features of *A. flavus* on PDA were initially white and turned yellowish green to light green (*Fig. 1A-B*). Some isolates have black sclerotia upon maturation. The colony's growth rates ranged between 0.77-0.83 cm/day at room temperature (25 ± 2ºC). The conidial heads appeared green with radiating and columnar measuring 30.0-100.0 µm in diameter (*Fig. 1C-D*). The conidiophores were smooth and moderate in length, measuring 800.0 µm. The vesicles were globose upon maturity with

TABLE 1
Distribution of *Aspergillus* and *Fusarium* isolated from cornmeal according to the state of origin.

States	Isolates							Total
	A. flavus	*A. nidulans*	*A. niger*	*A. fumigatus*	*F. semitectum*	*F. proliferatum*	*F. verticillioides*	
Pulau Pinang	26	0	4	0	2	1	1	34
Selangor	25	3	2	0	0	5	11	46
Melaka	11	8	0	0	0	0	0	19
Negeri Sembilan	3	0	0	0	0	0	1	4
Johor	17	1	2	0	0	0	1	21
Pahang	81	1	8	4	3	0	0	97
Terengganu	18	0	5	0	0	0	0	23
Sabah	35	0	0	0	5	0	0	40
Sarawak	25	1	3	1	0	0	0	30
Total	241	14	24	5	10	6	14	314
Percentage	76.8	4.5	7.6	1.6	3.2	1.9	4.5	100.0

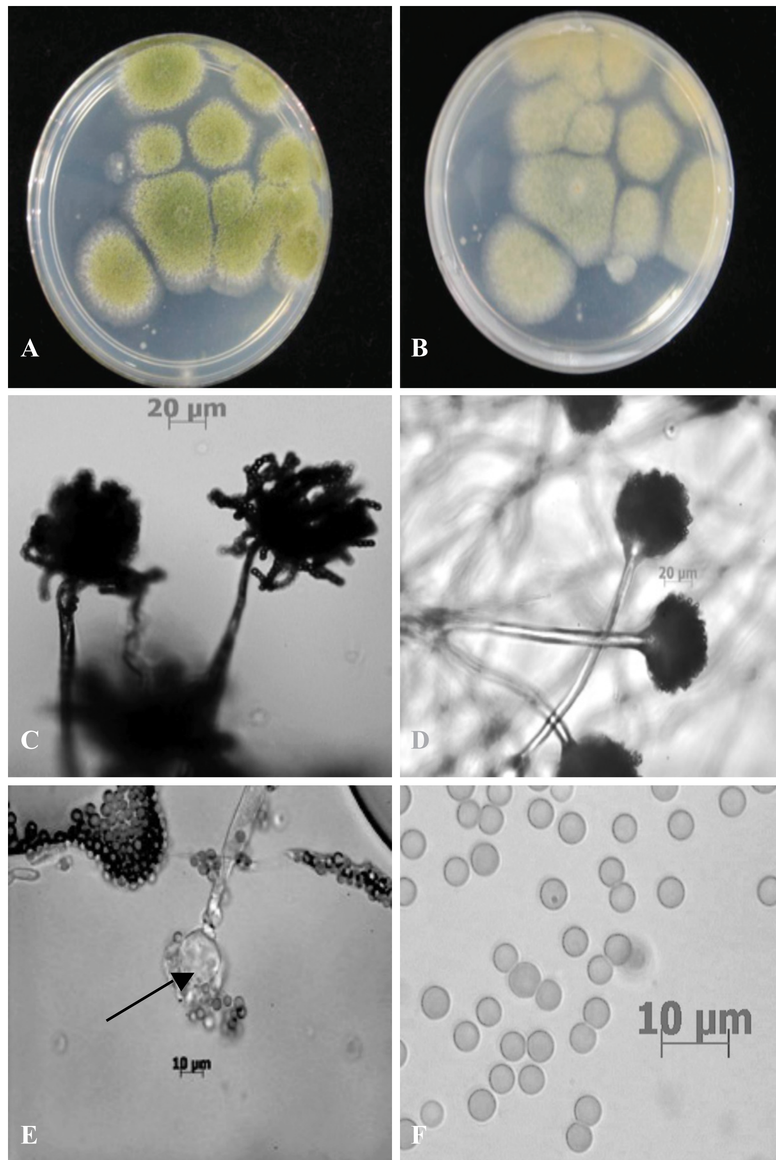

Fig. 1: Macroscopic and microscopic characteristics of A. flavus. *A-B: colony features on PDA, upper and lower surface, respectively; C-D: conidial heads; E: vesicle (arrow); F: conidia*

heads, and 20.0-45.0 µm in diameter, and with both uniseriate and biseriate sterigmata (*Fig. 1E*). The conidia are 3.0-6.0 µm in size with smooth cell wall (*Fig. 1F*). The morphological identification needs sufficient growth in order to evaluate the colony characteristics and microscopic features. Hence, PDA was used to accelerate the growth rate and the production of conidia, as reported by Diba et al., (2007).

A. niger isolates grew rapidly on PDA and were visibly white initially but they appeared

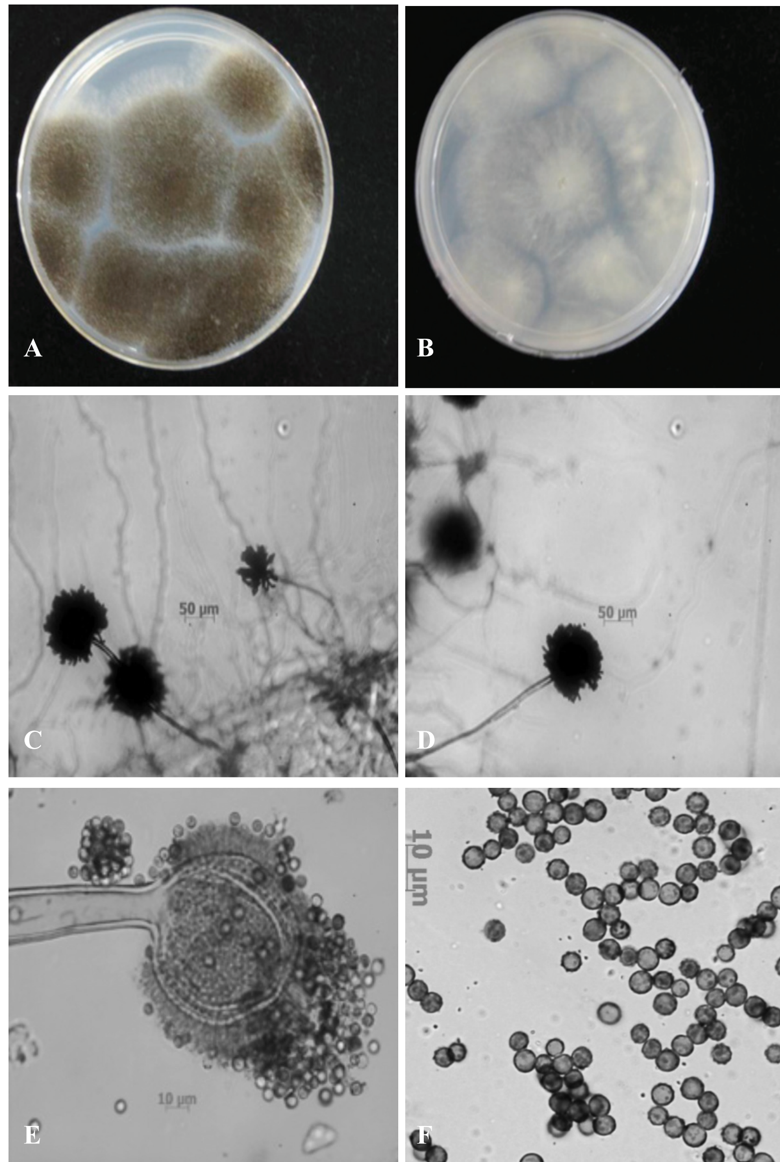

Fig. 2: Macroscopic and microscopic characteristics of A. niger. *A-B: colony features on PDA upper and lower surface, respectively; C-D: conidial heads; E: vesicle; F: coarsely roughened conidia*

black and powdery on the second day of incubatison (*Fig. 2A-B*). After three days, the growth rates were determined to be 0.73-0.83 cm/day. Some isolates grew rapidly and were uniformly distributed and scattered. Meanwhile, the conidial head was brownish black and it split into several irregular and regular columns of conidial chain (*Fig. 2C-D*). The diameter of the conidial heads ranged from 70.0-120.0 µm. The conidiophores were hyaline, brown in colour, measuring more than 500.0 µm. Vesicles were hyaline, globose, and also brown in colour, 30.0-75.0 µm in diameter with uniseriate sterigmata (*Fig. 2E*). The conidia were brown to black,

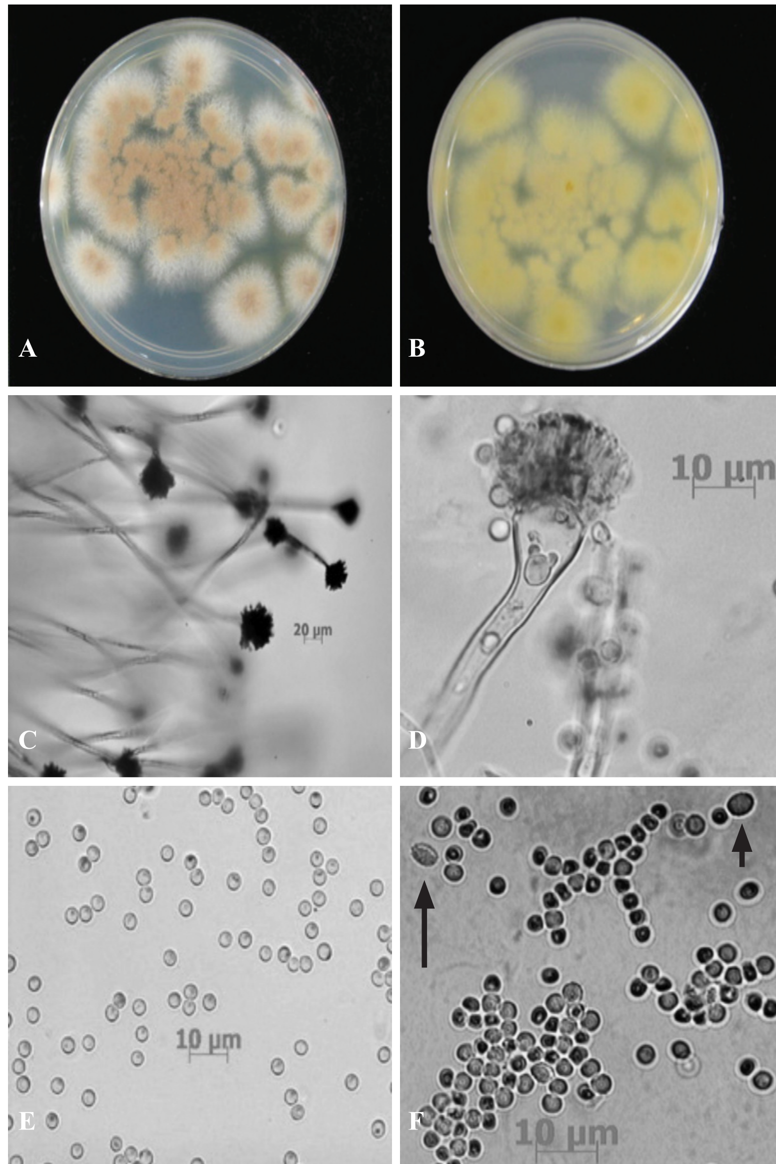

Fig.3: Macroscopic and microscopic characteristics of A. nidulans*. A-B: colony features on PDA upper and lower surface, respectively; C: conidia head; D: conidiophore with vesicle containing chains of conidia; E: conidia; F: smooth walled ascospores as indicated by arrows*

globose, very rough, and with a diameter ranging between 4.0-5.0 µm (*Fig. 2F*) (Raper & Fennell, 1965; Samson, 1979).

A. nidulans isolates initially appeared yellow and gradually turned orange and completely brown on matured cultures (*Fig. 3A-B*). The growth rates of the colony were 0.50-0.67 cm/day after three days of incubation. The conidial heads have short columnar, with a diameter of 40.0-80.0 µm (*Fig. 3C*). Conidiophores were

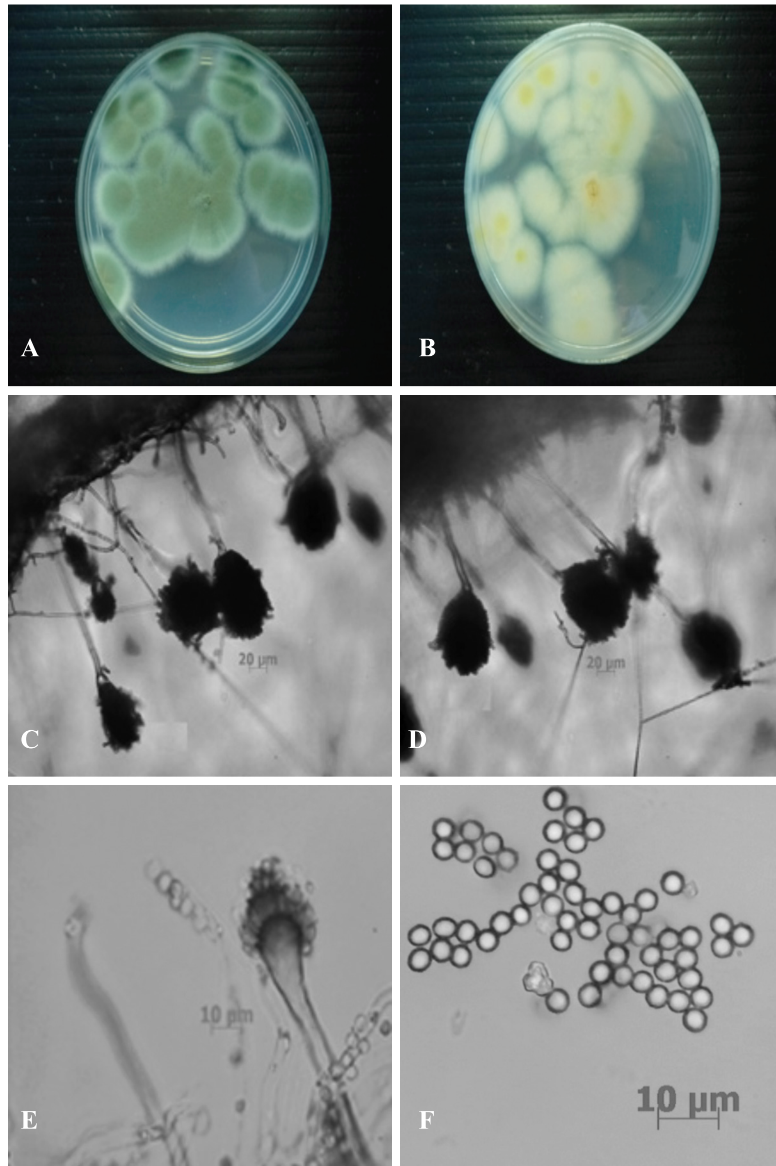

Fig. 4: Macroscopic and microscopic characteristics of A. fumigatus*. A-B: colony on PDA upper and lower surface, respectively; C-D: conidial heads; E: short conidiophores with vesicle; F: conidia*

moderate in length (less than 150 µm) and brown in colour. The vesicles were hemispherical in shape, small, globose and flattened at the apical part, and diameters ranging from 8.0-12.0 µm, with uniseriate and biseriate sterigmata (*Fig. 3D*). Meanwhile, conidia were light brown in colour, globose and rough walled and measuring at 3.0-4.0 µm in diameter (*Fig. 3E*). The ascospore have rough wall, reddish brown in colour, and with a diameter of 4.0-5.0 µm (*Fig. 3F*). The conidial heads of this species were small and compactly columnar (Raper & Fennell, 1965; Samson, 1979).

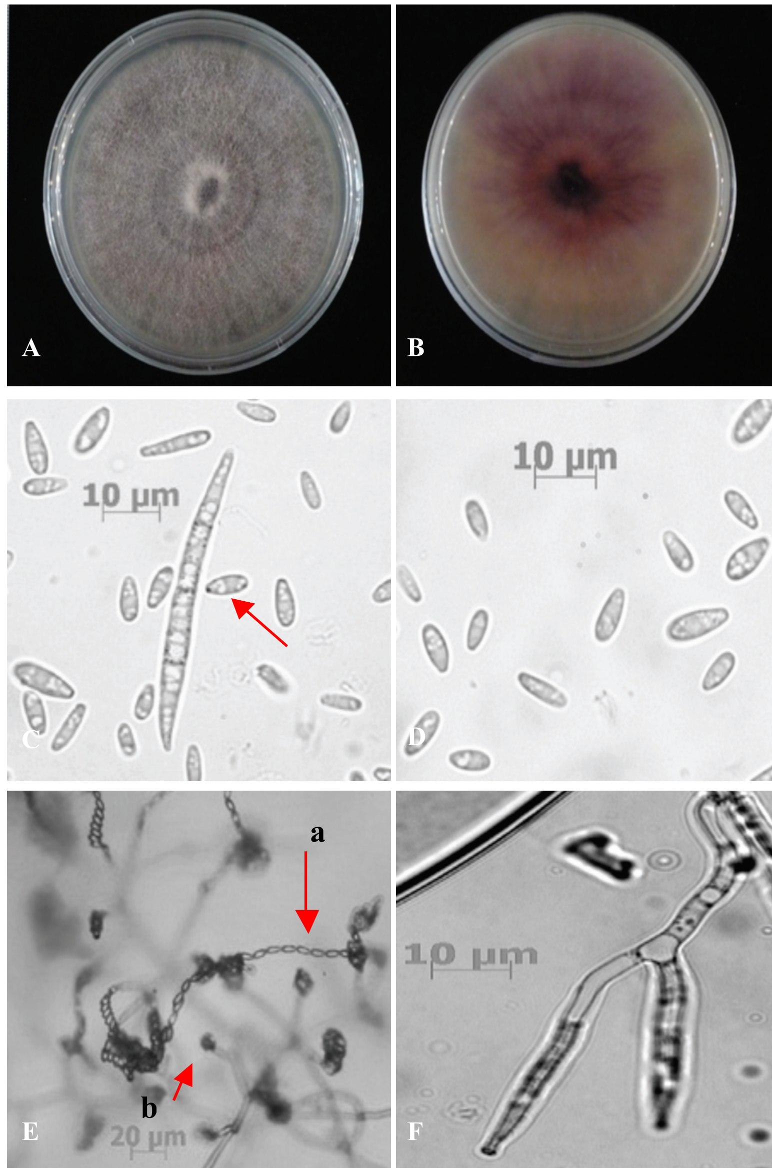

Fig. 5: Macroscopic and microscopic characteristics of F. verticillioides*. A-B: colony features on PDA upper and lower surface respectively; C: macroconidia (arrow); D: microconidia; E: microconidia forms long chains (a) and false head (b); F: branched monophialides*

The colony of *A. fumigatus* on PDA grew white initially and then turned dark green, while its old cultures turned smoky gray (*Fig. 4A-B*). The growth was rapid, i.e. at the growth rates of 0.47-0.67 cm/day after three days of incubation with dense colony. The conidial heads, measuring 70.0-80.0 µm with short conidiophores rising vertically from the hyphae (*Fig. 4C-D*). The conidiophores measured around 300.0 µm in length. The conidial head

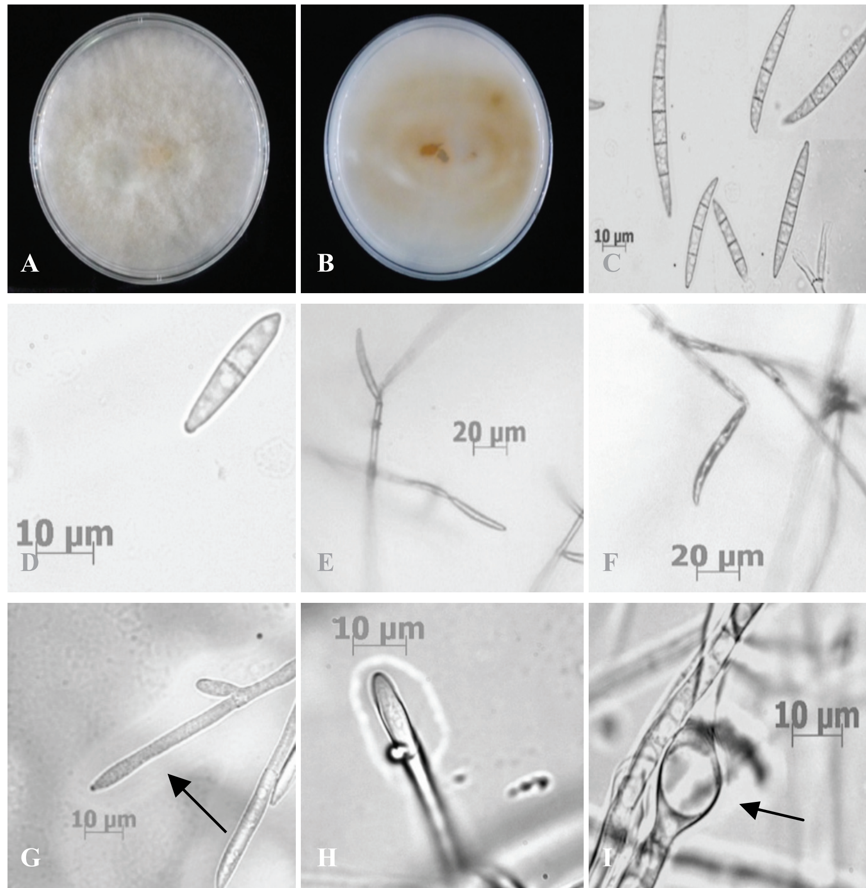

Fig. 6: Macroscopic and microscopic characteristics of F. semitectum*. A-B: colony features on PDA upper and lower surface, respectively; C: macroconidia; D: microconidia; E-F: mesoconidia froms singly on aerial hyphae; G: monophialides (arrow); H: polyphialides; I: intercalary chlamydospore (arrow)*

comprises a vesicle-like structure with size, 20.0-30.0 µm that holds the uniseriate sterigmata (*Fig. 4E*). Meanwhile, the conidia were green in colour and sub-globose with a diameter between 2.4-3.7 µm (*Fig. 4F*). *A. fumigatus* can be characterized by usually having subclavate vesicles (Samson *et al.,* 2006).

F. verticillioides isolates on the PDA formed aerial mycelia and produced violet pigments, ranging from a pinkish orange to dark violet (*Fig. 5A-B*). The growth rate of the colony ranged from 0.90-0.93 cm/day after three days. The macroconidia produced were long, slender, straight and thin walled, measuring around 37.0-55.0 µm x 4.0-4.2 µm, with three septates (*Fig.5C*). The apical cell of the macroconidia was curved and tapered, and the basal cell was notched. Microconidia were oval or abovoid with a flat base with 0-2 septate and 4.0-33.0 µm x 2.4-3.3 µm in size (*Fig. 5D*). The microconidia were abundant in aerial mycelia, formed long chains or false head (*Fig. 5E*) attached at branched monophialides (*Fig. 5F*). The cultures did not produce chlamydospores. Microconidia always formed from monophialides that occur in V-shaped pairs and were found in chains that are moderately long (Leslie & Summerell, 2006).

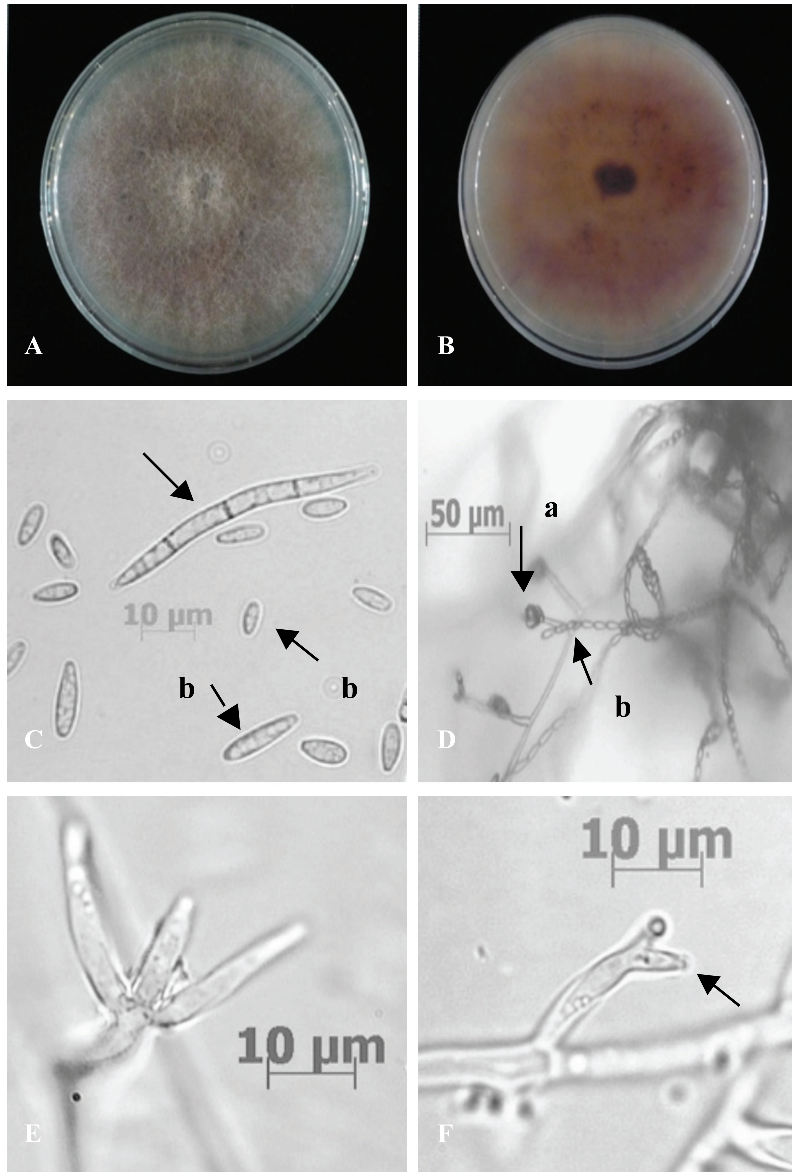

Fig. 7: Macroscopic and microscopic characteristics of F. proliferatum*. A-B: colony features on PDA upper and lower surface, respectively; C: macroconidia (a) and microconidia (b); D: microconidia in false head (a) and in chain (b); E: branched monophialides; F: polyphialides.*

The colony features of *F. semitectum* on the PDA exhibited a thick layer of aerial mycelia. The colony was initially white, while some produced light orange pigments, and turned beige and brown in matured cultures (*Fig. 6A-B*). The growth rates after three days were 0.80-0.93 cm/day. Macroconidia has a curved surface on vertical and a straight surface at the dorsal (*Fig. 6C*). The apical cell was curved and tapered and the basal cell was foot-shaped, measuring

TABLE 2
Diversity of *Aspergillus* species and *Fusarium* species in each sampling location

Locations	*Aspergillus* sp.		*Fusarium* sp.	
	Total	H'	Total	H'
Pulau Pinang	30	0.238	4	0.268
Selangor	30	0.238	16	0.335
Melaka	19	0.181	0	0
Negeri Sembilan	3	0.050	1	0.113
Johor	20	0.186	1	0.113
Pahang	94	0.366	3	0.230
Terengganu	23	0.204	0	0
Sabah	35	0.258	5	0.299
Sarawak	30	0.238	0	0

around 39.0-79.0 µm x 4.9-6.6 µm in size with 4-6 septate. Microconidia is oval-shaped with 1 septate (*Fig. 6D*). The size of microconidia is 20.0-37.4 µm x 4.0-4.2 µm. Single mesoconidia with 3 septates are abundance in the aerial mycelia, (*Fig. 6E-F*), measuring 25.0-37.4 µm x 4.0-5.0 µm. Conidiophores were monophialides (*Fig. 6G*) with/without polyphialides (*Fig. 6H*). Intercalary chlamydospore formed with diameter 22.0-26.0 µm (*Fig. 6I*) were also present. Mesoconidia were abundant in the aerial mycelia and were easily observed microscopically *in situ*, whereas microconidia are sparse and often difficult to find (Nelson *et al.,* 1983; Burgess *et al.,* 1994; Leslie & Summerell, 2006).

F. proliferatum producing aerial mycelia and pigmentation was violet, ranging from light to dark violet (*Fig. 7A-B*), and with growth rates of 0.97-1.07 cm/day. The macroconidia are thin-walled, slender and straight, with 3-5 septates (*Fig. 7C*). The apical cell was curved and measuring around 29.0-75.0 µm x 3.3-4.2 µm in size. The microconidia were abovoid with flattened base (*Fig. 7C*), 0-1 septate and formed in chains of moderate length and also in false heads (*Fig. 7D*), measuring 4.0-23.0 µm x 1.6-4.2 µm in size, while microconidia formed in chains of moderate length and in false heads borne from monophialides (*Fig. 7E*) and polyphialides (*Fig. 7F*). The chains of conidia were usually shorter than those formed by *F. verticillioides*. However, no chlamydospores were produced (Gerlach & Nirenberg, 1982).

The genus *Aspergillus* was abundantly found in the sample originating from Pahang, with a diversity index value of 0.366, followed by the sample from Sabah, with an index value of 0.258 (see Table 2). Meanwhile, the genus *Fusarium* was abundant in the samples from Selangor (0.335) and this was followed the samples from Sabah (0.299).

The diversity index based on Shannon-Weiner index showed that *A. nidulans* was the most abundant in the samples from Melaka, with 0.364 (Table 3). This was followed by *A. flavus* (0.332) and *A. niger* (0.332) in the samples from Selangor and Terengganu, respectively. Although *A. flavus* had a high percentage of occurrences (76.8%), it was found to be less diverse in the samples from Sabah (0.117). In addition, *A. fumigatus* was observed to be abundant in the samples from Pahang (0.131). The diversity of the *Fusarium* species indicated that *F. semitectum* was the most diverse in the cornmeal samples from Pulau Pinang and Sabah with index 0.167 and 0.260, respectively. This was followed by *F. proliferatum* in Selangor with an index of 0.242, whereas *F. verticillioides* was highly diverse (0.347) in the samples from Negeri Sembilan.

Based on the information gathered through the present study, it could be stated that the cornmeal was improperly stored and handled based on the presence of abundant microscopic fungi in the samples. This fact was due to the high diversity of *A. flavus* and *A. fumigatus*

TABLE 3
Diversity of Microscopic Fungi based on Shannon-Weiner Index

Locations	Aspergillus				Fusarium		
	A. flavus	*A. nidulans*	*A. niger*	*A. fumigatus*	*F. semitectum*	*F. proliferatum*	*F. verticillioides*
	*H’	*H’	*H’	*H’	*H’	*H’	*H’
Pulau Pinang	0.205	0	0.252	0	0.167	0.103	0.103
Selangor	0.332	0.177	0.135	0	0	0.242	0.342
Melaka	0.316	0.364	0	0	0	0	0
Negeri Sembilan	0.216	0	0	0	0	0	0.347
Johor	0.171	0.146	0.224	0	0	0	0.146
Pahang	0.151	0.05	0.205	0.131	0.108	0	0
Terengganu	0.192	0	0.332	0	0	0	0
Sabah	0.117	0	0	0	0.260	0	0
Sarawak	0.152	0.113	0.230	0.113	0	0	0

*Calculation: $pi = \frac{\sum \textit{Aspergillus} \text{ or } \textit{Fusarium} \text{ species in each state}}{\sum \textit{Aspergillus} \text{ or } \textit{Fusarium} \text{ in all states}}$

H’= pi(ln pi)

which are well known storage fungi that colonize post-harvested grains, specifically when provided with suitable conditions (Reddy *et al.,* 2009; Lacey, 1989). Conversely, *A. niger* is a saprophyte that grows readily on organic matters, as well as a pathogen that infects crops and stays dormant till storage (Raper & Fennell, 1965). According to Soriano and Dragacci (2004), amongst the corn-based products, cornmeal is one of the items that receives the mildest processing and treatment compared to other food stuff. Thus, it is very susceptible to fungal contamination, specifically air-borne fungi. The fungi that infected the cornmeal are capable of producing secondary metabolites that are responsible in causing hazardous diseases in both human and animal. Although the *Fusarium* species, such as *F. verticillioides* (Sacc.) Nirenberg (synonym, *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg (Torres *et al.,* 2001), are commonly known as plant pathogen, their invasion in the field could proceed till storage. *F. semitectum* is normally isolated from soil and possibly exists as soil inhabitants. Although this species is not regarded as significant plant pathogens, the species have been reported to cause diseases on several crops and widely distributed as saprophytes in soil, as well as on diseased tissues (Latiffah *et al.,* 2007). Moreover, there are occasions where other researchers managed to obtain *Fusarium* species in corn-based products, besides solely from corn plantation (Jimenez & Mateo, 1997; Pascale *et al.,* 2002). The prevalence of the *Fusarium* species is however lower compared to the *Aspergillus* species. Besides, the *Fusarium* species favours tropical and temperate areas to inhabit, therefore it has less adaptation to storage condition (Presello *et al.,* 2008).

CONCLUSION

Most of the *Aspergillus* and *Fusarium* isolates were obtained from the cornmeal samples from Pahang (H’=0.596), and this was followed by Selangor (H’=0.573). Meanwhile, the least number of isolates was obtained from Negeri Sembilan (H’=0.163). With the aid of the morphological identification, the diversity and

abundance of individual isolates of *Aspergillus* and *Fusarium* were attained instantly. The cornmeal samples were probably stored at a very poor condition since the diversity of storage fungi, specifically *A. flavus*, is tremendously high.

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REFERENCES

Burgess, L. W., Summerell, B. A., Bullock, S., Gott, K. P., & Backhouse, D. (1994). *Laboratory Manual for Fusarium Research*. Sydney: University of Sydney.

Cuenca, G., & Meneses, E. (1996). Diversity patterns of arbuscular mycorrhizal fungi associated with cacao in Venezuela. *Plant and Soil*, *183*, 315-322.

Diba, K., Kordbacheh, P., Mirhendi, S. H., Rezaie, S., & Mahmoudi, M. (2007). Identification of *Aspergillus* sp. using morphological characteristics. *Pakistan Journal of Medical Science*, *23*, 867-872.

Ezekiel, C. N., Odebode, A. C., & Fapohunda, S. O. (2008). Zearalenone Production by naturally occurring *Fusarium* sp. on maize, wheat and soybeans from Nigeria. *Journal of Biology and Environment Science, 6*, 77-82.

Gerlach, W., & Nirenberg, H. I. (1982). *The genus Fusarium: A pictorial atlas*. Berlin: Mitt. Biol. Bundes. Land- Forst. (Berlin - Dahlem).

Horn, B. W., Greene, R. L., & Dorner, J. W. (2000). Inhibition of Aflatoxin B1 Production by *Aspergillus* parasiticus Using Nonaflatoxigenic Strains: Role of Vegetative Compatibility. *Biological Control*, *17*, 147-154.

Hussein, H. M., Baxter, M., Andrew, I. G., & Franich, R. A. (1991). Mycotoxin production by *Fusarium* species isolated from New Zealand maize fields. *Mycopathologia, 113*, 35-40.

Jimenez, M., & Mateo, R. (1997). Determination of mycotoxins produced by *Fusarium* isolates from banana fruits by capillary gas chromatography and highperformance liquid chromatography. *Journal of Chromatography*, *778*, 363-372.

Lacey, J. (1989). Pre- and post-harvest ecology of fungi causing spoilage of foods and other stored products. *Journal of Applied Bacteriology Symposium Supplement*, 11-25.

Latiffah, Z., Mohd Zariman, M, & Baharuddin, S. (2007). Diversity of *Fusarium* species in cultivated soils in Penang. *Malaysian Journal of Microbiology, 3*, 27-30.

