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Research Management Centre (RMC)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://www.rmc.upm.edu.my
E-mail : ndeeps@admin.upm.edu.my
Tel : +603 8947 1622 /1620

UPM Press
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://penerbit.upm.edu.my
E-mail : penerbit@putra.upm.edu.my
Tel : +603 8946 8855/8854
Fax : +603 8941 6172

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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 is the official journal of Universiti Putra Malaysia. The abbreviation for *Pertanika* Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

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Research Management Centre (RMC),
1st Floor, IDEA Tower II, UPM-MTDC Technology Centre
Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
Tel: +603 8947 1622, 8947 1620
E-mail: ndeeps@admin.upm.edu.my

Publisher

The UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
Tel: +603 8946 8855, 8946 8854 • Fax: +603 8941 6172
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Review Article

Current ZP3-based Immunocontraceptive Vaccine for Free Ranging Wild Pest

Lo, S.C., Zeenathul, N.A.*, Sheikh Omar, A.R. and Mohd. Azmi, M.L.
Department of Veterinary Pathology and Microbiology,
Faculty of Veterinary Medicine, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
**E-mail: zeenathul@putra.upm.edu.my*

ABSTRACT

There is an overabundance of certain animal species that are considered as destructive and have reproductive control acts as a humane management of pest population. In particular, mammalian zona pellucida 3 (ZP3) has provoked a great interest as a potential antigen for immunocontraception. High levels of long-term infertility have been achieved in many species following ZP3-based immunization. This paper discusses the current updates on the ZP3-based immunocontraception for several major pest species of the world.

Keywords: Immunocontraception, pest control, zona pellucida 3

ABBREVIATIONS

BAC	:	Bacterial Artificial Chromosome
BMP15	:	Bone Morphogenic Protein 15
CHV	:	Canine Herpesvirus
CTL	:	Cytotoxic T Lymphocytes
DES	:	Diethylstilbestrol
FSH	:	Follicle Stimulating Hormone
fZP	:	Fox Zona Pellucida
GnRH	:	Gonadotrophin Releasing Hormone
GMCSF	:	Granulocyte-Macrophage Colony-Stimulating Factor
HCMV	:	Human Cytomegalovirus
ie1	:	Immediate Early 1 Region
ie2	:	Immediate Early 2 Region
IVF	:	In Vitro Fertilisation
IL-4	:	Interleukin 4
KLH	:	Keyhole Limpet Hemocynin
LH	:	Luteinizing Hormone
MBP	:	Maltose Binding Protein
MGA	:	Melengestrol Acetate
mZP	:	Mouse Zona Pellucida
MCMV	:	Murine Cytomegalovirus
NP	:	Nucleoprotein
OGP	:	Oviduct Glycoprotein
PZP	:	Porcine Zona Pellucida
PRL	:	Prolactin
rZP	:	Rat Zona Pellucida
SP56	:	Sperm Protein 56
SLP	:	Synthetic Late Pox Virus Promoter
TK	:	Thymidine Kinase
ZP	:	Zona Pellucida
ZP1	:	Zona Pellucida 1
ZP2	:	Zona Pellucida 2
ZP3	:	Zona Pellucida 3
ZPC	:	Zona pellucida C

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*Corresponding Author

INTRODUCTION

Reproductive control is important for the management of certain overabundant wildlife which has turned into pest species. Three basic available techniques for this purpose include surgical sterilization, hormonal contraception, and immunocontraception (Artois, 1997; Cowan and Tyndale-Biscoe, 1997; Sinclair, 1997). However, it is costly and impractical to conduct surgical sterilization for free ranging wildlife population. It involves invasive procedure, causes discomfort, pain, and poses the risk of infection. Hormonal contraception using highly conserved reproductive steroid sex hormones, such as gonadotropin releasing hormone (GnRH), follicle stimulating hormone (FSH), and luteinizing hormone (LH) to disrupt the oestrus cycle, is not species-specific. It often leads to undesirable side effects that raise ethical issues, such as premature termination of pregnancies or damages to non-reproductive tissues (Talwar, 1997; Delves, 2004; Ferro and Mordini, 2004), alterations in sexual and social behaviour of the target species (Tyndale-Biscoe, 1994; Tuyttens and Macdonald, 1998). Therefore, immunocontraception remains an attractive method to reduce and maintain the populations of these animals. In particular, immunocontraceptive vaccine prevents conception by stimulating the production of antibodies that bioneutralize proteins or hormones essential for reproduction. Meanwhile, the use of immunocontraception has been recognized to provide animal welfare benefits relative to alternative methods (Tyndale-Biscoe, 1994; Oogjes, 1997; Porton, 2005; Rutberg, 2005; Hardy and Braid, 2007). It provides alternatives to the on-farm (e.g. pig production industry) surgical removal of an animal's gonad which is usually undertaken without anaesthetic. For free ranging wildlife, immunocontraceptive control is preferable to most lethal methods of controlling populations by animal welfare proponents (Oogjes, 1997; Porton, 2005; Hardy and Braid, 2007). The application of this method is also suggested in areas where lethal control is restricted, such as at national parks.

Fertilization is a species-specific process (Hoodbhoy and Dean, 2004; Conner *et al*., 2005). Therefore, targeting the gamete proteins should provide an effective contraceptive vaccine with the least risk to non-target species. To date, the most widely tested immunocontraceptive vaccine for wildlife species is on the basis of developing antibodies to zona pellucida (ZP) (Jewgenow, 2009). Other potential immunocontraceptive antigens, particularly protein derived from sperm, appear less efficacious than ZP antigens (Naz *et al*., 2005; Hardy, 2007; Hardy and Braid, 2007). ZP is a unique extracellular glycoprotein matrix that surrounds mammalian oocyte through which the sperms must penetrate during the initial stages of fertilization. It is composed of three major glycoproteins, which are zona pellucida 1 (ZP1), zona pellucida 2 (ZP2) and zona pellucida 3 (ZP3). High levels of long-term infertility in females have been achieved in many species following immunization with native ZP and recombinant ZP3 antigens. In this paper, current updates on the ZP3-based immunocontraceptive vaccine for several world major pest species are discussed.

Rats

Rats have undeniable become major pests. In fact, they are the vectors of many diseases, such as plaque and leptospirosis. They destroy an estimated of 20% of the harvested crops of the world during storage and cause major damages to buildings and equipment. The current control methods used for wild rat population involve trapping, poisoning, and biological means. A biological rodenticide, using *Sarcocystis singaporensis,* has been reported (Wood, 1985; Wood and Chung, 2003). This protozoan alternates as a gut parasite of the reticulate python, passing back to rats which eat the snake feaces (Zaman and Colley, 1975). It causes a debilitating muscle infection, found to be lethal to rat (Wood, 1985). These population control methods are less than satisfactory due to the intrinsic capacity of these species to grow rapidly and replace those that have been killed.

Therefore, curbing the reproductive potential of the pest is a more effective approach in reducing the rat population densities.

In particular, Porcine ZP (PZP) has been extensively used for birth control studies due to its high availability at the abattoirs. Heteroimmunisation of PZP has been found to successfully reduce or inhibit fertility in several species (Wood *et al*., 1981; Mahi-Brown *et al*., 1985; Paterson *et al*., 1992). However, PZP has no fertility control effect on rats (Drell *et al*., 1984). Hence, 'self' ZP is required to suppress the fertility in rats. ZP3-based DNA immunocontraceptive vaccines have been previously developed in which the rat ZP3 glycoprotein (rZP3) is expressed under the control of the human cytomegalovirus (HCMV) immediate early promoter (Lai, 2004). The administration of this DNA vaccines resulted in reduction of an average litter size up to 90%, although the ZP3 antibody titre was significantly low. The ovarian dysfunction was characterized by excessive depletion of follicles and an increase in the number of oocyte-free cell clusters. In order to produce recombinant rZP3 that mimics the native glycoprotein in bulk, rZP3 cDNA was expressed in yeast. Vaccination with recombinant ZP3 protein, expressed by yeast cells stimulated strong antibody response, but no correlation between antibody titres and infertility was observed (Lai, 2004; Lo *et al*., 2009). Meanwhile, rZP3-DNA vaccine was co-administrated with Interleukin 4 (IL-4) to boost up the level of antibody response against ZP3 protein. However, a dramatic increase in the ZP antibody level did not enhance the efficacy but it further weakened its effectiveness in preventing fertilization.

An attempt to improve the potency of the rZP3-DNA vaccine is the incorporation of the Newcastle viral nucleoprotein (NP) to the DNA vaccine (Lai, 2004). The viral nucleoprotein served as an immunologic adjuvant in the form of fusion gene. Being an endogenous antigen, rZP3 is not highly antigenic on its own since it is recognized by the host as "self" molecules. The antigenicity of the rZP3 protein was greatly improved when conjugated with NP because the NP portion is of viral origin, and thereby rendering the entire protein foreign and evoking a stronger cytotoxic T lymphocytes (CTL) response and a high level of antigen specific antibodies, mainly IgG2a subclass via cross priming (Yankauckas *et al*., 1993; Lai, 2004). This may increase the anti-ZP3 antibody production without affecting the cell mediated immune response, unlike the enhancement of antibody response by IL-4 which is sometimes accompanied by a suppression of cell-mediated immune response that is important to ensure permanent infertility. Furthermore, this improved rZP3-DNA vaccine has been found to produce a significant enhancement on the levels of ZP3 antibody. However, despite high ZP3 antibody titres, the vaccinated rats produced normal litter size. This might be due to certain epitopes, specifically the sperm receptor sites or those possessing contraceptive activities might be masked during protein folding when the larger NP protein was expressed as recombinant protein resulting in less capability to prevent pregnancy.

Although rZP3-based DNA immuno-contaceptive vaccines has successfully reduced the number of rat populations to a certain extent, the need for booster vaccination raises both the economical and practical issues of using the DNA-based immunocontraceptive approach. Thus, viral vectors appear to be an ideal delivery system for an immunocontraceptive vaccine, especially for those antigens which are highly glycosylated and in which post-translational modification is important for the generation of immune responses to functionally important domains (Lai, 2004). The viral-based delivery system is practical and advantageous due to its long acting persisting nature in the populations even after a single exposure. Extensive *in vitro* studies on adenovirus and retrovirus ZP3 based immunocontraceptive vaccines have been successfully completed (Lo *et al*., 2006a: 2006b). Meanwhile, the experiment to test the effectiveness of these vaccines on reducing the rat population is underway.

Mice

Mice have also caused major damages worldwide. Globally, they have caused damages worth billions of dollars annually. Damages caused by mice in crops, such as rice (specifically in the temperate regions), currently form a major threat to humans. Wild mice in the southeastern grain-belt of Australia have been reported to cause great damages to crops and stored grain and have important social impacts on the rural communities (Redhead, 1988; Singleton *et al*., 1999: 2001; Ylönen, 2001). The current control methods used are apparently less than satisfactory. In more specific, these methods are either lethal, non-humane, and have caused negative side-effects through poisoning of non-target species. Thus, there is a need for continued evaluations and testing of novel approaches aimed at interrupting their fertility.

Mice heteroimmunized with porcine zona pellucida had no significant effect on reducing the mouse fertility (Sacco *et al*., 1981). Meanwhile, the immunization of certain mouse strains with ZP3 peptide (amino acid 330-342) stimulates autoimmune response and oophoritis (Rhim *et al*., 1992). The strain dependent nature reduces the usefulness of this ZP3 peptide in outbred mouse population (Millar *et al*., 1989; Rhim *et al*., 1992). In order to improve this vaccine, a synthetic peptide vaccine contained a 7-mer peptide represents amino acid 336-342 which is immediately adjacent to the most hydrophilic portion of ZP3 and partially overlaps a region that contains a potential amphipathic α helix coupled with a carrier protein, keyhole limpet hemocyanin (KLH) (Millar *et al*., 1989) was studied. Mouse ZP3 (mZP3) is too small to produce antibodies on its own, so it was coupled to a larger protein (KLH) to increase antibody production. This vaccine induces antibody responses to both mZP3 and KLH. It gives long-term contraception without exhibiting ovarian histopathology. Targeting functional regions of the antigens not only reduces the undesirable side-effects, but it also increases the specificity of the vaccine. Contraceptive peptide epitopes with reduced side-effects and increased species specificity have been described. The selected mouse specific immunocontraceptive peptides have been determined to play a key role in reproduction. Mouse specific immunocontraceptive polyepitope vaccines, containing mouse specific epitopes for ZP1, ZP3, sperm protein 56 (SP56) and proliferin fused to maltose binding protein (MBP) produced in bacterial expression system, were found to have caused 40% fertility decrease in inoculated mice (Hardy *et al*., 2002). Meanwhile, conjugated peptides containing SP56, granulocyte-macrophage colony-stimulating factor (GMCSF) and prolactin (PRL) elicited peptide-specific serum antibodies and reduced fertility by 50% (Hardy *et al*., 2004). Multiple peptide antigens could be coupled to immune enhancers to generate stronger immune responses. Furthermore, polyepitope vaccines closely mimic natural autoimmunity more closely, whereby the physiological effects require simultaneous immune responses against a number of different antigenic molecules (Bach *et al*., 1998; Hardy *et al*., 2004).

The viral-vectored immunocontraceptive vaccine was first demonstrated in mice using Ectromelia virus. The recombinant ectromelia virus expressed mouse zona pellucia 3 glycoprotein under the direction of a synthetic poxvirus early-late promoter (Jackson *et al*., 1998). A single inoculation with the recombinant virus into the footpad of BALB/c mice caused infertility in 70% of the mice for 5-9 months. Disruption of ovarian follicular development and the depletion of mature follicles without oophoritis were also observed (Jackson *et al*., 1998). Thus, to boost the immunological effect, a gene responsible for activation of interleukin 4 (IL-4) productions was incorporated into the recombinant ectromelia virus (Jackson *et al*., 2001; Ylönen, 2001). All the infected mice died one week after immunization. This recombinant virus was lethal without any contraceptive effect. Meanwhile, the addition of IL-4, which acts as a transmitter substance in inflammations, was aimed at strengthening the immune response. However, it had suppressed the function of the killer T cells to fight the inflammation and

infection of the treated mice and the expression of immune memory response (Ylönen, 2001).

Higher levels of infertility have been achieved by using recombinant murine cytomegalovirus (MCMV) to express the mZP3 glycoprotein at the immediate early 2 (*ie2*) region of the virus under the control of human cytomegalovirus immediate early (*ie1*) promoter (Chambers *et al*., 1999; Lloyd *et al*., 2003; Redwood *et al*., 2005; Hardy *et al*., 2006). MCMV is an attractive vector for fertility associated genes predominantly for its species-specificity and its capacity to infect mice with more than one strain of virus simultaneously (Booth *et al*., 1993; Shellam, 1994). It was also found to establish persistent and latent infection in mice with periodic reactivation (Osborn, 1982). A single inoculation of the recombinant MCMV expressing mZP3 antigen resulted in almost 100% infertility in mice up to 250 days. Persistent anti-ZP3 antibody production and profound changes of ovarian morphology have also been observed in the infected animals. However, in other studies, the innate resistance to MCMV in certain inbred mouse strains was found to significantly reduce the immunocontraceptive success (Chambers *et al*., 1999; Lloyd *et al*., 2007). Similarly, a prior exposure to MCMV could limit the immunocontraceptive effects of the recombinant MCMC vector expressing mZP3 (Gormana *et al*., 2008). The host resistant locus, *Cmv1* which controls the natural killer cell towards the MCMV was the key factor determining the vaccine efficacy. However, specific pathogen free outbred wild mice could be sterilized over 100 days to MCMV infection (Lloyd *et al*., 2007). This finding indicates that wild mice should be susceptible to recombinant MCMV-mZP3 infection. On the contrary, the recombinant MCMV-mZP3 showed no anti-fertility effects on rats. The lack of effect of the immunocontraceptive virus in closely related species indicates the species specificity of this vaccine in the non-host species (Smitha *et al*., 2005).