Leslie, J. F., & Summerell, B. A. (2006). *The Fusarium laboratory manual*. South Africa: Wiley-Blackwell Publishing.

Mubatanhema, W., Moss, M. O., Frank, M. J., & Wilson, D. M. (1999). Prevalence of *Fusarium* species of the Liseola section on Zimbabwean corn and their ability to produce the mycotoxins zearalenone, moniliformin and fumonisin B_1. *Mycopathologia*, *148*, 157-163.

Munkvold, G., Stahr, H. M., Logrieco, A., Moretti, A., & Ritieni, A. (1998). Occurrence of fusaproliferin and beauvericin in *Fusarium*-contaminated livestock feed in Iowa. *Applied and Environmental Microbiology, 64*, 3923-3926.

Nelson, P. E., Toussoun, T. A., & Marasas, W. F. O. (1983). *Fusarium Species: An Illustrated Manual for Identification*. University Park, Pennsylvania : Pennsylvania State University Press.

Papavizas, G. C. (1967). Evaluation of various media and antimicrobial agents for isolation of *Fusarium* from soil. *Phytopathology*, *57,* 848-852.

Pascale, M., Visconti, A., & Chelkowski, J. (2002). Ear rot susceptibility and mycotoxin contamination of maize hybrids inoculated with *Fusarium* species under field conditions. *European Journal of Plant Pathology*, *108*, 645-651.

Plattner, R. D., Ross, P. F., Reagor, J., Stedelin, J. and Rice, L. G. (1991). Analysis of corn and cultured corn for fumonisin B_1 by HPLC and GC/MS by four laboratories. *Journal of Veterinary Diagnosis and Investigation*, *3*, 357-358.

Presello, D. A., Botta, G., Iglesias, J., & Eyherabide, G. H. (2008). Effect of disease severity on yield and grain fumonisin concentration of maize

hybrids inoculated with *Fusarium verticillioides*. *Crop Protection*, *27,* 572-576.

Raper, K. B., & Fennell, D. I. (1965). *Description and morphology. The genus Aspergillus*. The Williams & Wilkins Company. (p. 17-29). United States of America: The Waverly Press.

Reddy, K. R. N., Reddy, C. S., & Muralidharan, K. (2009). Potential of botanicals and biocontrol agents on growth and aflatoxin production by *Aspergillus flavus* infecting rice grains. *Food Control*, *20*, 173-178.

Salleh, B., & Sulaiman, B. (1984). Fusaria associated with naturally diseasedplants in Penang. *Journal of Plant Protection in the Tropics*, *1*, 47-53.

Samson, R. A. (1979). A compilation of the *Aspergilli* described since 1965. *Studies in Mycology*, *18*, 1-38.

Samson, R. A., Hongs, S. B., & Frisvad, J. C. (2006). Old and new concepts of species differentiation in *Aspergillus*. *Medical Mycology*, *44*, 133-148.

Schollenberger, M., Muller, H. M., Rufle, M., Suchy, S., Plank, S., & Drochner, W. (2006). Natural occurrence of 16 *Fusarium* toxins in grains and feedstuffs of plant origin from Germany. *Mycopathologia*, *161*, 43-52.

Soriano, J. M., & Dragacci, S. (2004). Occurrence of fumonisins in foods. *Food Research International*, *37*, 985-1000.

Sewram, V., Nieuwoudt, T. W., Marasas, W. F. O., Shephard, G. S., & Ritieni, A. (1999). Determination of the mycotoxin moniliformin in cultures of *Fusarium subglutinans* and in naturally contaminated maize by high-performance liquid chromatography– atmospheric pressure chemical ionization mass spectrometry. *Journal of Chromatography*, *848*, 185-191.

Soriano, J. M., & Dragacci, S. (2004). Occurrence of fumonisins in foods. *Food Research International*, *37*, 985-1000.

Summerell, B. A., Salleh, B. and Leslie, J.F. (2003). A Utilitarian Approach to *Fusarium* Identification. *American Phytopathological Society, 87*, 117-128.

Torres, A. M., Reynoso, M. M., Rojo, F. G., Ramirez, M.L., & Chulze, S.N. (2001). *Fusarium* species (section Liseola) and its mycotoxins in maize harvested in northern Argentina. *Food Additives and Contaminants*, *18*, 836-843.

Vanara, F., Reyneri, A., & Blandino, M. (2009). Fate of fumonisin B_1 in the processing of whole maize kernels during dry-milling. *Food Control*, *20*, 235-238.

Zinedine, A., & Manes, J. (2009). Occurrence and legislation of mycotoxins in food and feed from Morocco. *Food Control*, *20*, 334-344.

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Utilization of Glucose Recovered by Phase Separation System from Acid-hydrolysed Oil Palm Empty Fruit Bunch for Bioethanol Production

Mohd Huzairi Mohd Zainudin[1], Nor'Aini Abdul Rahman[1], Suraini Abd-Aziz[1*], Masamitsu Funaoka,[3] Takanori Shinano[3], Yoshihito Shirai[2], Minato Wakisaka[2], and Mohd Ali Hassan[1]

[1]Department of Bioprocess Technology,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 Serdang, Selangor,Malaysia
[2]Department of Biological Functions and Engineering,
Graduate School of Life Science and Systems Engineering,
Kyushu Institute of Technology, 2-4 Hibikino,
Wakamatsu, Fukuoka, 808-0196 Japan
[3]Faculty of Bioresources,
Mie University, Kamihama, Tsu, Mie 514-8507, Japan
[*]E-mail: suraini@biotech.upm.edu.my

ABSTRACT

Oil palm empty fruit bunch (OPEFB) is one the most abundant lignocellulosic wastes produced throughout the year in the palm oil industry. A new process of separating lignocellulose components after acid hydrolysis (known as phase separation system) has been previously developed, by which lignin and carbohydrate can be completely and rapidly separated in 60 minutes between 25 and 30°C. In this process, cellulose is completely hydrolyzed to oligosaccharides and remains in the acid phase. The maximum glucose yield of 53.8% was obtained by hydrolysis, with 4% acid after autoclaving at 121°C for 5 minutes. This work focused on the separation of monosaccharide (glucose) from cellulose fraction, which was subsequently used as a substrate for ethanol production. For this purpose, different types of nitrogen sources were evaluated, with yeast extract as the best nitrogen source (93% of theoretical yield) as compared to palm oil mill effluent (POME) and sludge powder for the growth of acid tolerant *Saccharomyces cerevisiae* ATCC 26602. Batch and repeated batch fermentation of *S. cerevisiae* ATCC 26602 using OPEFB hydrolysate gave 0.46 g glucose g ethanol^{-1}, representing 87% of theoretical yield with a productivity of about 0.82 $g^{-1} l^{-1} h^{-1}$ and 0.48 g glucose g ethanol^{-1},representing 89% of theoretical yield with productivity of about 2.79 $g^{-1} l^{-1} h^{-1}$, respectively.

Keywords: Bioethanol, oil palm empty fruit bunch, phase separation system, acid hydrolysis, glucose

INTRODUCTION

Lignocellulose waste material, such as oil palm empty fruit bunch (OPEFB), is well known for its potential as a renewable resource for the production of food, feed and fine chemicals. Approximately 15 million tonnes of OPEFB is generated annually by palm oil mills in Malaysia (Rahman *et al*., 2006). In practice, OPEFB is burnt in incinerator to obtain bunch ash or is dumped for mulching in the oil palm plantation. With technologies such as diluted acid hydrolysis and enzymatic hydrolysis, cellulose from waste

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[*]Corresponding Author

materials is converted into glucose, leaving lignin as a by-product without any further use as value added-product. Most of lignins are burnt for power generation. Only a portion is used for various purposes, such as dispersant, pelletizing materials, molding stabilizers and concrete grinding additives (Funaoka *et al*., 1995). A new process of separating lignocellulose into lignin and carbohydrate moieties, with the conversion to highly reactive forms at room temperature, has been developed in previous study (Funaoka & Abe, 1989). This process includes phase-separation reaction system comprising of phenol derivatives and concentrated acid. In this process, lignocellulose material is rapidly separated into two main phases, called organic phase and aqueous phase. The acid solution is a solvent for carbohydrate and as a catalyst for the fragmentation and phenolation of lignin, while phenol derivatives act as phenolation agents, a barrier to minimize the attack of acid on the lignin and a solvent for the lignin fraction. Through this process, the lignin is converted into lignophenol. Lignophenol derivatives have currently been used for immobilizing proteins, specifically enzymes and they are also used in fibre composite (Funaoka, 1998). In order to develop the total usage of lignocellulosic materials, the carbohydrate (i.e. the by-product of the lignophenol) production can be utilized rather than left them unused. Currently, ethanol has gained a great interest as an alternative energy due to the concern of global warming and depletion of fossil fuel. Therefore, it is a good approach to convert these carbohydrates from biomass to ethanol. It has been estimated that OPEFB composed of 45-50% cellulose, a sugar polymer made of hexose sugar glucose (Rahman *et al*., 2006; Ariffin *et al*., 2008). The objective of this study was to utilize glucose recovered during phase separation process of acid-hydrolysed OPEFB for ethanol production.

MATERIALS AND METHODS

Sample Preparation

Shredded OPEFB was obtained from Seri Ulu Langat Palm Oil Mill, Dengkil, Selangor, Malaysia. The sample was degreased by soaking it with detergent overnight and washing was repeated until no trace of oil was observed. The sample was then placed in oven at 40°C. The sample was ground and sieved to 40 mesh. The extraction with ethanol:benzene (2:1) was done to remove extractives, such as terpene and tannin in the OPEFB fibre.

Phase Separation System

Ten ml *p*-cresol (a phenol derivative) was added to the treated OPEFB fibre and stirred for 10 minutes. Twenty ml of 72% sulfuric acid was then added to the mixture, and this was followed by vigorous stirring at room temperature (29-30°C) for 60 minutes. The mixture was then centrifuged at 3500 rpm, 25°C for 15 minute, as reported in the previous study by Funaoka *et al*. (1995).

Hydrolysis of Aqueous Phase for Substrate Preparation and Bioethanol Production

The aqueous phase from the phase separation process was separated and subjected to further treatment by adding water and hydrolyzed by boiling for 4 hours or autoclaving at 121°C for 5 minutes. The sample was then neutralized with $Ca(OH)_2$ and filtered to remove the precipitated $CaSO_4$. In addition, the sample was also concentrated using an evaporator (EYELA, Japan). The aqueous phase hydrolyzate was used as a substrate for ethanol production, as summarized in *Fig. 1*.

Microorganism and Medium

The yeast *Saccharomyces cerevisiae* ATCC 22062 was used for ethanol fermentation. It was kept at -30°C in Yeast Medium Broth (YMB) with 30% glycerol. The cells were transferred and maintained in the medium containing (in 1 L medium): 10.0 g glucose, 3 g yeast extract, 3 g malt extract, 5 g peptone and 20.0 g agar at 4°C and sub-cultured every 2 weeks. The growth medium consisted of (in 1 l medium) 30.0 g glucose, 5 g yeast extract and 3g peptone. Meanwhile, the fermentation medium consists of

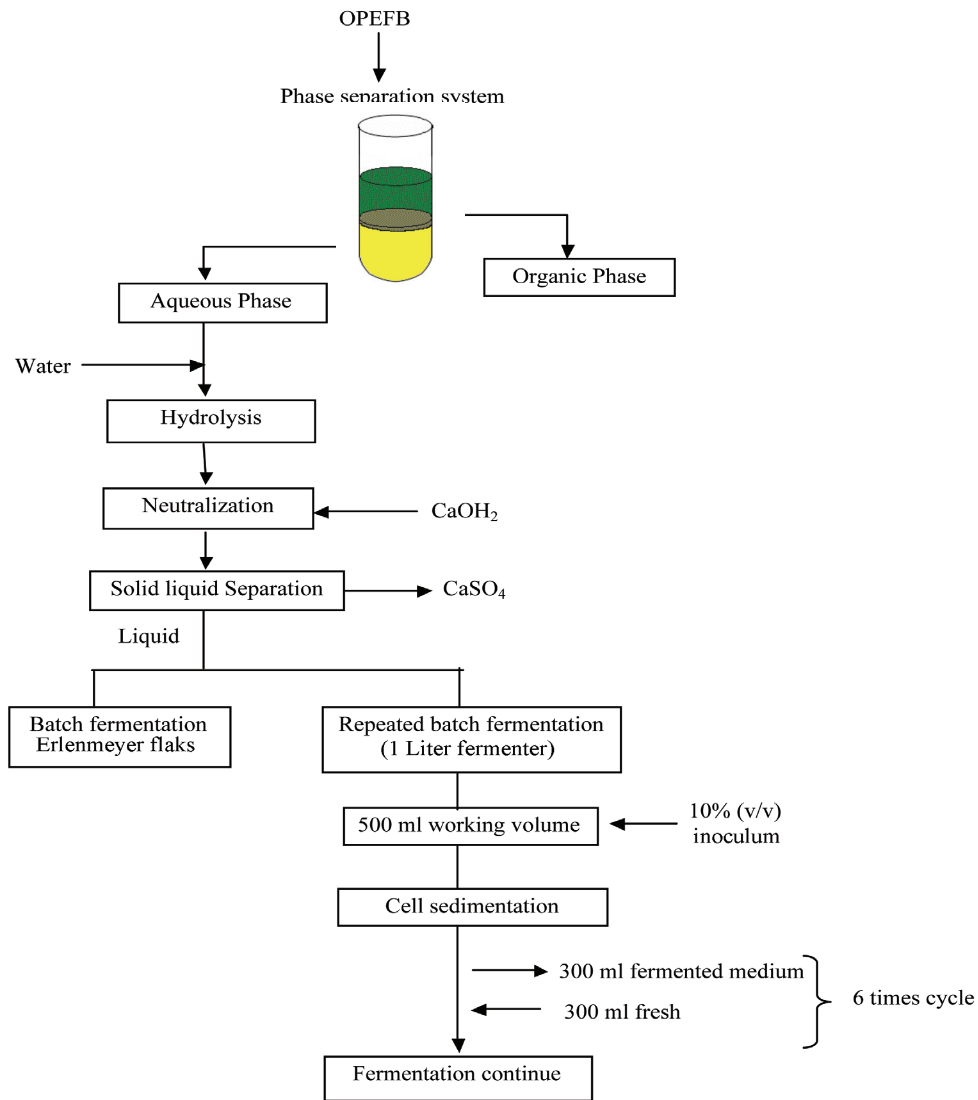

Fig. 1: Flow chart of the overall process for ethanol production from the OPEFB through phase separation system

hydrolysate containing (in 1 L medium) 5 g yeast extract, 5 g KH_2PO_4, 1.5 g NH_4Cl, 0.7 g Mg_2SO4, and 1.7 g KCl. The concentrations of glucose ranging from 32 g l^{-1} to 62 g l^{-1} were used. The fermentation medium was filter sterilized using 0.25µm membrane filter.

Samples Analysis

Determination of major components of extracted and non-extracted OPEFB was done by acid detergent fibre (ADF), acid detergent lignin (ADL) and neutral detergent fibre (NDF) (Wong *et al*., 2008).

The concentration of ethanol was analyzed using gas chromatography (Model GC-17A, Shimadzu) with BPX capillary column and flame ionization detection. Meanwhile, the temperature of the injector and detector was set at 150°C and 200°C, respectively. The oven temperature was maintained at 40°C for 1 minute and then raised to 130°C at a gradient of 20°C min^{-1}. Helium gas was used as the carrier gas at a flow rate of 0.5 mL min^{-1}. The ethanol standards were prepared using a mixture of commercial grade ethanol (Wako) and propanol as an internal standard. The amount of carbohydrates was determined according to the phenol sulfuric method (Liu *et al*., 1972). Viable cell, cell dry weight and glucose composition were measured in the fermentation process as described previously (Ma *et al*., 2007).

RESULTS AND DISCUSSION

Determination of the OPEFB composition

The results of the OPEFB composition in this study were compared with the previous study (Table 1). Nonetheless, the treatment of OPEFB by extracting with ethanol-benzene did not show significant changes on the major component of the OPEFB (Table 2). The composition of cellulose, hemicellulose and lignin of the extracted and non-extracted OPEFB were about 45.06%, 28.51% and 12.39% corresponding to the composition of the OPEFB done in a previous study by Rahman *et al*. (2006). The composition of the OPEFB was also compared with the other types of lignocellulosic materials (Table 3). It was found that the OPEFB cellulose composition was corresponded to the cellulose measured in hardwood (pine). However, the OPEFB lignin was found to correspond to the lignin in grass (switch grass). Therefore, it is suggested that the OPEFB could be categorized between hardwoods and grasses in term of their lignocelluloses content. Typically in hardwoods, the composition of cellulose, hemicellulose and lignin were about 40-50%, 15-30% and 10-25%, respectively (Malherbe & Cloete, 2000).

Phase Separation System

For a complete hydrolysis of lignocellulosic materials, the cellulose must be swollen by treatment with concentrated acid such as more than 65% of sulfuric acid or more than 85% of phosphoric acid (Mikame & Funaoka, 2006). For the OPEFB used in this study, the total sugar obtained from acid phase was about 0.73 g, which is 73% of raw material and 98% of total carbohydrate, after 60 minutes of treatment in the phase separation system. A previous study on separating lignocellulose of Yezo spruce (*Picea jezoensis*), through phase separation system by Mikame and Funaoka (2006) whereby the total sugars composition obtained after 60 minutes of treatment, was about 75% of raw materials and 104% of total carbohydrate (Table 4). Based on the simple total material balance (*Fig. 2*), a small part of carbohydrate was not hydrolyzed in which hydrolysis of cellulose was strongly dependent on the degree of crystallinity

TABLE 1
Major components of the oil palm empty fruit bunch (on a dry basis)

Main Fraction	Composition (%)		
	This study	Ariffin *et al*. (2008)	Rahman *et al*. (2006)
Cellulose	45.06	51.28	42.85
Hemicellulose	28.51	28.18	24.01
Lignin	12.39	15.17	11.70
Others (extractives, ash. etc)	14.04	5.37	21.44

The composition of the OPEFB obtained was compared with the values obtained from other references. Note: All data were average of duplicated samples.

TABLE 2
Major components of the oil palm empty fruit bunch, with and without extraction with ethanol-benzene

Main Fraction	With extraction	Without extraction
Cellulose	45.06	45.73
Hemicellulose	28.51	28.47
Lignin	12.39	12.07
Others (ash. etc)	11.03	10.72
Extractives	-	3.01

Note: All data were average of duplicated samples

TABLE 3
Major components of the types of lignocellulose materials (on a dry basis)

Main Fraction	Composition (%)			
	OPEFB	Softwood (Hybrid Poplar)	Hardwood (Pine)	Grasses
Cellulose	45.06	44.55	44.70	31.98
Hemicellulose	28.51	21.90	18.55	25.19
Lignin	12.39	27.67	26.44	18.13
Others (extractives, ash. etc)	14.04	5.88	10.31	24.7

References: Malherbe and Cloete (2002); Hamelink *et al*. (2005)

TABLE 4
Total sugar recovered from the phase separation process

	Total sugar recovered	
	% total carbohydrate	% raw materials
This study	73	98
Mikame and Funaoka (2006)	75	104

and swelling state of cellulose (Xiang *et al*., 2003). As cellulose contains high crystalline structure, hydrolysis of cellulose structure was not done completely. Therefore, some parts of cellulose, having a high crystalline structure, were not hydrolyzed and remained at interface. The release of glucose was suggested from hemicellulose and cellulose chain. Lignin, which had been transformed into lignophenol, was further purified. As lignophenol was one of the main products of this process, the yield was also calculated. The amount of lignophenol obtained was 0.13 g lignophenol g lignin^{-1}.

Aqueous Phase Hydrolysate as Substrate

In phase separation system, when the stirring of reaction mixture stopped, the system was quickly separated into the organic phase containing lignophenol derivatives and the aqueous phase containing partially hydrolyzed carbohydrates. Heating time and temperature were examined to

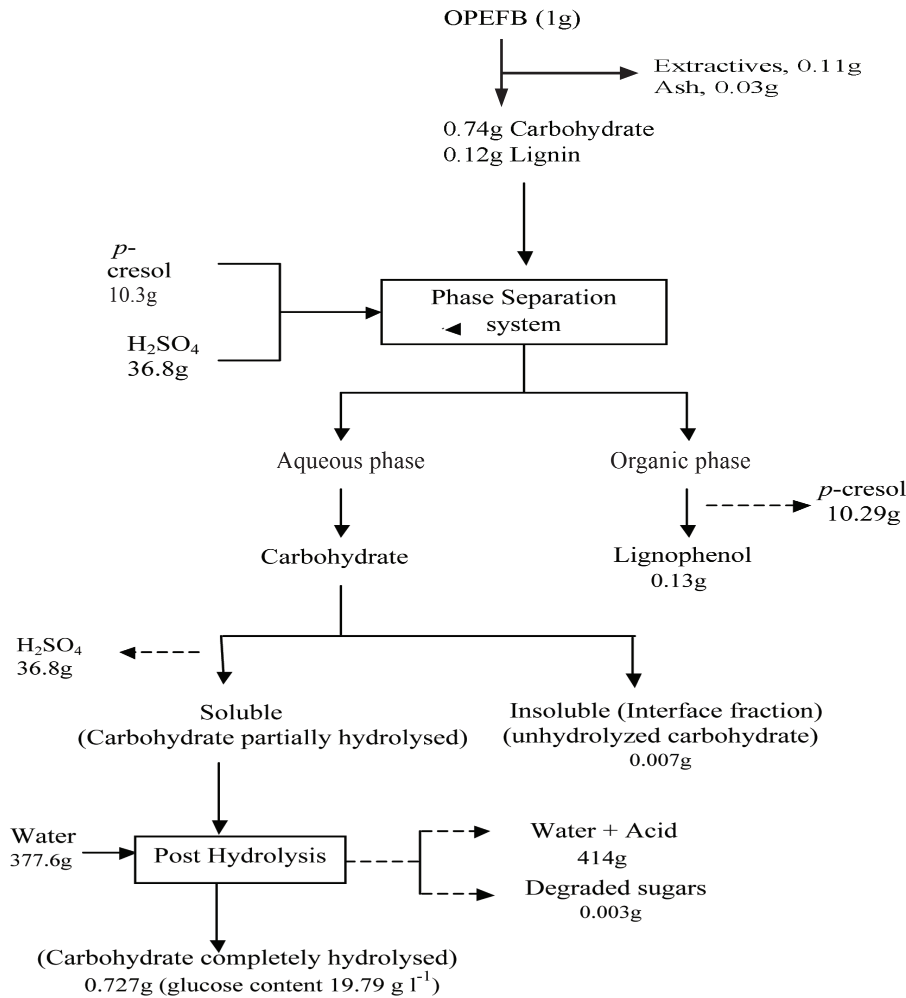

Fig. 2: The simplified materials balance of lignophenol and sugar in the phase separation system process

get maximum amount of glucose. The maximum amount of glucose obtained was 17.91 g l^{-1} yielding 79.1% based on cellulose and 48.1% based on raw material respectively, when hydrolysis occured at 100°C. It was reported that, 80% of the lignin was separated into the organic phase and 87% carbohydrates were in aqueous phase (Funaoka *et al*., 1995; Funaoka, 1998). With very small quantities of dissolved lignin, the hydrolysis process of cellulose to sugar becomes a simple process and can occur in a less acid concentration mixture (Iranmahboob *et al*., 2002). It was reported that the sugar present in aqueous phase consisted of a mixture of oligosaccharides with various molecular sizes (Mikame & Funaoka, 2006). Therefore,

the aqueous phase was subjected to hydrolysis after acid dilution in order to complete the hydrolysis. In the hydrolysis, heating is needed to accelerate the hydrolysis process in order to completely convert oligosaccharides into monosaccharides. Meanwhile, the production of glucose gradually increased by heightening the temperature. At high acid concentration and heating time, however, cellulose may be degraded and oxidized during the heating process. Thus, optimizing concentration of acid and heating time should be applied during the hydrolysis process.

Hydrolysis of Oligosaccharides in Aqueous Phase for Glucose Production

In order to obtain the maximum amount of glucose, different types of treatment on acid concentrations were examined (see Table 5). Based on the preliminary result, it was found that the best temperature of hydrolyzing carbohydrate in the aqueous phase was 100°C. The effective hydrolysis of carbohydrate is mostly depending on acid concentration and heating time. Meanwhile, the maximum amount of glucose obtained in the hydrolysis of aqueous phase was 19.8 g l^{-1} when autoclaved at 121°C with 4% acid and 18.5 g l^{-1} when boiling at 100°C with 3% acid. Nonetheless, only 13.7 g l^{-1} of the glucose was obtained for the hydrolysis using 4% acid by boiling at 100°C. The hydrolysis at 5% acid concentration for autoclaving at 121°C and boiling at 100°C gave the glucose concentration of 15.8 g l^{-1} and 14.9 g l^{-1}, respectively. The results showed that the hydrolysis efficiency by autoclaving gave a higher yield than boiling.

ETHANOL FERMENTATION

The Effect of Nitrogen Source on Ethanol Production

Yeast extract is typically used as a stimulating factor for yeast growth. Since yeast extract is rather expensive, therefore the effects of POME and sludge powder which are wastes from the oil palm industry were therefore evaluated together with yeast extract as nitrogen source in the batch culture in this study. Fifty g l^{-1} of technical grade glucose was used in this fermentation. The results showed that yeast extract was the best nitrogen source with ethanol production up to 93% of the theoretical yield. Meanwhile, POME gave 63% ethanol production based on the theoretical yield (Table 6). In the case of sludge powder, the ethanol yield was very low. Therefore, POME might have potential to be used as a nitrogen source as it is abundantly available in the oil palm industry.

TABLE 5
Different types of pre-treatment to maximize the production of glucose

Treatment	Yield (%) (g glucose g cellulose^{-1})	Yields (%) (g glucose g carbohydrate^{-1})	Glucose Concentration (g l^{-1})
a) Boiling			
3%	82.1	50.2	18.48
4%	60.7	37.1	13.66
5%	70.3	42.7	15.82
b) Autoclaved			
3%	57.0	34.8	12.82
4%	87.9	53.8	19.79
5%	66.4	40.6	14.94

The sample was treated through hydrolysis by boiling at 100°C for 4 hours or autoclaving at 121°C for 5 minutes.

TABLE 6
Ethanol production by *Saccharomyces cerevisea* ATCC 22062 with 50 g l^{-1} glucose using different nitrogen sources in the Erlenmeyer flask fermentation

Nitrogen source (5g l^{-1})	Ethanol concentration (g l^{-1})	Productivity of ethanol (g $l^{-1}h^{-1}$)	Ethanol yield (g ethanol g glucose^{-1})	Ethanol yield (% of theoretical)	Viable cell (cell ml^{-1})
Yeast extract	23.6	0.98	0.48	93	2.16×10^8
POME	14.9	0.47	0.32	63	7.8×10^7
Sludge powder	7.51	0.24	0.16	23	5.8×10^7

TABLE 7
Batch fermentation for ethanol production by *Saccharomyces cerevisea* ATCC 22062 in theErlenmeyer flask

Substrate	Productivity of ethanol (g $l^{-1}h^{-1}$)	Ethanol yield (g ethanol g glucose^{-1})	Ethanol yield (% of theoretical)	cell mas (g l^{-1})
Technical grade glucose	0.87	0.50	93	7.23
OPEFB glucose	0.82	0.46	87	3.63

Ethanol Production of Glucose from OPEFB in Batch and Repeated-batch Fermentation

The ethanol production by *Saccharomyces cerevisiae*, using glucose from OPEFB by batch fermentation, gave yield up to 82% (Table 7). In several studies, the presence of inhibitory factors in the acid hydrolysis of lignocellulose has been the main concern due to its toxicity in ethanol fermentation (Palmqvist *et al.*, 1999; Martinez, 2000). In this study, however, high yield of ethanol was produced and this indicated that small amounts of inhibitors were generated from the hydrolysis through the phase separation system. The extractive compounds, such as terpene and tannin generated during hydrolysis, and which are toxic to fermentation process, were removed through extraction by ethanol-benzene. In this study, the lignin and its degradation products such as polyaromatic compounds were separated from the hydrolysate in the phase separation process. Therefore, the effects of inhibitory compounds on ethanol fermentation have been reduced and the high yield of ethanol could be obtained. Previously, it was shown that the detoxification of diluted acid hydrolysate, using $CaOH_2$ to remove the inhibitory compound and the fermentability of the hydrolysate, was increased, with ethanol yield more than 90% (Milatti *et al.*, 2002). Through the repeated batch fermentation using 62 g l^{-1} OPEFB glucose, it was found that the maximum ethanol yield increased up to 88% of theoretical value. However, the productivity of ethanol production was found to be higher than in the batch fermentation, which was 2.79 g^{-1} l^{-1} h^{-1}. Six time of batch cycle had been done and showed consistent yields throughout the fermentation process (*Fig. 3*). It was suggested that the increased of productivity with repeated batch fermentation due to the adaptation of yeast on the hydrolysate condition.

CONCLUSION

Complete separations of lignin and carbohydrate were obtained in the phase separation system, comprising of phenol derivatives and concentrated acid. Based on the material balance, it was found that most of the lignin dissolved in organic phase and 0.73 g sugars

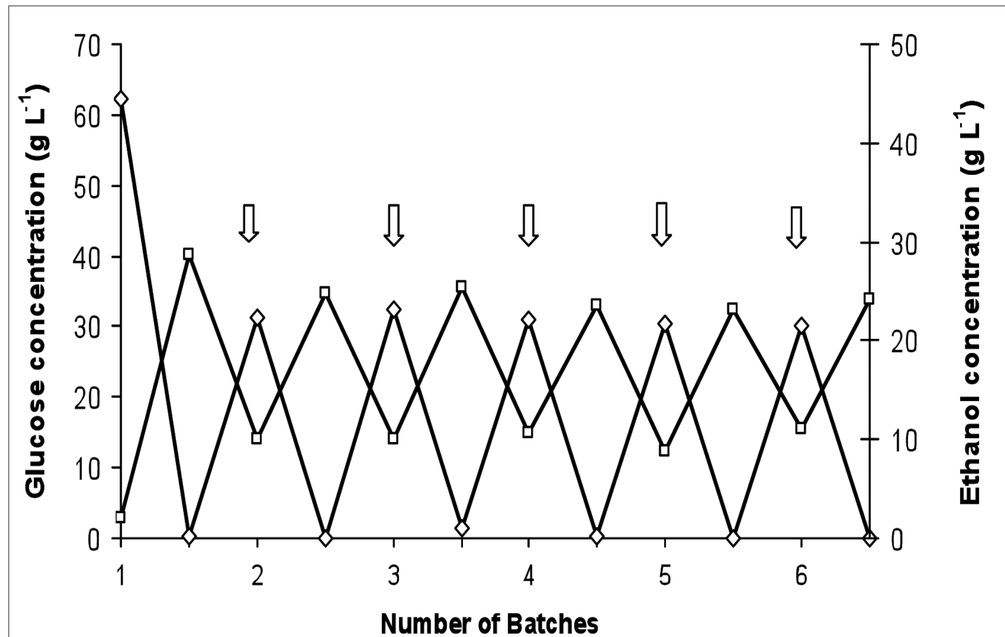

Fig. 3: Time course for ethanol fermentation using 1L bioreactor in repeated-batch fermentation. (EFB's glucose;, concentration of ethanol produced;) Arrows indicates the change of one batch to the next.

g carbohydrate^{-1} was dissolved in the aqueous phase. Meanwhile, oligosaccharides, which were mainly from cellulose in the aqueous phase, were successfully hydrolyzed in diluted acid to form glucose. The maximum glucose produced in the aqueous phase was 87.9% of cellulose content, which was 53.8% of raw material by autoclaving at 121°C for 5 minutes in 4% acid concentration. The sugar was fermented by *Saccharomyces cerevisiea* in the batch and repeated-batch fermentations to produce about 0.46 g glucose g ethanol^{-1} and 0.47 g glucose g ethanol^{-1}, with the productivity of about 0.82 g l^{-1} h^{-1} and 2.79 g l^{-1} h^{-1}, respectively. It was suggested that sugar, particularly glucose recovered from the OPEFB through phase separation process, had successfully been fermented to produce high yield of ethanol.

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REFERENCES

Ariffin, H., Hassan, M. A., Umi Kalsom, M. S., Abdullah, N., Ghazali, F. M., & Shirai, Y. (2008). Production of bacterial endoglucanase from oil palm empty fruit bunch by *Bacillus Pumilus* EB3. *Journal of Bioscience and Bioengineering, 3*, 231-2236.

Funaoka, M. (1998). A new type of phenolic lignin-based network polymer with the structure-variable function composed 1,1-diarylpropane units. *Polymer International*, *47*, 277-290.

Funaoka, M., & Abe, I. (1989). Rapid separation of wood into carbohydrate and lignin with concentrated acid-phenol system. *Tappi Journal, 72,* 145-149.

Funaoka, M., Matsubara, M., Seki, N., & Fukatsu, S. (1995). Conversion of native lignin to a highly phenol functional polymer and its separation from lignocellulosics. *Biotechnology and Bioengineering, 46*, 545-552.

Iranmahboob, J., Nadim, F., & Monemi, S., (2002). Optimizing acid hydrolysis: a critical step for production of ethanol from mixed wood chips. *Biomass Bioenergy, 22,* 401- 404.

Liu D., Wong, P. T. S., & Dutka, B. J. (1972). Determination of carbohydrate in lake sediment by modified phenol-sulfuric acid method. *Water Resource, 7*, 741-746.

Ma K. D., Wakisaka, M., Kiuchi, T., Praneetrattananon, S., Morimura, S., Kida, K., & Shirai, Y. (2007). Repeated-batch ethanol fermentation of kitchen refuse by acid-tolerant flocculating yeast under the non-sterilized condition. *Japan Journal of Food Engineering, 8*, 275–279.

Malherbe, S., & Cloete, T. E. (2002). Lignocellulose biodegradation: Fundamentals and applications. Review *Environmental Science and Biotechnology, 1*, 105-114.

Martinez, A., Rodriguez, M. E., York, S. W., Preston, J. F., & Ingram, L.O. (2000). Effects of $Ca(OH)_2$ treatments (“overliming”) on the composition and toxicity of baggase hemicellulose hydrolysate. *Biotechnology and Bioengineering, 69*, 526-536.

Mikame, K., & Funaoka, M. (2006). Conversion and separation pattern of Lignocellulosic carbohydrate through phase separation system. *Polymer Journal*, *38*, 694-702.

Milatti, R., Niklason, C., & Taherzadeh, M. J. (2002). Effect of pH, time and temperature of overliming on detoxification of diluted acid hydrolyzates for ethanol fermentation by *Saccharomyces cerevisiae*. *Process Biochemistry, 38*, 515-522.

Palmqvist, E., & Hahn-Hägerdal, B., (1999). Fermentation of lignocellulose hydrolysate. II: inhibitors and mechanisms of inhibition. *Bioresource Technology*, *74*, 25-33.

Rahman, S. H. A., Choudhury, J. P., & Ahmad, A. L. (2006). Production of xylose from oil palm empty fruit bunch fiber using sulfuric acid. *Biochemistry Engineering Journal, 30*, 97-103.

Wong, K. M., Rahman, N. R. A, Abd Aziz, S., Sabaratnam, V., & Hassan, M. A. (2008). Enzymatic of palm oil mill effluent solid using mixed cellulases from isolated fungi. *Research Journal of Microbiology, 6*, 474-481.

Xiang, Q., Lee, Y. Y., Pettersson, P. O., and Torget, R. W. (2003). Heterogeneous aspects of acid hydrolysis of α-cellulose. *Applied Biochemistry Biotechnology, 107*, 505-514.

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Effectiveness of Sticky Trap Designs and Colours in Trapping Alate Whitefly, *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae)

A. B. Idris[1]*, S. A. N. Khalid[2] and M. N. Mohamad Roff[3]
*[1]Centre for Insects Systematics,
School of Environmental and Natural Resource Sciences,
Faculty of Science and Technology, National University of Malaysia,
43600 Bangi, Selangor, Malaysia
[2]Department of Biology, Faculty of Education,
Thamar University, Sana'a P.O. Box 2614, Yemen
[3]Deputy Director/ Senior Principal Research Officer,
Pest and Disease Management Programme,
Horticulture Research Centre, MARDI HQ,
P.O. Box 12301, GPO, 50774 Kuala Lumpur, Malaysia
E-mail: idrisgh@ukm.my

ABSTRACT

A study to determine the effectiveness of sticky trap designs with different colours for trapping alate whitefly (WF), *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) was carried out at MARDI Station, Jalan Kebun, in Klang, Malaysia from September 2004 to August 2005. Trap designs (cylindrical, horizontal, and vertical), colours (yellow, blue, green, red, white and black) and time (sampling dates) gave significantly ($P<0.05$) different effectiveness of trapping the alate WF, as well as indication of its population abundance in the field. Both vertical and cylindrical traps were the most effective traps as significantly ($p<0.05$) higher numbers of alate WF caught on these traps than on traps of other designs. Meanwhile, yellow was the most attractive colour to alate WF, regardless of the trap design as it had the highest number of alates caught compared to the other colours. Comparatively, the vertical yellow trap was the most attractive and efficient trap to use in monitoring WF.

Key Words: Alate, *Bemisia tabaci*, trap, colour, population abundance, *Capsicum annum*

INTRODUCTION

Monitoring and surveillance are basic tools for developing effective pest-management system. Several researchers have highlighted the benefits of using different trapping methods for monitoring the population of homopterous insects. For example, Berlinger (1980), Gerling and Horowitz (1984), Musuna (1986) and Venugopal Rao *et al*. (1989) reported the effectiveness of using yellow sticky trap (YST) in monitoring the population of whitefly (WF) in the field.

The first step in managing whitefly (WF) is to determine the most accurate and effective monitoring system to measure the population levels of the insects (Byrne *et al.,* 1986). Heathcote *et al.* (1969) found that the counts of both suction traps and yellow sticky traps were more efficient than crop inspection for predicting the first seasonal flight of several aphid species. Burgess *et al*. (1979), Disney *et al*. (1982) and Southwood (1978) found that cylindrical sticky traps, which were placed at the height of 157.5 - 182.9 cm, caught more aphids than those

*Corresponding Author

placed at either 55.9 - 111.8 cm or 5.1- 35.6 cm. Besides, the highest number of aphids was recorded towards the end of the growing season, where traps that were placed at the lower canopy level (5.1 - 35.6 cm) caught 65% more aphids than those placed at other levels. Heathcote (1957) also found that the traps of different colours, designs, and placements caught various numbers of aphid species.

Byrne and Bishop (1979) reported that the number of *Myzus persicae* (Sulzer) caught in the yellow water pan trap was correlated with the number of potato leaves from the adjacent potato fields. Similarly, Gerling and Horowitz (1984) found that a high number of *B. tabaci* was captured in a horizontally placed trap compared to the cross-shaped vertical trap of similar size, and the catches were greater at the ground level. Mound (1962) found that *B. tabaci* was attracted to the blue-ultra violet and yellow part of the light spectrum. Several other researchers have suggested that this insect might be attracted to coloured traps (e.g. Webb & Smith, 1980; Patti & Rapisarda, 1981; Sharaf, 1982).

The purpose of this study was to evaluate the effectiveness of the sticky traps with different designs and colours in monitoring the WF population abundance in the field. It was hope that the information generated from this study would be able to support or help farmers to reduce and manage WF infestation in their crops.

MATERIALS AND METHODS

Study Site

This study was carried out at MARDI Research Station Jalan Kebun, in Klang, Selangor, Malaysia from September 2004 to August 2005. This research station is situated in a flat peat land that lies between 3°0'N latitude and 101° 30' E longitude, and 3.1m above the sea level.

Trap Design and Experimental Layout

In this study, three sticky trap designs were used (vertical, horizontal and cylindrical traps), as shown in *Fig. 1 A-C*). The size of the vertical and horizontal traps was 6 x 27 x 1 cm (width x length x thickness), and both the traps were made of rectangular flat wood; the vertical trap was attached to an erect wooden pole, while the horizontal trap was fixed onto a wooden pole at 90° angle. The cylindrical trap was made of transparent plastic container of 4 cm diameter and 30 cm length, and attached to an erected wooden pole. Four traps (replicates) of each type were painted with ICI Dulux paint in yellow, blue, green, red, white and black to act as the treatments. The traps were arranged in a row at a distance of 1.5 m apart from each other. All the traps were placed on a wooden pole that was installed 90 cm above the ground level within chilli (*Capsicum annum*) plot measuring of 8 m x 35.5 m and separated by 2 m apart between the plots. The treatments (18) were arranged in a randomized complete block design (RCBD) with four replicates. The traps were placed randomly in each block or chilli plot. These traps were first covered with transparent polyethylene plastic and then sprayed with sticky glue (Neopace™ Agricultural Chemical Malaysia Bhd.) to trap alate WF. The traps were repainted from time to time to ensure that their colours would not fade away.

Data Collection

The sampling of WF was done on a weekly basis from 0800 to 1000 h. On each sampling day, transparent polyethylene plastics were collected and marked with Y, R, BL, G, B, and W to represent trap colours (i.e. yellow, red, blue, green, black and white) and V, H and C to represent the trap designs (vertical, horizontal and cylindrical). A new transparent polyethylene plastic, sprayed with glue, was also put on each trap as a replacement. The collected plastics were brought back to the laboratory and the numbers of WF were counted. The alate WF was counted by placing the transparent plastic on a black polyethylene chloride (PVC) sheet and viewed using a 10-x magnifying glass.

Fig. 1: Samples of the trap design used in the study (A, B and C is a vertical, horizontal and cylindrical trap, respectively)

Statistical Analysis

Data on the number of WF per trap per week were pooled to obtain the number of WF per week. These were then transformed using $\sqrt{x + 0.5}$ for normalization before the analysis (Healy & Taylor, 1962). A three-way ANOVA (colours, sampling dates and trap's shape as the independent variables) was performed on the data to determine the differences in the numbers of WF caught in the different traps with different colours and at different sampling dates. When significant, the means were separated by Fisher's Protected Least Significant Difference ($p<0.05$). All the statistical analyses were carried out using SAS Institute 9.01 statistical software (SAS Institute Inc., 2002, Cary, NC, USA).

RESULTS

The interaction between trap designs, colour and sampling dates was found to be significant ($F=2.20$, df=510 and 1870, $P<0.05$) (Table 1) in catching WF. A significant interaction was also found between the trap designs and sampling dates ($F=13.59$, df=102 and 1870, $P = 0.05$), between the trap colour and sampling dates ($F=11.30$, df=255 and 1870, $P<0.05$), and between the trap colour and trap designs ($F=105.39$, df = 10 and 1870, $P<0.05$) (Table 1 and *Fig. 2*). The WF catches were significantly ($P<0.05$) higher in both the vertical and cylindrical yellow traps compared to other treatments (*Fig. 2*).

The mean number of WF caught in the traps with different colours, irrespective of trap

TABLE 1
Three-way ANOVA statistics for the mean number of alate whiteflies caught on different sticky trap designs and colours.

Source	*df*	*Sum of square*	*F- value*	*P - value*
Colour	5	563.97	905.70	<0.0001
Time	51	627.94	98.86	<0.0001
Shape of Traps	2	122.01	489.85	<0.0001
Colour x shape	10	131.25	105.39	<0.0001
Colour x Time	255	358.98	11.30	<0.0001
Shape x Time	102	172.57	13.59	<0.0001
Colour x shape x Time	510	139.71	2.20	<0.0001
Error	1870	232.89		

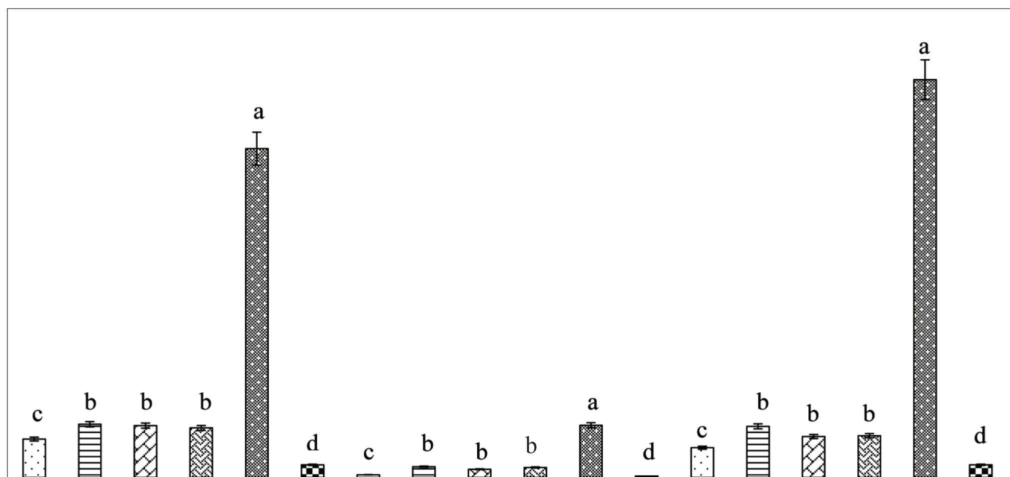

Fig. 2: Mean numbers of alate WF for different shapes and colours of traps from 1st September 2004 to 30th August 2005. Bars with different letters differ significantly at P<0.05.

designs, differed significantly (F=905.70, df=5 and 1870, P<0.05) (*Fig. 3*). In more specific, the yellow traps had significantly (P<0.05) higher number of WF caught compared to the other colour traps, whereas the mean number of WF caught was the lowest in the black traps (*Fig. 3*). Catches were intermediate for the traps of other colours.

The mean number of WF caught in the different trap designs, irrespective of the colours of the trap, was significantly (P<0.05) higher in both the vertical and cylindrical traps as compared to the horizontal ones (*Fig. 4*). However, the number of WF caught on the vertical trap did not differ significantly (P>0.05) with that of the cylindrical trap.

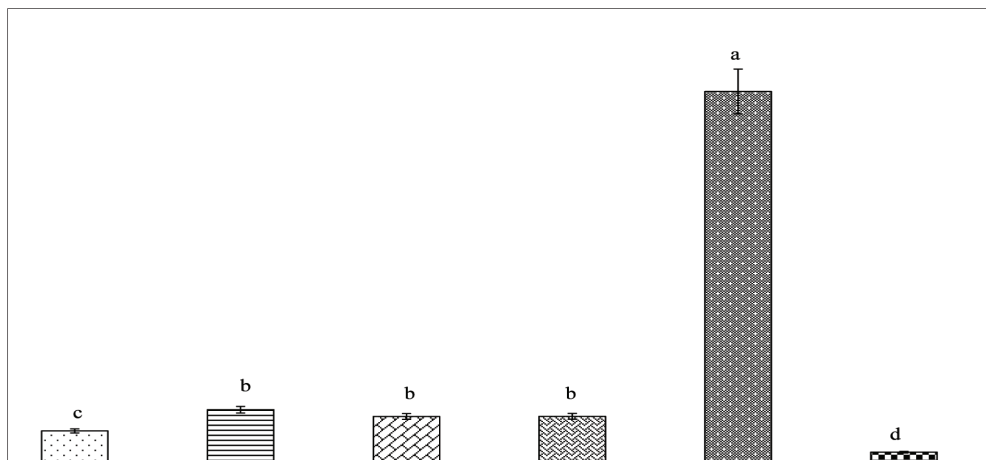

Fig. 3: The mean number of alate WF catches in different coloured traps from 1st September 2004 to 30th August 2005. Bars with different letters differ significantly at P<0.05.

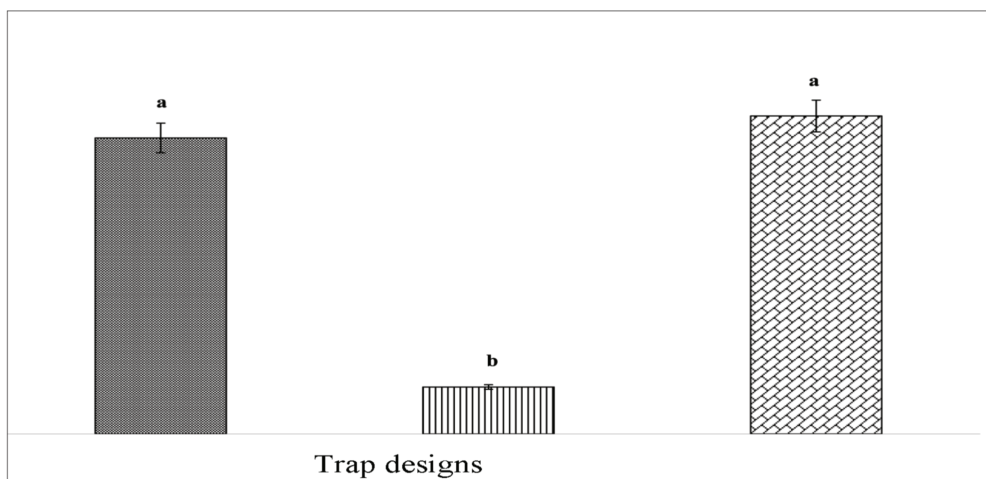

Fig. 4: A comparison of the total mean number of alate WF catches on the vertical, horizontal, and cylindrical traps from 1st September 2004 to 30th August 2005. Bars with different letters differ significantly at P<0.05.

DISCUSSION

The results showed that the WF catches in the yellow trap was the highest, and this was followed by red, green, blue, white, and black traps (*Fig. 3*). This is not surprise because a positive response to yellow colour is common to most foliage-seeking insects (Prokopy & Owens, 1983). Vaishampayan *et al*. (1975) and Southwood (1978) reported that yellow was found to be an efficient colour used in trapping insects such as Homopteran, Hymenopterans, Dipteran, and Thysanopteran compared to other colours. Furthermore, Venugopal Rao *et al.* (1991) reported that alate WF caught was highest in yellow sticky trap than those in the red, green, blue, black , yellow + red, yellow + green, yellow + white and yellow + blue (50:50) in the cotton field. Similar results were also reported

by Kirk (1984) who found that the numbers of flower thrips, glasshouse thrips and predatory flies caught were significantly higher in yellow traps than those of other colours. The possible reason is that the yellow traps create a contrast between the trap and the field background. This affects the optomotor of the insects' eyes and influences the landing response of the flying insects (Smith, 1976). The positive response to yellow pigment may be the characteristic of the majority of foliage-seeking insects, although there may be some variations (Greany *et al*., 1977) or exceptions (Saxena & Goyal, 1978). These insects may have mechanisms that enable the discrimination of foliage-like clues (which peak at 500 - 580 nm) (Kennedy *et al*., 1961). Yellow also reflects UV light, as well as other long wave and short wave lights (Halgren, 1970a, 1970b; Prokopy, 1972). The short wave light (500 - 580 nm) has been known to arrest the flying insects including WF (Halgren 1970a, 1970b; Prokopy, 1972). A lower number of catches by the traps of other colours (*Fig. 3*) indicated that WF responded weakly to these colours. This suggests that yellow is a good tool for sampling the WF population in chilli field. However, traps that were exposed for more than one day are often unreliable because of the dust and dirt coverage on the sticky material, and thus, a frequent replenishing is needed.

This study also indicated that the number of WF trapped in the vertical and cylindrical sticky traps was higher than in the horizontal traps (*Fig. 4*). Butler *et al*. (1986) and Youngman *et al*. (1986) also stated that vertical and cylindrical traps were more efficient in trapping insects than traps of other designs. However, Meyerdirk *et al.* (1986) reported that traps with horizontal surface attracted more WF than those with a vertical surface. Meanwhile, the lower number of WF caught in the horizontal trap in this study congruent with the report of Byrne *et al.* (1986). The high number of WF caught in both the vertical and cylindrical traps in this study might be due to the constant height of traps, which was placed at 90 cm above ground level. On the contrary, Byrne *et al*. (1986) placed the traps at ground level, i.e. 50 cm and 100 cm from the ground level. This result, nonetheless, differs from that of Gerling and Horowitz (1984) where the horizontal traps were found to have caught WF as low as the cylindrical trap.

The traps, regardless of its design (*Fig. 2*) and colour (*Fig. 3*), caught different numbers of WFs, indicating that the proximity of the open field generally has limited influence on the activity of WFs, even within the immediate surroundings. This finding is supported by Blackmer and Byrne (1993b), who reported that the resident populations of WF were found in the close proximity to their hosts, and rarely needed to move more than a few meters. Similar results were also reported for WF in cassava, whereby they were predominantly trapped in the crop canopy (Legg, 1994). This result is consistent with WFs being weak fliers with an estimated flight speed of only 0.2 ms^{-1} (Yano 1987) and a maximum climb rate of approximately 0.037 ms^{-1} (Blackmer & Byrne, 1993a). Two main flight activity categories have been suggested for WF. The first is trivial short heights, which occurs within and slightly above the plant canopy and is associated with 'vegetative' behaviours such as searching for mates, feeding and oviposition sites (Byrne *et al.*, 1996). The second type is the long-distance movement which occurs when alates WF leave the crop and are carried over by the wind (Blackmer & Byrne, 1993a; Southwood, 1978).

Hence, it can be stated that both the vertical and cylindrical yellow traps are the most effective tool for monitoring the WF population in the chilli field. Although WF is a very weak flier and always in the field feeding on the plants, it cannot be assumed that the surrounding vegetations and the abiotic factors do not have any influence on the alate WF flight activity. Thus, this needs further investigation.

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REFERENCES

Berlinger, M. J. (1980). A yellow sticky trap for whiteflies, *Trialeurodes vaporariorum* and *Bemisia tabaci* (Aleyrodidae). *Entmologia Experimentalis et Applicata, 27*, 98-102.

Blackmer, J. L., & Byrne, D. N. (1993a). Flight behaviour of Bemisia tabaci in a vertical flight chamber: effect of time of day, sex, age and host quality. *Physiological Entomology, 18*(3), 223-232.

Blackmer, J. L., & Byrne, D. N. (1993b). Environmental and physiological factors influencing phototactic flight of Bemisia tabaci. *Physiological Entomology, 18*(4), 336-342.

Burgess, N. R. H., Shuttleworth, A. E., & Chetwyn, K. N. (1979). Adhesive panels as a control method of the common cleg *Haematopota pluvialis* L. (Deptera: Tabanidae). *Journal of the Royal Army Medical crops, 125*, 156-161.

Butler Jr, G. D., Henneberry, T. J., & Wilson, F. D. (1986). Bemisia tabaci (Homoptera: Aleyrodidae) on Cotton: Adult Activity and Cultivar Oviposition Preference. *Journal of Economic Entomology, 79*(2), 350-354.

Byrne, D. N., & Bishop, G. W. (1979). Relationship of Green Peach Aphid Numbers to Spread of Potato Leaf Roll Virus in Southern Idaho. *Journal of Economic Entomology, 72*(6), 809-811.

Byrne, D. N., Von Bretzel, P. K., & Hoffman, C. J. (1986). Impact of Trap Design and Placement When Monitoring for the Bandedwinged Whitefly and the Sweetpotato Whitefly (Homoptera: Aleyrodidae). *Environmental Entomology, 15*(2), 300-304.

Byrne, D. N., & Bellows, T. S. (1991). Whitefly Biology. *Annual Review of Entomology, 36*(1), 431-457.

Byrne, D. N., Rathman, R. J., Orum, T. V., & Palumbo, J. C. (1996). Localized migration and dispersal by the sweet potato whitefly, *Bemisia tabaci*. *Oecologia, 105*(3), 320-328.

Disney, R. H. L., Erzinclioglu, Y. Z., deC. Henshaw, D. J., Howse D., Unwin D. M., Withers P., & Woods, A. (1982). Collecting methods and the adequacy of attempted founa surveys, with refrence to the Deptera. *Field Studies, 5*, 607-621.

Gerling, D., & Horowitz, A. R. (1984). Yellow traps for evaluating the population levels and dispersal patterns of *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae). *Annals of the Entomological Society of America, 77*, 753-759.

Greany, P. D., Agee, H. R., Burditt, A. K., & Chambers, D. L. (1977). Field studies on color preferences of the Caribbean fruit fly. *Anastrepha suspensa* (Diptera: Tephritidae). *Entomologia Experimentalis et Applicata, 21*(1), 63-70.

Halgren, L. A. (1970a). Take off behavior of greenbug, *Schisaphis graminum*, in the laboratory. *Annals of the Entomological Society of America, 63*, 712-715.

Halgren, L. A. (1970b). Flight behavior of greenbug, *Schisaphis graminum*, in laboratory. *Annals of the Entomological Society of America, 63*, 716-720.

Healy, M. J. R., & Taylor, L. R. (1962). Tables for power-low transformations. *Biometrika, 44*, 557-559.

Heathcote, G. D. (1957). The comparison of yellow cylindrical, flat and water traps, and of Johnson suction traps, for sampling aphids. *Annals of Applied Biology, 45*, 133-139.

Heathcote, G. D., Palmer, J. P., & Taylor, L. R. (1969). Sampling for aphids by traps and crop inspection. *Annals of Applied Biology, 63*, 155-166.

Kennedy, J. S., Booth, C. O., & Kershaw, W. J. S. (1961). Host finding by aphids in the field. *Annals of Applied Biology, 49*(1), 1-21.

Kirk, W. D. J. (1984). Ecologically selective coloured traps. *Ecological Entomology, 9*, 35-41.

Legg, J. P. (1994). *Bemisia tabaci*: The whitefly vector of cassava mosaic geminiviruses in Africa: an ecological perspective. *African Crop Science Journal, 2*, 437-448.

Meyerdirk, D. E., Coudriet, D. L., & Prabhaker, N. (1986). Population dynamics and control strategy for Bemisia tabaci in the imperial valley, California. *Agriculture, Ecosystems & Environment*, *17*(1-2), 61-67.

Mound, L. A. (1962). Studies on the olfaction and colour sensitivity of Bemisia tabaci (Genn.) (Homoptera, Aleyrodidae). *Entomologia Experimentalis et Applicata, 5*(2), 99-104.

Musuna, A. C. Z. (1986). A Method for Monitoring Whitefly, *Bemisia tabaci* (Genn.), in Cotton in Zimbabwe. *Agricultural Ecosystem and Environment, 17*, 29-35.

Patti, L., & Rapisaarda, C. (1981). Findings on the morphology and biology of aleyrodids injurious to cultivated plants in Italy. *Belletino di Zoologia Agraia e di Eachicolura, 16*, 135-190.

Prokopy, R. J. (1972). Response of apple maggot flies to reactangles of different colours and shade. *Environental Entomology, 1*, 720-726.

Prokopy, R. J., & Owens, E.D. (1983). Visual Detection of Plants by Herbivorous insects. *Annals Review of Entomology, 28*, 337-364.

Saxena, K. N., & Goyal, S. (1978). Host-plant relations of the citrus butterfly *Papilio demoleus* L.: Orientation and ovipositional responses. *Entmologia Experimentalis et Applicata, 24*, 1-10.

Sharaf, N. (1982). Parasitization of the tobacco whitefly, *Bemisia tabaci* Genn. (Homoptera: Aleyrodidae) on *Lantana camara* L. in the Jordan Valley. *Zeitscbrift fur Angewandte Entomologie, 94*, 263-271.

Smith, J. G. (1976). Influence of crop background on aphids and other phytophagous insects on brussel sprouts. *Annals of Applied Biology, 83*, 1-13.

Southwood, T. R. E. (1978). *Ecological methods with particular reference to the study of insect populations. 2nd Edition*. London, UK: Chapman and Hall.

Vaishampayan, S. M., Kogan, M., Waldbauer, G. P., & Woolley, J. T. (1975). Spectral specific responses in the visual behavior of the greenhouse whitefly *Trialeurodes vaporariorum* (Homoptera: Aleyrodidae). *Entomologia Experimentalis et Applicata, 18*(3), 344-356.

Venugopal Rao, N., Reddy, A. S., & K. Tirumala Rao (1989). A method to monitor whitefly (*Bemisia tabaci*) in cotton (*Gossypium hirsutum*). *Indian Journal of Agricultural Science, 59*, 459-461.

Venugopal Rao, N., Reddy, A. S., & K. Tirumala Rao. (1991). Monitoring of cotton whitefly, *Bemicia tabaci* Genn. with sticky traps. *Madras Agricultural Journal, 40*(1), 1 - 7

Webb, R. E., & Smith, F. F. (1980). Greenhouse whitefly control of an integrated regimen based on adult trapping and nymphal parasitism. *IOBC/WPRS Bulletin, 3*(3), 235- 246.

Yano, E. (1987). Quantitative monitoring techniques for greenhouse whitefly. *IOBC/WPRS Bulletin, 10*, 198-202.

Youngman, R. R., Toscano, N. C., Jones V. P., Kido K., & Natawick E. T. (1986). Correlations of seasonal trap counts of *Bemisia tabaci* (Homoptera: Aleyrodidae) in South-Western Californa. *Journal of Economic Entomology, 79*, 67-70.

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Aggregate Stability of Tropical Soils in Relation to Their Organic Matter Constituents and Other Soil Properties

Christopher Teh Boon Sung
Department of Land Management, Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia
E-mail: cbsteh@yahoo.com

ABSTRACT

This study was carried out to determine the distribution of organic matter and its constituents, as well as other soil properties, in various aggregate size fractions for four soil types in Malaysia, and to investigate the relationship and relative importance of these soil properties on aggregate stability. The four soil series with contrasting particle size distributions used were Munchong (Typic Hapludox), Melaka (Xanthic Hapludox), Rengam (Typic Paleudult), and Bungor (Typic Paleudult). The top soil, i.e. 0-15 cm of the soils of the four soil series was sampled and analyzed for their particle size distribution, aggregation, aggregate stability, organic matter, humic acids (HA), fulvic acids (FA), polysaccharides, functional groups of HA and FA (carboxylic, COOH, and phenolic-OH), and free Fe and Al oxides. Multiple linear regression revealed that silt, followed by free Fe oxides, fine sand, FA-OH, and HA-COOH, were the most important soil constituents to explain the observed differences in the aggregate stability between the four soil types. Generally, as the aggregate size decreased, the amount of clay, silt, OM, and free Fe oxides would also increase, while the aggregation and the amount of sand would decrease. As for the Rengam and Bungor series, the aggregate stability would generally increase with the decreasing aggregate size. Meanwhile, the observed differences in the amounts of HA, FA, and polysaccharides were mainly due to the differences in the soil types.

Keywords: Aggregate size, aggregate stability, fulvic acids, humic acids, humic substances, organic matter, polysaccharides

INTRODUCTION

Aggregate stability is a measure of soil aggregates resistance to breakdown by the destructive forces of water or wind. Consequently, aggregate stability is an important soil property used to evaluate the risk of soil erosion and deterioration of soil structure. Aggregate stability is dependent on many factors, particularly on organic matter, soil texture, and oxide contents (Zhang & Horn, 2001).

Although the importance of organic matter to improve soil aggregate stability is well known (Chenu *et al.*, 2000; Boix-Fayos *et al.*, 2001; Six *et al.*, 2004; Noellemeyer *et al.*, 2008), the experiments showing the beneficial effects of organic matter on aggregate stability have been varied. For instance, some workers (Chaney & Swift, 1984; Christensen, 1986) found a significant correlation between organic matter and aggregate stability, while others (Hamblin & Greenland, 1977; Dormaar, 1983; Li *et al.*, 2010) observed that it was the constituents of organic matter (polysaccharides, humic acids, or fulvic acids), rather than the total organic matter that were important to aggregate stability. There are also differences in the results in term of the type of organic matter constituents responsible for aggregate stability. Mehta *et al.* (1960) and

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Acton *et al.* (1963) noted that polysaccharides correlated positively with aggregate stability, but Chaney and Swift (1984, 1986) ascribed it to humic acids. Fortun *et al.* (1989), however, found that the combination of humic acids, whereas fulvic acids was the most effective in increasing aggregate stability. These varied results reveal the diverse nature of organic matter.

In addition, aggregate stability is also affected by aggregate size. As aggregate increases in size, its mass will rise more rapidly than its surface area because te surface area increases with the square of the radius, and the volume with the cube of the radius. Hence, gravity, counteracting adhesion, rises in proportion to the aggregate mass. Therefore, as the aggregate size increases, the adhesive forces remain constant, but the counteracting forces increase. Consequently, larger aggregates will eventually become increasingly less stable (Tisdall & Oades, 1982; Brady, 1990). Moreover, there is a difference between cementing agents responsible for the stability of macroaggregates (those larger than 250 μm) and microaggregates (<250 μm). Microaggregates are stabilized by persistent aromatic humic material associated with amorphous Fe and Al compounds, whereas the stability of macroaggregates are stabilized by the transient or temporary binding agents such as roots, hyphae, and microbial- and plant-derived polysaccharides (Tisdall & Oades, 1982). Nwadialo and Mbagwu (1991) used principal component analysis to reveal that the microaggregate stability of seven Italian soils of different pedogenesis was related mainly to soil mineralogy, while organic matter had been shown to have little effect.

The effects of organic matter constituents were found to vary between temperate and tropical soils. In the temperate soils, for instance, humic acids are more effective in increasing aggregate stability than fulvic acids, but in tropical soils including soils in Malaysia, it is fulvic acids that are usually more effective (Soong, 1980; Tajuddin, 1992; Theng *et al.,* 1989). Fulvic acids may perform better than humic acids for the tropical soils, and this is simply because there are more fulvic acids in the tropical soils than in the temperate soils (which have more humic acids than in tropical soils) (Theng *et al.*, 1989). In Peninsular Malaysia, for example, fulvic acids consist 75-90% of the organic carbon (Zainab, 1977). The humic acids are converted into fulvic acids at a faster rate in the tropical soils because of the higher organic matter turnover rate in tropical soils than in the temperate soils (Greenland *et al*., 1992).

Unfortunately, the relationship between aggregate stability and organic matter constituents are seldom studied for the soil series in Malaysia. Consequently, the objectives of this study were: 1) to determine the distribution of organic matter and its constituents, as well as other soil properties, in various aggregate size fractions for four soil types in Malaysia, and 2) to determine the relationship and relative importance of these soil properties on aggregate stability.

MATERIALS AND METHODS

Four soil series, commonly found in Malaysia, namely Munchong (Typic Hapludox), Melaka (Xanthic Hapludox), Rengam (Typic Paleudult), and Bungor (Typic Paleudult) were selected for this study. Based on the preliminary analyses, these soil series were selected because their particle size distributions are different from one another. The Munchong soil series (73% clay, 18% sand; land use: oil palm) and Melaka soil series (53% clay, 21% sand; land use: pine) were sampled from the farms at Universiti Putra Malaysia (2° 59’ 59” N and 101° 42’ 45” E), whereas the Rengam (41% clay, 52% sand; land use: rubber) and Bungor soil series (30% clay, 65% sand; land use: grassland) were sampled from the experimental sites of the Malaysian Rubber Board at Sungai Buloh (3° 9’ 30” N and 101° 33’ 33” E). Each soil series was sampled at the soil depth of 0-150 mm randomly around the field, bulked, and then air-dried for one week before any laboratory analysis was done.

Each of these four selected soils was then separated into three macro- and microaggregate size fractions, respectively, which were: 1) 1000-

2000 μm, 2) 500-1000 μm, 3) 250-500 μm, 4) 150-250 μm, 5) 50-150 μm, and 6) <50μm. Each aggregate size fraction was analyzed for the following (with three replications each): 1) particle size distribution (pipette method; Gee & Bauder, 1986), 2) aggregation, 3) aggregate stability, 4) organic carbon (Walkley-Black dichromate titration method, with organic matter content taken as 1.72 × organic carbon; Walkley & Black, 1934), 5) humic substances (humic acids and fulvic acids), 6) polysaccharides, 7) functional groups of humic substances, and 8) free Fe and Al oxides.

Aggregation was expressed in percentage, determined from the fraction of weight of a given aggregate size fraction to the weight of all the aggregates sized below 8.0 mm.

Meanwhile, aggregate stability was expressed using the index WDCS (water dispersible clay and silt). The method of Soil Survey Laboratory Staff (1992) was employed in the present study. Five grams of uncrushed soil (<2 mm) was added to 50 ml distilled water (ratio soil to water was 1:10), and end-over-end shaking for 30 minutes and 40 rounds-per-minute (rpm). The contents were then poured into a 1-liter measuring cylinder, and the volume was made up to one litre. The solution was gently stroked up-down to distribute the contents, and then left for four minutes for the undispersed aggregates and sand particles to settle to the bottom. The clay and silt particles were then siphoned at 10 cm depth using a 25 ml pipette. At the appropriate settling time, the clay particles were then siphoned at 10 cm depth using a 25 ml pipette. Later, these values were used to calculate the stability index WDCS in the following manner:

$$WDCS = \frac{\%\ dispersed\ clay\ and\ silt}{\%\ clay\ andf\ silt\ (from\ particle\ size\ analysis)} \times 100$$

The method by Norhayati and Verloo (1984) was used to extract, fractionate, and purify the humic substances (humic acids and fulvic acids) and polysaccharides. This method is also described in detail by Tajuddin (1992). The extracted humic acids and fulvic acids were each analyzed for the functional groups COOH and phenolic-OH, as described by Schnitzer (1986). Meanwhile, the phenolic-OH groups were determined indirectly by subtraction. Total acidity of the humic material was determined first (Schnitzer, 1986), and this was followed by calculating the phenolic-OH in the following manner: meq total acidity/g humic material - meq COOH groups/g humic material = meq phenolic-OH groups/g humic material.

Free iron and aluminium oxides were simultaneously extracted with citrate-bicarbonate-dithionite (CBD) solution, as described by Mehra and Jackson (1960). However, because the Al content was low, it was determined calorimetrically using the Aluminon method. The Al amount was read using the UV-Visible spectrophotometer at wavelength 530 nm.

Data was analyzed as a 4 x 6 CRD (Completely Randomized Design) factorial experiment: four soil series and six aggregate size fractions. For each of the parameters studied, the analysis of variance (ANOVA) was used to detect any significant effect of the main effects and their interaction. In order to determine the relationship between each of the three aggregate stability indices with the soil constituents, the multiple linear regression was used. All the statistical analyses (including ridge trace) were done using SPSS for Windows version 6.0 (SPSS Inc., Chicago).

RESULTS

Some of the measured soil parameters had to be transformed as they violated the assumptions of normality and homogeneity of variance. The following variables were transformed to correct these violations (skewness followed by kurtosis values are shown in brackets): silt (1.13, 0.82), very fine sand (2.61, 7.46), fine sand (2.34, 6.86), humic acids (HA) (1.82, 4.42), fulvic acids (FA) (2.66, 8.96), aggregation (0.98, -0.04), and WDCS (1.09, 0.57). These variables were transformed by ln(2X+e), where X is the variable to be transformed and e is the natural

logarithm. However, the carboxylic functional group of humic acids (HA-COOH) (0.75, -0.82) was transformed by ln(X).

The physical and chemical characteristics of the six aggregate size fractions for the four soil types are shown in Table 1. Meanwhile, the partial ANOVA results of the main and interaction effects are shown in Table 2, where η^2 (eta-squared), which is the proportion of the variation accounted for by the main and interaction effects, are shown in brackets. In short, η^2 measures the strength of an experimental effect.

The observed differences in the particle size distribution (clay, silt, and the various sand size fractions), organic matter (OM), and free Fe oxides were significant due to the three experimental effects (Table 2). This means that the amounts of clay, silt, sand, OM, and free Fe oxides in an aggregate would depend on the aggregate size, as well as the type of soil.