In a recent study, a range of protein or polyepitope antigens, associated with female reproductive processes expressed by MCMV, were tested for the sterilizing effects in mice (Redwood *et al*., 2007). The antigens tested were bone morphogenic protein 15 (BMP15), oviduct glycoprotein (OGP) and ubiquitin-tagged mZP3. Both BMP15 and OGP proteins were expressed within the reproductive system of female mice. In particular, the mZP3 was N-terminal ubiquitinated to improve CD8+ T cell responses and MHC class I antigen presentation (Rodriguez *et al*., 1997; Anderson and Barry, 2004). The experiment found that only full length mZP3 or ubiquitin-tagged mZP3 induced fertility in mice, but not BMP15 and OGP. Meanwhile, the expression of the mouse-specific polyepitope antigens by species specific vector, MCMV might improve the safety of viral-vectored immunocontraceptive vaccine.

European Red Fox (Vulpes vulpes)

The European red fox is one of the major vertebrate pests in Australia. The over-population of the European red fox has posed a major threat to the survival of endangered native fauna, acted as reservoirs for exotic diseases, and caused a considerable impact on lamb production. Conventional methods for fox population control are by natural predators, commercial trapping, shooting or poisoning with strychnine, phosphorus, arsenic or 1080 (sodium fluoroacetate) (Tyndale-Bisco, 1994). Poisoning with '1080' baits requires repeated applications which need to be delivered into remote and inaccessible regions. This poison is also highly susceptible to non-target animals, such as dogs, and is therefore not suitable to use in the urban areas (Strive *et al*., 2006).

Fox ZPC (fZPC) antigens have been expressed with baculovirus system due to the lack of variable glycosylation and low yields of recombinant product in both yeast and bacteria expression systems (Bradly *et al*., 1997).

To date, the fZPC-based immuno-contraceptive vaccine has been assessed in two viral vectors, namely the vaccinia virus and canine herpesvirus (CHV) vectors (Reubel *et al*., 2005; Strive *et al*., 2006). Recombinant

vaccinia-based rabies vaccine has been used in the past two decade in Europe and North America to mitigate and prevent the spread of rabies in the European red foxes without any undesirable side-effects (Brochier *et al*., 1991; Masson *et al*., 1996). Therefore, vaccinia virus was studied to evaluate their anti-fertility potential in foxes. The recombinant fZPC vaccinia virus was constructed under the control of a synthetic late poxvirus (SLP) promoter to drive the expression of fZPC which was inserted within thymidine kinase (TK) gene locus of vaccinia virus (Reubel *et al*., 2005). All the recombinant viruses expressed fZPC in the cell culture but were highly attenuated in foxes *in vivo*. None of the treated foxes raised antibodies specific to the transgenes fZPC. Moreover, repeated administration of recombinant fZPC has been found to fail to induce ZPC specific antibodies but it developed a pronounced immune response to vaccinia virus proteins. It has been reported that the inactivation of the vaccinia virus thymidine kinase gene, caused by the transgene insertion into the viral genome, leads to the attenuation of the virus *in vivo* (Buller and Palumbo, 1992). In contrast, self antigens that are derived from homologous tissues can be less immunogenic, specifically when they are linked toincompatible viral carrier or promoter (Dunbar *et al*., 2001; Guevara-Patino *et al*., 2003; Reubel *et al*., 2005). Meanwhile, the absence of infectious virus in foxes indicates abortive infection and probably lack of virally expressed late proteins, including transgene due to the synthetic late promoter which is active only at the late stages of vaccinia virus replication. This may cause insufficient transgene expression to induce adequate immune responses against transgenic proteins. Therefore, a high expression of the transgene is required to overcome the poor immune responses to anti-fertility antigens in foxes.

Canine Herpesvirus is another viral vector that has been assessed for use in foxes (Reubel *et al*., 2001). The bacterial artificial chromosome (BAC) system has been used for CHV to facilitate vaccine development (Strive *et al*., 2006). The BAC-derived recombinant fZP3-CHV vaccine was found to be highly attenuated *in vivo* and failed to induce antibody response against the fZPC protein, although high serum antibody levels against the CHV proteins were detected (Reubel *et al*., 2005; Strive *et al*., 2007). Attenuation was due to the presence of large amounts of additional BAC-related DNA incorporated into the viral genome and to the inactivation of the thymidine kinase gene (Strive *et al*., 2006).

Therefore, in order to improve BAC-derived CHV antifertility vaccines, the development of an improved TK positive, self-excisable CHV-BAC, that enables antigen expression from an intergenic region between the UL21 and UL22 genes within the CHV genome, was reported. In this study, the BAC-derived CHV-PZPC stably expressed the PZPC antigen (Strive *et al*., 2007). However, it was also highly attenuated *in vivo* and failed to induce anti-ZPC antibodies or infertility in foxes (Hardy *et al*., 2006).

European Rabbit (Oryctolagus cuniculus)

The European rabbit (*Oryctolagus cuniculus*) is an introduced species in Australia. High fertility rate and over-abundance of the European rabbit have turned this species into a major vertebrate pest species in Australia and other countries. The species has specifically caused significant losses in agricultural production and severe environmental degradation (Holland and Jackson, 1994; Gu *et al*., 2003: 2004). The current methods used to reduce of rabbit populations are inhumane, non-species specific and met with only partial success (Holland and Jackson, 1994; Tyndale-Biscoe, 1994). Therefore, fertility control was suggested.

The effects of alloimmunization (rabbit ZP) and heteroimmunization (porcine ZP) on the fertility in rabbits were studied (Wood *et al*., 1981). A rabbit that was heteroimmunized with PZP induces serum antibodies to rabbit ZP antigens and caused infertility with ovarian degeneration and endocrine dysfunction (Wood *et al*., 1981; Skinner *et al*., 1984; Kerr *et al*., 1999). Meanwhile, the rabbit that was alloimmunized with the rabbit ZP did not elicit

a significant immune response and normal offspring were obtained (Wood *et al*., 1981; Skinner, 1987). The fertility levels were also found to be lower than those of the controls, but there was a considerable variability between animals. The fertility reduction rate was also found to be dependent on the state of solubilization of zona prior to immunization (Wood *et al.*, 1981).

In other attempts, an immunization of the female rabbits with bacterially expressed rabbit ZP did not develop detectable antibody level (Dunbar *et al*., 1994; Prasad, 1995). A recombinant myxoma virus expressing rabbit zona pellucida C (ZPC) caused 70% infertility in immunized rabbits for 30-35 days (Mackenzie *et al*., 2006). In a recent study, recombinant myxoma virus expressing rabbit ZPC under the control of a synthetic early/late promoter induced short-term infertility in 90-100% of the treated rabbits (Hardy *et al*., 2006).

African Elephants (Loxodonta africana)

During the twentieth century, the number of African elephants (*Loxodonta africana*) declined dramatically as a result of over-hunting, poaching for ivory, and the loss of habitat through human cultivation and settlement. In 1989, bans were placed on all international trades of elephant products, whereas the protection offered by legislation and the establishment of protected areas has eventually led to a remarkable recovery in the number of elephants (Hanks, 2006). Unfortunately, efforts to nurture the elephant populations have resulted in elephant over-population in several southern and eastern African countries. The resulting high elephant densities in Southern Africa have lead to vegetation and habitat destruction, degradation of the appearance and ecological functioning of the landscape, and thereby reducing biological diversity. Methods such as culling, translocation, range expansion, manipulation of water sources, and contraception are options that have been used to reduce elephant densities (van Aarde and Jackson, 2007). However, culling is greatly opposed due to the ethical and animal rights concerns. Meanwhile, translocation is no longer a viable option due to high cost incurred, cumbersome, lack of suitable wildlife areas available and unrealistic to relocate thousands of animals per year (Colenbrander, 2003a).

With the increasing alarm, the assessment of elephant management in South Africa was conducted. Therefore, PZP immunocontraception for the elephant population control has been reported. The female elephants immunized with the whole porcine ZP developed antibodies that persisted for 12-14 months that were sufficient for an effective contraception in these animals (Fayrer-Hosken *et al*., 1997: 1999: 2000). In the first field trial, 56% of the vaccinated elephants were not pregnant (Bertschinger *et al*., undated). In the second trial, 80% of the inoculated elephants were not pregnant using a revised schedule (Bertschinger *et al*., undated). In more specific, the treated females did not conceive for up to 12 months. When a booster vaccination was given after a year, they remained sterile for up to two years with no deleterious effect on the ovary and cyclicity of the treated elephants (Fayrer-Hosken *et al*., 2000; Fayrer-Hosken, 2003). The trials have also proven that the vaccine is safe to use in pregnant elephants and it is reversible.

A follow-up phase was conducted at the Greater Makalali Private Game Reserve from 2000 and in the span of ~12 years. Forty-three percent of the treated female elephants underwent the 53-month inter-calving period with no early calving indicating 100% reproductive control (Delsink *et al*., 2003: 2004: 2007). This programme seemed to demonstrate that the PZP does not cause herd fragmentation, harassment by bulls, change in rank, and other negative behaviours. Interestingly, it has shown that the elephant anti-PZP antibodies preferentially recognize the theca cells in the primary and secondary follicles, but do not block fertilization in a porcine in vitro fertilization (IVF) system. Therefore, it is likely that the antibodies raised by PZP vaccination of elephant cows are not directed against the molecules involved in either the primary or secondary sperm-oocyte binding, but they exert their effects much earlier, i.e.

during the development of the follicle and zona pellucida (Colenbrander *et al*., 2003b).

One of the obstacles for a large population control is the need to administer multiple boosters to individual animals (Kirkpatrick *et al*., 1997; Turner *et al*., 1997; Kirkpatrick and Rutberg, 2001; Perdok *et al*., 2007). In order to overcome this hurdle, a single administration (one-shot), with multiple slow releases PZP pellet vaccine, has recently been developed (Frayne and Hall 1999; Kirkpatrick and Rutberg 2001; Kirkpatrick, 2003). All the captive elephants immunized with the one-shot vaccine were found to develop antibody titres that were better than with the conventional vaccine. In fact, this one-shot vaccine can greatly save the cost of contraception (single darting instead of triple darting) and provide a more feasible contraceptive treatment for larger populations of elephants.

White-tailed Deer (Odocoileus virginianus)

The over-population of the white-tailed deer (*Odocoileus virginianus*) has become a serious problem in many areas of the United States, particularly in the northern and eastern parts of the United States. Their adaptability, acute senses, and other physical attributes have enabled them to boom in the wilderness and metropolitan suburbs. Meanwhile, problems associated with over-abundance of white-tailed deer pose adverse effects to ecological communities, economic losses from crop damage, damages to ornamental plantings, damages to automobile, and injuries due to deer and vehicle collisions, as well as spread of Lyme disease from deer ticks (Anthony *et al*., 2000). Methods such as sport hunting, sharpshooting, and trap-and-kill have traditionally been used to maintain white-tailed deer populations. However, public opposition and municipal ordinances make these lethal methods illegal in some locations (Kellert, 1991). Surgical sterilization not only prolongs the breeding season of the female animals, it also requires licensed veterinarians and thus increases the cost incurred for this method. Thus, the fertility control seems to be a potential resolution to control the population of deer. Early studies of deer fertility control used synthetic steroid hormones, primarily melengestrol acetate (MGA), diethylstilbestrol (DES), administered either orally, through implant or infection (Harder and Peterle, 1974; Bell and Peterle, 1975; Matschke, 1977a, b: 1980; Roughton, 1979). However, this vaccine requires capture, immobilization and daily exposure of the agent to the animals. Deer are prey for several species, including human, and therefore, synthetic steroid contraceptives vaccine which resist biodegradation are unfavourable as a deer contraceptives agent.

Immunocontraception is a potential possibility for a more permanent solution. The first successful, remotely delivered immuno-contraception in captive, unrestrained white-tailed deer using porcine zona pellucida antigen was reported (Turner *et al*., 1992). All the PZP treated did not produce fawn but showed extended estrous cycle. The PZP protein was metabolized prior to ingestion or excretion; therefore, it could not be passed through food chain to the predators. In the first field test, single PZP inoculation provided limited contraceptive effect ($\leq$ 20%) and it was also found to yield full contraceptive effect (70-100%) with two inoculations. Meanwhile, a single annual booster inoculation in the second year reduced fertility to 20% thereafter. The second large scale field trial, with free-roaming deer that were acclimatized to human presence, revealed that only 28% of the does which had received the initial two inoculations produced fawns (Kirkpatrick *et al*., 1997).

The possibility for the use of one inoculation PZP vaccine in a two injection protocol was examined (Kirkpatrick *et al*., 1997). Does were given an initial inoculation of native PZP and an osmotic mini-pump that continuously released PZP for 28 days implanted subcutaneously in the neck. The continuous release of PZP elevated the antibody titres to contraceptive levels (Turner *et al*., 1996; Kirkpatrick *et al*., 1997).

The long-term effects of the PZP vaccine on the white-tailed deer were also studied. In particular, the PZP treated does have a 76%

reduction of fawning rate in 6 years. The deer remained infertile for 1-4 years after the booster. Infertility is directly related to the antibody titres to the PZP. In addition, an increase in estrous cycles also leads to prolonged breeding seasons in the treated does. The ovarian follicular cycle continued to produce ovulatory follicles in the PZP treated does but conception failed to occur. This suggests that the oocytes exposed to immune serum from PZP treated does is less capable of binding spermatozoa (Way *et al*., 1999; Killian and Miller, 2000). It is also possible that infertility results from the failure of a normal corpus luteum to develop sustain pregnancy due to the lower level of serum progesterone concentrations during the luteal phase (Miller *et al*., 1999).

In another study, the peptides that reacted with sera from infertile deer, including six peptides from ZP1 and six peptides from ZP3, were selected and produced to test the immunogenicity and immunocontraception in deer. The ZP3 peptides pin 46-54 (ZP3 279-332) induced high antibody titres towards PZP but does were found to have remained fertile. Meanwhile, ZP1 peptides pin 10–16 (ZP1 79–130) induced lower titres, but deer exhibited multiple estrus cycle and infertility. A less consistent response to PZP peptides as compared to whole molecules of native PZP was possibly due to the smaller peptides which did not contain sufficient T cell epitopes to ensure a sufficient binding by MHC and presentation to T cells to stimulate immunocontraception response (Miller and Killian, 2002). It is important to note that peptides will need to be coupled with a larger protein carrier or included sufficient T cells epitopes to provide sufficient T cells stimulation. Moreover, the three dimensional confirmation and glycosylation of the synthesized peptides may have been altered (Miller and Killian, 2002).