TABLE 1
Mean characteristics of the six aggregate size fractions for the four soil types (all values are in percentages, unless otherwise stated)

Soil series and aggregate size fractions (μm)	clay < 2 μm	silt 2-20 μm	sand > 50 μm	very fine sand 50-100 μm	fine sand 100-250 μm	aggregation	WDCS	free Fe oxides	free Al oxides
MUNCHONG									
1000-2000	71.15	7.67	21.19	2.34	5.41	12.24	11.93	3.92	0.16
500-1000	72.47	7.46	20.07	2.50	5.24	11.28	9.84	3.48	0.19
250-500	74.61	7.08	18.31	2.09	7.32	7.41	10.28	3.73	0.18
150-250	69.95	15.33	15.02	2.46	12.56	3.92	12.63	3.52	0.16
50-150	83.51	8.29	8.21	5.82	2.39	4.94	24.91	3.59	0.21
< 50	87.95	12.05	-	-	-	0.77	84.66	4.36	0.22
MELAKA									
1000-2000	49.42	21.72	28.86	5.53	6.00	9.02	44.33	3.26	0.14
500-1000	41.81	21.14	37.05	4.34	4.56	5.05	46.60	2.42	0.12
250-500	48.80	22.95	28.25	4.31	6.63	2.03	40.51	3.04	0.15
150-250	51.84	22.84	25.32	3.75	21.56	1.35	39.81	2.87	0.15
50-150	54.88	22.73	22.39	16.50	5.89	2.14	44.06	2.97	0.12
< 50	61.73	38.27	-	-	-	0.49	52.60	3.15	0.16
RENGAM									
1000-2000	37.28	1.13	61.59	1.88	5.38	21.91	35.06	1.36	0.05
500-1000	38.50	1.86	59.63	1.80	4.84	22.96	32.35	1.23	0.08
250-500	52.78	2.16	45.06	2.36	12.08	10.24	27.42	1.66	0.12
150-250	66.65	3.07	30.28	4.43	25.85	4.03	27.83	1.87	0.15
50-150	80.68	3.19	16.13	10.38	5.75	2.46	29.65	2.20	0.14
< 50	91.92	8.08	-	-	-	0.22	85.47	2.44	0.13
BUNGOR									
1000-2000	31.79	2.33	65.88	8.00	26.38	9.53	18.56	1.91	0.12
500-1000	25.79	1.49	72.71	5.64	17.16	18.91	15.15	1.55	0.13
250-500	22.52	2.09	75.38	4.11	24.87	23.23	16.25	1.42	0.11
150-250	30.13	2.52	67.35	6.32	61.03	11.12	12.86	1.82	0.18
50-150	50.33	4.08	45.58	26.69	18.89	7.46	10.90	2.60	0.15
< 50	82.18	17.82	-	-	-	0.98	74.17	3.38	0.20

TABLE 1 *(continued)*

Soil series and aggregate size fractions (μm)	OM %	HA mg /g	FA mg /g	HA-COOH meq /100g	HA-OH meq /100g	FA-COOH meq /100g	FA-OH meq /100g	Polysaccharides mg /g
MUNCHONG								
1000-2000	4.84	0.75	5.68	4.01	3.63	2.38	0.33	19.47
500-1000	4.91	1.11	9.05	2.35	3.51	1.53	1.37	11.34
250-500	4.79	1.04	2.08	2.48	3.39	7.38	1.34	8.06
150-250	5.04	1.44	3.06	2.54	2.93	3.67	0.44	12.98
50-150	5.60	1.13	2.62	2.57	2.92	2.71	2.28	8.44
< 50	6.21	0.57	2.39	4.38	3.58	3.40	4.80	7.77
MELAKA								
1000-2000	3.12	3.18	2.70	2.47	4.61	5.47	4.19	9.83
500-1000	2.92	7.62	2.94	1.97	2.20	6.05	1.45	6.18
250-500	2.87	4.57	3.15	2.25	3.00	3.07	2.31	6.09
150-250	2.61	2.01	2.24	2.62	4.78	6.08	4.22	9.81
50-150	2.76	2.36	2.62	2.69	5.28	3.85	0.47	8.13
< 50	3.11	2.47	2.55	2.53	3.03	6.46	6.57	11.68
RENGAM								
1000-2000	2.01	0.47	2.35	5.00	6.43	2.48	2.99	8.54
500-1000	2.10	0.32	2.50	4.74	1.07	4.39	1.03	7.41
250-500	2.75	0.39	2.18	4.76	3.86	4.97	8.19	7.05
150-250	3.65	0.64	2.08	4.21	8.02	6.72	5.46	10.17
50-150	4.54	0.50	2.60	5.56	0.25	5.22	3.49	14.43
< 50	5.30	0.57	2.38	4.76	1.25	6.13	0.28	10.25
BUNGOR								
1000-2000	3.57	2.50	2.04	2.90	5.24	5.01	5.61	8.52
500-1000	2.50	2.21	2.02	2.96	5.39	6.14	3.13	10.69
250-500	2.34	1.72	1.67	2.84	5.30	5.92	3.97	17.91
150-250	2.82	2.11	1.83	3.17	5.64	4.81	5.69	12.76
50-150	4.54	3.54	2.17	3.11	4.54	6.73	3.40	14.14
< 50	6.90	3.64	2.14	3.18	5.87	5.88	5.93	12.20

Generally, as the aggregate size decreased, the amount of clay, silt, OM, and free Fe oxides would increase (Table 1). As expected, the distribution of sand was the opposite of the distribution of clay. For all the soil series, the aggregate size fraction 50-150 μm had the highest amount of very fine sand, whereas the aggregate size fraction 150-250 μm had the highest amount of fine sand.

Like free Fe oxides, the observed differences in free Al oxides were also significantly affected by the three experimental effects (Table 2), but its distribution was less varied than that of free Fe oxides. Within the same soil, there was a weaker, less clearer trend that the amount of free Al oxides would increase with the decreasing aggregate size (Table 1). For the Melaka series, moreover, the amount of free Al oxides between the six aggregate size fractions were statistically similar to one another (Table 2), and that there was no significant differences in the amount of free Al oxides between the four soils in the aggregate size fraction 150-250 μm.

Aggregation (that is, the proportion of an aggregate size fraction to the whole soil <8 mm) would progressively decrease with the reducing aggregate size (Table 1). However, for all the soil series, the differences in the aggregation

for the smallest aggregate size fraction <50 µm between the four soils were not significant (Table 2).

Aggregate stability (WDCS) showed that the Melaka series was the least stable (Table 1). As for the average across all the six aggregate size fractions, the Bungor soil series was the most stable (that is, having the lowest WDCS), followed by the Munchong, and Rengam soil series. The least stable was the Melaka series. Additionally, the stability found in all the six aggregate size fractions in the Melaka soil series was insignificantly different from one other (Table 2). For all the soil series (except for the Melaka soil series), the WDCS would steeply increase for the smallest aggregate size fraction <50 µm (Table 1). As for the Rengam and Bungor series, there was a general trend that with decreasing aggregate size, aggregate stability would increase.

Nevertheless, aggregate size did not significantly influence the amount of humic acids in the soils (Table 2). The observed differences in the amount of humic acids between the soils and aggregate size fractions were only due to the main effect and the interaction effect of the soil series. Moreover, within the Munchong and Rengam series, the amount of humic acids was not significantly different between the six aggregate size fractions. Within the same soil series, the amount of humic acids between the six aggregate size fractions was generally similar to one another (Table 1). This is considering the fact that the soil series main effect could explain more of the total variation of humic acids than the interaction effect (Table 2). The observed differences in the amount of fulvic acids were significantly caused only by the differences in the soil series. Within the same soil, there were no significant differences in the amount of fulvic acids between the various aggregate size fractions. This was indicated by the insignificant effects by both the aggregate size main effect and the interaction effect.

Generally, there should be more fulvic acids than humic acids in tropical soils due to the higher organic matter turnover rate (Greenland *et al.*, 1992) which would convert humic acids into fulvic acids at a faster rate in the tropical soils than that in the temperate soils (Mendonça *et al.*, 1991). This study, however, showed that the amount of fulvic acids was significantly greater than humic acids for only the Munchong and Rengam soil series ($t=4.405$; $df=11$; $p<0.001$), but there were no significant differences between their amounts ($t=2.078$; $df=11$; $p<0.062$) for both the Melaka and Bungor soil series.

For the carboxylic functional groups in humic acids (HA-COOH), only the soil series main effect could explain the total observed variation (Table 2). Once again, this reveals that the observed differences in the HA-COOH amount were only due to the differences in the soil series. On the other hand, the amount of carboxylic groups in fulvic acids (FA-COOH) was influenced by the three experimental effects, of which the interaction effect was the greatest (Table 2). Generally, the amount of the COOH groups in fulvic acids was more than those of humic acids (Table 1). The COOH groups in fulvic acids ranged from 1.53 to 7.38 meq/100g soil, whilst the COOH groups in humic acids ranged from 1.97 to 5.56 meq/100 g soil.

Note that ANOVA was not done on phenolic-OH groups for either humic acid (HA-OH) or fulvic acids (FA-OH). As stated earlier, the amount of phenolic-OH groups was determined by subtracting the amount of COOH groups from total acidity. As the means of the three replications were used for the subtraction, phenolic-OH determination would not have any replications. Therefore, the explained variance (η^2) was also not done.

Finally, the observed differences in the amount of polysaccharides were significantly caused only by the differences in the soil types (Table 2); that is, the main effect of the soil series was significant at 5% level, which is in contrast to the insignificant aggregate size main effect and interaction effect.

Meanwhile, the correlations between aggregate stability and the soil constituents showed that very few constituents correlated significantly with aggregate stability (WDCS) (Table 3). In particular, only silt and polysaccharides correlated significantly with

TABLE 2
Partial ANOVA results of the main effects and interaction effect (measure of the experimental effect or *et*a-squared, η^2, in brack*et*s)

		Main effect		Interaction	
		Soil series (S)	Aggregate size (A)	(S x A)	Insignificant simple effects at
Param*et*er	MSE	F-statistic	F-statistic	F-statistic	5% level
Clay	3.78	1118.85** (0.986)	624.42** (0.985)	86.25** (0.964)	None
Silt	0.03	455.48** (0.966)	61.28** (0.865)	9.03** (0.738)	None
Sand	1.47	4507.49** (0.997)	771.46** (0.987)	132.83** (0.976)	None
Very fine sand	<0.01	1149.07** (0.989)	1535.87** (0.994)	74.11** (0.957)	None
Fine sand	<0.01	1748.66** (0.992)	995.55** (0.990)	38.65** (0.921)	None
Aggregation	0.04	81.81** (0.836)	185.50** (0.951)	12.89** (0.801)	(S) within <50µm
WDCS	0.02	189.25** (0.922)	147.74** (0.939)	20.32** (0.864)	(A) within Melaka
Free Fe oxides	0.03	452.64** (0.966)	60.14** (0.862)	11.00** (0.775)	None
Free Al oxides	<0.01	23.14** (0.591)	7.60** (0.442)	2.20* (0.407)	(S) within 150-250 µm; (A) within Melaka
OM	0.01	1487.93** (0.989)	769.63** (0.988)	165.89** (0.981)	None
HA	0.03	117.66** (0.880)	1.57^{ns} (0.141)	5.89** (0.648)	(A) within Munchong and Rengam
FA	0.05	7.19** (0.310)	1.86^{ns} (0.163)	1.01^{ns} (0.240)	All
HA-COOH	0.03	35.62** (0.817)	2.41^{ns} (0.334)	1.44^{ns} (0.474)	All
FA-COOH	1.50	10.86** (0.404)	3.24* (0.252)	4.79** (0.600)	(A) within Bungor
Polysaccharides	20.94	2.81* (0.149)	0.65^{ns} (0.064)	1.82^{ns} (0.362)	All

* $p<0.05$; ** $p<0.01$; ^{ns} not significant at 5% level

the WDCS. Their signs indicated that greater amounts of silt and polysaccharides would decrease and increase aggregate stability, respectively.

However, Lapin (1993) explained that the correlations and simple linear regressions do not reveal joint contributions of factors on the dependent variable. This also means that a factor may not correlate significantly to aggregate stability, but yet when its contribution is included with the effects of other factors, its contribution to aggregate stability may become significant. One way to reveal such joint contributions is by multiple linear regression.

TABLE 3
Correlations between aggregate stability and soil constituents

Parameter	WDCS
Clay	0.301^{ns}
Silt	0.461*
Sand	-0.094^{ns}
Very fine sand	0.043^{ns}
Fine sand	-0.332^{ns}
Free Fe oxides	0.095^{ns}
Free Al oxides	-0.039^{ns}
OM	0.097^{ns}
HA	0.088^{ns}
FA	-0.043^{ns}
HA-COOH	0.203^{ns}
HA-OH	-0.145^{ns}
FA-COOH	0.100^{ns}
FA-OH	0.177^{ns}
Polysaccharides	-0.430*

* $p<0.05$; ^{ns} not significant at 5% level

However, including all variables into a regression model was found to be inappropriate because there were symptoms of severe multicollinearity, whereby some variables were highly correlated or dependent on each other. Collinearity diagnostics were performed according to methods described by Belsley *et al.* (1980), Dougherty (1990), and Lardaro (1993). Meanwhile, VIF (variance inflation factors) were calculated for each variable, where any variables with VIF values above 4 or 5 revealed collinearity with others. The larger the VIF, the more a variable is dependent on others. This study found that more than half of the variables were affected by collinearity, with the largest being clay, OM, silt, free Fe oxides, and HA-COOH (all their VIF values exceeded 20). Moreover, the condition number of the collinearity matrix was 151.675, i.e. about five times more than the multicollinearity threshold of 30. As multicollinearity was present, the method of least squares applied to non-orthogonal (correlated) variables would produce poor estimates of the regression coefficients. One way to reduce multicollinearity is to remove unimportant variables from the regression equation. Nonetheless, usual variable selection methods (such as stepwise regression, forward selection, and backward elimination) would be unsuitable for highly correlated variables. In this study, multicollinearity was reduced by making the regressors orthogonal or nearly so with biased estimators using the ridge trace method (Hoerl & Kennard, 1970).

TABLE 4
Multiple regression coefficients for WDCS (criterion variable) at different stages of variable selection

	Variable selection stage	
Predictor	Full model	Final model
Clay	-0.015^{ns}	-
Silt	1.095*	0.794**
Very fine sand	-0.073^{ns}	-
Fine sand	-0.600*	-0.388**
OM	0.197^{ns}	-
HA	0.035^{ns}	-
FA	-1.183^{ns}	-
HA-COOH	1.466^{ns}	0.571*
HA-OH	0.080^{ns}	-
FA-COOH	-0.009^{ns}	-
FA-OH	0.042^{ns}	0.084**
Polysaccharides	-0.014^{ns}	-
Free Fe oxides	-0.682*	-0.599**
Free Al oxides	4.107^{ns}	-
Intercept	3.325*	3.607**
SSE	0.194	0.437
MSE	0.039	0.031
Standard error	0.197	0.177
Adjusted R^2	0.855	0.883
F-statistic	8.985*	29.694**
c.v. (%)	4.864	4.360

* $p<0.05$; ** $p<0.01$; ^{ns} not significant at 5% level

The multiple regression model for the WDCS is shown in Table 4. Although including all the variables into the model (that is, the full model) produced a significant regression model, a low SSE, and a high adjusted R^2, the regression coefficients would be inaccurate, as described earlier. Following the ridge trace method, two criteria were complied: (a) remove variables that are stable but with small prediction power, and (b) remove unstable variables that are driven to near zero values. Consequently, the following

variables were removed: clay, very fine sand, organic matter, humic acids, fulvic acids, HA-OH, FA-COOH, polysaccharides, and free Fe and Al oxides. Clay, for example, was removed as its values were unstable and were driven to almost zero value. Likewise, humic acids variable, though stable, was also removed as it had small predictive power. Meanwhile, selected variables were fitted into a new regression model. In order to further improve the model's fit, non-influential and multicollinear variables were removed, but they were tested if their removal would significantly inflate the SSE (sum of squared error) compared to the SSE of the full model before any removal. In this study, the Wald's test (Lardaro, 1993) was used. The best regression model was selected based on the following criteria; low SSE and MSE, high F-statistic, high adjusted R^2, low standard error of estimate, low standard error for each predictor variable and low correlations among predictor variables.

The final multiple linear regression model is presented in the last column in Table 4. The final model has the following variables: silt, fine sand, free Fe oxides, HA-COOH, and FA-OH. A proper variable selection greatly reduced the multicollinearity effects. The VIF values for all the variables were greatly reduced, and none of them exceeded the critical VIF value of 4 (Table 5). With multicollinearity reduced, the variables fitted better, showing improvement in the adjusted R^2, MSE, standard error of estimate, and F-statistic value (Table 4).

In Table 5, the five soil constituents contributed significantly to the regression model of WDCS, although each of their correlations with WDCS was low, and the highest was silt (r=0.37). These findings illustrated the fact that a variable might not correlate strongly or even significantly with WDCS, but would still contribute significantly when other variables were accounted with it in a multiple regression model. Higher values of WDCS were associated with higher values of silt, HA-COOH and FA-OH, suggesting that any increase in dispersibility (or decrease in aggregate stability) would be attributed to higher amounts of silt, HA-COOH, and FA-OH, but higher amounts of fine sand and free Fe oxides would be attributed to lower dispersibilities (or increases in aggregate stability).

The relative importance of a soil constituent on WDCS can only be determined by its semi-partial correlation to WDCS. Semi-partial r is the correlation of a variable to WDCS after the contributions of all other variables on WDCS are removed. The square of a variable's semi-partial r is then the decrement in R^2 if this variable is eliminated from the model. Therefore, the measure of a variable's "usefulness" in a regression model is its semi-partial r to the criterion variable, not its standardized regression coefficient (Darlington, 1990). Based on the values of the semi-partial r from Table 5, silt was found to be relatively the most important soil constituent in explaining WDCS, followed by free Fe oxides, fine sand, FA-OH, and lastly, HA-COOH.

DISCUSSION

Using the multiple regression has been shown to have improved the interpretation of the results by revealing joint contributions of soil constituents on aggregate stability. Though multicollinearity was a serious problem in this study, multicollinearity was also expected because this study dealt with many constituents that would interact or correlate with one another.

The multiple linear regression model (after correcting for multicollinearity) showed that dispersibility (WDCS) increased with the increasing amounts of silt, HA-COOH, and FA-OH. Meanwhile, the relationship between the latter two with aggregate stability contradicts with the common notion that functional groups of humic substances should reduce, not increase, dispersion.

The role by humic substances, however, is not always shown to be beneficial to aggregate stability. Several researchers have shown that increasing amounts of organic matter or its fractions (humic acids and fulvic acids) can promote dispersion (Gillman, 1974; Shanmuganathan & Oades, 1983; Mbagwu *et*

TABLE 5
Variables in the final regression model for the WDCS

Parameter	standard error of regression coefficient	r	Semipartial r	VIF
Silt	0.086	0.365	0.724	3.077
Free Fe oxides	0.072	-0.214	-0.655	2.455
Fine sand	0.078	-0.332	-0.391	1.934
FA-OH	0.027	0.100	0.243	1.969
HA-COOH	0.202	0.056	0.197	2.250
Intercept	0.542			

al., 1993; Nelson *et al.*, 1999). Organic matter and its fractions can increase dispersion because as more organic anions are sorbed onto colloid surfaces, they increase the negative charges on these surfaces, and thus, increase the diffuse layer of cations as well (Oades, 1984). With this increase, the net negative charge on the surfaces is also increased; thus, promoting dispersion. Therefore, the functional groups of humic substances, namely COOH and phenolic-OH that are mainly responsible for the binding of the humic molecules in clay minerals (Kononova, 1966; Schnitzer & Kodoma, 1977; Theng, 1979), may instead help to increase dispersion and lower aggregate stability.

Moreover, this study revealed that the contribution of FA-OH was more adverse on aggregate stability than of HA-COOH. Nayak *et al.* (1990) observed the same results in their study on three types of soil. They explained that increasing the amount of acidic groups (such as fulvic acids) would increase the inter-particle repulsion between clay and humic molecules. Furthermore, the more acidic groups will tend to be strongly hydrated, and this hydration energy is strong enough to disrupt bonds between clay and humus. As fulvic acids are more acidic than humic acids, fulvic acids would increase the inter-particle repulsion more and would yield stronger hydration energy than as humic acids would.

Silt explained the variability of the WDCS the most; however, its correlation was positive, indicating that increasing the amount of silt is associated with increasing dispersibility. The results from this study are in agreement with the findings by Voronin and Sereda (1976) and Soong (1977, 1980). The role of silt in aggregate stability is actually still unclear, while the correlations involving silt with aggregate stability have either been significantly positive or negative, and at times, the correlations are insignificant. For example, Levy *et al.* (1993) discovered that when the amount of clay and silt exceeded 15%, clay dispersion would exceed 40%. The soil series with less than 15% clay and silt, on the contrary, had less than 40% clay dispersion. Wustamidin and Douglas (1985), however, found silt to correlate positively and significantly with aggregate stability.

Free Fe oxides were the second most important contributor to the regression model to explain WDCS. Similarly, Zhang and Horn (2001) obtained a significant effect by free Fe oxides on aggregate stability. Free Fe oxides can increase the binding strength within aggregates by growing crystals between matrixes of particles; thus, producing a stable and non-dispersible matrix (Shadfan *et al.*, 1985). Similarly, free Fe oxides were also found to reduce the critical coagulation concentrations and clay swelling (Goldberg, 1989). The useful effects of Fe oxides have been demonstrated in many other ways, which include: 1) by significant correlations b*et*ween aggregate stability and Fe oxides (Arca & Weed, 1966; Murthi & Huang, 1987); 2) by electron optical observations of Fe oxides deposits on kaolinite platelets (Fordham & Noorish, 1983; Kitagawa, 1983); 3) by the dispersion of aggregated soils after the removal of their Fe oxides with reducing agents (McNeal *et al.*, 1968); and 4) by the aggregating effect of

added synthetic Fe oxides (Blackmore, 1973). Although free Al oxides are thought to be better than Fe oxides because free Al oxides has a platy structure and is more chemically stable, free Al oxides did not contribute significantly to aggregate stability in this study. This could be due to the low variability and amount of free Al oxides among the soils and aggregate size fractions.

After silt and free Fe oxides, fine sand was the most important constituent in explaining WDCS. In this context, the negative contribution of fine sand to dispersibility also means that with increasing amount of fine sand, aggregate stability is increased. This observation follows the well-known aggregate model by Emerson (1959), whereby for the formation of stable aggregates, fine sand particles are also required.

It is now known which soil constituents contributed significantly to explain aggregate stability. This can help to relate the distribution patterns of some soil constituents with dispersibility.

For the Rengam and Bungor series, as their aggregate size decreased, dispersibility, as measured by WDCS, would generally decrease. Similarly, Zhang and Horn (2001) also observed that for several Ultisols in China, aggregate stability would increase with decreasing aggregate size. This increasing trend in aggregate stability followed the general increasing trend for the amount of fine sand and free Fe oxides. With successively smaller aggregates, the amount of fine sand and free Fe oxides would generally increase, and subsequently increase aggregate stability. Nevertheless, the smallest aggregate size fraction of <50 μm for all the soil series (except for the Melaka series) was exceptional because its dispersibility was, in contrast, the highest. This was due to the sharp increase in the amount of silt in this aggregate size fraction. The acute increase in dispersion could also be explained by the sudden absence of finer-sized sand particles, especially the fine sand fraction. For larger aggregate size fractions, dispersibility was much smaller, and this was probably due to the combination of clay and finer sand particles to form relatively stable aggregates. With the absence of the finer sand particles for aggregates smaller than 50 μm, however, the stability of aggregates would decrease drastically (dispersing very easily). The importance of silt could also be seen for the Melaka soil which had the lowest aggregate stability and the highest amount of silt among the four soils.

The contributions of the organic matter and its fractions, polysaccharides, humic acids and fulvic acids, were each insignificant to explain the WDCS. This was due to the low variability of their amounts among the aggregate size fractions. This low variability among the aggregate size fractions is contrary to some previous work. Mendonça *et al.* (1991), for example, found that the macroaggregates in Oxisols had more fulvic acids and less humic acids than in microaggregates. On the other hand, Chakraborty *et al.* (1981) observed that with increasing aggregate size until 5.0 mm, the amount of both humic acids and fulvic acids increased, but the increase of humic acids was more than of fulvic acids. The distribution pattern of humic substances in the aggregates sizes would depend on the soil type and other environmental factors (Loveland & Webb, 2003). The whole soil used by Mendonça *et al.* (1991), for example, had more fulvic acids than humic acids, but the whole soils used by Chakraborty *et al.* (1981) had more humic acids than fulvic acids.

The distribution of the acidic functional groups of either humic acids or fulvic acids did not follow a particular trend among the aggregate size fractions. This could be the reason for the relatively low contributions of HA-COOH and FA-OH on aggregate stability.

CONCLUSIONS

In the present study, the multiple linear regression revealed that silt was the most important soil constituent to explain the observed differences in aggregate stability between the four soil types. This was followed by free Fe oxides, fine sand, FA-OH, and HA-COOH. With the increasing amounts of silt, FA-OH, and HA-COOH, aggregate stability would decrease, whereas

aggregate stability would instead increase with the increasing amounts of fine sand and free Fe oxides. Meanwhile, other soil constituents, such as clay, free Al oxides, humic acids, fulvic acids, and polysaccharides, did not significantly relate to aggregate stability.

Generally, as the aggregate size decreased, the amount of clay, silt, OM, and free Fe oxides would increase, whereas aggregation and the amount of sand would decrease. For the Rengam and Bungor soil series, aggregate stability would generally increase with the decreasing aggregate size.

The observed differences in the amounts of humic acids, fulvic acids, and polysaccharides were mainly due to the differences in soil types. Generally, within the same soil type, there were insignificant differences in the amount of humic acids, fulvic acids, and polysaccharides in the different aggregate size fractions.

Finally, the amount of fulvic acids in the Munchong and Rengam soil series was found to be significantly higher than the amount of humic acids, but the difference between their amounts was not significant for the Melaka and Bungor soil series.

REFERENCES

Acton, C. J., Rennie, D. A., & Paul, E. A. (1963). The relationship of polysaccharides to soil aggregation. *Canadian Journal of Soil Science, 43*, 201-209.

Arca, M. N., & Weed, S. B. (1966). Soil aggregation and porosity in relation to contents of free iron oxide and clay. *Soil Science, 101*, 164-170.

Belsley, D. A., Kuh, E., & Welsch, R. E. (1980). *Regression Diagnostics: Identifying Influential Data and Sources of Collinearity*. New York: John Wiley & Sons.

Blackmore, A. V. (1973). Aggregation of clay by the products of iron (III) hydrolysis. *Australian Journal of Soil Research, 11*, 75-82.

Boix-Fayos, C., Calvo-Cases, A., Imeson, A. C., & Soriano-Soto, M. D. (2001). Influence of soil properties on the aggregation of some Mediterranean soils and the use of aggregate size and stability as land degradation indicators. *Catena, 44*, 47–67.

Brady, N. C. (1990). *The Nature and Properties of Soils* (10th edn.). New York: Macmillan.

Chakraborty, G., Gupta, S. K., & Banerjee, S. K. (1981). Distribution of water-stable aggregates in some soils of West Bengal in relation to organic and inorganic constituents. *Journal of Indian Soil Science, 29*, 17-24.

Chaney, K., & Swift, R. S. (1984). The influence of organic matter on the stability of some British soils. *Journal of Soil Science, 35*, 223-230.

Chaney, K., & Swift, R. S. (1986). Studies on aggregate stability. II. The effect of humic substances on the stability of re-formed aggregates. *Journal of Soil Science, 37*, 337-343.

Chenu, C. Y., Bissonnais, L., & Arrouays, D. (2000). Organic matter influence on clay w*et*tability and soil aggregate stability. *Soil Science Society of America Journal*, 64, 1479-1486.

Christensen, B. T. (1986). Straw incorporation and soil organic matter in microaggregates and particle size fractions. *Journal of Soil Science, 37*, 125-135.

Darlington, R. B. (1990). *Regression and Linear Models*. New York: McGraw Hill.

Doormar, J. F. (1983). Chemical properties of soil and water-stable aggregate after sixty-seven years of cropping to spring wheat. *Plant and Soil, 75*, 51-61.

Emerson, W. W. (1959). The structure of soil crumbs. *Journal of Soil Science, 10*, 235-243.

Fordham, A. W., & Norrish, K. (1983). The nature of soil particles particularly those reacting with arsenate in a series of chemically treated samples. *Australian Journal of Soil Research, 21*, 455-477.

Fortun, C., Fortun, A., & Almendros, G. (1989). The effect of organic materials and their humified fractions on the formation and stabilization of soil aggregates. *The Science of the Total Environment, 81/82*: 561-568.

Gee, G. W., & Bauder, J. W. (1986). Particle-size analysis. In A. Klute (Ed.), *Methods of soil analysis. Part 1. Physical and mineralogical*

methods (2nd edn.) (pp. 383-411). Wisconsin: American Society of Agronomy.

Gillman, G. P. (1974). The influence of net charge on water dispersible clay and sorbed sulphate. *Australian Journal of Soil Research, 12*, 173-176.

Goldberg, S. (1989). Interaction of aluminum and iron oxides and clay minerals and their effect on soil physical properties: a review. *Communication in Soil Science and Plant Analysis*, *20*, 1181-1207.

Greenland, D. J., Wild, A., & Adams, D. (1992). Organic matter dynamics in soils of the tropics - from myth to complex reality. In R. Lal and P.A. Sanchez (Eds.), *Myths and science of soils of the tropic. SSSA Special Publication no. 29*. (pp. 17-33). Wisconsin: Soil Science Society of America, Inc. and American Society of Agronomy, Inc.

Hamblin, A. P., & Greenland, D. J. (1977). Effect of organic constituents and complexing metal ions on aggregate stability of some East Anglian soils. *Journal of Soil Science, 31*, 203-215.

Hoerl, A. E., & Kennard, R. W. (1970). Ridge regression: biased estimation for nonorthogonal problems. *Technometrics, 12*, 55-67.

Kitagawa, Y. (1983). Goethite and hematite in some soils from the Amazon region. *Soil Science and Plant Nutrition, 29*, 209-217.

Kononova, M. M. (1966). *Soil Organic Matter. Its nature, its Role in Soil Formation and in Soil Fertility* (2nd edn.). Oxford: Pergamon Press.

Krishna Murthi, G. S. R., & Huang, P. M. (1987). Influence of constituents on the stability of mechanical separates of soils representing major taxonomic orders. *Applied Clay Science, 2,* 299-308.

Lapin, L. L. (1993). *Statistics for Modern Business Decisions* (6th edn.). Fort Worth: Dryden Press.

Lardaro, L. (1993). *Applied Econometrics*. New York: Harper Collins.

Levy, G. J., Eisenberg, H., & Shainberg, I. (1993). Clay dispersion as related to soil properties and water permeability. *Soil Science, 155*, 15-22.

Li, H., Chun, Y. W., Wen, F. T., Hong, Q. H., Chong, F. C., & Ming, K. W. (2010). Distribution of organic matter in aggregates of eroded Ultisols, Central China. *Soil & Tillage Research, 108*, 59-67.

Loveland, P., & Webb, J. (2003). Is there a critical level of organic matter in the agricultural soils of temperate regions: a review. *Soil & Tillage Research, 70*, 1-18.

Mbagwu, J. S. C., Piccolo, A., & Mbila, M. O. (1993). Water-stability of aggregates of some tropical soils treated with humic substances. *Pedologie, 43*, 269-284.

McNeal, B. L., Layfield, D. A., Norvell, W. A., & Rhoades, J. D. (1968). Factors influencing hydraulic conductivity of soils in the presence of mixed salt solution. *Soil Science Society of America Proceedings, 32*, 187-190.

Mehra, O. P., & Jackson, M. L. (1960). Iron oxide removal from soils and clays by a dithionite-citrate system buffered with sodium bicarbonate. *Clays and Clay Minerals*, *7*, 317-327.

Mehta, N. C., Streuli, H. Muller, M., & Deuel, H. (1960). Role of polysaccharides in soil aggregation. *Journal of Science, Food and Agriculture, 11*, 40-47.

Mendoça, E. de S., Filho, W. M., & Costa, L. M. (1991). Organic matter and chemical characteristics of aggregates from a red-yellow Latosol under natural forest, rubber plant, and grass in Brazil. In W. S. Wilson (Ed.), *Advances in soil organic matter research: The impact on agriculture and the environment. Special Publication no. 90*. (pp. 185-195). Cambridge: The Royal Society of Chemistry

Nayak, D. C., Varadachari, C., & Ghosh, K. (1990). Influence of organic acidic functional groups of humic substances in complexation with clay minerals. *Soil Science, 149*, 268-271.

Nelson, P. N., Baldock, J. A., Clarke, P., Oades, J. M., & Churchman, G. J. (1999). Dispersed clay and organic matter in soil: their nature and associations. *Australian Journal of Soil Research, 37*, 289-315.

Noellemeyer, E., Frank, F., Alvarez, C., Morazzo, G., & Quiroga, A. (2008). Carbon contents and aggregation related to soil physical and biological properties under a land-use sequence in the semiarid region of central Argentina. *Soil & Tillage Research, 99*, 179–190.

Norhayati, M., & Verloo, M. (1984). Characterization of organic matter in four soils of Peninsular

Malaysia. I. Extraction, fractionation and purification of humic substances. *Journal of Rubber Research Instute of Malaysia, 32*, 30-39.

Nwadialo, B. E., & Mbagwu, J. S. C. (1991). Analysis of soil components active in microaggregate stability. *Soil Technology, 4*, 343-350.

Oades, J. M. (1984). Soil organic matter and structural stability: mechanisms and implications for management. *Plant and Soil, 76*, 319-337.

Schnitzer, M. (1986). Organic matter characterization. In A. Klute (Ed.), *Methods of soil analysis. Part 1. Physical and mineralogical methods* (2nd edn.) (pp. 581-594). Wisconsin: American Society of Agronomy.

Schnitzer, M., & Kodama, H. (1977). Reactions of minerals with soil humic substances. In J. B. Dixon, and S. B. Weed (Eds.), *Minerals in soil environments* (2nd edn.) (pp. 741-700). Wisconsin: Soil Science Society of America.

Shadfan, H., Dixon, J. B., & Calhoun, F. G. (1985). Iron oxide properties versus strength ferruginous crust and iron-glaebules in soils. *Soil Science, 140*, 317-325.

Shanmuganathan, R. T., & Oades, J. M. (1982). Effect of dispersible clay on the physical properties of the B horizon of a red-brown earth. *Austrialian Journal of Soil Research, 20*, 315-324.

Six, J., Bossuyt, H., De Gryze, S., & Denef, K. (2004). A history of research on the link between (micro) aggregates, soil biota, and soil organic matter dynamics. *Soil & Tillage Research, 79*, 7–31.

Soil Survey Laboratory Staff (1992). *Soil Survey Laboratory Methods Manual*. Soil survey investigations report no. 42. Washington: US Dept. of Agriculture, Soil Conservation Service & Soil Survey Staff.

Soong, N. K. (1977). Soil aggregation in Peninsular Malaysia soils and its influence on growth of *Pueraria phaseoloides*. In *Proceedings conference on chemistry and fertility of tropical soils* (pp. 38-49), Kuala Lumpur: Malaysian Society of Soil Science.

Soong, N. K. (1980). Influence of soil organic matter on soil aggregation of soils in Peninsular Malaysia. *Journal of Rubber Research Institute of Malaysia, 28*, 32-46.

Tajuddin, A. (1992). *Influence of Organic Matter on Soil Aggregation and Erodibility of Some Malaysian Soils*. (M. Sc. Agric. Thesis dissertation). Universiti Pertanian Malaysia, Serdang, Selangor, Malaysia

Theng, B. K. G. (1979). Interactions between montmorillonite and fulvic acid. *Geoderma, 15*, 243-251.