Although several previous studies reported the success of the PZP immunocontraception in white-tailed deer with minimal health side-effect but Curtis *et al*. (2007) identified pathophysiology resulting from the PZP treatment. Most of the does were detected with granulomas at injection sites, even after 2 years of the treatment. The majority of the treated does developed eosinophilic oophorities in ovaries. The ovarian ZP antigens presented to the immune system stimulated release of chemokines which attracted eosinophils. Then, the eosinophils stimulated inflammatory response in the ovary (Curtis *et al*., 2007). It was found that the does without oophoritis revaccinates, with the last booster, had the greatest reduction in normal secondary follicles. This condition has been related to inhibited ovarian function, abnormal cycling, and suppressed progesterone levels (Skinner *et al*., 1984; Mahi-Brown *et al*., 1988; Dunbar *et al*., 1989; Sacco *et al*., 1991; Dunbar *et al*., 2001; Stoops *et al*., 2006; Curtis *et al*., 2007).

Efforts to control overabundant of the white-tailed deer with a long lasting single dose contraceptive vaccine, SpayVac®, have been carried out. SpayVac® consist liposome with encapsulated intact PZP. A single dose of SpayVac® was highly effective for more than 2 years but the practical application is limited to small enclosed areas due to high labour cost (Locke *et al*., 2007).

CONCLUSIONS

It can therefore be concluded that immunocontraception is an attractive and ethically supported alternative for animal control as it is more humane and less invasive. The effectiveness of this particular method has been studied in captive and small population of free-ranging wildlife animals. Nonetheless, further research is needed to safely apply immunocontraception to over-abundant free ranging wildlife populations. Parameters that require crucial considerations are needed with regard to the distribution of the species under study, whether large scale or localised control is desired, and the issue of directed specificity with regard to other species. Antigens that are highly conserved between the species should be avoided. Besides, specificity can also be built within the vaccine which might include the target antigen or epitopes, microbial or other delivery vector. Ideally, a potential immunocontraceptive

vaccine that induces infertility should not cause ovarian dysfunction and significant health side-effects. Different vaccine delivery systems, such as dart delivery, disseminating viral vectors and oral bait systems, have been assessed to ensure that the immunocontraceptive vaccine is capable of inducing a long-lasting immune response to a high percentage of the target wildlife population. Moreover, it is vital that all public concerns and legal requirements in relation to the risks of disseminating genetically modified organism are adequately addressed during the development phase and prior to any environmental release. If issues such as long-term efficacy and safety, the requirement for booster vaccinations, the use of adjuvants and high production cost can be resolved, immunocontraceptive vaccines will then be able to provide consistent, long-term infertility after a single inoculation, and offer realistic alternatives to free ranging wildlife population control in the near future.

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Review Article

Cottonseed Oil as Health Oil

Savanam Chandra Sekhar[1*] and Bhaskara Rao, V.K.[2]
[1]St. Ann's College of Engineering & Technology, Chirala, Vetapalem – 523 187, Prakasam District, Andhra Pradesh, India
[2]Post Graduate Department of Commerce & Management Studies, V.R.S & Y.R.N College, Chirala – 523 157, Prakasam District, Andhra Pradesh, India
[*]E-mail: tejachandrasekhar_1973@yahoo.co.in

ABSTRACT

There are varieties of cooking oil and fat available today and the claims made by them are, at best, confusing. On one side are the traditional ghee, mustard oil, coconut oil, and gingelly oil. Then, there are the used-for-decades vanaspati and groundnut oil, as well as the relatively newer kinds of vegetable oil ranging from cottonseed, sunflower, safflower, corn, canola, soybean, and palm to various blends. In particular, cottonseed oil performs better than other oil as it lasts a long time and stores well by withstanding higher temperature for food items due to its high antioxidant content. For instance, chips and snacks fried in cottonseed oil may maintain a longer shelf life. It is a good option for preparing healthier foods. The purpose of this paper is to highlight the value of addition cottonseed oil in the food industry and presents an insight into other contemporary edible oil. It is important to note that buying the right oil for health has become a big deal.

Keywords: Cottonseed oil, edible oil, fatty acids, gossypol, health oil, trans fat, vegetable oil

INTRODUCTION

Cottonseed oil, a by-product of cottonseed, is a valuable source of edible oil. The whole cottonseed contains 15-20% oil and about 30-38% of kernel, depending on the quality of seed and the species. Meanwhile, the free fatty acid and quality of oil depend on the weather prevailing during the time that cotton stands in the fields after coming to maturity. Hence, the quality of oil varies from place to place and season to season. In more specific, the quality of oil is high in dry seasons and low when the seed is exposed to wet weather in the fields or handled or stored with high moisture. Crude cottonseed oil has a better condition stability due to the presence of segment named gossypol (Bambawale *et al*., 2004).

Cottonseed oil must be refined to remove gossypol, a naturally occurring toxin that protects the cotton plant from insect damage (Kanoi, 2005). Therefore, unrefined cottonseed oil is sometimes used as a pesticide. The oil with practically no gossypol is pale yellow in colour and rich in Vitamin-E and can be used directly as a cooking medium, as well as for manufacturing of vanaspati. The keeping quality of oil is also quite good and is comparable with other edible oil (Alderks, 1948).

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[*]Corresponding Author

COTTONSEED OIL PRODUCTION SCENARIO IN INDIA

The total production of cottonseed oil (Washed) in India, i.e. both from undecorticated crushing and scientific processing, was estimated to be 851,000 tonnes during the period between November 2008 and October 2009 as compared to 934,000 tonnes between 2007 and 2008. It clearly revealed that cottonseed oil production was lagging behind the estimated potentiality of 1.17 million tonnes during 2008-09 as against 1.29 million tonnes during 2007-08, if all cottonseed available for processing was scientifically processed. Of course, this is due to huge amount of cottonseed is being crushed through traditional methods in India (Tripp & Pal, 2000). As per an estimate, about 9 million tonnes of cottonseed produced in India could yield approximately 1.5 million tonnes of oil (Irani *et al*., 2000). The nutritional value of cottonseed oil is around 9 kcal/g, while the average digestibility is around 97%, which is comparable to that of the soybean, safflower and sunflower oil (Achaya, 1990).

As of 2008, oilseed was grown on 26.54 million hectares and produced 28.82 million tonnes, with an average productivity of 1086 kgs/ha. The net domestic availability of edible oil was only 7.2 million tonnes as against the actual consumption of 11.5 million tonnes and the gap of 4.3 million tonnes had to be imported, and this posed a great drain on the Indian foreign exchange. It was projected that by 2020 A.D, India would have to produce 66 million tonnes of oil seeds to meet the oil demand of 21.8 million tonnes. Otherwise, India has to helplessly depend on imports (Persaud and Landes, 2006). This is a big challenge before India.

TRADITIONAL METHOD RESULTS IN SIGNIFICANT LOSS IN THE PRODUCTION OF COTTONSEED OIL

More than 95% of the cottonseed processed in India is through traditional (crude) method,

TABLE 1
Production of oilseed/oil and net domestic availability of edible oil in India (2002-07)

(in 00000 metric tonnes)

	Oilseeds	Oil
Groundnut	48.6	11.3
Rapeseed-mustard	74.4	22.0
Soybean	99.9	14.2
Sunflower	14.4	3.9
Sesame	5.8	1.8
Niger seed	0.7	0.2
Safflower	2.2	0.7
Castor	7.9	3.2
Linseed	1.8	0.5
Coconut	-	4.5
Cottonseed	-	6.3
Rice bran	-	7.0
Solvent extracted oil	-	3.5
Tree & Forest origin	-	1.2
Less: Export of industrial	-	7.8
Net domestic availability of edible oils		72.4
Import		42.2
Actual consumption		114.6

Source: AICOSCA News Letter, Mumbai, February 2009, p.4

wherein the seeds are simply crushed through mechanical means without undergoing the pre-process formalities of delinting or dehulling, etc. (Barwale *et al*., 1999). The process is rather primitive in nature and yields only about 12-13% crude oil, which is inferior in term of quality and dark in colour. Moreover, it produces about 80-85% cake which has low nitrogen and high fibre content (Jayaraman, 2004). The process also results in passing of about 6 to 7% oil in oilcake, which is considered as a waste. It is a myth that oil content in the cottonseed cake raises milk yield or fat percentage in the milk. In addition, oil is generally required to generate energy. The energy is generated while cattle carry on rumination of the fibrous portion of the feed. The ruminant have a specialized digestive system with compound four compartment stomach. Therefore, the oil content in oilcake is virtually not of much use to the cattle (Santhanam and Sundaram, 1999). There is a loss around 500,000 to 600,000 tonnes of cottonseed oil every year due to the practice of undecorticated cottonseed cake feeding to the cattle (Bhale, 1999).

FAT FACTOR

Cooking oil is pure fat obtained from plants or animals. Whether from plants or animals, one gram of cooking oil or fat provides about nine calories. Experts recommend a total of 20 gm or four teaspoons of oil for an adult. The type of oil being consumed determines a person's risk of heart disease. All cooking oil is made up of a combination of fatty acids, which may be saturated, mono-unsaturated or poly-unsaturated. Their relative proportions classify the oil as saturated, mono-unsaturated or poly-unsaturated (Dohlman *et al*., 2003).

Saturated fat found in animal fat like ghee, butter, lard (fat in meat) is solid at room temperature and raises LDL (bad) cholesterol in blood (Tomek and Peterson, 2001). At the same time, it reduces HDL (good) cholesterol. Good for infants and young children, the fat is a definite no-no for heart health. Plant oils like palm oil (palmolein) and coconut oil are also very high in saturated fats and should be totally avoided (Cheng Hai, 2002).

On the other hand, both Mono-Unsaturated Fatty Acids (MUFA) and Poly-Unsaturated Fatty Acids (PUFA) are considered 'good' fat because they lower total LDL cholesterol. Ideal cooking oil requires a balance between MUFA and PUFA levels. Also, since omega-3 fatty acids are extremely good for the heart, selecting an oil rich in omega-3s is therefore important. Thus, which oil is good for cooking?

Mustard oil, canola (rapeseed) oil, olive oil and groundnut oil have the best combination of good and bad fatty acids. Meanwhile, gingelly oil is another excellent option. Soybean, corn, sunflower and safflower oils have low saturated fatty acid contents, but their MUFA content is lower than the PUFA content, which is not desirable.

Since time immemorial, mustard oil is used in India and recent research lauds mustard oil as ideal. A study by the Harvard School of Medicine, All India Institute of Medical Sciences, New Delhi, and St. John Hospital, Bangalore, found that chances of heart disease drop by nearly 70 per cent when mustard oil is used for cooking (World Bank, 1997). Moreover, its nutritional benefits are comparably better with the much-hyped olive oil at one-sixth the cost. Cooking in mustard oil (especially the cold-pressed or Kachchi ghani variety) could be the wisest health investment one can make.

Cottonseed oil contains about 50% essential poly-unsaturated fatty acid (linoleic acid) against about 30% in the traditional oil, which is required in human diet as it is not biosynthesized in human body and prevents coronary arteries from hardening. It is safe and suitable for human consumption. It is one of the few oil types which are in the "OK FOOD" list of American Heart Association (AHA) and considered as "Heart Oil". The oil is nutritive as certified by AHA. As stated earlier, cottonseed oil has high level of natural antioxidants. These antioxidants make cottonseed oil a natural preservative that contribute long fry life and shelf life for many different food sources.

Cottonseed oil is moderately rich in MUFA and PUFA and it is also an ideal cooking medium. An alternative product from refinery is a partially processed cottonseed oil and sold as Washed Oil. It is ideal oil in the manufacture of margarine (Butter substitute) and bakery products.

Another advantage is that it does not allow speedy blackening of coronary arteries by forming hard pellets of cholesterol. In view of the above, cottonseed oil should be made mandatory to be used in the form of blend, either with oil or with hydrogenated vanaspati. It can also be supplied in the form of encapsulated material.

The fatty acids are essential for synthesis of various hormones, without which, the internal vital organs of the human body cannot function properly. The fatty acid profile of various edible oil types is given in Table 2.

WHY IS THE COTTONSEED OIL HIGHLY ACCEPTABLE AS AN EDIBLE OIL?

Even though cottonseed oil is darker than soybean, peanut and other traditional oil types in colour, the impurities and pigments are readily removed by modern refining and bleaching techniques to produce lighter colour. It possesses properties that make it suitable for processing in salad oil. The proportion of highly saturated glycerides is such that when the oil is chilled slowly, the higher melting glycerides separate out and can be readily removed by filtration which does not get crystallized when held at 40° to 45°F. The high melting portion is generally utilized in blended oils for shortening or in hydrogenated products. It contains traces of fatty acids with instauration greater than linoleic acid. On hydrogenation, the instauration decreases and stability is further increased. Unlike soybean oil, cottonseed oil has greater resistance to flavour reversion. The stability is also due to the presence of antioxidants, namely tocopherols (Srinivasan, 2004).

TO FRY IN

The oil is primarily used as a medium for frying and for manufacturing of hydrogenated vegetable products, cooking, and salad dressings and production of soap. Now, which oil is the best for frying? The high heat during frying decomposes or breaks down the oil producing cancerous substances. So, the best oil for frying is one that can withstand the high temperature without foaming and smoking. Groundnut and gingelly oil are especially good for frying.

All said and done, the best bet is to use a variety of oils judiciously. A combination of several oil types ensures a healthy intake of all important fatty acids. It may also wise to rotate the good oil types over the months. Thus, use mustard, sesame, canola or olive oil (extra light or refined) for cooking, groundnut oil for frying, and olive oil (extra virgin) for salads and pasta. As for cooking oil, less is more.

Speaking of fats, one should not ignore the latest demon on the health scene, i.e. trans fats or vegetable oil that have been partially hydrogenated. Meanwhile, food manufacturers add hydrogen to liquid vegetable oil and heat them. As a result, the oil is hardened and the shelf life is increased. Food made with such partially hydrogenated fat has better texture, flavour, taste and spreadability.

Cottonseed oil is commonly used in manufacturing potato chips and other snack food. Along with soybean oil, it is very often partially or fully hydrogenated. The growing consensus is that in hydrogenated (trans fats) form, these oil types are very unhealthy. Cottonseed oil was the first oil to be hydrogenated in mass production, originally intended for candle production, and also as a food. In part because regulations apply differently to non-food crops, it has also been suggested that cottonseed oil may be highly contaminated with pesticide residues; however, insufficient testing has been done to prove this. Cotton (oil) is also one of the big four (soy, corn, rapeseed/canola, and cotton) genetically modified crops grown around the world. Fried foods and fast food chains commonly use cottonseed oil and vegetable oil blends to fry everything.

TABLE 2
Fatty acid composition of various edible oil

Oil source	Fatty acids (%)						
	Myristic (14:0)	Palmitic (16:0)	Stearic (18:0)	Others	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)
Cottonseed	0.79	24.70	2.20	-	20.87	50.76	-
Groundnut	-	13.69	1.96	3.28*	52.13	28.94	-
Sunflower	0.38	4.27	5.46	-	49.41	40.48	-
Safflower	1.50	3.00	1.00	-	33.50	61.00	-
Til	-	10.02	5.85	-	40.11	44.02	-
Soybean	-	10.33	3.86	-	26.52	52.92	6.37
Corn	-	14.98	1.31	-	34.12	49.59	-
Mustard	-	2.10	0.39	3.010*	10.31	13.80	11.52
Coconut	18.76	8.38	2.18	62.17*	6.96	1.55	-
Palm	1.50	45.00	4.00	-	39.00	10.50	-

* This includes caproic, caprylic, capric, lauric, arachidic, dehenic and lignoceric, either all or a few in different properties:

Palmitoleic (16:1)	Cottonseed 0.68%	Mustard 0.26%
Elcosenoic (20:1)	Mustard 7.39%	
Decosenoic (22:1)	Mustard 51.2%	

Source: AICOSCA News Letter, Mumbai, June 2008

WHY IS TRANS FAT BAD?

The processed food industry loves trans fats (Pray *et al*., 2001). One can find it in biscuits, crackers, cookies, breads and buns, breakfast cereals, bread spreads and salad dressings, cakes and cake mixes, microwave popcorn, pizza, burgers and French fries, heat-and-eat curries, artificial creamers, chocolates and candy, mithai and ice cream.

If trans fat makes food so tasty, why are they bad? Trans fat fouls up the body's entire machinery, and it causes weight gain and excessive abdominal fat – both risk factors for heart disease and diabetes. Moreover, it increases LDL cholesterol, which clogs up arteries, and lowers the HDL cholesterol, which will otherwise take HDL cholesterol to the liver where it would be broken down and excreted. In fact, trans fat interferes with metabolism of fats and elevates blood triglyceride levels. If one continues to eat trans fat-rich foods, it sets the stage for a heart attack.

Ironically, there is no safe limit for trans fat. Some amounts of trans fat are found in whole milk and meat, but this is too small an amount. Trans fat that is found naturally in animal fats is much less harmful than that found in partially hydrogenated oil.

In its natural un-hydrogenated state, like all vegetable oil, cottonseed oil has no cholesterol. It also contains no trans fatty acids. However, it contains over 50% omega-6 fatty acids and only trace amounts of omega-3 fatty acids, and the imbalance is considered unhealthy if not used in moderation or balanced elsewhere in the diet. Furthermore, this poly-unsaturated fat can potentially go rancid during the extraction process.

THE FACTS OF COTTONSEED OIL

Cottonseed oil is extracted from cottonseed. It plays a vital role in terms of adding value than any other by-products of cottonseed. In USA, it is a part of diet for well over a century. Until the 1940's, it was the major vegetable oil produced in the United States. Now, it ranks third in volume after soybean and corn oil, representing

about 5-6% of the total domestic fat and oil supply. The average annual production crosses more than 1 billion pounds in the United States.

Cottonseed oil has many food applications. As salad oil, it is used in mayonnaise, salad dressings, sauces, and marinades. As cooking oil, it is used for frying in both commercial and home cooking. As shortening or margarine, it is ideal for baked goods and cake icings. It is primarily used in the U.S. as a salad or cooking oil. About 56% is consumed in that category, while about 36% goes into baking and frying fats, and a small amount into margarine and other uses.

Cottonseed oil has a mild, nut-like taste, clear, with a light golden colour, flavour stability and is used as a yardstick for measuring flavour and odour qualities in other oil. Meanwhile, refined and deodorized cottonseed oil is one of the purest food products available. Few food types can be as highly cleaned and refined, and still maintain their nutritional quality. Cottonseed oil meets the government's strict standards for purity. Unlike some oils, cottonseed oil does not deteriorate or "revert" rapidly in flavour when it is used at high temperatures. Owing to acceptability of reducing saturated fat intake, it is considered as one of the few unsaturated oil, like safflower, corn, soybean, canola, and sunflower seed oil.

Cottonseed oil has a 2:1 ratio of poly-unsaturated to saturated fatty acid. It is described as naturally hydrogenated because its fatty acid profile generally consists of 70% unsaturated fatty acids, including 18% mono-unsaturated (oleic) and 52% poly-unsaturated (linoleic), and 26% saturated (primarily palmitic and stearic). These make the oil stable for frying without the need for additional processing or the formation of trans fatty acids.

Cottonseed oil does not have to be as fully hydrogenated for many purposes as some of the more poly-unsaturated oil. When it is partially hydrogenated, however, its mono-unsaturated fatty acid actually increases. When hydrogenated to a typical Iodine Value of about 80, for example, its fatty acid profile shifts to 50% mono-unsaturated, 21% poly-unsaturated, and 29% saturates all well within current diet/ health guidelines.

Cottonseed oil is rich in tocopherols. These natural antioxidants, which have varying degrees of vitamin-E activity, also contribute to its stability giving products that contain it a long shelf life. It can be found as an ingredient in many food products and is available on the grocery shelf only in limited areas. Cottonseed oil is light, non-oily consistency and high smoke point, making it the most desirable for stir fry cooking and other oriental dishes, as well as for frying fish.

NEGATIVE IMPACTS OF COTTONSEED OIL

The renowned nutrition expert, Dr. Andrew Weil, says cottonseed oil contains both naturally occurring toxins and pesticide contaminants. Cottonseed oil is generally extracted by using harsh chemical solvents and heat which may alter the chemistry of the oil. Most nutritionists are still uncertain about the long-term implications of these changes. Cottonseed oil is high in Vitamin E, which is an antioxidant. Antioxidants present in cottonseed oil work against the free radicals that cause cell damage aging. Nonetheless, it has very low amounts of heart-healthy omega-3 and mono-unsaturated fats.

In addition, cottonseed oil contains gossypol, a substance that has been shown to cause sterility in rats. For this reason, it has been used in parts of the world as a contraceptive and cottonseed oil has been seen as a threat to men's fertility. A 2006 study done at the University of Lecce, Italy, 'proteinaceous diet inhibits gossypol-induced spermatotoxicity" showed that gossypol in cottonseed oil is not an effective contraceptive, because if combined with most proteins, gossypol no longer causes infertility. Gossypol still has toxins that decrease spermatogenesis and sperm motility in men. This is a topic that should be brought up with fertility doctors because cottonseed oil is a very commonly used ingredient in many foods.

COTTONSEED OIL: INDIAN PERSPECTIVE

The survey has confirmed that cottonseed oil is a significant source of income for the community in the main cotton producing districts of India and that within the local market cottonseed is valued for cooking oil. In terms of gender, cotton is a crop which is almost exclusively the domain of women. The crop is gathered by women and children and processed by them. Cottonseed oil is enshrined in the domestic culture of women and is used as source of edible oil which has less fatty contents and good for health. Men are mainly involved in the storing and marketing of cottonseed oil (Sharma, 2006). Research has found that the high involvement of women in purchasing and consumption of cottonseed oil is likely to be the most lucrative commercial activity of the oil market. Therefore, any increase in demand for cottonseed oil would have a considerable positive economic impact on their livelihoods.

For dietary and food security importance, cottonseed oil is highly prized as health oil during the lean season and is an important source of fat for most of the villages in the northern districts. Thus, cottonseed oil is one of the best sources of cooking oil.

WHOLESOME TIPS

The maxim 'Health is Wealth' advocates that people are health conscious and subjected to follow certain tips to keep their health in good condition. As a general rule, cook wholesome meals at home with healthy oils. When shopping, avoid food that contains shortening, hydrogenated or partially hydrogenated oil as an ingredient. Since it is now mandatory to put trans fats content on the label, choose food with no trans fat. At the same time, avoid eating at roadside dhabas, takeaways, and halwais. Restrict eating out to once a month. Avoid gravies, creamy sauces, and salad dressings. Opt for stir-fried over fried and grilled over curried.

CONCLUSIONS

The utilization of cottonseed oil for human consumption should receive immediate attention in India for meeting the shortage of edible oil (Mehta, 2006). It contains more than 50% of poly-unsaturated fatty acids and is very ideal in human diets. Cottonseed oil is very popular in USA (Young and Westcott, 2000). However, in India, it is used to a very small extent. Therefore, efforts are to be made on a war footing to popularize its use in our country, which can eventually result in stoppage of import of other edible oil, at least to some extent. Efforts are also needed to popularize cultivation of varieties of cotton with high percentage of oil. Nonetheless, most of the people need to curb the total amount of fat in their diet (Morris, 2002). A combination of oil ensures a healthy intake of all important fatty acids. Therefore, use mustard, sesame, canola or olive oil (extra light or refined) for cooking, groundnut oil for frying, and olive oil (extra virgin) for salads and pasta. With cooking oil, less is more.

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Short Communications

Physical Characteristics and Weight Relationship of *Gigantochloa scortechinii* (Buluh Semantan) 1-, 2- and 3-Year Old Natural Stand Bamboos

Azmy Hj. Mohamed[1*], Sulaiman Othman[2], Razak Wahab[3] and Rokiah Hashim[2]
[1]Faculty of Forestry,
[1]Institute of Tropical Forestry and Forest Products (INTROP),
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Universiti Sains Malaysia, 11800 Minden, Penang, Malaysia
[3]School of International Tropical Forest, Universiti Malaysia Sabah,
Lock Bag 2073, 88999 Kota Kinabalu, Sabah, Malaysia
[*]E-mail: drazmy2002@yahoo.com.sg

ABSTRACT

Physical characteristics, such as average number of internodes, internode length, culm wall thickness, circumference of internodes, and weight of culm, branch and leaf of 1-, 2- and 3-year old culms were determined. Data were gathered from 47 to 62 of 1-, 2- and 3-year old culms in Kedah, Peninsular Malaysia. Significant linear relationships between weight and solid volume of the bamboo were observed for all the various ages studied. In addition, their corresponding regression equations were also determined. The 3-year old culm had the biggest mean for all the characteristic features, except for the Diameter-breast height (Dbh) and the culm wall thickness at the base. This was followed by the 2-year old culm. Meanwhile, regression of the height and culm weight on the Dbh gave the best regression line for 1 year old culm in terms of its r-squared value of 84 and 86 percent in comparison to the 2 and 3-year old culm.

Keywords: Culm age-internodes, Diameter-breast height (Dbh), weight, volume

INTRODUCTION

Most of Malaysian bamboos are found in logged-over areas and disturbed forests (Burton, 1979; Ng and Mohd Noor, 1980). Out of 50 cultivated and wild bamboo species in the forest, only 14 types of bamboo have been exploited for their culms and shoots (Wong, 1989).

The cottage industries in Malaysia use bamboo on an organized scale. For this purpose, some bamboo species, such as *Gigantochloa scortechinii* (buluh semantan) and *G. wrayi* (buluh beti), have been used in the manufacturing of poultry cages, shade blinds and barbeque sticks, vegetables baskets, incense sticks, and tooth picks (Wong, 1989; Azmy, 1989). In particular, *Gigantochloa scortechinii* (buluh semantan) has been an important bamboo commercial species used in the utilization of bamboo products in the country. In addition, the culms can be used to produce high value-added products such as laminated panelling for parquets and furniture.

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[*]Corresponding Author

It is important to note that bamboos vary considerably in size, depending on their species, locality and vigour of the clump (Krishnaswamy, 1956). Meanwhile, certain attributes, such as stem size and wall thickness, influence the range of their usage (Wong, 1982). The strength, straightness, lightness combined with hardness, variation in size, ease with which they can be propagated and the short period taken to mature and the available for harvesting have rendered bamboos immense uses (Sharma, 1982).

The anatomical structure of a bamboo culm determines its properties and assesses the characteristics of the culm in relation to its end product (Abd. Latif, 2001). The anatomical properties are insignificantly correlated with the age and height of culm (Abd. Latif, 1993). For instance, the growth towards the maturation stage of *Gigantochloa scortechinii* began at the age of 3.5 years and above (Norul Hisham *et al*., 2006).

A study on the physical characteristics and weight relationship of *Gigantochloa scortechinii* (buluh semantan), based on various age levels, has not been reported in Peninsular Malaysia. Therefore, this study is an attempt to give comprehensive information on the characteristic qualities of the most important natural stand bamboos in Peninsular Malaysia for cottage and commercial industries references. Additionally, using regression techniques, the weight-volume relationships were also determined based on various ages.

MATERIALS AND METHODS

The study was conducted in north Peninsular Malaysia, whereby culms of 3 various ages were selected, namely 55 culms of 1 year old, 47 culms of 2 year old and 62 culms of 3 year old, taken from Nami, located in Kedah, Peninsular Malaysia (see *Fig. 1*). The culm age of *Gigantochloa scortechinii* was determined by tagging it four years earlier at an experimental area during the shoot's sprouting stage. The required data were gathered from the freshly harvested culms: (1) for each whole culm, the circumferences of the node and the internode were measured from the base upward at an interval of five nodes; (2) the length of each internode was also measured from the base upward and the number of internodes was noted for all the culms in each age; (3) the culm's wall thicknesses of the base and apex were measured using a vernier caliper to the nearest tenth of a centimetre. The average culm wall thickness on all four directions and the average Dbh were also determined. The Dbh was determined at 1.3m above the ground.

For each age, the culms were cut into four equal sections, and these are 0 to 25, 25 to 50, 50 to 75, and 75 to 100% of the whole culm. The culm wall thickness, fresh weight and solid volume were determined from each section. The culms were cut into four equal sections to determine them to the nearest estimate of the culm wall thickness. In addition, the branch and leaf weight of each culm were also measured. The solid volume of each equal section was computed using the formula by Tandug and Torres (1985). The formula is given below:

$$V = \frac{(A1 - A2) + (a1 - a2)}{2} \times L$$

where

V = solid volume of each section of the culm (cm^3);
A1 = area of the large end of the section (cm^2);
A2 = area of the large end of the hollow portion (cm^2);
a1 = area of the small end of the section (cm^2);
a2 = area of the small end of the hollow portion (cm^2);
L = length of the section (cm).

The total green weight (kg) was derived by summing up the values obtained for each section. Meanwhile, the total volume and weight for each culm were determined and used in the fitted regression. The branch and leaf weight of each culm were also measured according to each age. The culms were harvested during the dry season and the green weight was recorded in the forest.