Theng, B. K. G., Tate, K. R., & Sollins, P. (with Norhayati, M., Nadkarni, N., and Tate III, R.L.) (1989). Constituents of organic matter in temperate and tropical soils. In D. C. Coleman, J. M. Oades and G. Uehara (Eds.), *Dynamics of soil organic matter in tropical ecosystems* (pp. 5-32). Hawaii: University of Hawaii.

Tisdall, J. M., & Oades, J.M. (1982). Organic matter and water-stable aggregates in soils. *Journal of Soil Science, 33*, 141-163.

Voronin, A. D., & Sereda, N. A. (1976). Composition and structure of the microaggregate fractions of certain type of soils. *Moscow University Soil Science Bulletin, 31*, 100-107.

Walkley, A., & Black, I. A. (1934). An examination of the Degtjareff method for determining soil organic matter and a proposed modification of the chromic acid titration method. *Soil Science, 37*, 29-38.

Wustamidin, L., & A. Douglas (1985). Aggregate breakdown in relation to raindrop energy. *Soil Science, 139*, 239-242.

Zainab, H. (1977). *Complexing Ability of Soil Organic Matter with Mineral Elements and Interactions with Nutrients*. (M. Sc. Agric. Thesis dissertation). State University of Ghent, Belgium.

Zhang, B., & Horn, R. (2001). Mechanisms of aggregate stabilization in Ultisols from subtropical China. *Geoderma, 99*, 123-145.

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Susceptibility of Bagworm *Metisa plana* (Lepidoptera: Psychidae) to Chlorantraniliprole

Chua Chin Kok[1], Ooi Kok Eng[1], Abdul Rahman Razak[2], Adzemi Mat Arshad[2] and Paula G. Marcon[3]

[1]DuPont Malaysia Sdn Bhd,
Malaysia Field Research Station, Lot 2385, Mukim 3, Lahar Tiang,
13200 Kepala Batas, Seberang Perai Utara, Pulau Pinang, Malaysia
[2]Faculty Agro-Technology & Food Science,
Universiti Malaysia Terengganu,
21030 Kuala Terengganu, Terengganu, Malaysia
[3]DuPont Crop Protection, Stine-Haskell Research Center,
1090 Elkton Road, Newark - DE, 19711, USA
**E-mail: Chin-Kok.Chua@mys.dupont.com*

ABSTRACTS

Bagworm (*Metisa plana*) is an ubiquitous pest in oil palm plantations. Seven insecticides were evaluated for their effectiveness in controlling *M. plana* using a leaf dip bioassay. The evaluation assessed the speeds of action, susceptibility of different instars, ovicide and ovi-larvicide activity of chlorantraniliprole. The lowest LC_{50} (0.25ppm) was found with chlorantraniliprole and trichlorfon, followed by thiamethoxam with 0.70 ppm, indoxacarb with 0.72 ppm, cypermethrin with 0.90 ppm, and for monocrotophos with 15.03 ppm. The highest LC_{50} (18.58 ppm) was found for Bacillus thuringiensis, which was approximately 74 times larger than trichlorfon and chlorantraniliprole. Meanwhile, the speed of action of these insecticides on *M. plana* larvae was also found to differ. Trichlorfon (1900.0 ppm), chlorantraniliprole (50.0 ppm) and cypermethrin (75.0 ppm) were among the three fastest acting insecticides evaluated, with respective LT_{50} values of 12.66, 17.04 and 28.63 minutes and larval mortality of 19.91, 47.27 and 53.06 minutes after exposure to the chemicals. *Bacillus thuringiensis* (324.0ppm) was the slowest acting insecticide, requiring more than 2000 minutes to kill 50% of *M. plana* larvae. The first three instars of *M. plana* larvae were very susceptible to chlorantraniliprole, with LC_{50} below 1 ppm, as compared to LC_{50} of 1.91ppm and 9.62ppm for the 4th and 5th instar larvae. Chlorantraniliprole had low to moderate ovicidal effects on *M. plana*, which caused egg mortality to range from 27.50% to 72.50%, but it was shown to be highly toxic on the neonates emerging from the eggs.

Keywords: Rynaxypyr®, chlorantraniliprole, *Metisa plana*, oil palm.

INTRODUCTION

Bagworm (*Metisa plana*) is one of the most destructive pests in oil palm plantations. Outbreaks of bagworm occur frequently (Ho, 1998). Bagworm has a short life cycle and can have several generations within the narrow span of time (Yap, 2005). Hence, appropriate pest management strategies must be taken to control bagworms and to maintain a healthy and productive oil palm plantation. Foliar application of insecticide is still the best option for bagworm control among oil palm growers.

The concept of integrated pest management (IPM) includes the use of selective insecticides

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*Corresponding Author

(when necessary) to keep the pest population below the economical threshold, and the choice of insecticides that is the least toxic to humans and the environment (Wood, 1971). Currently, organophosphate and pyrethroid are still widely used for bagworm control. Newer alternative effective insecticides, with favourable toxicological and environmental profiles, could be valuable additional tools for oil palm growers.

Chlorantraniliprole (Rynaxypyr®) is a novel insecticide in the anthranilic diamides class. It is a potent and selective activator of insect ryanodine receptors, which are critical for muscle contraction. When the ryanodine receptor in insects is activated, calcium homeostasis in the cell is affected, and this leads to feeding cessation, lethargy, muscle paralysis and ultimately death of the insects (Lahm *et al*., 2007). Chlorantraniliprole is a highly selective insecticide with low mammalian toxicity. The acute oral dosage on rat (LD_{50}) is > 5000mg kg^{-1} (DuPont Crop Protection, 2007). In addition, chlorantraniliprole has demonstrated an excellent efficacy and long lasting control on a broad spectrum of Lepidopteran species in several crops.

In order to manage insecticide resistance, monitoring of pest populations for their susceptibility to various insecticides should be carried out at regular intervals. For comparison of susceptibility, the baseline data for various insecticides must be generated prior to widespread use and they should also be easily available for comparative studies. However, there has been no baseline study of bagworm susceptibility to insecticides in Malaysia to date. *Metisa plana*, just like other Lepidopteran species (like diamondback moth and beet armyworm), has a high potential for the development of resistance to the commonly used insecticides.

The objectives of this study were to: (1) determine the baseline susceptibility of *M. plana* to chlorantraniliprole as compared to other commercial insecticide currently being used in oil palm plantations, (2) evaluate the speed of action of chlorantraniliprole in comparison with current standards on *M. plana* control, (3) understand the level of susceptibility of different larval instars of *M. plana* to chlorantraniliprole, and (4) evaluate the ovicidal and ovi-larvicidal activities of chlorantraniliprole against *M. plana.*

MATERIALS AND METHODS

Insect Preparation

Cocoons of *M. plana* were collected in an oil palm estate and maintained in a growth chamber at 25 – 28 °C. All the insects were reared on oil palm leaves, i.e. their natural food source, until they were ready for bioassays. The larvae of the F1 generation were used for the bioassay studies.

Experiment 1: Susceptibility of M. plana to chlorantraniliprole and other insecticides

The insecticides used in this study are listed in Table 1. The rate (g ai ha^{-1}) of commercially available insecticides in Malaysia tested was based on the recommendations for each product on oil palm, except for chlorantraniliprole, indoxacarb and thiamethoxam. Each insecticide was diluted with distilled water to obtain seven different concentrations (namely, 0, 0.3, 1, 3, 10, 30 and 100 ppm). Consequently, thirty-two healthy 2nd instar larvae were used for each insecticide treatment in the current study.

Leaf-dip bioassay method was used for assaying bagworm susceptibility in this study. An oil palm leaf was collected from healthy oil palm seedling planted in a greenhouse at the DuPont Malaysia Field Research Station (MFRS) under insecticide free conditions. The midrib was removed and the leaf lamina was retained. Rectangular leaf pieces, measuring 40 x 50 mm, were cut from the leaf lamina. The rectangular pieces were individually dipped for 10 seconds in the insecticide solutions, air-dried in the laboratory for 1 hour, and placed in polystyrene trays consisting of 32 cells. One larva was gently released into each cell unit. Meanwhile, distilled water was used to treat the control leaf cut. The cells were sealed with transparent plastic lids. Observed under the stereoscope, the larvae were scored dead if they were unable to move after gentle probing

TABLE 1
Insecticides used in the bioassay

IRAC Insecticide Group	Active Ingredient	Formulation
Group 28: Ryanodione receptor modulator	Chlorantraniliprole	Water-Soluble Concentrate (SC)
Group 22: Voltage dependent sodium channel blockers	Indoxacarb	Water-Soluble Concentrate (SC)
Group 3: Sodium channel modulators	Cypermethrin	Emulsifiable Concentrate (EC)
Group 1: Acetylcholinesterase inhibitors	Trichlorfon	Soluble Powder (SP)
Group 4: Nicotinic acetylcholine receptor agonists	Thiamethoxam	Water-Dispersible Granule (WG)
Group 1: Acetylcholinesterase inhibitors	Monocrotophos	Emulsifiable Concentrate (EC)
Group 11: Microbial disruptors of insect midgut membranes	Bacillus thuringiensis	Water-Dispersible Granule (WDG)

with a small paint brush. The mortality of these larvae was recorded 96 hours after they had been released into the cells.

Experiment 2: The speed of action of chlorantraniliprole on Metisa plana

The speed of action of chlorantraniliprole on *M. plana* was evaluated based on time to stop feeding and time to kill, using five insecticides belonging to distinct chemical classes (Table 2).

Once again, the leaf-dip bioassay method (as described Experiment 1) was used in for this experiment. Thirty-two healthy 2nd instar larvae were used to evaluate each insecticide. While preparing for the bioassay, larvae were starved for 24 hours prior to exposure to the leaf cuts. This procedure standardized the nutritional status of the groups of insects to be tested and ensured immediate initiation of feeding as soon as the larvae were transferred to the cell units with leaf cuttings.

To evaluate the speed of action of the insecticides, each individual larva was observed under the stereoscope from the time it was placed on the leaf cut. Time zero (T_0) was defined as the time when each individual larva initiated feeding on the leaf cut. Time 1 (T_1) was defined as the time when each individual larva permanently stopped feeding. Time to complete feeding cessation and kill (T_{total}) was calculated as (T_{total}) = (T_1) - (T_0). The observations were recorded at 5, 15, 30, 45, 60, 75, 90, 105, 120, 180, 240, 300, 360, 720, 1440, 2880, 4320 and 5760 minutes. Larvae from each treatment at 0, 1, 2, 3, 4 days were fixed in FAA solution and processed for SEM observation.

TABLE 2
Treatment list for the speed of action study

No.	Treatment	Formulation	Rate (ppm)
1	Chlorantraniliprole	5SC	12.5
2	Chlorantraniliprole	5SC	25.0
3	Chlorantraniliprole	5SC	50.0
4	Indoxacarb	15SC	50.0
5	Cypermethrin	5EC	75.0
6	Trichlorfon	95SP	1900.0
7	Bacillus thuringiensis	54WG	324.0

Speed of Action Categorization

In this study, LT_{50} obtained by probit analysis was categorized into five categories: (I) very fast speed of action = insecticides that stopped feeding or killed *M. plana* within 30 minutes of exposure; (II) fast speed of action = insecticides that stopped feeding or killed *M. plana* in less

than 3 hours (180 minutes) of exposure; (III) moderate speed of action = insecticides that allowed feeding or survived for more than 3 hours (180 minutes) but less than 6 hours (360 minutes) of exposure; (IV) slow speed of action = insecticides that allowed feeding or survived for more than 6 hours (360 minutes) but less than 24 hours (1440 minutes) of exposure; and (V) very slow speed of action = insecticides that allowed feeding or survived for more than 24 hours (≥ 1440 minutes) of exposure.

Preparation for Scanning Electron Microscopy (SEM)

The internal organs of larvae of different instars were viewed under the scanning electron microscope (JOEL 6310). The cuticle of the abdomen of the instars was sliced longitudinally using a sharp razor blade under a stereo-binocular microscope. The larvae with the sliced abdomen were fixed in FAA for 24 hours, washed in 1% cacodylate buffer and post fixed in 1% cacodylate buffered osmium tetroxide for two hours. The larvae were dehydrated in a graded series of alcohol concentrations (i.e. 30, 50, 70, 90, 95 and 100% in Belzers D30, respectively), and Critical Point Drier Balzers using carbon dioxide liquid as an intermediate fluid. Critical point dried samples were mounted on cylinder stubs and sputter coated with gold, and then viewed with scanning electron microscope JEOL 6310 at an acceleration voltage of 10 or 15kV.

Experiment 3: Susceptibility of different instars of M. plana larvae to chlorantraniliprole

Five different instars (1st, 2nd, 3rd, 4th and 5th) of *M. plana* were used in this study. The leaf-dip bioassay method was employed in the study, and the details were as previously described under Experiment 1. Seven different concentrations of chlorantraniliprole (i.e. at 0, 0.3, 1, 3, 10, 30 and 100 ppm) were obtained by diluting the insecticide with distilled water. Each treatment was evaluated using thirty-two healthy larvae.

Experiment 4: Ovicidal and ovi-larvicidal effect of chlorantraniliprole on Metisa plana

Eggs of *M. plana* used in this experiment came from F1 population which was obtained as described above. One day-old eggs were used for the ovicidal and ovi-larvicidal study. To determine the toxicity of chlorantraniliprole and other insecticides to *M. plana* eggs, the eggs were exposed to 12.5 ppm, 25.0 ppm and 50.0 ppm of chlorantraniliprole, 75.0 ppm of cypermethrin and 1900.0 ppm of trichlorfon (Table 3). A total of ten eggs were used per replicate, with four replications per treatment. To ensure uniformity, the eggs collected from the same egg mass were used in each replication. These eggs were dipped in the insecticide solutions for five seconds and air dried for one hour under laboratory conditions. The undipped eggs were used as control. Each replication of ten treated eggs was placed in a 20 ml transparent screw-capped vial. The total number of hatched and unhatched eggs, as well as dead and alive larvae was counted under the stereomicroscope on day 12.

TABLE 3
A treatment list for the ovicidal and ovi-larvicidal study

No.	Treatment	Formulation	Rate (ppm)
1	Chlorantraniliprole	5SC	12.5
2	Chlorantraniliprole	5SC	25.0
3	Chlorantraniliprole	5SC	50.0
4	Cypermethrin	5EC	75.0
5	Trichlorfon	95SP	1900.0
6	Untreated check	-	-

Statistical Analysis

For Experiments 1, 2 and 3, the mortality data were subjected to probit analysis to calculate median lethal concentration (LC_{50}), LC_{90} and Fiducial limits. The probit analysis was carried out using the DuPont Dose Response probit analysis software version 2.0.

For Experiment 4, however, all the treatment data were subjected to ANOVA using FieldPro Biodata Management software, in which the means for each treatment were separated ($P \leq 0.05$) using Duncan's Multiple Range Test.

RESULTS

Experiment 1: The Susceptibility of M. plana to Chlorantraniliprole and Other Insecticides

The probit analyses of the susceptibility of *M. plana* 2nd instar larvae to the treated insecticides are presented in Table 4. The lethal concentrations (LC_{50} and LC_{90} values) of *M. plana* varied greatly between the insecticides tested, suggesting that the level of *M. plana* susceptibility to each insecticide differed greatly. The LC_{50} values of trichlorfon and chlorantraniliprole were 0.25 ppm, followed by 0.70 ppm for thiamethoxam, 0.72 ppm for indoxacarb, 0.90 ppm for cypermethrin, and 15.03 ppm for monocrotophos. The highest LC_{50} was observed for *B. thuringiensis,* i.e. at 18.58ppm, and approximately 74 times more than trichlorfon and chlorantraniliprole (0.25 ppm). It is important to note that the mortality in the untreated control for all the treatments was less than 5% after 96 hours.

The LC_{90} value for chlorantraniliprole was the lowest among the seven groups of insecticides, i.e. at 0.64 ppm, followed by cypermethrin, indoxacarb, trichlorfon and monocrotophos at 4.69 ppm, 6.20 ppm, 46.53 ppm, 198.41 ppm, respectively. Meanwhile, Thiamethoxam and *B. thuringiensis* have the highest LC_{90} values at 371.58 ppm 302.65 ppm, respectively, the value which are more than 500 times higher than that of chlorantraniliprole.

Experiment 2: The Speed of Action of Chlorantraniliprole on Metisa plana

Time to Stop Feeding

The highest percentage of larvae that stopped feeding within the first 5 minutes of exposure to the insecticides was found with 50.0 ppm chlorantraniliprole (15.63%), followed by 1900.0 ppm trichlorfon (9.38%) and 25 ppm chlorantraniliprole (3.13%), as shown in Table 5. After 30 minutes of exposure to 50.0 ppm chlorantraniliprole and 1900.0 ppm trichlorfon, 78.13% of *M. plana* stopped feeding. The percentage of the larvae that stopped feeding increased with the time of exposure to pesticides. After 60 minutes, 93.75% of *M. plana* stopped feeding after being exposed to 1900.0 ppm trichlorfon and 75.0 ppm cypermethrin. The descending order of the feeding cessation of *M. plana* larvae to the remaining insecticides after 60 minutes of exposure are 87.50% for

TABLE 4
Dosage-mortality response of the susceptibility bagworm, *Metisa plana* to chlorantraniliprole, indoxacarb, cypermethrin, trichlorfon, thiamethoxam, monocrotophos and *Bacillus thuringiensis,* after 96 hours of insecticide exposure

Insecticides	Slope	Intercept	Chi-Square	LC_{50} (ppm)	LC_{50} (ppm) Fiducial Limit Lower 95% CL	Upper 95% CL	LC_{90} (ppm)	LC_{90} (ppm) Fiducial Limit Lower 95% CL	Upper 95% CL
Chlorantraniliprole	3.142	1.878	0.013	0.25	0.12	0.33	0.64	0.47	1.40
Indoxacarb	1.374	0.192	12.830	0.72	0.03	2.10	6.20	2.13	804.87
Cypermethrin	1.791	0.078	2.648	0.90	0.60	1.26	4.69	3.07	9.06
Trichlorfon	0.565	0.251	4.999	0.25	0.02	0.72	46.53	15.85	492.53
Thiamethoxam	0.470	0.072	0.291	0.70	0.08	1.90	371.58	72.54	26929.22
Mococrotophos	1.143	-1.346	7.512	15.03	9.80	24.58	198.41	95.56	633.28
Bacillus thuringiensis	1.057	-1.342	9.772	18.58	6.55	105.84	302.65	66.18	62467.42

TABLE 5
Time to stop feeding response of *Metisa plana* to chlorantraniliprole, indoxacarb, cypermethrin, trichlorfon, and *Bacillus thuringiensis,* after 96 hours of insecticide exposure

Time (Minutes)	Treatment (Percentage of larvae stop feeding)						
	12.5ppm Chlorantraniliprole	25.0ppm Chlorantraniliprole	50.0ppm Chlorantraniliprole	50.0ppm Indoxacarb	75.0ppm Cypermethrin	1900.0ppm Trichlorfon	324.0ppm *B. thuringensis*
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	3.13	15.63	0.00	0.00	9.38	0.00
15	9.38	18.75	34.38	3.13	6.25	71.88	0.00
30	34.38	43.75	78.13	3.13	62.50	78.13	0.00
45	43.78	50.00	84.38	3.13	81.25	90.63	0.00
60	53.13	65.63	87.50	3.13	93.75	93.75	0.00
75	56.25	71.88	90.63	6.25	93.75	93.75	3.13
90	56.25	71.88	93.75	9.38	96.88	93.75	6.25
105	68.75	71.88	93.75	9.38	100.00	93.75	6.25
120	68.75	75.00	100.00	15.63	100.00	93.75	6.25
180	81.25	81.25	100.00	40.63	100.00	96.88	6.25
240	96.88	96.88	100.00	53.13	100.00	100.00	9.38
300	100.00	96.88	100.00	56.25	100.00	100.00	9.38
360	100.00	100.00	100.00	65.63	100.00	100.00	9.38
720	100.00	100.00	100.00	71.88	100.00	100.00	9.38
1440	100.00	100.00	100.00	75.00	100.00	100.00	28.13
2880	100.00	100.00	100.00	78.13	100.00	100.00	84.38
4320	100.00	100.00	100.00	87.50	100.00	100.00	87.50
5760	100.00	100.00	100.00	100.00	100.00	100.00	90.63

50.0 ppm chlorantraniliprole, 65.63% for 25.0 ppm chlorantraniliprole, 53.13% for 12.5 ppm chlorantraniliprole, 3.13% for 50.0 ppm indoxacarb, and the feeding of larva continued on to 324.0 ppm *B. thuringiensis* treatment after 60 minutes. Nonetheless, the feeding completely stopped after 105 minutes of exposure to 75.0 ppm cypermethrin, 120 minutes and 240 minutes to 50.0 ppm chlorantraniliprole and 1900.0 ppm trichlorfon, respectively. The first cessation of feeding of the larvae on *B. thuringiensis* treated leaf cut was observed 75 minutes after exposure and 28.13% of the larvae stop feeding after 1440 minutes (1 day) of exposure. All larvae ended feeding within 96 hours exposure to the insecticides, with the exception of larvae which were exposed to 324.0 ppm *B. thuringiensis* and only 90.63% ended feeding.

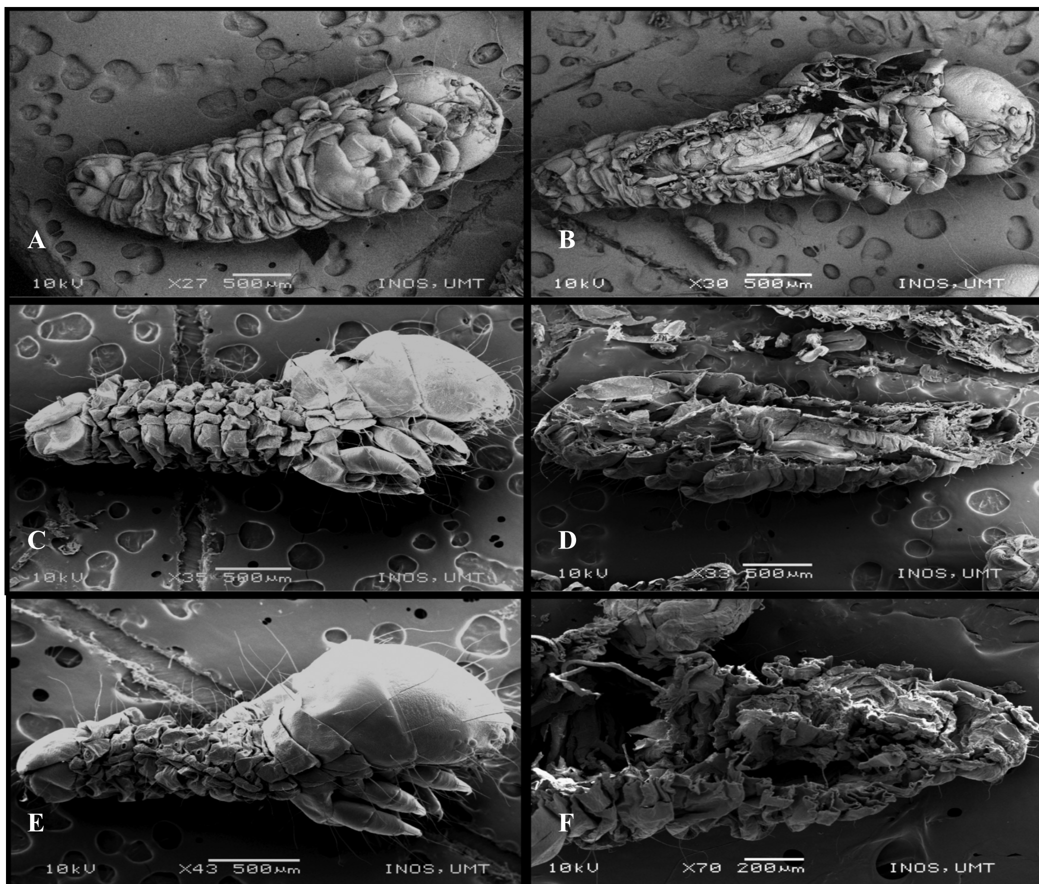

Fig. 1: SEM micrographs of, (A); fresh larva (ventral view; X 27), (B); showing the internal organs of fresh larva (ventral view; X 30), (C); larva exposed to chlorantraniliprole after 1 day of bioassay (side view; X 35), (D); showing the internal organs of day 1 after being exposed to chlorantraniliprole larva (ventral view; X 33), (E): a larva exposed to chlorantraniliprole after 3 days of bioassay (side view; X 43), and (F); showing the internal organs of larva after day 3 of exposure to chlorantraniliprole larva (ventral view; X 70).

TABLE 6
Time to kill response of *Metisa plana* to chlorantraniliprole, indoxacarb, cypermethrin, trichlorfon, and *Bacillus thuringiensis,* after 96 hours of insecticide exposure.

Time (Minutes)	Treatment (Percentage of larvae mortality)						
	12.5ppm Chlorantraniliprole	25.0ppm Chlorantraniliprole	50.0ppm Chlorantraniliprole	50.0ppm Indoxacarb	75.0ppm Cypermethrin	1900.0ppm Trichlorfon	324.0ppm *B. thuringensis*
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	6.25	0.00
15	0.00	0.00	15.63	0.00	0.00	31.25	0.00
30	0.00	15.63	21.88	3.13	15.63	78.13	0.00
45	9.38	37.50	37.50	3.13	31.25	78.13	0.00
60	21.88	37.50	56.25	3.13	50.00	90.63	0.00
75	31.25	53.13	78.13	3.13	81.25	93.75	3.13
90	34.38	56.25	84.38	6.25	87.50	93.75	3.13
105	34.38	56.25	84.38	9.38	96.88	93.75	3.13
120	40.63	56.25	84.38	9.38	100.00	93.75	3.13
180	62.50	71.88	96.88	12.50	100.00	93.75	3.13
240	68.75	78.13	100.00	31.25	100.00	96.88	9.38
300	96.88	81.25	100.00	53.13	100.00	100.00	9.38
360	96.88	96.88	100.00	56.25	100.00	100.00	9.38
720	96.88	100.00	100.00	65.63	100.00	100.00	9.38
1440	96.88	100.00	100.00	68.75	100.00	100.00	9.38
2880	100.00	100.00	100.00	71.88	100.00	100.00	59.38
4320	100.00	100.00	100.00	75.00	100.00	100.00	84.38
5760	100.00	100.00	100.00	75.00	100.00	100.00	87.50

The time to stop feeding response of *M. plana* 2nd instars larvae is presented in Table 7. The larvae exposed to 1900.0 ppm trichlorfon produced the lowest LT_{50} value of 12.66 minutes and this was followed by 17.04 minutes for 50.0 ppm chlorantraniliprole, and 28.63 minutes for 75.0 ppm cypermethrin. The difference of the LT_{50} value between the lowest LT_{50} value of 1900.0 ppm trichlorfon and the second lowest of 50.0 ppm chlorantraniliprole was extremely close, i.e. less than 5 minutes. The difference between the second and third lowest LT_{50} value of 50.0 ppm chlorantraniliprole and 75.0 ppm cypermethrin was more than 10 minutes. The LT_{50} values for 25.0 ppm and 12.5 ppm chlorantraniliprole were at 42.76 and 58.48 minutes, respectively. Meanwhile, the LT_{50} value of 50.0 ppm indoxacarb was slightly higher at 402.91 minutes. At 324.0 ppm, *B. thuringiensis* produced the highest LT_{50} value on *M. plana* larvae at 1451.36 minutes, with 114.6 and 85.1 times higher than 1900.0 ppm trichlorfon and 50.0 ppm chlorantraniliprole, respectively.

When looking at LT_{90} values, 75.0 ppm cypermethrin had the lowest LT_{90} value with 55.56 minutes, followed by 56.13 minutes for 1900.0 ppm trichlorfon, 62.36 minutes for 50.0 ppm chlorantraniliprole, 189.40 minutes for 25.0 ppm chlorantraniliprole, 205.18 minutes for 12.5 ppm chlorantraniliprole and 2714.97 minutes for 50.0 ppm indoxcarb. Meanwhile, 324.0 ppm *B. thuringiensis* still ranked the highest, with the LT_{90} value at 7756.25 and/ or about 5.3 days.

Time to Kill

The mortality time of *M. plana* after exposure to the insecticides is presented in Table 6. The findings revealed that the exposure to trichlorfon (1900.0 ppm) killed 6.25% of the larvae within the first 5 minutes and increased to 31.25% within the first 15 minutes. The killing time of the larvae exposed to the insecticides was observed to vary. After 60 minutes of chemical exposure, 1900.0ppm trichlorfon still ranked the highest, with the percentage of mortality at 90.63%, and this was followed by 50.0 ppm chlorantraniliprole and 75.0 ppm cypermethrin with about 50% mortality. The larvae were killed within 120 minutes of exposure to 75.0 ppm cypermethrin as compared to 240 minutes with 50.0 ppm chlorantraniliprole and 300 minutes with 1900.0 ppm trichlorfon. A longer exposure time was required for 100% kill, with a lower concentration of chlorantraniliprole, 720 minutes and 2880 minutes at 25.0 ppm and 12.5 ppm, respectively. Both 50.0 ppm indoxacarb and 324.0 ppm *B. thuringiensis* achieved only 75.00% and 87.50% kill when the experiment was terminated after 5760 minutes (4 days) of exposure to the tested insecticides.

Table 8 shows the LT_{50} and LT_{90} values of all the treatments tested in terms of the time to kill. The probit analysis result showed that the LT_{50} and LT_{90} values of larvae exposed to 1900.0 ppm trichlorfon remained at the lowest, with 19.91 and 75.80 minutes, respectively. The LT_{50} and LT_{90} values of 50.0 ppm chlorantraniliprole and 75.0 ppm cypermethrin were between 47 to 53 minutes and 91 to 126 minutes, respectively. At 25.0ppm and 12.5ppm, Chlorantraniliprole was ranked the fourth and fifth lowest LT_{50} and LT_{90} values, with the LT_{50} values at 85.59 and 130.31 minutes and LT_{90} values at 326.06 and 372.69 minutes, respectively. The LT_{50} and LT_{90} values of 50.0ppm indoxacarb were 764.01 and 7367.64 minutes, respectively. At 324.0 ppm, *B. thuringiensis* remained the highest with the LT_{50} and LT_{90} values of 2093.82 and 11941.65 minutes.

Speed of Action Categorization

Base on LT_{50} time to stop feeding and probit kill (Tables 7 and 8), the speed of action index of the 5 groups of pesticides was categorized as follows; for feeding cessation, Category I had 1900 ppm trichlorfon, 50 ppm chlorantraniliprole and 75 ppm cypermethrin; Category II had 25 ppm and 12.5 ppm chlorantraniliprole; Category IV had 50 ppm indoxacarb; and Category V had 324 ppm *B. thuringiensis*. As for time to kill, Category I had 1900ppm trichlorfon, Category II had 50 ppm chlorantraniliprole and 75 ppm cypermethrin, Category III had 25 ppm and 12.5 ppm chlorantraniliprole, Category IV had 50 ppm indoxacarb, and Category V had 324ppm *B. thuringiensis*.

TABLE 7

Time to stop feeding response of *Metisa plana* to chlorantraniliprole, indoxacarb, cypermethrin, trichlorfon, and *Bacillus thuringiensis,* after 96 hours of insecticide exposure.

Insecticide	Rate (ppm)	Slope	Intercept	Chi-Square	LT_{50}	LT_{50} Fiducial Limit		LT_{90}	LT_{90} Fiducial Limit	
						Lower 95% CL	Upper 95% CL		Lower 95% CL	Upper 95% CL
Chlorantraniliprole	12.5	2.351	-4.154	10.229	58.48	49.54	67.72	205.18	168.82	264.72
Chlorantraniliprole	25.0	1.983	-3.234	6.866	42.76	34.69	51.07	189.40	152.56	250.42
Chlorantraniliprole	50.0	2.274	-2.801	5.396	17.04	12.97	21.15	62.36	50.88	79.80
Indoxacarb	50.0	1.546	-4.029	32.030	402.91	298.57	568.02	2714.97	1644.85	5669.24
Cypermethrin	75.0	4.451	-6.485	3.836	28.63	24.48	32.55	55.56	48.55	66.06
Trichlorfon	1900.0	1.981	2.185	10.980	12.66	8.98	16.44	56.13	44.66	73.87
Bacillus thuringensis	324.0	1.760	-.5.566	27.409	1451.36	1069.53	2088.80	7756.25	4758.21	15820.94

TABLE 8

Time to kill response of *Metisa plana* to chlorantraniliprole, indoxacarb, cypermethrin, trichlorfon, and *Bacillus thuringiensis,* after 96 hours of insecticide exposure.

Insecticide	Rate (ppm)	Slope	Intercept	Chi-Square	LT50	LT50 Fiducial Limit		LT90	LT90 Fiducial Limit	
						Lower 95% CL	Upper 95% CL		Lower 95% CL	Upper 95% CL
Chlorantraniliprole	12.5	2.808	-5.939	27.170	130.31	109.49	156.07	372.69	286.54	552.56
Chlorantraniliprole	25.0	2.206	-4.263	9.970	85.59	73.34	99.01	326.06	261.76	435.86
Chlorantraniliprole	50.0	3.000	-5.024	8.906	47.27	40.48	53.96	126.40	107.92	155.15
Indoxacarb	50.0	1.302	-3.754	33.396	764.01	528.33	1203.79	7367.64	3821.47	20122.63
Cypermethrin	75.0	5.450	-9.400	5.259	53.06	47.86	57.97	91.18	82.07	104.97
Trichlorfon	1900.0	2.207	-2.867	11.187	19.91	15.31	24.54	75.80	61.99	96.94
Bacillus thuringensis	324.0	1.694	-5.628	26.080	2093.82	1646.67	2773.40	11941.65	7923.48	20735.16

TABLE 9
Probit analysis of dosage-mortality response of the susceptibility bagworm, *Metisa plana* to different instars after 96 hours insecticide exposure.

Instar	Slope	Intercept	Chi-Square	LC50	LC50 Fiducial Limit		LC90	LC90 Fiducial Limit	
					Lower 95% CL	Upper 95% CL		Lower 95% CL	Upper 95% CL
1st	1.418	1.834	4.812	0.05	0.02	0.08	0.40	0.23	0.95
2nd	3.142	1.878	0.013	0.25	0.12	0.33	0.64	0.47	1.40
3rd	2.761	1.073	0.351	0.40	0.22	0.58	1.18	0.81	2.61
4th	1.257	-0.355	3.242	1.91	1.17	3.39	20.02	8.51	143.22
5th	2.154	-2.118	3.869	9.62	6.38	12.57	37.85	27.25	68.77

Structural Changes in Metisa plana Larvae after Exposure to Chlorantraniliprole

The surface of the folded structure of fresh larval abdomen was formed by columnar cells (*Fig. 1A*). Organs of the larvae, such as spiracles, setae and prolegs were clearly defined. The longitudinal section of *M. plana* larva showed that the larval midgut was the longest portion of the alimentary canal, lying convoluted within the larval body cavity (*Fig. 1B*).

When the larva was exposed to chlorantraniliprole after 1 day of bioassay, 96.88 % was obtained (Table 6). The SEM micrograph in *Fig. 1C* showed that the surface morphology of the larva was preserved, but with a less uniformed shape. The spiracles, setae and prolegs were still visible although they were not clearly discernible. The alimentary canal remained intact and the larval intestine shrunk from approximately 100 µm to 35 µm in diameter (*Fig. 1D*). The compact helical shaped intestine was loosening into a straight empty space in the internal body cavity.

After 3 days of bioassay, no survival was obtained and the structure was severely disintegrated. The abdominal body wall fractured and the normal columnar cell disappeared (*Fig. 1E*). Internally, the integrity of the organs started to lose, and the intestine was completely destroyed leaving an empty abdomen (*Fig. 1F*).