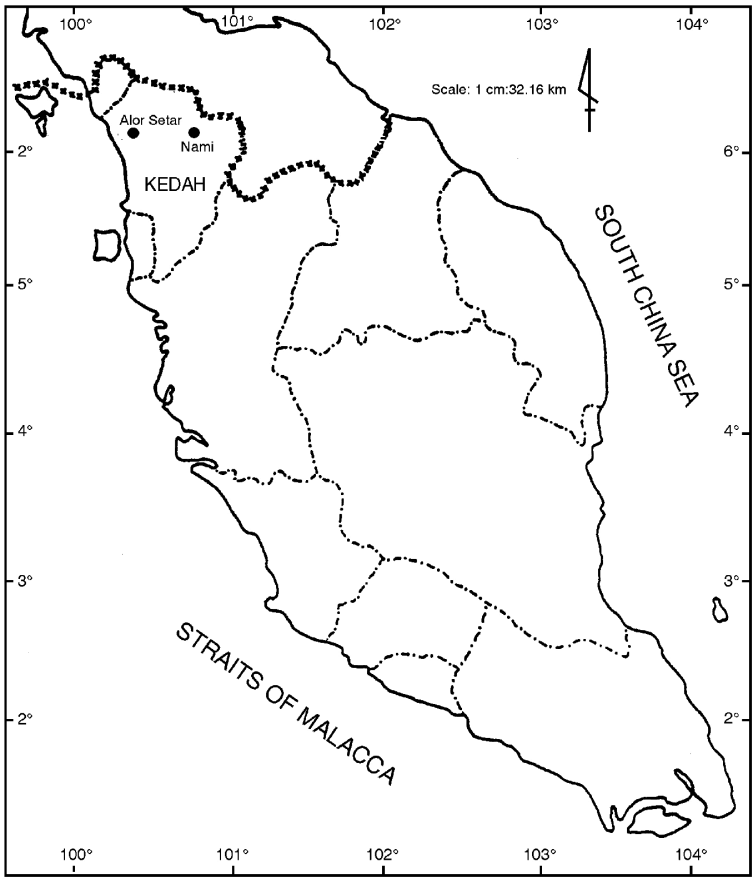

Fig. 1: Location map of study

RESULTS AND DISCUSSION

Measurement Attributes

1 year old culms

55 culms of 1 year old *G. scortechinii* that were gathered for the study had the mean culm lengths ranging from 11.5 to 13.9 m, as depicted in Table 1. The average internode was 33.8 cm, with the shortest at both ends (apex and base) and the longest in the middle. A normal culm of the 1 year old had relatively thick walls all throughout and become gradually thinner towards the top. The thickest section was located at the base, and this ranged from 0.4 to 0.7 mm (Table 1). It was large at the base and gradually tapered towards the top. The mean Dbh was 9.5 cm, while the mean culm wall thickness at the base was 0.4 mm. The mean culm length and the mean number of internodes were 11.5 m and 33.3 cm, respectively. In addition, the mean average internode length and the mean average circumference were 33.8 and 4.7 cm, respectively. The mean culm weight was 10.7 kg, while the mean branch and leaf weight were 0.7 and 1.1 kg, respectively (Table 2).

2 year old culms

47 culms selected in this study had a mean length of 12.3 m (Table 1). The mean number of the internodes was 35.1 cm whereas their

TABLE 1
The physical characteristics of *Gigantochloa scortechinii* culm (1- 3 year old)

Year	No. of samples	Culm length (m)		No. of internodes (cm)		Average internode length (cm)		Average circumference of internodes (cm)		Dbh (cm)		Culm wall thickness at base (mm)	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
1	55	11.5	2.0	33.3	3.5	33.8	2.5	4.7	1.2	9.5	1.8	0.4	0.3
2	47	12.3	1.9	35.1	3.3	35.3	2.9	5.3	1.14	10.0	1.4	0.5	0.4
3	62	13.9	1.6	35.6	2.9	37.2	2.5	5.7	1.1	10.2	1.2	0.7	0.6

mean average internode length was 35.3 cm. Meanwhile, the mean average circumference of the internodes was 5.3 cm. The mean Dbh and culm wall thickness at the base were 10 cm and 0.5 mm, respectively. The 2 year old culms had a mean weight of 13.2 kg, and this was found to be 0.8 kg for the branch and 1.9 kg for the leaf (Table 2). Its standing culm does not have any culm sheath available. As presented in Table 2, the mean volume for the 2 year old culm was 177.2 cm^3.

3 year old culms

Sixty two samples of the culms had a mean length of 13.9 m. From Table 1, all the mean physical characteristics of the 3 year old culms tended to be higher than those of the 1 and 2 year old culms. Apparently, the mean volume for the 3 year old culms was greater than the culms of other ages, i.e. 219.1 cm^3 (*see* Table 2).

Measurement Attributes of Natural Stand Bamboos According to Age

The measurement attributes of the natural stand bamboos undertaken in this study were determined according to their various ages of 1, 2, and 3 years and above. A simple linear regression was used to retrieve the relationship between heights versus Dbh. From Table 3, it was observed that the linear regression equation for the one year old culms gave the best equation in terms of the correlation coefficient of 84 percent of its r-squared compared to the 2 and 3 year old culms with merely 77 percent and 76 percent, respectively. On the contrary, the 3 year old culms gave a poor coefficient of correlation. The regression of height versus Dbh was significant for all the culms of the 3 years covered, except for the 3 year old culms whereby they had a significant value of 1.981 at 0.05 level (*see* Table 3).

Meanwhile, the regression equation of the culm weight versus Dbh for the 1 year old also gave the best equation, as presented in Table 4, with 86 percent of its r-squared. The 3 year old culms, once again, gave the lowest value for 79 percent of their r-square. All the regressions were found to be significant for the culms of 1, 2, and 3 year old and above. The T-values were 13.0, 74.3 and 62.5, respectively. Thus, it was significant at 0.05 level.

TABLE 2
Volume and weight of *Gigantochloa scortechinii* culm (1-3 year old)

Year	No. of samples	Weight of culm (kg)		Branch weight (kg)		Leaf weight (kg)		Volume (cm^3)	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
1	55	10.7	2.6	0.7	1.0	1.1	0.7	147.5	10.0
2	47	13.2	2.7	0.8	0.7	1.9	0.9	177.2	11.0
3	62	15.8	2.8	0.9	0.8	2.03	1.03	219.1	9.7

TABLE 3
The regression equations of the height on Dbh for 1, 2 and 3 year old *Gigantochloa scortechinii* culms

Year	Y = a + bx	r^2 (%)	T values
1	Y= 0.425433 + 1.93322x	0.84	11.60
2	Y= 0.286244 + 1.89367x	0.77	8.09
3	Y= 4.47962 + 1.31436x	0.56	1.98

TABLE 4
Regression equations of the culm weight on Dbh for 1, 2 and 3 year old *Gigantochloa scortechinii* culms

Year	Y = a + bx	r^2 (%)	T values
1	Y = -9.69221 + 3.38073x	0.86	13.00
2	Y = -13.1258 + 4.15221x	0.87	74.3
3	Y = -21.1764 + 5.09095x	0.79	62.5

Based on the above results, the significant regressions could therefore be used in predicting the height and culm weight from the Dbh measurement. In particular, a higher r-squared would give us more confidence in the prediction.

Weight Volume Relationship

The relationship between the green weight and solid volume of the culms of different ages (Table 5) was also determined using the regression method in this study. It was found that the 3 year old had the heaviest mean culm, branch, and leaf weight (15.8 kg) compared to the 1 and 2 year old culms, with merely 0.9 and 2.03 kg, respectively. The mean volume for 1 year old culms was 147.5, and this was 177.2 for the 2 year old culms and 219.1 cm^3 for the 3 year old culms. The highest volume was found for the 3-year old culms.

The regression results presented in Table 5 indicate a highly positive linear relationship between the volume and weight of the bamboo culms of all ages. In more specific, the highest coefficient of determination of the r-squared was 86, which was obtained for the 1 year old culms. The fitted regression lines are shown in *Fig. 2*.

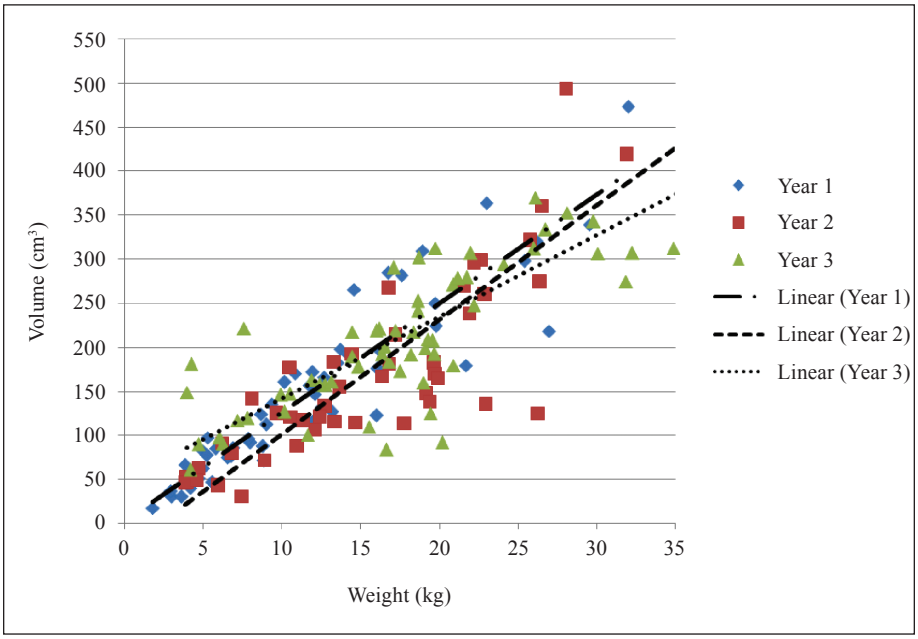

Fig. 2: Fitted regression lines of volume on weight for the 1, 2 and 3 year old culms

TABLE 5
Regression equations of the volume on weight of each culm age

Year	Y = a + bx	r^2 (%)
1	Y = 12.385 + 2.032x	0.86
2	Y = 13.003 + 29.462x	0.75
3	Y = 9.276 + 48.519x	0.72

where Y = volume (cm^3); x = weight (kg); r^2 = coefficient of determination

CONCLUSIONS

Among the culms of the three ages studied, the 3-year old ones had the highest mean for all the characteristics, except for the Dbh and the wall thickness at the base. Meanwhile, the 2-year old culm was second, followed by the 3-year old culms in terms of all the characteristics except for the wall thickness.

The mean diameter-breast height and the wall thickness at the base of the culms of all ages seemed to be bigger and thicker in relation to the age factor. This is probably because both these characteristics only expanded and grew in their culm wall thickness at the age beyond 1 year old, as compared to the growth and thickening of the culm walls which occur at the early establishment stage, i.e. from the shoot sprouting stage until almost 3-4 months, and up to its utmost height. They become mature culms at three year old and above.

As for the 3-year old culm, it had the longest mean internode length. Based on the distinguished features, the 3 year old culm is suitable for making chopsticks, barbeque sticks, toothpicks, and other bamboo products (Azmy, 1989). Based on the physical characteristics of bamboos, moreover, the 3 year old culm is much better in terms of its length, internode length as well as Dbh and circumference. Thus, at this age, the utilization of culm is much preferred compared to culms of other age (i.e. 1 and 2 years old).

Significant positive linear relationships between green weight and solid volume of all the culm ages were observed for all the culms of different ages undertaken in the study. Therefore, the relationship between the measurement characteristics and weight-volume for all the culms of different ages is important in determining their yields and uses.

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Diversity in Rice Genotypes under Salt Affected Soil Based on Multivariate Analysis

A. Anandan*, R. Eswaran and M. Prakash
Department of Genetics and Plant Breeding, Faculty of Agriculture, Annamalai University, Chidambaram – 608 002, Tamil Nadu, India
**E-mail: anandanau@yahoo.com*

ABSTRACT

Diversity of forty four salt tolerant rice genotypes from different geographic regions were assessed using Mahalanobis D^2 and principal component analysis (PCA). The D^2 statistics grouped the genotypes into 12 distinct clusters. Consisting of 19 genotypes, Cluster I was the largest cluster, followed by Cluster II with nine genotypes. Meanwhile, Clusters III, IV, VI, VII, IX, and XII were mono-genotypic clusters. The maximum intercluster distance was exhibited between Cluster IX and X (144.91), followed by Clusters II and X (131.87), as well as clusters VII and X (126.27). The number of grains per panicle (42.71%), followed by the grain yield per plot (29.81%), was the major contributor to the total divergence. The PCA revealed that axes 1 and 2 accounted for 82.88% and 11.14% of the variance, respectively. The highest contributing variable was the number of grains per panicle in PC1 and the plant height in PC2. The genotypes from more than one place of origin were grouped in one cluster, whereas the genotypes from one state were grouped in more than one cluster. Both D^2 and PCA revealed that the morphometric diversity was based on the pedigree and independent of geographical origin. Hybridization among the genotypes which had the maximum inter-cluster distances could produce heterotic combinations and wide variability in segregating generations for many beneficial traits.

Keywords: Morphometric diversity, Mahalanobis D^2, *Oryza sativa*, principal component analysis, rice

INTRODUCTION

The presence of excess salt is one of the most widespread soil toxicity problems in many rice growing areas. In particular, it accounts for 8.5 million hectares of land in India and the yield reduction is estimated at 30-50% (Babu *et al*., 2005). The success of any breeding programme is dependent on the available genetic divergence in the crop. Rice germplasm is known to be a rich source of salt tolerant genes (Yeo and Flowers, 1982) for improving salinity tolerance in high yielding varieties.

A narrow genetic base among released cultivars and the practice of using elite line x elite line crosses have been implicated in slowing the rate of genetic advance for yield (Lal and Rana, 2000). Meanwhile, divergent parents in a cross have a greater scope of obtaining heterotic F_1s and a broad spectrum of variability in the segregating generations in any varietal improvement programme (Arunachalam, 1981). Hence, analysis of genetic relationships among extant genotypes is an important component of crop improvement programme as it provides information about genetic diversity and helps to stratify breeding populations.

*Corresponding Author

Clustering germplasm into various groups, using hierarchical or non-hierarchical algorithms based on the multivariate statistical techniques and sampling from within discrete groups, is a common method used for maximizing diversity. Thus, the present investigation was designed and carried out to: (i) investigate the extent of genetic diversity in saline tolerant rice genotypes, (ii) identify promising genotypes for future utilization in hybridization for developing saline tolerant genotype with high yield, and (iii) estimate the nature and magnitude of the relationship between quantitative traits associated with yield under salinity environment.

MATERIALS AND METHODS

In the present study, forty four genetically diverse salt tolerant genotypes of rice taken from the different regions of India were investigated (Table 1). It is important to note that these genotypes were grown in saline soil with electrical conductivity (EC) of 2.83 ds m^{-1} during the dry seasons of 2006 and 2007. The experiments were conducted at the experimental farm of Plant Breeding (11° 24' N latitude and 79° 44' E longitude, + 5.79 m ASL), located in Annamalai University, Tamil Nadu, India. The seeds were sown in a raised nursery bed with good irrigation water, whereas the 25-day old seedlings were transplanted at the field. The experiment was laid out in a randomized block design with three replications, using 20 x 20 cm spacing in 10 m^2 plots. Crop management corresponded to intensive farming methods with full insecticide and fungicide cover. The recommended fertilizer doses of 150 kg nitrogen ha^{-1}, 50 kg phosphorus ha^{-1}, and 50 kg potash ha^{-1} were applied. Manual weeding was carried out twice. Observations were recorded on ten randomly selected plants in each experimental unit for seven characters, namely, days to 50% flowering, plant height, number of productive tillers, panicle length, number of grains per panicle, yield per plot, and percentage of survival. Meanwhile, the data gathered from the two years were pooled in the analysis and subjected to estimate genetic divergence. The D^2 analysis was done as per Mahalanobis's D^2 –statistic and extended by Rao (1970). The estimation of the D^2 values was according to the following formula:

$$D^2 = \sum\sum w^{ij} \left(\overline{x}_i^1 - \overline{x}_i^2\right)\left(\overline{x}_j^1 - \overline{x}_j^2\right)$$

Where, w^{ij} is the inverse of variance and co-variance matrix.