Experiment 3: The Susceptibility of Different Instars of M. plana Larvae to Chlorantraniliprole

The results from the probit analysis of dosage-mortality response of all *M. plana* instars larvae are presented in Table 9. No mortality was recorded in the untreated larvae after 96 hours of feeding. In the chlorantraniliprole treated leaves, the LC_{50} values of the 1st to 5th instars larvae were 0.05ppm, 0.25ppm, 0.40ppm, 1.91ppm and 9.62ppm, respectively; whereas the respective LC_{90} values were 0.40ppm, 0.64ppm, 1.18ppm, 20.02ppm and 37.85ppm. These results show that the LC_{50} and LC_{90} values of the first three instars larvae of *M. plana* were generally low (i.e. below 1 ppm), except for the LC_{90} value of 3rd instar larvae which was at 1.18 ppm. Meanwhile, the LC_{50} and LC_{90} values of the 4th and 5th instar larvae increased drastically from 1.91 ppm to 9.62 ppm and from 20.02 ppm to 37.85 ppm, respectively. These results also showed that the 1st, 2nd and 3rd instars larvae were very susceptible to chlorantraniliprole, with relatively 100% mortality and 96 hours exposure to the 3ppm chemical dipped leaf cut; however, the 4th and 5th instars larvae needed a higher concentration of 30 ppm to obtain 100% mortality. Therefore, the results clearly confirmed the greater LC_{50} and LC_{90} values in the older instars than the younger instars. The LC_{50} and LC_{90} values increased drastically beyond the 4th instar larvae stage.

TABLE 10
Percentage of ovicidal and ovi-larvicidal responses of *Metisa plana*.

Treatment	Rate (ppm)	Ovicidal (%)	Ovi-larvicidal (%)
Chlorantraniliprole	12.5	35.00^{b}	100.00^{a}
Chlorantraniliprole	25.0	72.50^{ab}	100.00^{a}
Chlorantraniliprole	50.0	27.50^{b}	100.00^{a}
Cypermethrin	75.0	100.00^{a}	100.00^{a}
Trichlorfon	1900.0	100.00^{a}	100.00^{a}
Untreated check	-	27.50^{b}	32.50^{b}

Note:
1. Ovicidal effect was calculated according to the number of unhatched eggs. The number of effective larval penetration was used to determine the total effect (egg survival).
2. Values within the column having the same superscripts are not significantly different at $P \leq 0.05$.

Experiment 4: The Effect of Chlorantraniliprole on Ovicidal and Ovi-larvicidal of Metisa plana

The results for the ovicidal and ovi-larvicidal effects of the tested pesticides are presented in Table 10. There was a significant difference for the ovicidal response between the treatments. 75.0 ppm cypermethrin and 1900.0 ppm trichlorfon have very strong ovicidal effects resulting in 100.00% unhatched eggs. On the contrary, *M. plana* had low to moderate ovicidal responses to chlorantraniliprole at 12.5 ppm to 50.0 ppm with 27.50% to 72.50%, respectively.

Chlorantraniliprole showed strong ovi-larvicidal property. The total mortality of unhatched eggs (ovicidal) plus immediate death of larvae during and after hatching reached 100.00%. Therefore, the ovi-larvicidal effect of all the treatments was significantly higher than the untreated control.

DISCUSSION

This study has showed that among all the current insecticides in the market, chlorantraniliprole is the most potent insecticide against *M. plana* in oil palm plantations. Based on the LC_{50} and LC_{90} values of 0.25ppm and 0.64ppm, the relative susceptibility of the *M. plana* larvae to chlorantraniliprole was 7.3 times higher than cypermethrin (ranked the second best). Likewise, chlorantraniliprole was found to be 9.6, 72.7, 310.0, 472.8 and 580.5 times more effective to indoxacarb, triclorfon, monocrotophos, *B. thuringiensis* and thiamethoxam, respectively. A low LC_{50} value of chlorantraniliprole was also reported with other pests, such as *Scirphophaga incertulas* (0.03ppm), *Plutella xylostella* (0.11ppm) and *Spodoptera exigua* (0.20ppm) in Malaysia (Kamar *et al*., 2008). Cypermethrin was reported to be the most effective insecticide against the 4th instar bagworm (*Auchmophila kordofensis*) larvae on *Acacia* with the LC_{50} and LC_{90} values of 7.04 ppm and 34.76 ppm, respectively, and these are better than chlorpyrifos and spinosad (Kowkab *et al.*, 2008). However, the current study further revealed that chlorantraniliprole performed even more potent than cypermethrin on *M. plana*. In addition, several synthetic pythrethroid products have shown field failure in the official spraying program against Lepidoptera species, such as *Spodoptera littoralis* (Temerak, 2002). *Spodoptera* and *Helicoverpa* species have been found to develop resistance to synthetic pyrethroids such as cypermethrin (El-Dahan *et al.*, 1985, Tikar *et al.*, 2004). Incidences of *H. armigera* resistance to cypermethrin have frequently been reported in India (Tikar *et al.*, 2004).

Resistance monitoring is an important component for developing IRM strategies. The strategy of insecticide resistance management (IRM) emphasizes on the judicial use of

insecticides to minimize selection for insect resistance and to increase the life-span of the insecticidal molecules. In the recent years, increasing efforts have been made towards incorporating IRM strategies into the larger realm in most of the agrochemical industry. However, the IRM programme for *M. plana* on oil palm is still lacking.

Chlorantraniliprole is a new insecticide with a novel mode of action and it is also effective on lepidopterous pests that have developed resistant to other insecticides. Apparently, chlorantraniliprole has constantly provided a good control on leaf folders and stem borers in rice, as well as beet army worm and diamondback moth in vegetables after several years and numerous crops trials (Kamar *et al*., 2008). Efforts must be made to preserve this high activity; for example, by not applying insecticides in the same chemical class to consecutive generations of the same pest, or by not using those insecticides over more than 50% of the crop time (Insecticide Resistance Action Committee, 2008).

The ability and speed to cease feeding have become one of the critical indicators of insecticidal potency for discovering and developing novel chemical classes of insecticides. Chlorantraniliprole was among the fastest-acting insecticides for feeding cessation (Category I), but not the fastest to kill *M. plana* (Category II). The SEM micrograph showed that chlorantraniliprole had destroyed the internal organs of the larvae. The stomach poisoning effect of chlorantraniliprole was manifested in the deformation of the surface, and disintegration of the intestine. When the cells and the internal organs are damaged, the larvae became lethargic and ultimately die (Lam *et al*., 2007). Chlorantraniliprole was also reported to have caused the fastest feeding cessation on *Plutella xylostella*, *Trichoplusia ni*, *Spodoptera exigua* and *Helicoverpa zea* as compared to emamectin benzoate, indoxacarb, methoxyfenozide and metaflumizone (Hannig *et al*., 2009).

The susceptibility level of chlorantraniliprole gradually decreased with the succeeding instars, as reflected in the values of LC_{50} and LC_{90} in Table 9. The values of LC_{50} and LC_{90} drastically increased with the 4th instar larvae. The larger the larvae, the harder they are to kill with chemicals. Apparently, the best time to apply chlorantraniliprole for *M. plana* control is during the vulnerable stage of the neonate 1st to 3rd instars when the larvae are small and actively feeding on leaves (LC_{50} ranging from 0.05 ppm to 0.40 ppm). The control becomes difficult and less effective with later instars (close to pupation stage), such as 5th instar and above. Moreover, *M. plana* larvae are active feeders from the 1st to 3rd instars, but they slow down near to pupation at the 4th and 5th instar. The LC_{50} of 0.40ppm was sufficient to kill up to the 3rd instar. A higher concentration was required to give a similar result at the 4th and 5th instars. The result of this study concurred with the report of Kumar and Kumar (2008) on *B. thuringiensis* against *Helicoverpa armigera*. The neonates (i.e. the 1st instar) were the most susceptible to *B. thuringiensis* than other later instars. Therefore, the timing of application in the field is very critical to obtain the optimum control. The first 28.0 days of the 1st to 3rd instars larval period are the best time to apply chlorantraniliprole against *M. plana* on oil palm.

The mode of insecticidal action in insect eggs is not well understood and at least two types of mortality have been associated with the death of the developing insect. The embryo in the egg may be killed (true ovicidal effect) and further development (embryogenesis) is halted or the larva dies as it feeds on the chorion during eclosion, an "ovi-larvicidal" effect (Temarak, 2003). Chlorantraniliprole has low to moderate ovicidal effect on *M. plana*, ranging 27.50% to 72.50%, but is potent against emerging neonates. The combined effects against the eggs and larvae of *M. plana* can contribute to its efficacy in the field. Similar finding was also reported on the low ovicidal effect but high ovi-larvicidal effect of chlorantraniliprole on *Lobesia botrana*

(Claudio *et al.*, 2009). The ovi-larvicidal activity of chlorantraniliprole against *M. plana* may increase its benefits in situations where the *M. plana* outbreak occurs over time.

CONCLUSION

Based on the findings of the current study, it can be concluded that Chlorantraniliprole provides a new standard for good residual control of leaf feeders in the tropical climate, and is a good addition to the insecticide resistance management (IRM) strategies and integrated pest management (IPM) of *M. plana* in oil palm. This is based on its low LC_{50} against the larvae of *M. plana,* and also its times of stop feeding and kill (Category I and Category II). Moreover it has demonstrated good ovi-larvicidal and residual properties, and can be regarded as a novel insecticide.

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REFERENCES

Claudio, I., Gianfranco, A., Gino, A., Valerio, M., & Federica, T. (2009). Effects of chlorantraniliprole on eggs and larvae of *Lobesia botrana* (Denis & Schiffermuller) (Lepidoptera: Tortricidae). *Pest Management Science*, *65*, 717-722.

DuPont Crop Protection. (2007). *Rynaxypyr ™ Insect Control.* DuPont Technical Bullent. DuPont Crop Protection, Newark, D.E., USA.

El-Dahan, A. A., El-Guingy, M. A., & Bakry, N. M. (1985). Efficacy of certain insecticides against a field strain of cotton leafworm *Spodoptera littoralis* (Boisd) during 1980 season. *Bulletin Entomology Society in Egypt*, *13*, 1-7.

Hannig, G. T, Ziegler, M., & Marcon, P.G. (2009). Feeding cessation effects of chlorantraniliprole, a new anthranilic diamide insecticide, in comparison with several insecticides in distinct chemical classes and mode-of-action groups. *Pesticide Management Science*, *65*, 969-974.

Ho, C. T. (1998). Safe and efficient management systems for plantation pests and diseases. *The Planter, 74*, 369-385.

Insecticide Resistance Action Committee. (2008). Insecticide Resistance Management: Global Guidelines for IRAC Group 28 (Diamide) Insecticides.

Kamar, S., Chua, C. K., & Ooi, K. E. (2008). *Prevathon™ – A novel insecticide in controlling wide spectrum of Lepidopteran pests in various economically important crops.* Paper presented at the 7th MAPPS International Conference on Plant Protection in the Tropics. Malaysian Plant Protection Society, Malaysia.

Kowkab, F. E. B., Yousif, O. H. A., & Nabil, H.H.B. (2008). Susceptibility of bagworm *Auchmophila kordofensis* Rebel (Lepidoptera: Psychidae) to the three insecticides groups Spinosad, Chlorpyrifos and Cypermethrin. *Resistant Pest Management Newsletter, 17*(2), 19-26.

Kumar, S., & Kumar, H. (2008). Susceptiblity of different instars of *Helicoverpa armigera* to *Bacillus thuringiensis* insecticidal proteins. *Resistant Pest Management Newsletter, 17*(2), 30-32.

Lahm, G. P., Stevenson, T. M., Selby, T. P., Freudenberger, J. H., Cordova, D., Flexner, L., Bellin, C. A., Dubas, C. M., Smith, B. K., Hughes, K. A., Hollingshaus, J. G., Clark, C. E., & Benner, E. A. (2007). Rynaxypyr™: A new insecticidal anthranilic diamide that acts as a potent and selective ryanodine receptor activator. *Bioorganic & Medicinal Chemistry Letters*, *17*, 6274-6279.

Temerak, S. A. (2002). Historical records of field cotton leafworm (*Spodoptera littoralis*) resistance to conventional insecticides as influenced by the resistance programs in Egypt. *Resistant Pest Management Newsletter, 12*(1), 7-10.

Temerak, S. A. (2003). Differential susceptibility of pink and spiny bollworms to the ova-larvicidal activity of spinosad, a nutural metabolite of the Actinomycete *Saccharopolyspora spinosa* with special reference to solve the field failure of thiodiocarb in the current resistance rotation spraying program in Egypt. *Resistant Pest Management Newsletter*, *13*(1), 42-46.

Tikar, S. N., Nimbalkar, S. A., Satpute, N. S., Moharil, M. P., & Rao, N. G. V. (2004). Pyrethroid resistance in *Helicoverpa armigera* (Hubner) (Lepidoptera:Noctuidae) in Western Vidarbha Region of India. *Resistant Pest Management Newsletter*, *14*(1), 5-8.

Wood, B. J. (1971). Development of integrated control programs for pests of tropical perennial crops in Malaysia. In C. B. Huffaker (Ed.). *Biological Control* (pp. 422 – 457). N.Y.: Plenum Press.

Yap, T. H. (2005). A review on the management of Lepidoptera leaf-eaters in oil palm: Practical implementation of integrated pest management strategies. *The Planter*, *81*(954), 569-586.

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Effect of Breed on *cis*-9, *trans*-11 and *trans*-10, *cis*-12 Conjugated Linoleic Acids (CLA) Concentrations in Milk Fat of Dairy Cattle and the Relationship of These CLA with other Unsaturated C_{18} Fatty Acids

M. A. Yassir[1], A. K. Arifah[1*], H. Yaakub[2], A. Zuraini[3], Z. A. Zakaria[3] and M. N. Somchit[3]

[1]Faculty of Veterinary Medicine,
[2]Faculty of Agriculture,
[3]Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia,
43400 Serdang, Selangor, Malaysia
**E-mail: arifah@vet.upm.edu.my*

ABSTRACT

Much attention has been given to unsaturated carbon 18 fatty acids in milk, particularly conjugated linoleic acids (CLAs) which have a beneficial effect on human health. This study was undertaken to investigate the effect of breed on *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers in the milk fat of dairy cattle and their relationship with other unsaturated carbon 18 fatty acids. Mafriwal (n=15) and Jersey (n=15) cows were at mid-lactation period, grazed on pasture and given 5.5kg of concentrate per head daily. The composition of milk fatty acid was determined using gas chromatography after the extraction of milk fat using the modified Folch's method. The results showed that breed had an effect on *cis*-9, *trans*-11 CLA deposition in milk fat. The level of *cis*-9, *trans*-11 CLA in milk fat of Mafriwal was significantly higher ($P<0.05$) than that of the Jersey cows, while the levels of *trans*-10, *cis*-12 CLA were not significantly different between the two breeds. The levels of *cis*-9, *trans*-11 CLA were positively correlated with the concentration of *trans* 11-octadecenoic ($C_{18:1}$), *cis* 9-octadecenoic ($C_{18:1}$) and octadecatrienoic ($C_{18:3}$) acids. A positive correlation was also observed between the levels of *trans*-10, *cis*-12 CLA and octadecatrienoic ($C_{18:3}$) acid in milk fat. These results indicated that breed selection could be used to improve the quality of milk for human consumption.

Keywords: Breed, Dairy cattle, Conjugated linoleic acids, milk fat, Unsaturated carbon 18 fatty acids.

INTRODUCTION

Conjugated linoleic acids (CLAs) are a mixture of positional and geometric isomers of linoleic acid (LA) containing conjugated double bonds. Furthermore, each double bond can be in the trans or cis configuration. Therefore, various forms of CLA are possible (Sehat *et al*., 1998; Yurawecz *et al*., 1999). *Cis*-9, *trans*-11 and *trans*-10, *cis*-12 are the more active CLA isomers and they have been found to be predominant in ruminant meat and dairy products. These CLA isomers have been receiving particular attention in the past decade because they are natural food components claimed to possess important health benefits. These beneficial effects include anti-carcinogenic (Ip *et al*., 1994; Guo *et al*., 2007), anti-atherogenic (Lee *et al*., 1994; Valeille *et al*., 2006), anti-obesity (Lee *et al*., 2006), anti-diabetic (Ryder *et al*., 2001; Belury *et al*., 2003) and immune system enhancement (Ntambi *et al*., 2002). *Cis*-9, *trans*-11 CLA is

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*Corresponding Author

produced primarily in the mammary gland from desaturation of vaccenic acid (*trans*-11 $C_{18:1}$) via Δ9-desaturase enzyme. In addition, fatty acid is produced as an intermediate throughout the incomplete biohydrogenation of dietary linoleic acid ($C_{18:2}$) to stearic acid ($C_{18:0}$) in the rumen, while *trans*-10, *cis*-12 CLA in cows' milk fat arise directly from ruminal production and cannot be produced endogenously (AbuGhazaleh *et al*., 2007).

Manipulation of the cow's diet, particularly the type of supplemental fat, is the most popular way to change or improve the fatty acid content in cows' milk fat; however, this particular approach confers certain disadvantages, among which is the fact that it ignores the animal genetic effect on milk fatty acid composition. Indeed, any change or improvement due to diet is not permanent, because if the feed supplementation is changed, the additional nutritional quality will also be changed. Improvement in animal genetic is persistent and has the advantage of generating additional value through selection. Various studies have been conducted on the effects on cow breeds. In addition, the differences in the milk-fat composition in Holstein and Jersey breeds were investigated by White *et al*. (2001) and Mele *et al*. (2007), and another study was carried out by Talpur *et al*. (2006) for White Thari and Red Sindhi breeds. Most of the published studies on CLA were performed on animals kept under temperate climate. Therefore, cattle kept under hot and humid tropical conditions (like in Malaysia) were postulated to contain different CLA concentrations in their milk fat. Indeed, the current compositional information on CLA in milk fat has only been conducted in a few countries, not including Malaysia.

A lot of attention has also been given to other unsaturated fatty acids (USFAs), such as unsaturated carbon 18 fatty acids in cows' milk fat, which seem to be favourable for human health (McGuire *et al*., 2000). Many studies have investigated the phenotypic correlation of these fatty acids and other milk fatty acid contents in milk fat, suggesting that animal selection based on the fatty acid profile may be possible (Peterson *et al*., 2002; AbuGhazaleh *et al*., 2003; Soyeurt *et al*., 2006). It is also critical to clarify whether CLA isomers have any relationship with other unsaturated carbon 18 fatty acids. Therefore, this study focused on the effect of breed on the levels of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers and their relations with other unsaturated carbon 18 fatty acids in the milk fat of Mafriwal and Jersey cows fed with the same diet.

MATERIALS AND METHODS

Animals and Milk Samples

This study was carried out in one herd of Mafriwal and Jersey dairy cattle at the Institut Haiwan in Kluang, Johor, Malaysia. This farm, which served as a main farm for animal selection and development, belongs to the Department of Veterinary Services (DVS), Ministry of Agriculture and Agro-based industries, Malaysia. Milk samples were obtained from thirty lactating cows of Mafriwal (n=15) and Jersey (n=15) at the mid-lactation period of 123.5±16.4 days in milk, within the same parity (1.7 ±0.7). Each cow was fed on pasture and supplemented with 5.5 kg concentrate per day. The milk samples were obtained after a complete individual milking for each cow and kept at -20ºC prior to analysis for fatty acid composition using gas chromatography (GC) (Agilent technologies Inc., USA).

Total Lipid Extraction

Lipid extraction from the milk samples was performed according to Folch *et al*. (1957). Milk samples (3 mL) were mixed with chloroform-methanol (2:1, v/v) solution (40 mL) with vigorous shaking for 5 minutes and allowed to stand for 12 hours with occasional shaking. The mixture was then filtered into another separating flask, and this was followed by an addition of 10 mL of normal saline. The lower chloroform phase was subsequently recovered and evaporated using rotary evaporator (Heidolph®, Germany). Then, 100 µL of internal standard (4mg/mL of heneicosanoic acid [C_{21}] in chloroform methanol) was added to the extracted

fat that had been mixed previously with 5 mL of chloroform-methanol prior to fatty acid methyl ester (FAME) preparation.

FAME Preparation and Analysis

A method by Wijngaarden (1967) was used to prepare the FAME from the milk sample. The extracted lipid was dried using nitrogen steady flow, and 2 mL of potassium hydrochloride was added. The mixture was then heated for 10 minutes in a boiling water bath with occasional shaking, and 2 mL of 14 % boron-trifluoride in methanol was added after cooling of the mixture. The mixture was reheated again for 20 minutes, and 4 mL of deionized water and 4 mL petroleum ether were added after cooling of the mixture. Finally, 2 µL of FAME was injected into the gas chromatography (GC) fitted with HP-88 silica capillary column (60 m, 0.25 mm id, 0.20 µm film thickness) (Agilent technologies Inc., USA) after separating the petroleum phase containing FAME by centrifugation.

The individual FAME peak was identified according to the similar retention time by using known external standard. The quantitative analysis was carried out based on the proportional comparison of the chromatographic peak areas between an identified fatty acid and the known internal standard.

Statistical Analysis

The differences in the milk parameter between the two breeds were assessed using independent *T*-test after verification of the normal distribution of the data. The correlations between the two CLA isomers and other unsaturated carbon 18 fatty acids were analyzed using Pearson's correlation (SPSS 15 software package). Significant differences were tested at $P < 0.05$ level.

RESULTS

Conjugated Linoleic Acids

Conjugated linoleic acid concentrations in milk fat from the two breeds of cattle are expressed in *Fig. 1*. Total CLA (*trans*-10, *cis*-12 + *cis*-9, *trans*-11 CLA) content in milk fat of Mafriwal was significantly higher ($P<0.05$) than that of the Jersey cows (3.87 and 2.55 mg/g of total fatty acids, respectively). However, the mean values of *trans*-10, *cis*-12 CLA of 0.3 and 0.25 mg/g of the total fatty acids for the Mafriwal and Jersey cows, were respectively not significantly ($P>0.05$) different. Meanwhile, the mean value of *cis*-9, *trans*-11 CLA in the milk fat of Mafriwal was significantly higher ($P<0.05$) than that of the Jersey cows (3.5 and 2.3 mg/g of the total fatty acids, respectively). The possible ratios between *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers were estimated to be 1:6 to 1:13 (*trans*-10, *cis*-12/ *cis*-9, *trans*-11) in the milk fat from the two breeds.

Unsaturated Carbon 18 Fatty Acids

The mean values of unsaturated carbon 18 fatty acid compositions of the milk fat from the two breeds are summarized in Table 1. The dominant fatty acid observed among the unsaturated carbon 18 fatty acid in the milk fat of Mafriwal and Jersey cows was *cis*-9 octadecenoic acid ($C_{18:1}$) (17.03 g/100g), followed by *trans*-11 octadecenoic ($C_{18:1}$) (0.55 g/100g), *cis*-9, *trans*-11 CLA (0.29 g/100g), octadecatrienoic ($C_{18:3}$) (0.1 g/100g) and *trans*-10, *cis*-12 CLA (0.03 g/100g).

TABLE 1
The mean values conjugated linoleic acids and other unsaturated carbon 18 fatty acids (g/100g) in the milk fat of mid lactation Mafriwal and Jersey cows

Fatty acids		Mean ±SD	Range
$C_{18:1}$	*cis*-9 octadecenoic	17.03 ±5.9	6.48 - 32.6
$C_{18:1}$	*trans*-11 octadecenoic	0.55 ±0.2	0.15 - 1.04
$C_{18:2}$	*cis*-9, *trans*-11 CLA	0.29 ±0.1	0.18 - 0.58
$C_{18:2}$	*trans*-10, *cis*-12 CLA	0.03±0.008	0.015 - 0.051
$C_{18:3}$	Octadecatrienoic	0.1 ±0.05	0.05 - 0.28

Values represent mean ± SD (n=30). SD= standard deviation

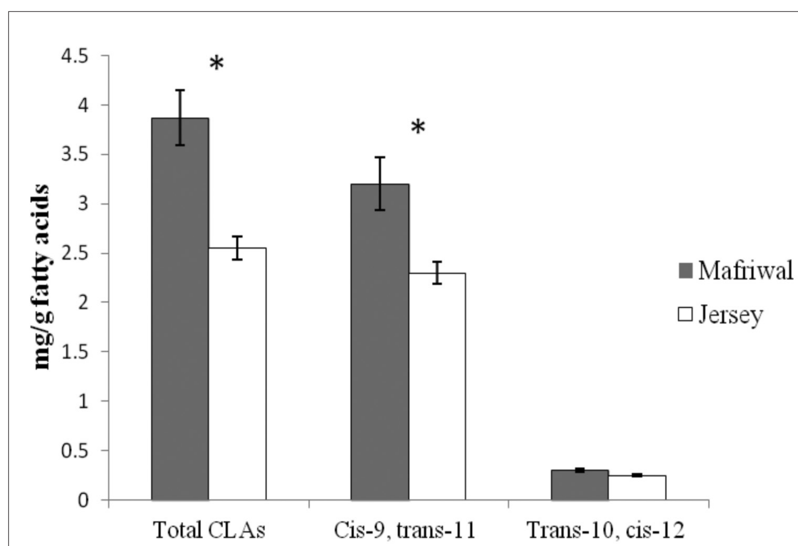

Fig. 1: Conjugated linoleic acid contents of milk fat for mid lactation Mafriwal and Jersey cows. Values are mean (n=15) ± SEM. An asterisk (*) at the top of a column indicates that a significant difference at P < 0.05 level was detected between the two breeds. Total CLAs= cis-9, trans-11 CLA + trans-10, cis-12 CLA.

Conjugated Linoleic Acid Correlations

Significant positive correlations were observed between *cis*-9, *trans*-11 CLA and *trans* 11-octadecenoic ($C_{18:1}$) acid (r=0.540, P<0.05), *cis* 9-octadecenoic ($C_{18:1}$) acid (r=0.554, P<0.05), and octadecatrienoic ($C_{18:3}$) acid (r=0. 808, P<0.05) (*Fig. 2*). Meanwhile, positive correlations were also observed between *trans*-10, *cis*-12 CLA and *trans* 11-octadecenoic ($C_{18:1}$) acid (r=0.299), *cis* 9-octadecenoic ($C_{18:1}$) acid (r=0.198), and octadecatrienoic ($C_{18:3}$) acid (r=0. 537, P<0.05), but the correlation was significant only with octadecatrienoic ($C_{18:3}$) acid (*Fig. 3*).

DISCUSSION

In this study, the total amount of CLAs in the milk fat of Mafriwal was significantly higher than that of the Jersey cows, although the mean values of *trans*-10, *cis*-12 CLA which had been shown to be effective in various cancer models (Hubbard *et al*., 2003; Masso-Welch *et al*., 2004), were not significantly different (0.3 and 0.25 mg/g of total fatty acids, respectively). Meanwhile, Mafriwal cows had significantly higher (P<0.05) level of *cis*-9, *trans*-11 CLA in their milk fat than that of the Jersey cows (3.5 and 2.3 mg/g of total fatty acids, respectively). The values of *cis*-9, *trans*-11 CLA were lower than those reported by Talpur *et al*. (2006) for White Thari and Red Sindhi cows, but were comparable to the figures reported earlier by White et al. (2001) for Holstein and Jersey cows. The disparity of the CLA deposition in the milk fat between Mafriwal and Jersey cows probably attributed to the tissue quantity and activity of Δ9-desaturase enzyme, which converted vaccenic acid (*trans*-11 $C_{18:1}$) to *cis*-9, *trans*-11 CLA by desaturation process in the mammary gland. The possible ratios of *trans*-10, *cis*-12/ *cis*-9, *trans*-11 CLA isomers were estimated to be 1:6 to 1:13 in milk fat from the two breeds. In other words, the *cis*-9, *trans*-11 CLA concentration in the milk fat of cows in this study was approximately 6 to 13 times more than the *trans*-10, *cis*-12 CLA concentration. *Trans*-10, *cis*-12 CLA in cow milk fat arises directly from ruminal production

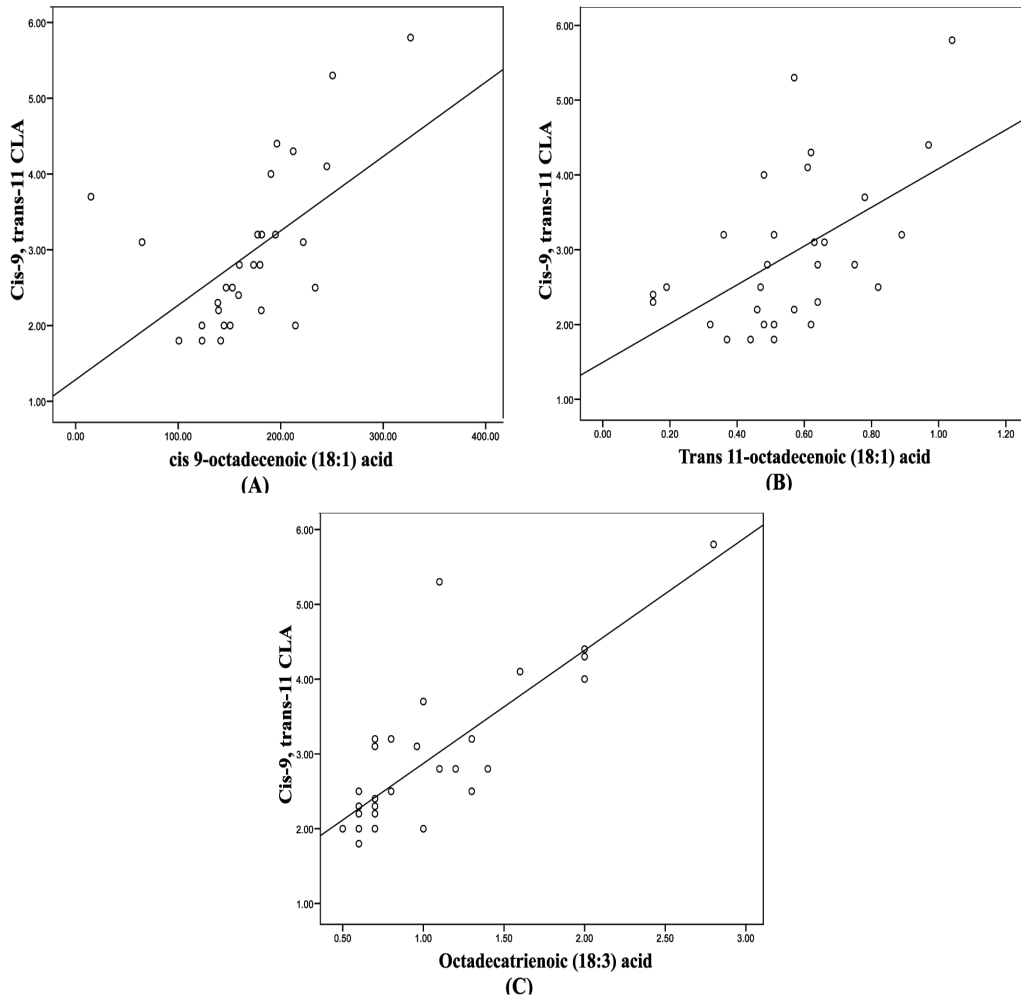

Fig. 2: The relationship between cis-9, trans-11 CLA isomer and cis 9-octadecenoic ($C_{18:1}$) acid (A), trans 11-octadecenoic ($C_{18:1}$) acid (B), and octadecatrienoic ($C_{18:3}$) acid (C) in milk fat of mid lactation Mafriwal and Jersey cows (n=30) as depicted by scattered plot and the best fitting line.

and could be produced endogenously while larger portions of *cis*-9, *trans*-11 CLA are of endogenous origin, synthesized by Δ9-desaturase enzyme from trans-11 $C_{18:1}$ (vaccenic acid), an intermediate in the rumen biohydrogenation of linoleic and linolenic acids (Corl *et al*., 2001). This could be the reason for the lower proportion of *trans*-10, *cis*-12 in cow milk fat as compared to *cis*-9, *trans*-11 CLA isomer.

Among the other unsaturated carbon 18 fatty acids in the present study, a higher proportion of *cis*-9 octadecenoic acid ($C_{18:1}$) was observed in the milk fat of Mafriwal and Jersey cows. The value of *cis*-9 octadecenoic acid ($C_{18:1}$) observed was comparable to that reported earlier by Mele *et al*. (2007) for Holstein cows, but lower than the values reported by White *et al*. (2001) and Kelly *et al*. (1998) for Holstein and Jersey cows.

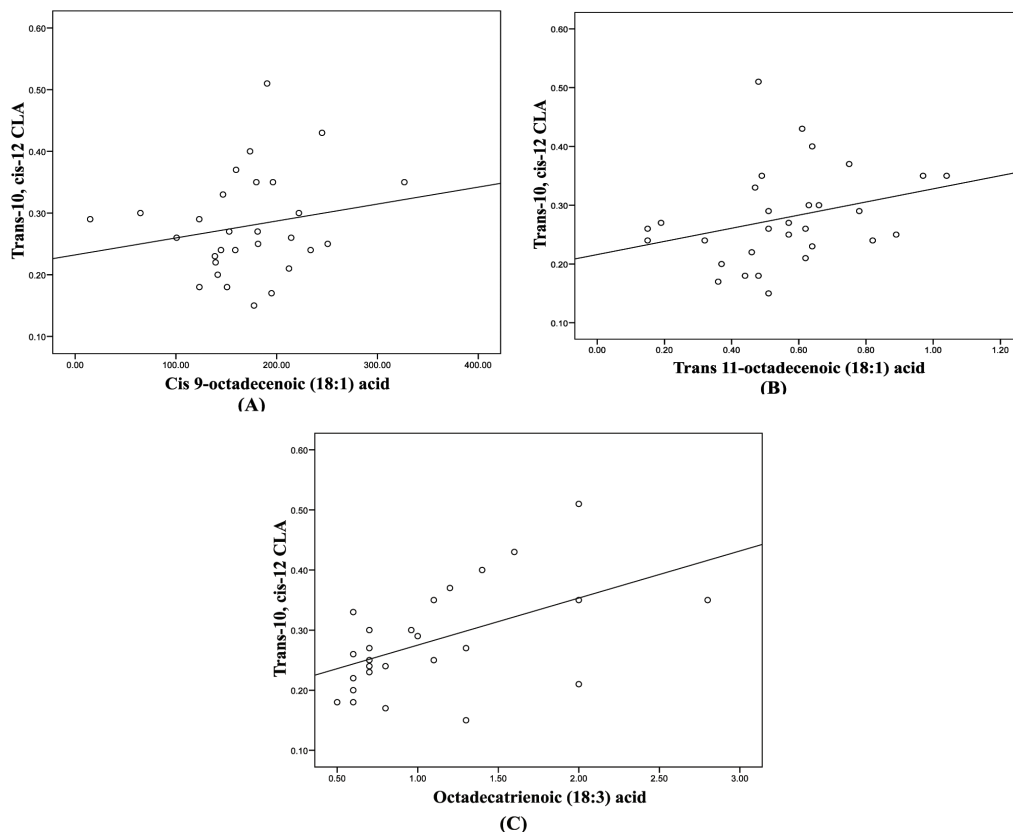

Fig. 3: The relationship between trans-10, cis-12 CLA isomer and cis 9-octadecenoic ($C_{18:1}$) acid (A), trans 11-octadecenoic ($C_{18:1}$) acid (B), and octadecatrienoic ($C_{18:3}$) acid (C) in milk fat of mid lactation Mafriwal and Jersey cows (n=30) as depicted by scattered plot and the best fitting line

The mean values of *trans*-11 octadecenoic ($C_{18:1}$) and octadecatrienoic ($C_{18:3}$) in the milk fat of Mafriwal and Jersey cows were lower than those reported earlier by White *et al*. (2001) for Holstein and Jersey cows, and Talpur *et al*. (2006) for White Thari and Red Sindhi cows. All the fatty acids, with 18 carbon chains in bovine milk fat, were derived from circulating preformed fatty acids (Bauman *et al*., 1974). These fatty acids originate from dietary and microbial lipids absorbed via the digestive tract and incorporated into chylomicrons and very low-density lipoproteins (VLDL) in the intestine and liver, respectively, and the mobilized body fat storage. In the mammary capillaries, triglycerides in VLDL and chylomicrons are hydrolyzed into free fatty acids and glycerol by lipoprotein lipase (LPL), along with adipose-derived non-esterified fatty acids (NEFA), are taken up by the mammary epithelial tissue (Davies *et al*., 1983), and this process may control the deposition of these fatty acids in cow milk fat.

There is an interest in the relationships of CLA isomers to other unsaturated carbon 18 fatty acids in cows' milk fat. In this study, *cis*-9*, trans*-11CLA, was positively correlated with *trans* 11-octadecenoic ($C_{18:1}$) acid, and this finding is in agreement with the work by Peterson *et al*. (2002). Significantly positive correlations were also observed between *cis*-9*, trans*-11 CLA and *cis* 9-octadecenoic ($C_{18:1}$) acid, *cis*-9*, trans*-11

CLA, and octadecatrienoic ($C_{18:3}$). These correlations are consistent with the findings of a previous work by AbuGhazaleh *et al.* (2003). Similarly, a significant positive correlation was also observed between *trans*-10, *cis*-12 CLA and octadecatrienoic ($C_{18:3}$) acid. These various correlations indicated that the concentrations of CLA isomers and other unsaturated carbon 18 fatty acids in cows' milk fat are related, and these relationships are important because they suggest that based on fatty acid profile, animal selection may be possible.

CONCLUSIONS

Within the limits of the conditions of this study, the results indicated that for dairy cattle, breed had an effect on CLA deposition in milk fat, implying that breed selection could be used as a tool to get better milk quality in terms of CLA content for human consumption. Furthermore, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers were correlated positively with some other unsaturated carbon 18 fatty acids, and this indicated that the levels of CLA in cows' milk fat could be increased by increasing the contents of these specific C_{18} fatty acids, possibly through nutritional manipulation.

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REFERENCES

AbuGhazaleh, A., & Jacobson, B. (2007). The effect of pH and polyunsaturated C18 fatty acid source on the production of vaccenic acid and conjugated linoleic acids in ruminal cultures incubated with docosahexaenoic acid. *Animal Feed Science and Technology, 136*(1-2), 11-22.

AbuGhazaleh, A., Schingoethe, D., Hippen, A., & Kalscheur, K. (2003). Milk conjugated linoleic acid response to fish oil supplementation of diets differing in fatty acid profiles. *Journal of Dairy Science, 86*(3), 944-953.

Bauman, D., & Davis, C. (1974). Biosynthesis of milk fat. *Lactation: A comprehensive treatise* (pp. 31-75). NY: Academic Press.

Belury, M. A., Mahon, A., & Banni, S. (2003). The conjugated linoleic acid (CLA) isomer, t10c12-CLA, is inversely associated with changes in body weight and serum leptin in subjects with type 2 diabetes mellitus. *Journal of Nutrition, 133*(1), 257-260.

Corl, B. A., Baumgard, L. H., Dwyer, D. A., Griinari, J. M., Phillips, B. S., & Bauman, D. E. (2001). The role of Δ9-desaturase in the production of cis-9, trans-11 CLA. *The Journal of Nutritional Biochemistry, 12*(11), 622-630.

Davies, D., Holt, C., & Christie, W. (1983). The composition of milk. *Biology of lactation* (pp. 71-117). Amsterdam: .Elsevier.

Folch, J., Lees, M., & Stanley, G. H. S. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *Journal of Biological Chemistry, 226*(1), 497-509.

Guo, D. D., Moon, H. S., Arote, R., Seo, J. H., Quan, J. S., Choi, Y. J., & Cho, C. S. (2007). Enhanced anticancer effect of conjugated linoleic acid by conjugation with pluronic F127 on MCF-7 breast cancer cells. *Cancer Letters, 254*(2), 244-254.

Hubbard, N. E., Lim, D., & Erickson, K. L. (2003). Effect of separate conjugated linoleic acid isomers on murine mammary tumorigenesis. *Cancer Letters, 190*(1), 13-19.

Ip, C., Singh, M., Thompson, H. J., & Scimeca, J. A. (1994). Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Research, 54*(5), 1212-1215.

Kelly, M., Kolver, E., Bauman, D., Van Amburgh, M., & Muller, L. (1998). Effect of intake of pasture

on concentrations of conjugated linoleic acid in milk of lactating cows. *Journal of Dairy Science, 81*(6), 1630-1636.

Lee, H. Y., Park, J. H., Seok, S. H., Baek, M. W., Kim, D. J., Lee, K. E., Paek, K. S., Lee, Y., & Park, J. H. (2006). Human originated bacteria, *lactobacillus rhamnosus* PL60, produce conjugated linoleic acid and show anti-obesity effects in diet-induced obese mice. *BBA-Molecular and Cell Biology of Lipids, 1761*(7), 736-744.

Lee, K. N., Kritchevsky, D., & Parizaa, M. W. (1994). Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis, 108*(1), 19-25.

Masso-Welch, P. A., Zangani, D., Ip, C., Vaughan, M. M., Shoemaker, S. F., McGee, S. O., & Ip, M. M. (2004). Isomers of Conjugated Linoleic Acid Differ in Their Effects on Angiogenesis and Survival of Mouse Mammary Adipose Vasculature. *The Journal of Nutrition, 134*(2), 299-307.

McGuire, M., & McGuire, M. (2000). Conjugated linoleic acid (CLA): A ruminant fatty acid with beneficial effects on human health. *Journal of Animal Science, 77*, 1-8.

Mele, M., Conte, G., Castiglioni, B., Chessa, S., Macciotta, N., Serra, A., Buccioni, A., Pagnacco, G., & Secchiari, P. (2007). Stearoyl-coenzyme A desaturase gene polymorphism and milk fatty acid composition in Italian Holsteins. *Journal of Dairy Science, 90*(9), 4458-4465.

Ntambi, J. M., Choi, Y., Park, Y., Peters, J. M., & Pariza, M. W. (2002). Effects of conjugated linoleic acid (CLA) on immune responses, body composition and stearoyl-CoA desaturase. *Applied Physiology, Nutrition, and Metabolism, 27*(6), 617-627.

Peterson, D., Kelsey, J., & Bauman, D. (2002). Analysis of variation in cis-9, trans-11 conjugated linoleic acid (CLA) in milk fat of dairy cows. *Journal of Dairy Science, 85*(9), 2164-2172.

Ryder, J., Portocarrero, C., Song, X., Cui, L., Yu, M., Combatsiaris, T., Galuska, D., Bauman, D. E., Barbano, D. M., Charron, M. J., Zierath, J. R. & Houseknecht, K. L. (2001). Isomer-specific antidiabetic properties of conjugated linoleic acid. *Diabetes, 50*(5), 1149-1157.

Sehat, N., Kramer, J. K. G., Mossoba, M. M., Yurawecz, M. P., Roach, J. A. G., Eulitz, K., Morehouse, K. M. & Ku, Y. (1998). Identification of conjugated linoleic acid isomers in cheese by gas chromatography, silver ion high performance liquid chromatography and mass spectral reconstructed ion profiles. comparison of chromatographic elution sequences. *Lipids, 33*(10), 963-971.

Soyeurt, H., Dardenne, P., Gillon, A., Croquet, C., Vanderick, S., Mayeres, P., Bertozzi, C., & Gengler, N. (2006). Variation in Fatty Acid Contents of Milk and Milk Fat Within and Across Breeds. *Journal of Dairy Science, 89*(12), 4858-4865.

Talpur, F. N., Bhanger, M., & Khuhawar, M. (2006). Comparison of fatty acids and cholesterol content in the milk of Pakistani cow breeds. *Journal of Food Composition and Analysis, 19*(6-7), 698-703.

Valeille, K., Férézou, J., Parquet, M., Amsler, G., Gripois, D., Quignard-Boulangé, A., & Martin, J.-C. (2006). The Natural Concentration of the Conjugated Linoleic Acid, cis-9,trans-11, in Milk Fat Has Antiatherogenic Effects in Hyperlipidemic Hamsters. *The Journal of Nutrition, 136*(5), 1305-1310.

White, S., Bertrand, J., Wade, M., Washburn, S., Green Jr, J., & Jenkins, T. (2001). Comparison of fatty acid content of milk from Jersey and Holstein cows consuming pasture or a total mixed ration. *Journal of Dairy Science, 84*(10), 2295-2301.

Wijngaarden, D. (1967). Modified rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Anal.Chem, 39*, 848-849.

Yurawecz, M. P., Sehat, N., Mossoba, M. M., Roach, J. A. G., Kramer, J. K. G., & Ku, Y. (1999). Variations in isomer distribution in commercially available conjugated linoleic acid. *European Journal of Lipid Science and Technology, 101*(8), 277-282.

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Biocontrol Agent *Trichoderma harzianum* Strain FA 1132 as An Enhancer of Oil Palm Growth

Laila Naher[1], Soon Guan Tan[2*], Umi Kalsom Yusuf[1], Chai-Ling Ho[2] and Faridah Abdullah[1]

[1]Department of Biology,
Faculty of Science,
[2]Department of Cell and Molecular Biology,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
**E-mail: sgtan_98@yahoo.com*

ABSTRACT

Agricultural products are mostly and adversely affected by environmental pollution caused by chemical residues of pesticides which are used for plant disease management. Consequently, researchers look for alternative approaches of disease control such as biocontrol agents. The results of this study showed that conidial suspension of the soil borne fungus *Trichoderma harzianum* strain FA 1132 can control *Ganoderma boninense* which causes basal stem rot (BSR) disease in oil palm. The conidial suspension treatment was applied by using *Trichoderma*-incorporated surface mulch. The disease severity index value (DSI) showed that *Ganoderma* infected the root as early as at week 5, with a DSI value of 8.3%, while physical symptoms appeared in leaf at week 8. However, no disease symptom was observed in *T. harzianum* strain FA1132 treated plants and it also markedly increased oil palm root and leaf weights.

Key words: Biocontrol effect, *Trichoderma harzianum* strain FA 1132, *Ganoderma boninense*

INTRODUCTION

The soil-borne fungus, *Trichoderma harzianum*, is known as an early invader of roots which rapidly multiplies in an ecological niche of the roots. It has potential to be a biocontrol agent against many soil-borne pathogens, specifically for *Ganoderma boninense* which causes basal stem rot (BSR) disease of oil palm. The disease and its causal pathogen were first reported in 1931 by Thompson to infected old age oil palm trees (Latiffah *et al*., 2005). In the 1960, the disease was found in younger palms of 10-15 years (Turner, 1981). Currently, *Ganoderma* was reported to cause infection in oil palms as young as 1-2 years of age (Azhar *et al*., 2008).

The available control measures for BSR diseases, such as cultural practices or fungicides, were unsatisfactory due to the fact that *Ganoderma* has various resting stages like melanised mycelium, basiodio-spores and pseudosclerotia (Izzati & Abdullah, 2008). By the time the disease symptoms appear, about more or less 50% of the palm's internal tissues have already rotted. Therefore, fungicides cannot cure such badly infected palms. In order to combat this characteristic, the best approach to control BSR disease is by biological control and the utilization of BSR resistant oil palm plants. It has been established that the biological control of plant pathogens is an alternative approach to decrease the strong dependence of modern

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*Corresponding Author

agriculture on chemical fungicides which cause environmental pollution.

Trichoderma is one of the most exploited fungal biocontrol agents in agriculture for the management of plant diseases caused by a wide spectrum of fungal pathogens (Elad, 2000; Mathivanan *et al*., 2000). *Trichoderma* sp. typically grows toward the hyphae of other fungi, coils about them in a lectin mediated reaction, and degrades the cell walls of the target fungi by the secretion of different lytic enzymes. This process of mycoparasitism limits the growth and activity of plant pathogenic fungi (Almeida *et al*., 2006).

Many in-house trials and field trials have been conducted using various *Trichoderma* spp. which showed a high efficacy for controlling *Ganoderma* infection in oil palms (Abdullah *et al*., 1999; Sariah *et al*., 2005; Susantho *et al*., 2005), and *Trichoderma* spp. are being commercialized for use in the protection and growth enhancement of a number of crops (Samules, 1996). Harman (2004; 2006) stated that *Trichoderma* sp. could control soil-borne pathogens, enhance plant growth and stimulate plant defense mechanism. Therefore, *Trichoderma* sp. has been acknowledged as a potential biocontrol agent for a broad range of plant pathogens in agriculture.

The efficacy of *T. harzianum* strain FA 1132 as a biocontrol agent for BSR disease was already proven by previous studies based on leaf data alone (Izzati, 2008; Sundram, 2008). Therefore, the main aim of this study was to determine whether *T. harzianum* strain FA 1132 only controlled BSR disease or it could also enhance oil palm health. Hence, this study measured both the root and leaf weights separately, determined the chlorophyll concentrations in the treated plants, as well as ascertained the disease severity index (DSI) values of *Ganoderma* infection in root and leaf separately when the palms were inoculated with *T. harzianum* and *G. boninense,* either separately or together.

MATERIALS AND METHODS

The Carriers for Trichoderma mulch Preparation

T. harzianum strain FA 1132 was collected from the Mycology and Plant Pathology Laboratory, Department of Biology, Universiti Putra Malaysia (UPM). Tricho-carrier was prepared by using palm pressed mesocarp fibres as organic compost. The whole fibres were washed through running tap water. After that, around 300 g per packet of palm pressed mesocarp fibres was placed into heat resistant polypropylene bags and autoclaved at 121°C, 1.04 kg/cm^2 for 45 min. Then, seven days cultured *T. harzianum* (strain FA 1132) conidial suspension was added into the sterilized palm fibres. Subsequently, the inoculated carrier bags were incubated in a dark chamber, with the temperature of 28 ± 2°C for 2 weeks.

Gano-wood Block Preparation

Gano-wood blocks were prepared by using wood from rubber tree (*Hevea brasiliensi*). The wood was cut into 2.5 x 2.5 x 5 cm^3 sized block each and washed by running tap water before soaking them in distilled water overnight. They were put into plastic bags and autoclaved at 121°C, 15 psi for 45 min. Then, 100 ml of potato sugar agar (PSA) was transferred into the plastic bags containing sterilized block and the blocks were autoclaved once again. Seven day old culture of *Ganoderma boninense* strain PER71, obtained from Malaysian Palm Oil Board (MPOB, Bangi, Malaysia), was cut into pieces and transferred into the cooled PSA containing sterilized block. The blocks were incubated for 10-12 weeks (Abdullah *et al*., 2001).

Experimental Design and Plant Treatment

A glass house trial plant treatment was carried out for 8 weeks using a completely randomized design with three biological replicates. A total of 108 five-months old oil palm seedlings (Dura X Pisifera) were obtained from Sime Darby Seeds & Agricultural Services Sdn. Bhd. (42700

Banting, Selangor, Malaysia). The plants were divided into four treatments: (1) Control oil palm seedlings without any inoculation (C); (2) *Ganoderma* inoculated oil palm seedlings (G); (3) *Trichoderma* inoculated oil palm seedlings (T); and (4) *Trichoderma* and *Ganoderma* together inoculated oil palm seedlings (GT).

Khairuddin's (1990) method was used for the artificial inoculation of *Ganoderma* onto the oil palm seedlings. In brief, a *Ganoderma* colonized wood block was placed in direct contact with the roots of one oil palm plant grown in a garden pot, which was then covered with soil; after that, around 600g Tricho-carrier mulch was placed onto the surface of the soil contained in the pot.

Preparation of Trichoderma Conidial Suspension

In addition to the surface mulch, the *Trichoderma* treated plants were also periodically given *Trichoderma* conidial soil drench. Conidial suspension was prepared from a 7-day old culture plate of *Trichoderma*. The conidia of FA 1132 were briefly harvested in 10 ml distilled water and gently removed with an L-shaped glass rod. The mixture was filtered using Whatman no. 1 filter paper to separate the mycelial debris and was topped up with distilled water to make 1 litre of conidial suspension. The fresh conidial suspension was poured at a rate of 1 litre per plant onto treatments T and GT, with *Trichoderma* carrier mulch, once every 2 weeks.

Trichoderma Colony Forming Unit (cfu) Measurements from Control and Treatment Soils

Samples from the control and treatment soils were recorded by using a cock borer, with a length of 34 cm and a diameter of 3 cm. The sampling was done at 5 cm and 15 cm soil depths for each treatment/ control. Meanwhile, a 10 g soil sample was mixed in 100 ml autoclaved distilled water by shaking it in an orbital shaker at 100 rpm for 15 minutes. Later, this mixture was diluted through serial dilution until 10^{-3} and 1 ml of 10^{-3} diluted soil was transferred onto a Petri plate and 9 ml of Rose Bengal Agar (RBA) was added onto the plate. The plates were gently swirled and incubated at room temperature (28±1°C). The cultures were done in three replicates. The cfu measurements were counted and recorded after 5 days.

Chlorophyll Determination

The concentrations of chlorophyll a (Chla), chlorophyll b (Chlb) and total chlorophyll were analyzed following the method described by Arnon (1949). N, N-dimethylformamide (DMF), was used for chlorophyll determination by immersing the leaves in the solvents. Leaf samples from the control and treatments were used for the determination of total chlorophyll. The ratio for the extraction was 10% (w/v). After that, the extract was kept in the dark for 48 hours at 4°C prior to spectroscopic examination.

The concentrations of Chlorophyll a and Chlorophyll b were measured using a UV-nanophotometer (IMPLEN, Nanophotometer, Malaysia) at the wavelengths of 663 nm and 645 nm. The concentrations of Chl a, Chl b and total chlorophyll in the leaf tissues were calculated according to the following equations:

$$\text{mg chlorophyll a/ litre} = 0.0127\,(\text{O.D.}_{663}) - 0.00269\,(\text{O.D.}_{645})$$

$$\text{mg chlorophyll b/ litre} = 0.0229(\text{O.D.}_{645}) - 0.00468\,(\text{O.D.}_{663})$$

Where, O.D is the optical density at that wavelength.

$$\text{Total chlorophyll} = [\text{Chl a}] + [\text{Chl b}]\ \mu g/g.$$

Root and Leaf Weight Determinations

The root and leaf samples from the control and treatments were taken at 2, 5, and 8 weeks of post-inoculation. The seedlings were uprooted and washed with running tap water. The leaves and roots were both dried with paper towels. The control and treated seedling roots and leaves were excised using a pair of clean scissors and their weights were also taken.

Assessment of Disease Signs and Symptoms

Ganoderma infection in the oil palm starts from the root tissues. This study assessed the signs and symptoms of the disease from the root to shoot by destructive sampling at every time point. The signs and symptoms of the disease were examined based on the BSR with some modifications (Izzati & Abdullah, 2008). A disease index value of 0 means that all plants are healthy, while the values between 0 - 100 represent a range of severity. The Mathematical formula of disease severity index (DSI), based on the observation of signs and symptoms of the disease class of the infected plants, gives numerical values ranging from 0 to 4 (Izzati & Abdullah, 2008) as in Table 1.

TABLE 1
Disease signs and symptoms corresponding to the disease class (Izzati & Abdullah, 2008)

Class	Disease sign and symptoms
Class 0	Healthy plant with green leaves and no mycelial development on any part of the plant.
Class 1	Formation of white mass of mycelia on any part of the plant, with or without chlorotic leaves.
Class 2	Appearance of 3 or more chlorotic leaves.
Class 3	Formation of sporophores or basidioma on any part of the plant with chlorotic leaves.
Class 4	Appearance of well-developed basidioma on plants showing at least 50% dried leaves and the plant drying up, is dying or is already dead.

Formula of Disease severity index (DSI) = $$\frac{\sum (A \times B) \times 100}{\sum n \times 4}$$

Where, A= Disease classes (0, 1, 2, 3 and 4).
B= Number of plants showing disease per treatment.
n= total number of plants per treatment, with class 4 represents the highest class of assessment.

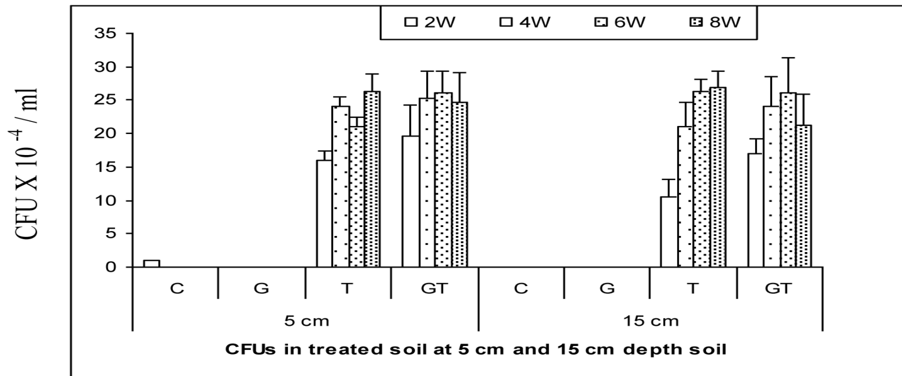
Statistical Analysis

All the data were analyzed using the SPSS software version 17.0. The t-test was carried out on cfu/g between 5 cm and 15 cm of soil depth. The chlorophyll amount, root and leaf weight data were subjected to test for analysis of variances (ANOVA). The mean differences were determined by the Duncan's homogeneity subsets if F tests were significant at 95% probability.

RESULTS AND DISCUSSION

Estimation of T. harzianum Colony Forming Units in Soil

The distribution of the cfu/g soil over 8 weeks is shown in *Fig. 1*. However, the initial load of *Trichoderma* cfu conidial suspension in the surface mulch was 3 x10^{11}, and more or less the same loading was used at the subsequent time points. The result showed that among the treatments T and GT at 5 cm and 15 cm depth soil, the cfu/g values for both were almost similar after 2 weeks. At week 2, the cfu/g at 15 cm depth soil was low in treatment T. On the other hand, it increased dramatically after 2 weeks. The control (C) and *Ganoderma* treated plant (G) soil samples were checked to observe the presence of any *Trichoderma* or other soil fungi. At week 2, only the 5 cm deep control soil had a few colonies, but after that, none was observed in the following samplings. Meanwhile, no significant difference was found when the t-test was carried out on treatments T and GT between 5 cm and 15 cm depth soil cfu/g, the p value was 0.886 and 0.09, respectively. Similar results were also obtained in the oil palm which had been treated with *T. harzianum* strain FA 1132 and the cfu/g decreased at 15 cm depth soil compared to 5 cm depth soil after 14 weeks (Sundram *et al*., 2008). Although the reason for this was not clear, it might involve the timing of the sporulation of the fungi. In this study, the cfu/g did not decrease until the end of the experiment at week 8 and at 15 cm depth soil. Unfortunately, Sundram *et al*. (2008) did no



Legend:
C: Control plant
G: *Ganoderma* infected plant
T: *Trichoderma* treated plant
GT: *Ganoderma* + *Trichoderma* treated plant

Fig. 1: T. harzianum *colony forming units recorded until 8 weeks post inoculation with each sampling taken before the application of* T. harzianum *conidial suspension. Error bar is standard error.*

t report any finding at week 8 of their experiment for us to compare the results of the current study with.

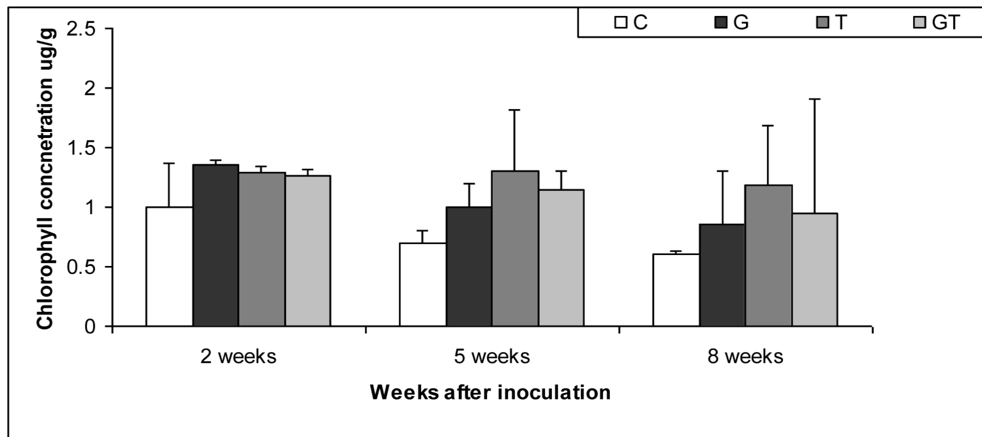
Determination of Chlorophyll Concentration

Chlorophyll concentrations were determined at 2, 5, and 8 weeks of post-inoculation (Figure 2). It is crucial to note that there was no statistically significant difference found between the treatments by the ANOVA test. Even though the concentration of chlorophyll at week 2 was high in *Ganoderma* infected oil palm, it dramatically reduced at the subsequent time points. The concentration of chlorophyll in the *Trichoderma* treated oil palm was slightly reduced after 2 weeks.

Therefore, the current study found that the concentration of chlorophyll was not increased at the time points of weeks 2, 5 and 8 in the *Trichoderma harzianum* strain FA 1132 treated plants (T and GT) but the concentration was higher than that of the control (C) plants and *G. boninense* treated plants (G) after 2 weeks. However, the concentration of chlorophyll was increased by *T. harzianum* strain 1295-22, BR16 and *T. virens* strain R42 in cucurbitaceous seedling plants; on the contrary, the concentration in the plants treated with *T. harzianum* strain Zts428 was not increased in the same study (Lo & Lin, 2002). The increase of chlorophyll in plants may depend on fungal strains.

Estimation of Root and Leaf Weights

The ANOVA test showed that the root weights (*Fig. 3*) of the G (*Ganoderma* alone) treated plants were significantly different from C, T and GT plants at 2, 5 and 8 weeks of post-inoculation, while the weight of the leaf (*Fig. 4*) was significantly different at week 8, indicating that *Ganoderma* infected the root within 2 weeks and the disease spread to the basal tissue and leaf within 8 weeks. At 8 weeks of post-inoculation, *Ganoderma* sporophore was observed on the root, while basal tissue and some bottom leaves had already turned yellow from this infection (*Fig. 4* and *Fig. 5*). However, no infection was observed in the plants treated with *Ganoderma* and *Trichoderma* together. Moreover, it was



C = Control, G = *Ganoderma*, T = *Trichoderma*, GT = *Ganoderma* +*Trichoderma*

Fig. 2: Chlorophyll concentrations in the treated leaf samples. Error bar is standard deviations.

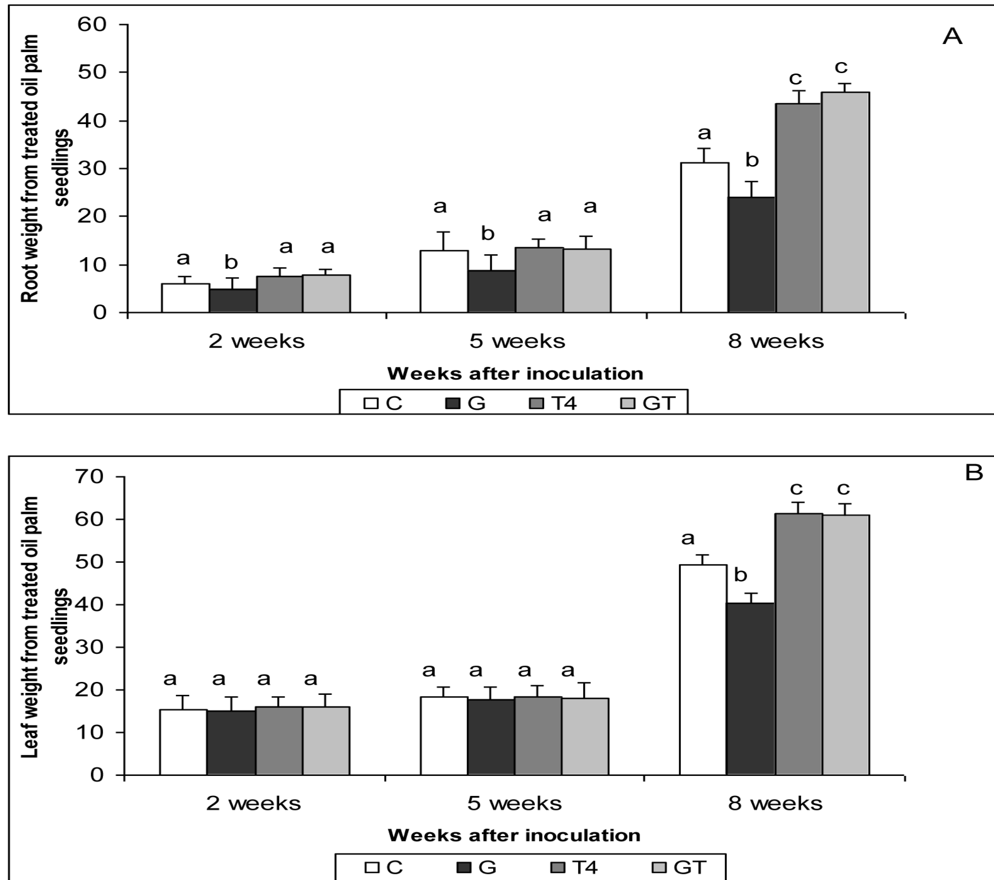
found that in either the *Trichoderma* alone (T) or together with the *Ganoderma* (GT) treated plants, the root and leaf weights increased gradually compared to the control (C) plant and the plants treated with *Ganoderma* alone (G). However, it is also well known that *Trichoderma* species enhances plant growth (Baker, 1991; Kleifeld & Chet, 1992; Sivan *et al*., 1984). This study found that *T. harzaianum* strain FA1132 increased oil palm leaf and root weights compared to that of the control plants (C) and the plants infected with *Ganoderma* alone (G). Meanwhile, the root and leaf weights were significantly different between the treatments. An enhanced root growth by the application of *Trichoderma* sp was also observed in the previous studies on other plants. For example, *T. harzianum* increases plant vigour of bentgrasses and Cron plant's root and shoot growth (Lo *et al*., 1997; Bojrkman *et al*., 1994).

Nonetheless, the mechanism of plant growth induction by *Trichoderma* spp. is not clear. There may be a few factors involved for its beneficial effects on plant growth, such as: (1) controlling deleterious microorganisms, (2) stimulating plant growth factors like plant hormones, (3) increasing nutrient up take or enhancing availability of the necessary nutrients, and (4) reducing the concentrations of soil substances which are inhibitory to plant growth (Kleifeld & Chet, 1992; Wang, 2000; Windham, 1986; Lo & Lin, 2002).

Disease Severity Index

Treatment G (*Ganoderma* alone) showed disease symptoms as early as week 5 (*Fig. 6*) in the root, with a DSI value of 8.3%. Nevertheless, no disease symptoms were observed in control (C), *Trichoderma* alone (T), and *Ganoderma* + *Trichoderma* (GT) at any time point in either tissue (*Fig. 4*) at all time points in both tissues (Table 2).

Trichoderma spp are well established as biocontrol agents for several plant diseases (Harman *et al*., 1989; Lo *et al*., 1997; Papavizas, 1985). This study aimed to study the disease development and the control of the disease at its early stage of infection. The researchers found that *T. harzianum* strain FA1132 could control *G. boninense* as well as increase oil palm root (A) and leaf (B) growth (Figure 3). Some previous studies have proven *Trichoderma* spp to have high efficacy for controlling *G. boninense* infection in oil palms (see Abdullah *et al*., 1999; Sariah *et al*., 2005; Susantho *et*



Legend:
C: Control plant
G: *Ganoderma* infected plant
T: *Trichoderma* treated plant
GT: *Ganoderma*+*Trichoderma* treated plant

Fig. 3: Weight of oil palm seedlings roots (A) and leaves (B) after the treatments. Duncan's homogeneity test was used to define ranked classes. Means with the same letter are not significantly different. Error bars represent standard deviations.

al., 2005). Meanwhile, *T. hazrianum* strain FA1132 was found to be an efficient biocontrol agent of *Ganoderma* for BSR disease of oil palm (Abdullah *et al*., 2003; Izzati *et al*., 2008; Sundram *et al*., 2008). However, previous studies only observed the leaf portion. Thus, this study estimated the DSI values in the root and leaf separately by destructive sampling because by the time the external disease symptoms appeared, most of the internal tissues had already died. The authors observed a DSI value of 8.3% in the root at week 5, whereas no disease symptom was recorded in the leaf at week 5. In the leaf, the disease symptoms were first observed at week 8 with a DSI value of 11.11%, whereas at week 8, most roots had already been damaged with a DSI value 16.66%. Sundram *et al.* (2008) and Izzati (2008) only recorded the disease symptoms at weeks 12 and 14 and obtained the DSI values of 16.67% and 12.5%, respectively, in the leaf tissues alone. Later, their time periods were compared to the

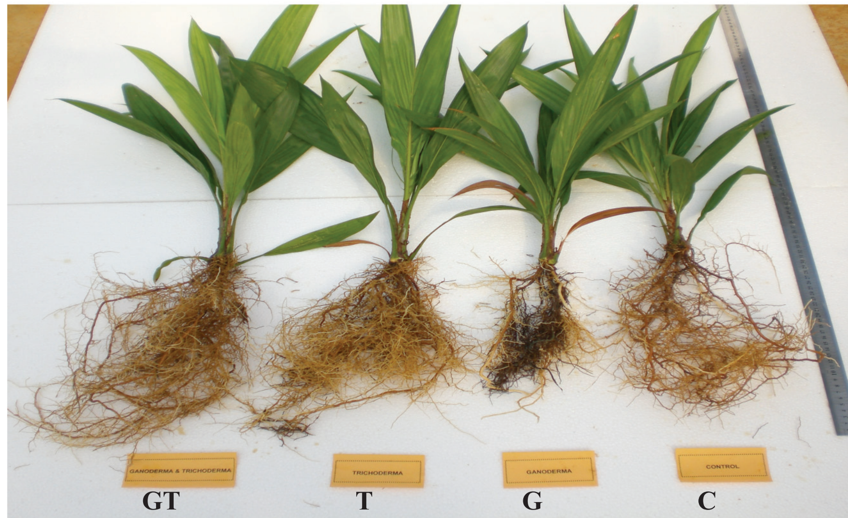


*Fig. 4: Treated plants after post-inoculation. C - control plant, G -*Ganoderma *infected plant, T -* Trichoderma *inoculated plant and GT -* Ganoderma + Trichoderma *together treated plant.*

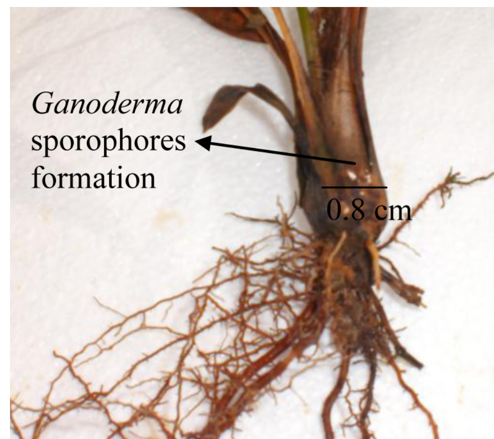


Fig. 5: Ganoderma *sporophores formation on plants. Scale bar is an equal amount to 0.8 cm.*

time points used in this study which aimed at the earlier stages of BSR disease. There might be differences in the pathogen growth and environmental effects for disease development (George, 2005). As mentioned earlier, most of the tissues were already internally infected by *G. boninense* when the disease symptoms appeared physically. However, information on early plant infection is still lacking. Therefore, to deal with this lack of information, this study observed *G. boninense* infection by destructive sampling and found that within one month, the plant roots were already infected but it was only at two months that the physical symptoms appeared in the leaf. Nevertheless, no disease symptom was observed in the leaf and root tissues until the end of the experiments in the current study, i.e. when the plants had simultaneously been infected with

TABLE 1
Disease Severity Index (DSI) values over 8 weeks of inoculation

Treatments	Disease severity index (%)					
	2 weeks		5 weeks		8 weeks	
	Root	Leaf	Root	Leaf	Root	Leaf
C	0.00	0.00	0.00	0.00	0.00	0.00
G	0.00	0.00	8.3	0.00	16.66	11.11
T	0.00	0.00	0.00	0.00	0.00	0.00
GT	0.00	0.00	0.00	0.00	0.00	0.00

G. boninense and *T. harzianum*. The treatments with *T. hazrianum* strain FA1132 conidial suspension were given at every 2-week intervals up to the end of the experiment. Therefore, the treatment with *T. harzianum* is a good approach for controlling *G. boninense* infection.