Furthermore, $_{the}$ computation of the D^2 values was reduced to simple summation of the differences in the mean values of various characters of two populations, i.e. Σd^2_i. Therefore, the correlated variables were first transformed to the uncorrelated ones so as to work out the D^2 values.

$$D^2 = \sum d_i^2 = \sum \left(Y_i^j - Y_i^k\right)^2$$

Where, Y is the uncorrelated variable which varied from i = 1 to x, i.e. the number of characters. Y_i^j and Y_i^k represented the transformed uncorrelated mean of the i^{th} character for the genotypes *j* and *k*, respectively. The genotypes were then grouped on the basis of minimum generalised distance using the Tocher's method as described by Rao (1970), and this was followed by the principal component analysis (PCA). These analyses were performed using the software Windowstat 7.5 (Indostat Services, Hyderabad, India).

RESULTS AND DISCUSSION

Based on the D^2 analysis, the genotypes under saline soil condition could be grouped into 12 clusters (Table 2). Cluster I comprised of 19 genotypes, whereas Cluster II had nine genotypes. The genotypes from a few states (Andhra Pradesh, Haryana, Maharastra, Orissa, and Uttar Pradesh) or with similar pedigree were grouped in the same cluster, as revealed by Clusters I and II. This is in agreement with the findings reported by Arunachalam and Ram (1967). In contrast, some genotypes taken from Andhra Pradesh, Maharastra, and West Bengal were grouped in Cluster V,

TABLE 1
List of rice genotypes selected for diversity analysis, with their parentage and place of origin

S. No.	Genotype code	Parentage	Origin
1	G1	KDML 105 x IR 4630-22-2-5-1-3 x IR 20925-33-3-1-28	Haryana, India
2	G2	Savithri x Lunishree	Orissa, India
3	G3	Jaya x Lunishree	Orissa, India
4	G4	Savithri x Lunishree	Orissa, India
5	G5	Mahsuri x Ormundakan	Orissa, India
6	G6	TCCP 266-249-B-B-3 x IR 262-43-8-1	Uttar Pradesh, India
7	G7	IR 55182-3B-14-3-2-2- x IR 4499-29-2-2-2	Uttar Pradesh, India
8	G8	IR 70804-9-NDR-3-7-91	Uttar Pradesh, India
9	G9	IET 8320 x PNL 1	Maharastra, India
10	G10	PNL 2 x IET 8320	Maharastra, India
11	G11	PNL -2 x IET 8320	Maharastra, India
12	G12	Jaya x CSR 23	Haryana, India
13	G13	IR 72 x CSR 23	Haryana, India
14	G14	Mahsuri x Madhukar 105	Uttar Pradesh, India
15	G15	IR 68661-16-8 x NDR-2-B-2-1	Uttar Pradesh, India
16	G16	Usar 1 x Mahsuri	Uttar Pradesh, India
17	G17	Selection from Kalanamak	Uttar Pradesh, India
18	G18	CSR 21 x CSR 10	Haryana, India
19	G19	IR 64 x IR 4630-22-2-5-1-3 x IR 9764-45-2-2	Haryana, India
20	G20	IR 64 x PNL 2	Maharastra, India
21	G21	IET 13845 x GJ 11	Maharastra, India
22	G22	IR 28 x Chakrakonda	Orissa, India
23	G23	Mahsuri x Chakrakonda	Orissa, India
24	G24	Jaya x Lunishree	Orissa, India
25	G25	Jaya x Lunishree	Orissa, India
26	G26	CSR 23 x CSR 10	Haryana, India
27	G27	Bipasa x Kalojira	West Bengal, India
28	G28	Pankaj x SR 26B	West Bengal, India
29	G29	Nonabokra x IR 36	West Bengal, India
30	G30	Mutant of IR 4630-22-2-5-1-3 x Pokkali	Andhra Pradesh, India
31	G31	IET 9993 (MS) x N 52	Andhra Pradesh, India
32	G32	CSR 3 x Kasturi	Andhra Pradesh, India
33	G33	CSR 3 x Kasturi	Andhra Pradesh, India
34	G34	CSR 3 x Kasturi	Andhra Pradesh, India
35	G35	CSRL-04-2366	Uttar Pradesh, India
36	G36	CSRL-01-IR 75	Uttar Pradesh, India
37	G37	CSRL-01-IR 97	Uttar Pradesh, India
38	G38	IET 9993 (MS) x BM 47	Andhra Pradesh, India
39	G39	Pankaj x SR 26B	West Bengal, India
40	G40	CSR 1 x Basmati 370 x CSR 5	Haryana, India
41	G41	Nona Bokra x IR 5657-33-2	Haryana, India
42	G42	CST 7-1	Haryana, India
43	G43	TN1 x T141	Andhra Pradesh, India
44	G44	IR 578-172-2-2 x BR-1-2-B-19	Tamil Nadu, India

TABLE 2
Clustering pattern of rice genotypes based on the D2 analysis

Cluster No.	No. of genotypes	Genotypes
I	19	G18, G19, G42, G36, G37, G16, G22, G31, G27, G38, G6, G32, G25, G7, G20, G29, G33, G12 and G15
II	09	G13, G30, G23, G8, G2, G1, G10, G9 and G26
III	01	G34
IV	01	G4
V	04	G21, G39, G28 and G43
VI	01	G35
VII	01	G44
VIII	02	G5 and G17
IX	01	G11
X	02	G3 and G41
XI	02	G24 and G40
XII	01	G14

i.e. containing four genotypes (Sinha *et al.*, 2001). The remaining genotypes were scattered into monogenic clusters, namely III, IV, VI, VII, IX, and XII. This seems to suggest that geographical distribution is not necessarily related to genetic divergence (Sarawgi and Shrivastava, 1996). For instance, the scattering of the genotypes from the same geographic region into different clusters may be due to genetic heterogeneity (different genes producing identical phenotypes), genetic architecture and history of selection (Murty and Arunachalam, 1966). In particular, Clusters VIII, X and XI comprised of two genotypes each. The genotypes belonging to these three clusters originated from the states of Haryana, Orissa and Uttar Pradesh. These findings are in an agreement with that of other similar rice studies conducted by Ratho (1984), Rathore *et al.* (2001) and Sharma *et al.* (2008). The grouping of the genotypes from the different states into a single cluster might be due to unidirectional selection practiced by the breeders or free exchange of germplasm among them.

The intracluster distance varied from 0.00 to 40.16, as shown in Table 3. The maximum distances for Clusters XI, V, X, VIII, I, and II were 40.16, 37.12, 36.16, 35.9, 34.54, and 33.73, respectively. Intracluster distance was zero in Clusters III, IV, VI, VII, IX, and XII since each contained only a single genotype. Meanwhile, the minimum intercluster distance was obtained between Clusters III and IV (24.95), and this was followed by Clusters III and V (38.99). The maximum intercluster distance was observed between Clusters IX and X (144.91). The figures stated for II vs. X and VII vs. X were 131.87 and 126, respectively. The presence of high intercluster distanced genotypes permits the selection of divergent parents. This avoids the selection of parents from genetically homogeneous clusters, and thus reducing the breeding of a population with the likelihood of a narrow genetic base. Higher genetic distance between the clusters suggested a wide diversity among the genotypes. The crosses made between the genotypes from the above clusters might give useful transgressive segregants (Sharma and Bhuyan, 2004). According to Rieseberg *et al*. (1999), transgressive segregation is the production of F_2 or later-generation hybrid progeny with phenotypes that could fall outside the phenotypic range of the parental populations from which they were derived. Meanwhile, the improvement of self-pollinated crops is connected with the production of homozygous

TABLE 3
Intra- (bold) and inter-cluster distances for 12 clusters in rice in relation to Table 2

Cluster No.	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
I	**34.54**	50.78	45.41	45.29	72.73	42.05	52.85	83.55	53.75	107.78	88.69	56.33
II		**33.73**	76.01	67.73	106.60	51.08	79.09	102.46	60.36	131.87	114.45	69.51
III			**0.00**	24.95	38.99	45.35	55.74	55.38	78.51	80.15	75.68	63.18
IV				**0.00**	53.40	39.18	59.44	46.03	76.88	89.43	92.01	73.74
V					**37.12**	74.93	68.17	66.15	99.13	79.23	78.03	85.53
VI						**0.00**	76.54	66.54	79.01	97.62	92.84	49.80
VII							**0.00**	99.00	41.83	126.27	101.35	79.91
VIII								**35.90**	119.79	75.67	104.30	102.87
IX									**0.00**	144.91	112.82	82.60
X										**36.16**	66.05	116.27
XI											**40.16**	93.27
XII												**0.00**

selections superior to the parental genotypes. It is widely known that the frequency of the transgression effects in homozygous populations is dependent on cross combinations, i.e. on the genotypes of crossing parents. The characters contributing more to the divergence are given greater emphasis for deciding on the cluster for the purposes of further selection and the choice of patterns for hybridization (Jagadev *et al*., 1991). The differences between the genotypes for the number of grains per panicle, grain yield per plot and percentage of survival were more pronounced as compared to the other traits (Table 4). In particular, the number of grains per panicle seems to be the most important as the percentage of its contribution was the maximum

TABLE 4
Mean performance of different clusters with respect to different traits in rice

Cluster / traits	Days to 50% flowering	Plant height	No. of productive tillers/plant	Panicle length	No. of grain/ panicle	Grain yield/ plot	Survival percent
I	86.86	104.02	19.33	29.20	109.91	5.28	61.37
II	85.63	92.47	17.46	25.52	78.41	1.91	61.34
III	86.33	87.22	14.83	28.10	166.33	6.15	62.00
IV	92.00	110.17	17.33	28.13	165.00	4.06	66.34
V	87.79	113.56	21.96	29.25	190.58	8.70	62.77
VI	77.17	121.53	27.50	28.45	132.67	2.39	61.17
VII	96.67	109.16	16.50	25.03	110.33	9.51	72.28
VIII	90.75	134.84	17.33	26.14	220.75	2.12	64.30
IX	97.83	94.30	22.83	26.48	59.00	7.71	66.78
X	85.58	131.38	17.67	30.21	222.33	5.13	34.85
XI	82.67	113.90	24.83	26.13	144.33	9.12	31.33
XII	60.33	93.85	20.50	26.50	102.33	5.78	60.67
Contribution to diversity (%)	7.08	1.16	1.59	0.42	42.07	29.81	17.86

(42.07%) in its genetic divergence. This was followed by the grain yielded per plant and the percentage of survival (De and Rao, 1987; Chaturvedi and Maurya, 2005).

The cluster mean values showed a wide range of variations for all the traits undertaken in this study (Table 4). In more specific, Cluster VIII was characterized with the high mean values for plant height and the number of grains per panicle. Meanwhile, Cluster XI exhibited a high mean for the number of productive tillers per plant and grain yield per plot. Clusters X, I, and V had the highest mean of panicle length. The single genotypic clusters were quite different from the other clusters by either the highest or the lowest value for a particular character. The monogenotypic Cluster XII had a low mean for days to 50% flowering and plant height. Cluster VI had a high mean for productive tillers per plant and percentage of survival. The putative parents for a systematic crossing programme should belong to diverse clusters which are characterized by a large intercluster distance. Such genotypes have genes with different magnitude of effects and higher probability in term of the chances to obtain recombinants outside the range of parents. Besides the high genetic divergence, considering the contribution of different characters towards the total divergence and the magnitude of cluster means for different characters should also be given due importance in the selection of genotypes for hybridization programme. Based on the above data, the genotypes belonging to Clusters X (G3 and G41), VI (G35), VII (G44), and XII (G14) could therefore be used in the hybridization programme so as to obtain useful recombinants for saline environmental condition.

The graphical representation of the PCA analysis was applied to identify the genetic diversity among the genotypes and the traits responsible for the main source of variability. The first and second principal components (PC) accounted for 82.88% and 11.14% of the variance, respectively (Table 5). Since the first two PC accounted for about 94.02% of the total variability, a two dimensional representation of the relative position of the varieties in the biplot graph was found adequate.

The 82.88% variation in the first PC was mainly due to the variation in the number of grains per panicle. Loadings (latent vector) with large absolute values corresponding to the variables should have a greater discriminating ability. The first PC was positively correlated with all the characters, except for the percentage of plant survival. The largest absolute value for plant height in the second PC indicated that this trait was mainly responsible for explaining 11.14% of the total variance. Meanwhile, the second PC was positively correlated with the

TABLE 5
Principal components for rice genotypes based on seven characters

Parameters	PC1	PC2	PC3	PC4
Eigen values	102366	13755	3934	1717
Percentage variance (%)	82.88	11.14	3.19	1.39
Cumulative variance (%)	82.88	94.02	97.21	98.60
Character		Latent vectors (loadings)		
Days to 50% flowering	0.009	-0.010	0.150	0.979
Plant height	0.218	-0.972	0.080	-0.023
No. of productive tillers/plant	0.005	-0.009	-0.013	-0.127
Panicle length	0.016	-0.003	0.029	0.000
No. of grain/panicle	0.974	0.222	0.034	-0.013
Grain yield/plot	0.014	0.004	-0.041	0.065
Survival percent	-0.053	0.073	0.984	-0.145

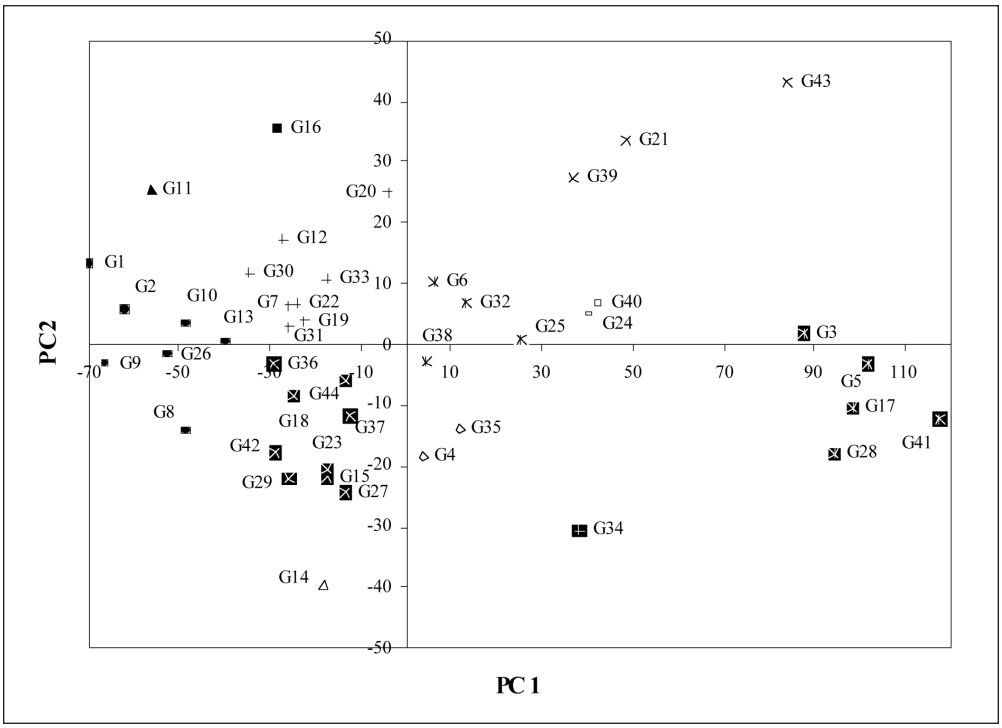

Fig. 1: Spatial distribution of 44 rice genotypes for the first two principal components. Legend – ⊠ cluster I, ● cluster II, ✚ cluster III, ◊ cluster IV, × cluster V, ⊠ cluster VI, ▲cluster VII, □ cluster VIII, Δ cluster IX, ■ cluster X, + cluster XI and ✳ cluster XII

number of grains per panicle, percentage of plant survival, and grain yield per plot.