CONCLUSIONS

In this study, *G. boninense* was found to infect the roots within 2 weeks but the disease only appeared in the leaves at week 8. It was observed that *T. harzianum* (strain FA1132) was not only efficient for controlling the BSR disease but it also enhanced chlorophyll concentration, as well as the weights of root and leaf. Therefore, *T. harzianum* mulch and its conidial suspension can be used as fertilizers to increase oil palm health and protect the plant from BSR disease.

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REFERENCES

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

Abdullah, F., Jayanthi, N., Illias, G. N. M., & Malik, N. (2001). *Properties of substrate inocula and plant hosts in disease extablishment by Ganoderma boninense*. Paper presented at the 2001 PIPOC International Palm Oil Congress – Agriculture. Malaysia Palm Oil Board, Kuala Lumpur, Malaysia.

Abdullah, F., Illias, G. N. M., Vijava, S. K., & Leong, T. T. (1999). Diversity of *Trichoderma* and its in vivo efficacy agains *Ganoderma boninense*. In Z. Sidek, S.K. Bong, C.A. Ong and A.K Husan (Eds.), *Sustainable Crop Protection Practices in the Next Millennium* (p. 137-140). Kota Kinabalu, Sabah, Malaysia.

Almeida, F. B. D. R., Cerqueira, F. M., Silva, R. D. N., Ulhoa, C. J., & Lima, A. L. (2007). Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evolution of coiling and hydrolytic enzyme production. *Biotechnology Letter*, *29*, 1189-1193.

Ariffin, D., Singh, G., & Lim, T. K. (1989). *Ganoderma* in Malaysia-Current Status and Research Strategy. In S. Jalani and D. Ariffin (Eds.), *Proceedings of the 1989 PORIM International palm oil Development Conference-Module II: Agriculture* (p. 249-297). Palm Oil Research Institute of Malaysia, Bangi, Selangor, Malaysia.

Arnon, D. I. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in beta ulgaris. *Plant Physiology*, *24*, 1-14.

Baker, R. (1991). Induction of rhizosphere competence in the biocontrol fungus *Trichoderma*. In D.L. Keister and P.B. Cregan (Eds.), *Rhizosphere and plant growth*, (p. 221-228). Kluwer Academic Publishers.

Bjorkman, T., Price, H. C., Harman, G. E., Ballerstein, J., & Nielsen, P. (1994). Improved performance of shrunken-2 sweet corn using *Trichoderma harzianum* as a bioprotectant. *Horticulture Science*, *29*, 471.

Elad, Y. (2000). Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. *Crop Protection*, *19*, 709-714.

George, N. A. (2005). *Plant pathology*. USA: Elsevier Academic Press.

Harman, G. E., Taylor, A. G., & Staz, T. E. (1989). Combining effective strains of *Trichoderma harzianum* and solid matrix priming to improve biological seed treatments. *Plant Disease*, *73*, 631-637.

Ilias, G. N. M. (2000). *Trichoderma and its efficacy as a bio-control agent of basal stem rot of oil palm* (Elaeis guineensis *Jacq*.). (Doctoral dissertation). Universiti Putra Malaysia, Selangor, Malaysia.

Izzati, N. A. M. Z., & Abdullah, F. (2008). Disease suppression in *Ganoderma* -infected oil palm seedlings treated with *Trichoderma harzianum*. *Plant Protection Science*, *44*, 101-107.

Khairuddin, H. (1990). *Basal stem rot of oil palm: Incidence, etiology and control*. (Master of Agriculture Thesis). Universiti Pertanian Malaysia, Selangor, Malaysia.

Kleifeld, O., & Chet, I. (1992). *Trichoderma harzianum* - interaction with plants an effect on growth response. *Plant and Soil*, *144*, 267-272.

Latiffah, Z., Harikrishna, K., Tan, S. G., Abdullah, F., & Ho, Y. W. (2005). Random amplified polymorphic DNA (RAPD) and random amplified microsatellite (RAMS) of *Ganoderma* from infected oil palm and coconut stumps in Malaysia. *Asia Pacific Journal pf Molecular Biology and Biotechnology*, *13,* 23-34.

Lo, C. T., & Lin, C. Y. (2002). Screening strains of *Trichoderma* spp. for Plant growth enhancement in Taiwan. *Plant Pathology Bulletin*, *11*, 215-220.

Lo, C. T., Nelson, E. B., & Harman, G. E. (1997). Improved the biocontrol efficacy of *Trichoderma harzianum* 1295-22 for controlling foliar phases of turf disease by spray applications. *Plant Disease*, *81*, 1132-1138.

Mathivanan, N., Srinivasan, K., & Chelliah, S. (2000). Biological control of soil-borne diseases of cotton, eggplant, okra and sunflower by *Trichoderma viride*. *Journal of Plant Diseases and Protection*, *107*, 235–244.

Papavizas, G. C. (1985). *Trichodema* and *Gliocladium*: Biology, ecology and potential for biocontrol. *Annual Review Phytopathology*, *22*, 23-54.

Samuels, J. G. (1996). *Trichoderma*: a review of biology and systematics of the genus. *Mycology Research*, *10*, 923-935.

Sariah, M., Choo, C. W., Zakaria, H., & Norihan, M.S. (2005). Quantification and Characterization of *Trichoderma* spp. from different ecosystems. *Mycopathologia*, *159*, 113-17.

Sivan, F. M., Ziegle, J. S., & Kloepper, J. W. (1984). A method of assessing the root colonization capacity of *Trichoderma harzianum*. *Journal of Genetics Microbiology*, *137*, 23-30.

Sundram, S., Abdullah, F., Ahmad, Z.A.M., & Yusuf, U. K. (2008). Efficacy of single and mixed treatments of *Trichoderma harzianum* as biocontrol agents of *Ganoderma* basal stem rot in oil palm. *Journal of Oil Palm Research*, *20*, 470- 483.

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

Wang, C., Kinll, K., Glick, B. R., & Defago, G. (2000). Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its gacA derivative CHA96 on their growth-promoting and disease-suppressive capacities. *Canadian Journal of Microbiology*, *46*, 898-907.

Windham, M. T., Elad, Y., & Baker, R. (1986). A mechanism for increased plant growth induced by *Trichoderma* spp. *Phytopathology*, *76*, 518-521.

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Bioaccumulation of Heavy Metals (Cd, Pb, Cu and Zn) in *Scylla serrata* (Forsskal 1775) Collected from Sungai Penor, Pahang, Malaysia

Kamaruzzaman, B. Y.*, Akbar John, B., Maryam, B. Z., Jalal, K. C. A. and Shahbuddin, S.

Department of Biotechnology, Kulliyyah of Science,
International Islamic University Malaysia,
Jalan Sultan Ahmad Shah, Bandar Indera Mahkota,
25200, Kuantan Pahang, Malaysia
**E-mail: kama@iium.edu.my*

ABSTRACT

A study was conducted to assess the bioaccumulation levels of heavy metals (copper, zinc, cadmium and lead) in different body parts (carapace, claw, walking legs and intestinal tract) of the common mud crab *Scylla serrata* collected from Sungai Penor, Pahang, on January 2009. Accumulation of metal was determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Average Cu concentrations in carapace, claws, walking legs and gut were 43.83 ± 16.43μgg^{-1}, 21.54 ± 7.14μgg^{-1}, 28.2 ± 12.76μgg^{-1} and 57.06 ± 13.47μgg^{-1}, respectively. Meanwhile, average Zn concentrations in carapace, claws, walking legs and gut were 387.38 ± 17.89μgg^{-1}, 376.62 ± 21.91μgg^{-1}, 361.92 ± 26.68μgg^{-1} and 496.31 ± 20.59μgg^{-1}, respectively. Since Zn and Cu are the precursors of most enzymatic activities, all the body parts have significantly higher tendency to accumulate Zn and Cu. It was apparent that Zn concentration was higher in crab body parts, followed by Cu. It was also observed that intestinal track had higher levels of metals than other body parts. Since high calcium content inhibits lead uptake into the gut, a large portion of the lead burden was sequestered in the carapace. Average Pb concentrations in carapace, claws, walking legs and gut were observed to be 7.17 ± 0.46μgg^{-1}, 6.27 ± 0.75μgg^{-1}, 6.52 ± 0.37μgg^{-1} and 2.27 ± 0.82μgg^{-1}, respectively. Among the analyzed heavy metals, Cd concentration was low in all the body parts of the crab. Average Cd concentrations in carapace, claws, walking legs and gut were 0.68 ± 0.05μgg^{-1}, 0.42 ± 0.05μgg^{-1}, 0.35 ± 0.04μgg^{-1} and 0.13 ± 0.05μgg^{-1}, respectively. Exoskeleton absorbs higher level of Cd than internal gut region, but the accumulation of Pb was higher in gut region than the exoskeleton of the crab. It was evident from this study that all the heavy metal (Cd, Pb, Cu and Zn) accumulations in *Scylla serrata* were higher than the international standard Maximum Permissible Level (MPL). Hence, a detailed investigation needs to be addressed on this issue to determine the pollution status in crabs inhabiting along the Sungai Penor waters.

Key words: Bioaccumulation, Heavy metal, ICP-MS, *Scylla serrata,* Sungai Penor

INTRODUCTION

The natural aquatic systems may extensively be contaminated with heavy metals released from domestic, industrial and other man-made activities (Velez & Montoro, 1998; Conacher *et al.,* 1993). Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Vosyliene & Jankaite, 2006; Farombi *et al.,* 2007). Thus, determination of harmful and toxic substances

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*Corresponding Author

in water sediments and biota will give direct information on the significance of pollution in the aquatic environment (Hugget *et al.,* 1973). Among other, estuaries and coastal zones receive pollutant input from both specific and non-specific sources, especially such ecosystems as seaports, cities or other industrialized coastal areas that receive chronic input of metals. Since many species of crustaceans inhabit estuaries, numerous studies have aimed at examining the bioaccumulation and effects of various toxicants in these animals (Bryan, 1971; Rainbow, 1990).

Meanwhile, heavy metals from natural and anthropogenic sources are continually released into aquatic ecosystem and they cause serious threats because of their toxicity, long persistence, as well as bioaccumulation and biomagnifications in the food chain (Kamaruzzaman *et al*., 2007). The fate of heavy metals introduced by human activities into the aquatic ecosystems has recently become the subject of wide spread concern, since they become toxic beyond the tolerable limits (Rainbow, 1995). However, metals can be excreted from the tissues of aquatic animals. It was reported that there are several factors influencing the elimination of metals from the tissues of aquatic animals (Skejelkvale *et al.,* 2001). These include period of exposure, surrounding temperature, interacting agents, age and the metabolic activity of the animal, and biological half life of metals. Metal elimination routes are more numerous than uptake routes; however, metal accumulation is more rapid than its elimination, and this is probably due to the presence of metal binding proteins in the tissues (Soegianto *et al.,* 1999).

Studies carried out on various fish species have shown that heavy metals may alter the physiological activities and biochemical parameters both in the tissues and in the blood (Soegianto *et al.,* 1999). The toxic effects of heavy metals have been reviewed, including their bioaccumulation (Tinsley, 1979). The organisms developed a protective defence against the deleterious effects of essential and inessential heavy metals and other xenobiotics that produce degenerative changes like oxidative stress in the body (Bahadorani & Sepehr, 2008).

It has been documented that the industrial and domestic dumping of sewage mostly contain plastic wastes contribute to the pollution at mangrove forests and their surrounding water body (Kamaruzzaman *et al*., 2009). In addition, the decomposition of litter gives harmful effects on marine life by increasing level of phytoplankton which in turn deoxygenates the water and adversely affects water quality. Furthermore, the lack of proper planning and management leads to drastic increase in pollutant level in this area.

Mud crab (*Scylla serrata*) is one of the important fishery commodities in Southeast Asian countries. This species is a very popular seafood and has a high commercial value, and it is extensively used as a candidate species in aquaculture practices. Their distribution was recorded in the present sampling site (Sungai Penor) which is situated along the east coast of Malaysia, facing the South China Sea in the state of Pahang. Their feeding behaviour will lead to a higher accumulation of toxic metals in their body parts which are biomagnified through food chain. It was also proposed as a biomonitor species for the current RWQPP Marine Monitoring Programme because of its capacity to bioaccumulate a range of contaminants, and its significance as a target species for subsistence, commercial and recreational fisheries. They have limited territorial ranges and are large enough to provide ample tissue for chemical analysis (Ryan, 2003). Various studies have been carried out on heavy metal accumulation in mud crabs but similar studies from Malaysian waters on this very species are still scanty. Hence, the present research was conducted to examine the bioaccumulation level of selected heavy metals in various parts of common mud crab (*S. serrata*) sampled from Sungai Penor, Pahang, Malaysia.

MATERIALS AND METHODS

Sampling Site, Sample Collection and Preparation

Sungai Penor (3^0 39’ 10.13’’ N, $103^0$21’ 15.77’’ E), which is located in Kuantan, Pahang,

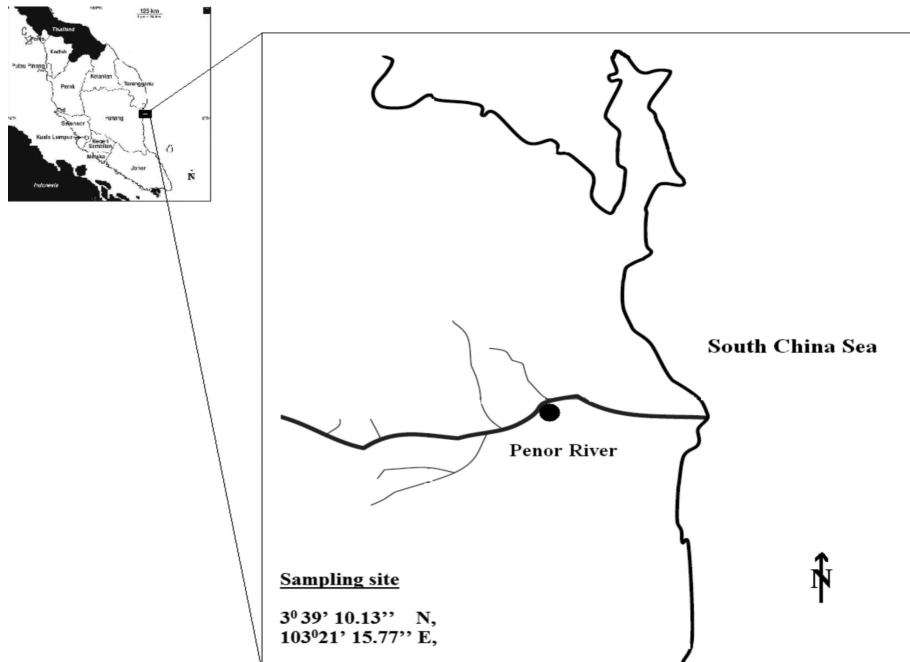

Fig. 1: Location of the sampling site at Sungai Penor, Kuantan, Pahang, Malaysia

Malaysia (*Fig. 1*), is threatened by a series of pollutants, such as industrial discharges and domestic sewage, discharged from nearby fishermen villages. A total of 65 crab traps were set (at every 50 metres) along Penor River for overnight during January 2009. Samples were collected on next day and identified using a standard reference (Keenan *et al.,* 1998) prior to storage at -20^{0}C. A total of 15 crabs were analyzed based on their weight and size according to carapace width (CW) which varied between 100 – 540g and 8.5 – 14.4 cm, respectively. Prior to the analysis, the crabs were cleaned with running tape water and thawed at room temperature. The carapace, claws, walking legs and gut region (after removing the gut contents) of each crab were dissected by using a pair of sterile stainless steel scissors. All the parts were replicated and transferred to Petri dishes. The tissues were dried in an oven for three days at 60 ^{0}C. The desired constant dry weight (0.5g) of each sample was obtained after three days of drying process.

Acid digestion and ICP-MS Analysis

The acid digestion method was performed to digest the samples; this involved heating 0.5g of dried tissues of crabs in a Teflon beaker with mixed concentrated acids (Hydrogen Peroxide (H2O2), Nitric acid (HNO3), hydrochloric acid (HCl) and sulphuric acid (H2SO4) in the ratio of 1:1 (Kamaruzzaman et al., 2007). After the digestion process, hundred times of dilution were performed using Mili-Q water before the samples were analyzed using Inductively Coupled Plasma Spectrophotometer (ICP-MS). The values of the heavy metal concentrations in the tissues were calculated based on their dry weights as this discounts the variability due to the inner part differences in the moisture content of organisms. The international certified standards (DORM-2) by the National Research Council of Canada and a blank in replicates were used to control the accurateness of this procedure, and the percentage of recovery was between 95 – 105%. Analysis of Variance (ANOVA) statistical test was performed to check

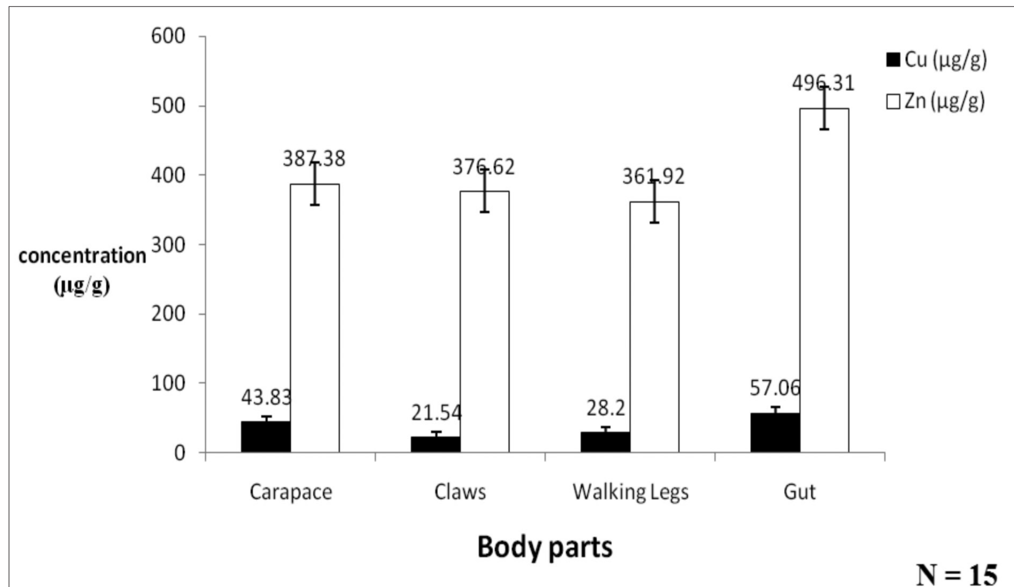

Fig. 2: The average concentrations of Zn and Cu (µgg^{-1} dry weight) in the different body parts of Scylla serrata.

the significance in the bioaccumulation of metals in the different body parts.

RESULTS AND DISCUSSION

The concentrations of metals in all the 15 crabs were analyzed using ICP-MS with the dilution factor of 10. Significant differences in metal accumulation were observed in different body parts of the crabs (P<0.05). The concentrations of Zn and Cu were observed to be higher in the gut region of the crab (469.31 and 57.06 µgg^{-1}, respectively), followed by carapace (387.38 and 43.83 µgg^{-1}, respectively).

The average Zn accumulation was higher in the claws (376.62 µgg^{-1}) as compared to the walking legs (361.92 µgg^{-1}), but the Cu accumulation was comparatively higher in the walking legs than the claws of *S. serrata*. It was apparent that Zn concentration was higher in the crab's body parts, followed by Cu. It was interesting to note that intestinal track had a higher level of metal concentration than the other body parts (*Fig. 2*). In the present study, heavy metal accumulation in med crab flowed in Zn > Cu > Pb > Cd order. This pattern of accumulation is also in agreement with that of the previous studies (see Krishnamurti, 1998; Srinivsa, 2007). The high Zn concentration observed in the crab's body parts than Cu concentration was probably due to major role played by these metals as a precursor in most enzymatic activities and hence, all the body parts have a high tendency to accumulate higher levels of these metals (Kamaruzzaman *et al.,* 2009).

It is worth highlighting that copper and zinc are essential trace metals in crabs but they may be toxic at high concentrations (Phillips & Rainbow, 1993). The haemolymph of crabs, in particular, contains high concentrations of copper which is involved in oxygen transport. These essential trace metals (copper and zinc) are highly regulated within crabs over a wide range of bio-availabilities, while non-essential metals such as cadmium are usually accumulated in proportion to availability. Mud crabs may therefore be useful biomonitors of non-essential metals, such as arsenic, mercury, cadmium and selenium, but more research is still needed in combination with direct water and/or sediment

samples to confirm this. It was also observed that the accumulations of Cu and Zn were higher in the gut region of the crab compared to other body parts and the accumulations of Pb and Cd were lesser in the gut region compared to other organs in the case of *S. serrata*. It was also evident from this study that heavy metal accumulation in *S. serrata* was higher than the international standard references (Table 1).

TABLE 1
Maximum Permissible Level of heavy metal concentrations in aquatic crustacean species enforced by Food and Drug Administration (FDA, 2001), Singapore Food Regulations (1990), Food Standards Australia (2002) and European Union Commission Regulation (2001)

	Heavy metal concentration (in $\mu g g^{-1}$)			
	Vital elements		Toxic elements	
	Cu	Zn	Pb	Cd
FDA (2001)	100	150	1.5	0.2
SFR (1990)	-	-	2	1
FSA (2002)	-	-	2	2
EUCR (2001)	-	-	1	1
	Comparison with the present study			
Present Study	6.38-79.38	325.39-523.21	1.49-7.84	0.05-0.78

Ololade *et al.* (2008) suggested that the crabs are the bottom feeders and generally expected to concentrate more heavy metals than the mobile fish. The variation in metal uptake is also an indication of the degree to which the species pick up particulate matter from the surrounding water and sediment while feeding. A higher level of metal concentration was observed in the gut region and this was probably due to the feeding behaviour of the crabs. A sex specific difference in the metal uptake by the crabs was also observed (Sastre *et al.,* 1999). The male crabs, which are the common residents of low saline area, are expected to have higher metal concentrations in the body compared to the female crabs which generally exhibit seasonal migratory behaviour, especially for reproductive purposes that might help them in exchange of metallic ions into the clean environment. Therefore, the male crabs are more likely to accumulate higher amounts of heavy metals than the female crabs (Ololade *et al.,* 2008). Due to the small sampling size, the sex and size related heavy metal concentrations in crabs were not established in this study. However, Poovachiranon (1991) observed the high levels of Cu and Zn in young crabs were probably due to the faster growth rate of young crabs and the feeding behaviour. It was also due to the food preference by the young and adult crabs, whereby young crabs prey more on aquatic benthic invertebrates, while the larger crabs prefer plant materials (Poovachiranon, 1991). Meanwhile, various studies have shown that aquatic invertebrates tend to accumulate high level of heavy metals (Janczur *et al*., 2000). Thus, determination of harmful and toxic substances in water sediments and biota gives direct information on the significance of pollution in the aquatic environment (Hugget *et al.,* 1973). It is believed that the Cu accumulated in the crabs is due to their feeding habits, even though there is no clear evidence indicating the Cu dietary transfer, and many studies have demonstrated that diet is the most important route of Cu accumulation in aquatic animals (Sindaigaya *et al*., 1994). Decapode crustaceans, including crabs, maintain relatively low and constant body level of Zn when exposed to a range of dissolved Zn concentrations in seawater because of the low integument permeability of the metals. Hence, a detailed study is needed to determine the sex and size related metal accumulations in the med crabs inhabiting Sungai Penor, Kuantan.

Cadmium is widely distributed at low level in the environment and most food has an inherently low level of Cd which has been shown to bind to the protein and accumulate significantly in higher level (FDA, 2001). The present study revealed that the average concentrations of Pb and Cd were higher

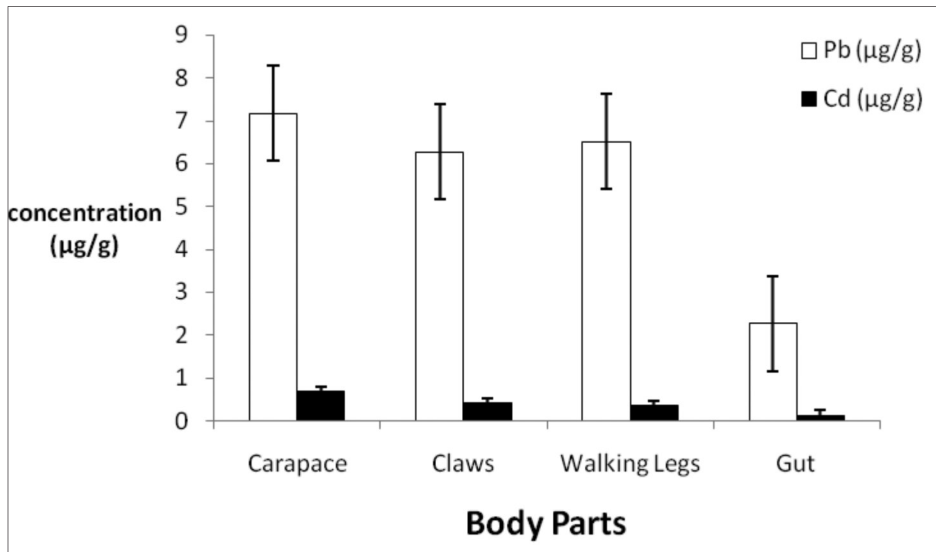

Fig. 3: The average concentrations of Pb and Cd (µgg^{-1} dry weight) in the different body parts of Scylla serrata.

($p<0.05$) in carapace (7.17 and 0.68 µgg^{-1}, respectively) but lower in the gut region (2.27 and 0.13 µgg^{-1}, respectively), indicating that the carapace has higher exposure and tendency to accumulate more concentrations of both metals than the gut region. Slight changes in the average Pb accumulation was observed and it was higher in the walking legs (6.52 µgg^{-1}) compared to the claws (6.27 µgg^{-1}). Nevertheless, the Cd accumulation was comparatively higher in the claws than in the walking legs of *S. serrata*. It is also evident from the study that the carapace showed a high tendency to accumulate more Pb and Cd concentrations compared to other body parts (*Fig. 3*).

Ploetz *et al*. (2007) reported that Cd level is almost 10 times higher in shell fish than in finfish. In this study, the high levels of Pb and Cd were observed in the carapace compared to the other parts of the crab, and these were probably due to the calcium content having a high affinity towards these heavy metals (Du Preez *et al*., 1993; Reinecke *et al.,* 2003). Nevertheless, periodic molting could help in eliminating Pb and Cd levels in the exoskeleton.

The observed levels of selected heavy metals in the mud crabs from the present study were higher than the maximum permissible levels (MPL) set by various international consortiums. Although these food materials are processed (heating, cooking) before consumption, the effects of processing could be minimal, since the heavy metals are non-degradable. Thus, consumption of these heavy metal contaminated crabs may result in bioaccumulation of toxic metals in the human system and may lead to adverse health effects (Bergback *et al.,* 1992; Koller *et al.,* 2004).

CONCLUSION

The accumulation of heavy metals (Cu, Zn, Pb and Cd) in crabs is to a considerable extent, and it is relatively more than that reported from other regions in the literature. The high bioaccumulation of these metals is believed to be occurring due to the rigorous anthropogenic input of bioaccumulative contaminants into the aquatic environment. It was evident from this study that heavy metal accumulations in *S. serrata* were higher than almost all the international standard levels. Detailed and extensive studies should address this issue to determine the bio-accumulative heavy metal

concentrations in edible parts of mud crab and find out the suitability of it flesh for human consumption.

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REFERENCES

Ashraj, W. (2005). Accumulation of heavy metals in kidney and heart tissues of Epinephelus microdon fish from the Arabian Gulf. *Environmental Monitoring Assessment, 101*(1-3), 311-316.

Bahadorani, S. (2008). *The effects of heavy metal toxicity and oxidative stress on genetically-compromised models of* Drosophila melanogaster. (Doctoral dissertation) York University, Toronto, Canada.

Bergback, B., Anderberg, S., & Lohm, U. (1992). Lead load: Historic pattern of lead use in Sweden. *Ambio, 21*(2), 159-165.

Bryan, G. W. (1971). The effects of heavy metals (other than mercury) on marine and estuarine organisms. *Proceedings of Royal Society of London, Series B, Biological Science* (1934-1990), *177*, 389-410.

Conacher, H. B., Page, B. D., & Ryan, J. J. (1993). Industrial chemical contamination of foods. *Food Addit Contam, 10*(1), 129-143.

Du Preez, H. H., Steenkamp, V. E., & Schoonbee, H. J. (1993). Bioaccumulation of Zinc and lead in selected tissues and organs of the freshwater crab, *Potamonautes warreni*. *Science of the Total Environment, Supplement, 134*, 469-478.

European Union Commission Regulation (EUEC), (2001). No 466/2001 of 8 March 2001.

Farombi, E. O., Adelowo, O. A., & Ajimoko. Y. R. (2007). Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African Cat fish (*Clarias gariepinus*) from Nigeria Ogun river. *International Journal of Environmental Research and Public Health, 4*(2), 158-165.

FDA. (2001). *Fish and Fisheries Products Hazards and Controls Guidance* (3rd ed). Centre for Food Safety and Applied Nutrition, US Food and Drug Administration.

Food Standards Australia, (2002). *Issue 53*, Standard 1.4.1 and 1.4.2.

Hugget, R. J., Bencher, M. E., & Slone, H. D. (1973). Utilizing metal concentration relationships in the eastern oyster (Craostrae virginica) to detect heavy metal pollution. *Water Research, 7*, 451-460.

Janczur, M., Kozlowski, J., & Laskowski, R. (2000). Optimal allocation, Life history and heavy metal accumulation: Dynamic programming model. In Kamenga and R. Laskowski (Eds.). Demography in Ecotoxicology (pp. 179 – 197). UK: Wiley Chichester.

Kamaruzzaman, B. Y., Ong, M. C., Jalal, K. C. A., Shahbudin, S., & Mohd Nor. O. (2009). Accumulation of Lead and Copper in *Rhizophora apiculata* from Setiu mangrove forest, Terengganu. *Malaysian Journal of Environmental Biology*, *30*(5), 821-824.

Kamaruzzaman, B. Y., Zaleha, K., Ong, M. C., & Wilson, K. Y. S. (2007). *Copper and Zinc in Three Dominant Brackish Water Fish Species from Paka Estuary, Terengganu, Malaysia*. *Malaysian Journal of Science, 26*(2), 65-70.

Keenan, C. P., Davie, P. J. F., & Mann, D. L. (1998). A revision of the genus *Scylla*de Haan, 1833 (Crustacea: Decapoda: Brachyura: Portunidae). *The Raffles Bulletin of Zoology*, *46*(1), 217-245.

Koller, K., Brown, T., Spurgeon, A., & Levy, L. (2004). Recent development in low level exposure and intellectual impairment in children. *Environmental Health Perspective, 112*(9), 987-994.

Krishnamurti, A. J., & Vijayalakshmi, R. N. (1998). Concentration of metals in shrimps and crabs from Thane-Bassein creek system, Maharastra. *Indian Journal of Marine Science,* 28, 92-95.

Ololade, I. A., Lajide, L., Amoo, I. A., & Oladoja, N. A. (2008). Investigation of heavy metals contamination of edible marine seafood. *African Journal of Pure and Applied Chemistry*. *2*(12), 121-131.

Phillips, D. H., & Rainbow, P. S. (1993). *Biomonitoring of trace aquatic contaminants*. London: Chapman and Hall.

Ploetz, D. M., Fitts, B. E., & Rice, T. M. (2007). Differential Accumulation of Heavy Metals in Muscle and Liver of a Marine Fish, (King Mackerel, *Scomberomorus cavalla* Cuvier) from the Northern Gulf of Mexico, USA. *Bulletin of Environmental Contamination and Toxicology, 78*, 134-137.

Poovachiranon, S. (1991). Biological studies on the mud crab (Scylla serrata) of the mangrove ecosystem in the Andaman Sea. *Report of the Seminar on the Mud Crab Culture and Trade.* pp. 49-57.

Rainbow, P. S. (1990). Heavy metal levels in marine invertebrates In R. W. Furness, and P.S. Rainbow (Eds.). *Heavy metals in the marine environment*. Boca Raton, Florida: RC Press, Inc.

Rainbow, P. S. (1995). Biomonitoring of heavy metal availability in the marine environment. *Marine Pollution Bulletin, 31*, 183-192.

Reinecke, A. J., Snyman, R. G., & Nel, J. A. J. (2003). Uptake and distribution of Lead (Pb) and Cadmium (Cd) in the freshwater crab, *Potamonautes perlatus* (Crustacea) in the Eerste river, South Africa. *Water Air and Soil Pollution, 145*, 395-408.

Ryan, S. (2003). *Queensland mud crab fishery: ecological assessment*. In Report Series: Queensland Department of Primary Industries, pp. 55.

Sastre, M. P., Reyes, P., Ramos, H., Romero, R., & Rivera, J. (1999). Heavy metals bioaccumulation in Puertorichen blue crab (*Callinetes sapidus*). *Bulletin of Marine Science, 64*, 209-217.

Sindaigaya, E., Cauwenbergh, R. V., Robberecht, H., & Deelstra, H. (1994). Copper, Zinc, Manganese, Iron, Lead, cadmium, mercury and Arsenic in fish from Lake Tanganyika, Burundi. *Science of the Total Environment, 144,* 103-115.

Singapore Food Regulations, (1990). Cap. 283, 1st Ed., Singapore.

Skejelkvale, B. L., Andersen, T., Fjeld, E., Mannio, J., Wilander, A., Johansoon, K., Jensen, J. P., & Moiseenko, T. (2001). Heavy metal surveys in Nordic Lakes: Concentrations, geographic patterns and relation to critical limits. *Ambio, 30*(1), 2-10.

Soegianto, A., Charmantier-Daures, M., Trilles, J. P., & Charmantier, G. (1999). Impact of cadmium on the structure of gills and epipodites of the shrimp *Penaeus japonicus*. *Aquatic Living Recourses, 12*(1), 57–70.

Srinivasa, R., Bhavesh, M., Sunil, D., Manish, J., Leena, K., Venkatrama, K., Susarla, S., Shaik, B., Gadde, R., & Prashant, B. (2007). Bioaccumulation of heavy metals in some commercial fishes and crabs of Gulf of Cambay, India. *Current Science, 92*(11), 1489-1491.

Tinsley, I. J. (1979). *Chemical concepts in pollutants behaviour*. J. Willey and Sons Inc. NY. Niger Delta Environmental Survey (NDES), Phase I Report, Vol. 1. Environmental and Socio-economic characteristic (revised edition).

Velez, D., & Montoro, R. (1998). Arsenic speciation in manufactured seafood products: a review. *Journal of Food Protection, 61*(9), 1240-1245.

Vosyliene, M. Z., & Jankaite. A. (2006). Effect of heavy metal model mixture on biological parameters of rainbow trout *Oncorhynchus mykiss*. *Ekologija, 4*, 12-17.

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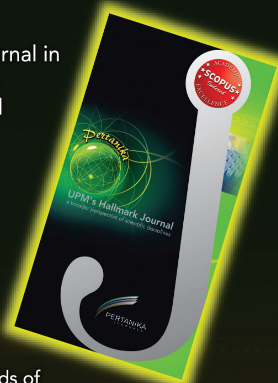

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