Fig. 1 shows that the clusters were distinctly delineated to their respective positions that are similar to their position in the D^2 analysis (Tyagi *et al.*, 1999). In particular, genotypes G3 and G41 of Cluster X and G11 of Cluster IX were situated opposite to each other, indicating a considerable divergence between them. Just like the D^2 analysis, the PCA also clustered the genotypes based on their pedigree and not by their geographic origin. Thus, it was suggested that the selection of lines for hybridization programme should be based on genetic diversity rather than geographic distance.

CONCLUSIONS

Hierarchical and non-hierarchical algorithms, based on the multivariate statistical techniques, are common methods used by breeders to identify diverse genotypes for developing varieties that suit the target environment. Thus, it provides a chance to obtain recombinants resulting from recombination of favourable genes. In the present study, the results indicated that the genotypes selected from Clusters X, VI, VII, and XII could be used in hybridization programme. The genotypes from these clusters exhibited the maximum diversity with respect to the aggregate effects of the characters, such as earliness, high productive tillers per plant, longest panicle, maximum number of grains per panicle, high survival percentage, and high grain yield per plot. Therefore, the segregants from the above clusters would yield promising genotypes for salt affected soil with high survival percentage and yield.

The PCA and D^2 statistic exhibited a high level of variability among the genotypes and

it also allowed the selection of highly diverse genotypes which differ in their phenotype performance.

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Short-term Changes in the Soil Physical and Chemical Properties due to Different Soil and Water Conservation Practices in a Sloping Land Oil Palm Estate

Christopher Teh Boon Sung[1*], Goh Kah Joo[2], Law Chu Chien[1] and Seah Tiong Seng[1]

[1]Department of Land Management, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[2]Applied Agriculture Research, Locked Bag 212, Sg. Buloh Post Office, Sg. Buloh, Selangor, Malaysia

[*]E-mail: cbsteh@yahoo.com

ABSTRACT

The effectiveness of mulching materials, empty fruit bunches (EFB) and EFB mat (Ecomat) and the construction of soil trenches (silt pits) as soil water and nutrient conservation methods, have yet to be compared against one another in any single study. Moreover, as compared to the EFB studies, much less has been studied on the effectiveness of Ecomat and silt pit to improve soil properties and conserve water. Thus, this study was undertaken to compare the effects of the EFB, Ecomat, silt pit, and control (stacked pruned oil palm fronds) on several soil properties at soil depths of 0-150 and 150-300 mm, over a period of six months, at an oil palm estate with a hill slope of 6°. This study found that in just a period of six months, there were significant effects of the four treatments on the soil chemical and physical properties. Overall, the EFB was found to be the best treatment to improve the chemical properties of soil in both depths (CEC, Ca, Mg, K, P, C, and pH). However, both the EFB and Ecomat gave similar values for the soil available water content and aggregate stability. The mean daily total soil water content (up to 1 m depth) for the EFB, Ecomat, and control were found to be insignificantly different from one another, but silt pit had the statistically lowest total soil water content. The soil water distribution under the Ecomat mulches was rather uniform throughout the soil depths (up to 1 m), whereas in the EFB and silt pit treatments, the soil water tended to be concentrated at the upper soil layers until 0.6 m depth, with the concentration of water restricted to a shallower depth for silt pit as compared to the EFB. As for the control, water concentrated mostly below 0.5 m depth. This study is on-going, but the results have so far indicated that the EFB, followed by Ecomat, is the best soil and water conservation method, particularly to improving the chemical properties of soil. Ecomat, due to its lower nutrient content than EFB, generally did not improve the soil chemical properties by as much as the EFB. The poorer performance of the silt pit, as compared to the EFB and Ecomat (and to the control in some cases), was because the silt pit walls were observed to be easily collapsible, and in turn, silt pits became increasingly shallow and less effective to trap runoff over time.

Keywords: Empty fruit bunches, Ecomat, silt pit, oil palm, soil and water conservation, hill slopes, organic matter

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[*]Corresponding Author

INTRODUCTION

New oil palm plantations today are often being limited to marginal lands which include those in hilly, steepland areas. These areas are frequently related to soil erosion and run off losses caused by excessive rain falls. In order to reduce soil and water losses by erosion, terraces are often built. Nonetheless, hill cutting activities to construct these terraces cause not only compacted soils but also reduce soil fertility because the fertile, top soils are physically removed from the area.

Nowadays, some oil plantations have forsaken the hill terracing practice and are planting oil palms on non-terraced hill slopes. Therefore, in order to reduce water and nutrient losses, several methods are used. One of them is the use of empty fruit bunches (EFB) as a natural mulching material. The beneficial effects of the EFB in improving soil properties and oil palm growth and yield have been well documented, among other by Chan and Goh (1978), Lim and Pillai (1979), Khoo and Chew (1979), Chan *et al*. (1980), Singh *et al*. (1981), Loong *et al*. (1987), Lim and Chan (1987), Zaharah and Lim (2000), as well as Lim and Zaharah (2002). In term of fertiliser use, one tonne of EFB is equivalent to 7 kg of urea, 2.8 kg of rock phosphate, 19.3 kg of muriate of potash, and 4.4 kg of kieserite (Singh *et al*., 1999).

Nevertheless, one well-known disadvantage of EFB is that it is bulky, making its transportation, storage, and distribution rather difficult and expensive. One recent method is to compress the EFB into a mat or carpet known as Ecomat. According to Yeo (2007), Ecomat is produced by shredding the EFB into its raw fibre and then combed out, after which EFB undergoes a high pressure hydraulic press to remove impurities, such as water, sludge, and oil traces. The EFB is then dried, using high temperature, to about 15% gravimetric water content, before it is trimmed to the required size and packed for shipping. Being less bulky, storage, transportation, and handling of Ecomat is therefore much easier and cheaper than the EFB. Moreover, Ecomat is more marketable and a better choice as a mulching material for landscaping purposes in urban areas because it is more aesthetically pleasing than the EFB. The use of Ecomat gained a wide public attention; for example, China imported Ecomat from Malaysia as a landscaping mulching material to be used during the Beijing Olympics in 2008.

In addition, the use of Ecomat has shown to be beneficial in improving soil properties and crop growth. MPOB (2003), as well as Khalid and Tarmizi (2004), reported that young oil palms planted on hill terraces with Ecomat mulching showed higher growth rates and higher uptake of N, P, and K nutrients than those without it. Several studies conducted in China have shown increased soil water content due to Ecomat mulching as compared to without it. Xin-Fu (2004), for example, reported higher water contents by 17.4% and 8.9% in the 0-200 mm and 200-400 mm soil depths, respectively. Similarly, Liu *et al*. (2005) reported a higher increase in soil water content by 44.3% in the 0-200 mm depth. Both these studies also reported that Ecomat helped to cool the soil during summer and to warm the soil during winter. In an unpublished study by the Beijing Forestry and Parks Department of International Cooperation, conducted from 2002 to 2006, Ecomat mulching was found to have increased soil water content by 35.5% after two years, N by 3.5% and 6.7% in the summer and winter periods, respectively, and K by between 20 to 128.6% as compared to bare soil alone.

Other than the EFB and Ecomat, another current method used to conserve soil water and nutrients on oil palm hill slopes is to construct silt pits, where long and wide trenches are dug into the soil somewhere between the planting rows and in perpendicular to the hill slope so that these trenches will collect runoff water and soil. The idea is that these silt pits will act as storage areas, and preserve the soil water and nutrients which will otherwise lose through run-off. These trenches will then help to redistribute the collected water and nutrients back into the plant roots after a rainfall event.

Unlike the studies on EFB, much less has been carried out on the effects of silt pit

in improving soil properties. Among other, Murtilaksono *et al*. (2008) compared two soil conservation methods, namely silt pitting and bund terracing, against control (i.e. without any conservation methods) on increasing oil palm fresh fruit bunch (FFB) yield. They found that although silt pit had significantly given higher FFB yield (23.6 tons ha^{-1}) than the control (20.8 tons ha^{-1}), it was the plots with the bund terracing method that had produced the significantly highest FFB yield (25.2 tons ha^{-1}). In an earlier study by Soon and Hoong (2002), soil loss via runoff was found to have probably reduced significantly (by as much as five times lesser) by stacking the oil palm fronds along the hill contour rather than stacking them without any order. Furthermore, by combining the silt pitting method with the contour frond stacking method, it reduced soil loss further by 10.5%. Although silt pitting reduced soil loss significantly, the researchers found no significant effect at the 10% level of silt pitting on most of the oil palm vegetative growth properties (palm height, number of fronds, total number of leaflets, rachis length, leaf dry, weight, and petiole area), even after three years. Similarly, silt pitting was found to insignificantly affect the leaf nutrient contents (N, P, K, Ca, and Mg) at the 10% level during the same period. The treatments only had a significant effect on the FFB yield during the third year; however, the plots with the silt pitting method had unexpectedly lower FFB yield than those without any conservation methods (control).

Although much has been researched on the effects of EFB (but to a much lesser degree for Ecomat and silt pit) on the properties of soil, there is no single study, to the researchers' knowledge, that compares the effects of the three soil and water conservation methods on soil properties. Thus, it was the main objective of this paper to compare the effects of four soil and water conservation methods (namely, control, EFB, Ecomat, and silt pit) on several soil chemical and physical properties at a sloping land oil palm estate. This paper reports the results of the first six months of the field experiment.

MATERIALS AND METHODS

A field experiment was setup in an oil palm (*Elaeis guineensis*) site at Balau Estate (2.9325° N; 101.8822° E), located in Semenyih, Selangor. The study area has a slope of 6°, and under the USDA Taxonomy classification, the soil is classified as a Typic Paleudult (Rengam series), as the soil has a sandy clay texture (37% clay, 7% silt, and 56% sand). Meanwhile, the average bulk density for all the treatment plots at 0-150 mm soil depth was 1.62 Mg m^{-3}, while the organic carbon for 0-150 and 150-300 mm soil depth were found to be 1.14% and 1.05%, respectively. The oil palm trees in the study area at the time of the experiment were about eight years old, and the trees were planted with 8 m × 8 m spacing between them.

The field experimental design had four treatments and three blocks (replications), as shown in *Fig. 1*. The treatments were control (CON) (normal field practice where pruned fronds were heaped on the soil surface), empty fruit bunches (EFB), Ecomat (ECO), and silt pit (PIT). Each block was equally divided into four plots, whereby each plot was measured to 8 m x 8 m and with a gap of 8 m between two plots. The number of palms per plot was one, and each treatment was randomly assigned to a plot for each block. Each of the three blocks was located at different hill elevations, and the hill slope was the same for all the blocks, i.e. at 6°.

The application of the EFB and Ecomat treatments and the construction of the silt pits began in February 2006. In the middle of each EFB treatment plot, empty fruit bunches (rate of 1000 kg EFB $palm^{-1}$ $year^{-1}$) were heaped as a single layer on the ground. Likewise, in the middle of each Ecomat treatment plot, four Ecomat carpets (2 m × 2 m long and wide, and 0.02 m thick) were arranged in a single layer on the ground. The silt pits were constructed by digging a trench along the hill contour, measuring 1 m wide, 4 m long and 0.5 m deep. The silt pits were located in the middle of each silt pit treatment plot.

The field data collection was started in March 2006 and continued every month.

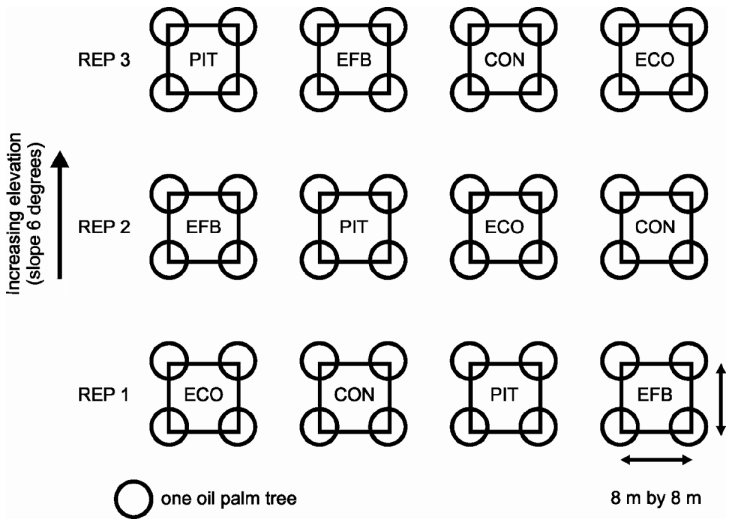

Fig. 1: Field experimental design

Thus, the data presented in this paper are for six months, ending in August 2006. A total of soil samples, from 0-150 and 150-300 mm soil depths, were randomly collected at several points in a plot. The air-dried soil samples were then analysed for their pH (1:2.5 soil to water ratio), cation exchange capacity (CEC) (using 1M ammonium acetate, pH7.0, (Lim, 1975), whereas the leachate was collected to determine the concentration of cations by atomic absorption spectrometry (Ca and Mg) and flame photometer (K), total N (Kjedahl method; Bremner and Mulvaney, 1982), available P (Bray and Krutz no. II, Molybdenum Blue method; Olsen and Sommer, 1982), organic C (combustion method; McKeague, 1976), bulk density (core ring method; Blake and Hartge, 1986), aggregate stability (wet-sieving method; Kemper and Rosenau, 1986), and aggregation (dry-sieving method; Kemper and Rosenau, 1986). Soil aggregate stability and aggregation were expressed as the mean weight diameter (MWD) in unit mm (Kemper and Rosenau, 1986). Meanwhile, soil water retention was measured using the pressure plate and membrane technique (Richards, 1947) to determine the available water content for plants (i.e. the difference between the water content at field capacity and permanent wilting point) and the slope of the soil moisture characteristic curve.

The above data were analysed using ANOVA (analysis of variance), according to the split-split block experimental design, with three replications and three main factors, namely the four treatments (treatment factor), the two soil depths (space factor), and the six monthly collection periods (time factor). The main plot, subplot, and sub-subplot were the time factor, treatment factor, and space factor, respectively. Meanwhile, the mean separation test was by the least significance difference (LSD) method at 5% level. The data analysis was done using the statistical software SPSS ver. 17 (SPSS Inc., Chicago).

The soil water content from every treatment plot was measured at the soil depths of 0.1, 0.2, 0.4, 0.6 and 1.0 m, using a soil water profile probe (PR1, Delta-T, Cambridge, England). The PR1 probe measures the soil water content based on the capacitance method. This probe consists of both a transmitter and a receiver, whereby the transmitter emits a low-powered signal of about 100 MHz that can be detected by the probe's receiver. The frequency of the signal, however, will change depending on the amount of water in the soil. In more specific, the more water a

soil has, the more the frequency of this signal will be changed. By detecting this amount of change, the PR1 probe will then determine the corresponding soil water content.

In this study, soil water measurements were done between 6:00 to 7:00 hours each day, beginning from 1 March 2006 and ending on 14 May 2006. These measurement dates corresponded to the day of year (DOY) 60 to 137 (where Jan. 1 = 1, Jan. 2 = 2, Feb. 1 = 32, and so on). Nonetheless, soil water measurements ended earlier than expected due to the fault in the PR1 probe. As a result, this paper only reports the data for the soil moisture only for the first three months.

The soil water content between the treatments were first analysed by calculating their individual daily total soil water content up to 1 m soil depth. The one-way ANOVA and LSD were then used to detect the significant mean differences between the daily total soil water content of the four treatments. This was unlike the ANOVA done for analysing the other soil properties. Meanwhile, the soil water content was not analysed as a split-split block design mainly because of missing data due to the fault in installing the PR1 probe for the second replication of the control plot. This problem could only be rectified about a month into the field experiment. Finally, the daily rainfall data were collected using a portable weather station (Watchdog Model 700, Spectrum Technologies Inc., Illinois).

RESULTS AND DISCUSSION

Changes in the Soil Chemical Properties

The ANOVA revealed that the four soil conservation practices had a significant effect on all the measured soil chemical properties (Table 2). The interaction effect, Treatment (T) × Month (M) × Depth (D), was significant at least at the 5% level for almost all the soil chemical properties; CEC, exchangeable Ca, Mg and K, available P, organic C, and C:N. This indicates that the effect of the four conservation practices on the seven soil properties would vary according to time and soil depth. Only the total N and soil pH did not have any significant, T × M × D, interaction effect at the 5% level. As for N, the interaction effect T × M was significant at the 1% level, and the interaction effects T × D and T × M were significant for the pH at 1% and 5% levels, respectively.

The mean separation test using the LSD method at the 5% level showed that the EFB generally produced the highest values of CEC, Ca, Mg, K, P, C, and pH in both the soil depths and for all the months covered as compared to the other three treatments (Table 2). Meanwhile, the Ecomat treatment generally gave the second highest readings for these soil properties. The exceptions were observed for the P and C contents in the subsoil; both the EFB and Ecomat produced similar P and C contents in this lower soil depth for a given month.

The highest N contents in both the soil depths for all the months were derived from the EFB and Ecomat treatments. For a given soil depth and month, both the EFB and Ecomat gave similar N content. This was followed by the control and the silt pit treatment.

The soil C:N ratio for both the soil depths in all the treatments remained between 8 and 20 throughout the study. Any organic materials with a C:N ratio greater than 30 favours immobilisation, supplying C to the soil; however they may cause a reduction in plant-available N. In contrast, the organic materials with a C:N ratio below 20 will favour N mineralization and supply N to the soil. A C:N ratio of about 25 is often regarded as the point where immobilisation and mineralization are in balance. The LSD method showed that the silt pit treatment had given the highest C:N ratio for both the soil depths (mean C:N ratio for both depths were 15) as compared to the other treatments (Table 2). Similarly, LSD also showed that for a given soil depth, the C:N ratios in the control, EFB, and Ecomat plots were generally similar to one another (their means for both the soil depths were 10).

These results have so far supported the beneficial effects of the EFB mulching on the soil properties. The benefits of the EFB mulching have been known since 1934 (Abdullah *et al*.,

1987). Numerous studies have shown that a direct EFB application had increased vegetative growth, nutrition, and yield of oil palm (Chan and Goh, 1978; Lim and Pillai, 1979; Khoo and Chew, 1979; Singh *et al*., 1981; Loong *et al*., 1987; Lim and Chan, 1987; Lim and Zaharah, 2002), as well as increased the organic matter content, pH, and nutrient content of soil (Chan *et al*., 1980; Zaharah and Lim, 2000; Lim and Zaharah, 2002).

It is well known that the main constraints to the EFB application are the high cost of transportation, storage, and distribution (due to the bulkiness and weight of EFB), as well as the potential of EFB to harbour pests and diseases (Turner and Gillbanks, 1974; Hartley, 1980). Converting EFB into a thinner and lighter material such as Ecomat is therefore attractive because this material is apparently easier and cheaper to transport, store, and distribute.

However, the results from this study have indicated that the EFB was generally better than the Ecomat in improving the chemical properties of soil. Although Ecomat is made solely from EFB fibres, the nutrient contents of Ecomat have been reported to be significantly lower than that of EFB (Table 1). Meanwhile, Wan Asma (2006) reported that the processing of EFB into Ecomat has caused Ecomat to lose most of the original benefits of the EFB. This loss of nutrients is most probably caused by the high pressure and heat required to convert and compress the EFB into a thinner and lighter material (Ecomat). In this study, nevertheless, the Ecomat treatment was generally found to be better than both the silt pit and control in improving the chemical properties of soil.

However, the silt pit treatment was not better than either the EFB or Ecomat in improving the soil chemical properties. Additionally, as compared to the control, the effects of the silt pit treatment on improving the chemical properties of soil were found to be better in some cases and worse in others. In more specific, the LSD showed that generally silt pit had given higher readings than the control for both soil depths for the CEC, K, and C. As for the soil properties N and pH, however, the control gave higher readings for both the soil depths compared to the silt pit. Both the silt pit and control generally gave similar readings for Ca (top soil) and Mg (subsoil).

As mentioned earlier, silt pit had the highest C:N ratio than the other three treatments. The mean C:N ratio for the silt pit for both the soil depths were 15 compared to only 10 for the other treatments. As stable organic matter had a C:N ratio between 10 and 12 (Pierzynski *et al*., 2005), a higher mean C:N ratio for the silt pit plots suggested that their soil organic matters were relatively fresher and less stable than those in the other treatment plots. Norton *et al*. (2003) found that the steeper the gradient of a hill, the larger the soil's C:N ratio. The researchers attributed this particular observation to the fast removal of organic materials from the steep slope due to fast run-off, leaving relatively fresher and higher C:N organic materials in

TABLE 1
Chemical characteristics of the EFB, Ecomat, and pruned fronds

Properties (% dry matter)	EFB[1]	Ecomat[2]	Fronds[3]
Total C	61.20	33.85	50.43
Total N	0.86	0.55	0.79
Total P	0.16	0.39	0.08
Total K	2.21	2.59	2.26
Total Ca	0.48	0.22	0.48
Total Mg	0.37	0.21	0.10

[1] Rosenani and Wingkis (1999)
[2] Wan Asma (2006)
[3] The present study

TABLE 2
Soil properties in all the treatments, expressed as means of three replicates

Properties[1], units	Depth, mm (D)	Treatment [2] (T)	Month[3] (M)					
			1	2	3	4	5	6
CEC, cmol (+) kg^{-1}	0-150	CON	6.390 (0.006)	6.440 **a** (0.006)	6.973 **a** (0.007)	6.943 **a** (0.003)	7.013 (0.009)	7.033 (0.003)
T × M × D **		EFB	7.490 (0.006)	7.487 (0.003)	7.803 (0.003)	7.553 (0.003)	7.563 (0.003)	7.587 (0.003)
(F = 4.806, df = 15)		ECO	7.147 (0.009)	7.163 (0.012)	7.190 (0.001)	7.200 (0.009)	7.263 **a** (0.009)	7.270 (0.006)
		PIT	6.710 (0.390)	6.830 **a** (0.330)	6.947 **a** (0.432)	6.923 **a** (0.320)	6.983 a (0.293)	7.377 (0.604)
	150-300	CON	6.037 (0.003)	6.180 (0.006)	6.293 (0.007)	6.337 **a** (0.003)	6.433 **a** (0.009)	6.560 (0.012)
		EFB	7.303 (0.003)	7.313 (0.003)	7.320 (0.006)	7.353 (0.003)	7.353 (0.009)	7.367 (0.003)
		ECO	6.857 (0.019)	6.887 (0.006)	6.890 (0.006)	6.910 (0.006)	6.943 (0.012)	6.977 (0.009)
		PIT	6.423 (0.443)	6.427 (0.447)	6.443 (0.433)	6.457 **a** (0.447)	6.467 **a** (0.442)	6.480 (0.445)
K, cmol (+) kg^{-1}	0-150	CON	0.080 **a** (0.001)	0.090 (0.006)	0.100 (0.010)	0.333 (0.018)	0.477 (0.015)	0.670 **a** (0.006)
T × M × D **		EFB	0.090 **a** (0.001)	0.407 (0.003)	0.627 (0.003)	0.847 (0.003)	0.850 (0.003)	0.857 **b** (0.003)
(F = 5.541, df = 15)		ECO	0.080 **a** (0.001)	0.187 **a** (0.009)	0.377 (0.009)	0.463 (0.006)	0.680 (0.006)	0.840 **b** (0.071)
		PIT	0.077 **a** (0.007)	0.210 **a** (0.095)	0.320 (0.155)	0.530 (0.161)	0.613 (0.118)	0.667 **a** (0.097)

Table 2 (Continued)

	150-300	CON	0.070 **a** (0.001)	0.080 **a** (0.001)	0.097 (0.003)	0.320 (0.012)	0.413 (0.009)	0.560 **a** (0.006)
		EFB	0.077 **a** (0.003)	0.303 (0.003)	0.573 (0.003)	0.717 (0.003)	0.717 (0.003)	0.737 (0.003)
		ECO	0.070 **a** (0.001)	0.113 **a** (0.012)	0.267 **a** (0.012)	0.470 (0.010)	0.567 (0.009)	0.627 (0.003)
		PIT	0.080 **a** (0.001)	0.180 (0.065)	0.287 **a** (0.147)	0.430 (0.140)	0.483 (0.113)	0.580 **a** (0.075)
Ca, cmol (+) kg^{-1}	0-150	CON	0.100 **a** (0.001)	0.150 **a** (0.006)	0.290 (0.006)	0.427 (0.009)	0.587 **a** (0.003)	0.630 **a** (0.006)
T × M × D **		EFB	0.100 **a** (0.001)	0.237 (0.003)	0.483 (0.003)	0.507 (0.003)	0.640 (0.006)	0.710 (0.006)
(F = 7.571, df = 15)		ECO	0.100 **a** (0.001)	0.180 **b** (0.006)	0.287 (0.009)	0.463 (0.015)	0.580 **a** (0.015)	0.677 (0.003)
		PIT	0.113 **a** (0.007)	0.177 **ab** (0.032)	0.353 (0.069)	0.387 (0.057)	0.520 (0.060)	0.633 **a** (0.038)
	150-300	CON	0.090 **a** (0.001)	0.130 (0.006)	0.247 **a** (0.003)	0.387 (0.009)	0.413 **a** (0.009)	0.457 **a** (0.003)
		EFB	0.070 **a** (0.001)	0.147 (0.003)	0.390 (0.006)	0.353 (0.003)	0.427 **a** (0.009)	0.583 (0.003)
		ECO	0.080 **a** (0.001)	0.083 (0.003)	0.123 (0.003)	0.293 **a** (0.009)	0.460 (0.006)	0.497 (0.009)
		PIT	0.083 **a** (0.007)	0.120 (0.015)	0.273 **a** (0.054)	0.300 **a** (0.030)	0.373 (0.030)	0.450 **a** (0.065)

Table 2 (Continued)

Mg, cmol (+) kg^{-1}	0-150	CON	0.080 **a** (0.001)	0.100 **a** (0.001)	0.123 **a** (0.033)	0.127 (0.028)	0.223 **a** (0.007)	0.293 **a** (0.003)
T × M × D **		EFB	0.080 **a** (0.001)	0.180 (0.006)	0.253 (0.003)	0.290 (0.006)	0.320 **b** (0.003)	0.343 (0.003)
(F = 8.232, df = 15)		ECO	0.087 **a** (0.003)	0.100 **a** (0.001)	0.120 **a** (0.003)	0.253 (0.009)	0.303 **b** (0.009)	0.307 **a** (0.007)
		PIT	0.087 **a** (0.003)	0.130 (0.025)	0.173 (0.044)	0.207 (0.042)	0.223 **a** (0.048)	0.250 (0.045)
	150-300	CON	0.080 **a** (0.001)	0.090 **a** (0.001)	0.077 **a** (0.003)	0.137 **a** (0.003)	0.150 **a** (0.010)	0.153 **a** (0.003)
		EFB	0.070 **a** (0.001)	0.083 **a** (0.003)	0.157 (0.003)	0.200 (0.006)	0.213 (0.009)	0.253 (0.003)
		ECO	0.080 **a** (0.001)	0.083 **a** (0.003)	0.093 **ab** (0.003)	0.140 **a** (0.006)	0.157 **a** (0.003)	0.197 (0.003)
		PIT	0.077 **a** (0.003)	0.087 **a** (0.003)	0.103 **b** (0.023)	0.123 **a** (0.038)	0.123 (0.044)	0.157 **a** (0.047)
P, ug g^{-1}	0-150	CON	22.873 **a** (0.032)	23.667 **a** (0.337)	30.443 **a** (0.023)	32.907 **a** (0.032)	38.547 **a** (0.094)	40.013 **a** (0.009)
T × M × D **		EFB	21.860 **abd** (0.035)	22.373 **ab** (0.033)	38.987 (0.012)	39.553 (0.327)	41.433 **b** (0.052)	43.293 (0.052)
(F = 10.343, df = 15)		ECO	20.407 **bc** (0.033)	20.917 **b** (0.029)	23.963 (0.012)	36.393 (0.015)	39.427 **ab** (0.015)	41.347 **a** (0.029)
		PIT	21.543 **acd** (0.146)	23.057 **a** (0.358)	29.543 **a** (4.714)	32.927 **a** (3.467)	34.093 (3.623)	40.367 **a** (1.457)

Table 2 (Continued)

	150-300	CON	13.997 **a** (0.015)	14.313 (0.013)	15.923 (0.028)	26.383 **a** (0.026)	29.590 **a** (0.049)	33.850 **a** (0.035)
		EFB	19.563 **b** (0.019)	21.403 **a** (0.015)	22.963 **a** (0.052)	31.390 **b** (0.031)	35.633 (0.061)	36.723 **b** (0.012)
		ECO	19.007 **b** (0.023)	19.610 **a** (0.027)	22.617 **a** (0.027)	29.410 **b** (0.020)	30.667 **a** (0.047)	35.957 **b** (0.045)
		PIT	15.580 **a** (1.980)	17.603 **a** (1.889)	20.297 (1.337)	24.757 **a** (3.297)	30.877 **a** (2.377)	32.690 **a** (2.020)
N, ug g^{-1}	0-150	CON	0.097 (0.003)	0.103 (0.003)	0.120 (0.001)	0.107 (0.003)	0.130 (0.001)	0.133 (0.003)
T × M **		EFB	0.117 (0.003)	0.123 (0.003)	0.133 **a** (0.003)	0.130 (0.006)	0.157 **a** (0.003)	0.153 **a** (0.003)
(F = 3.982, df = 15)		ECO	0.137 (0.003)	0.133 (0.003)	0.137 **a** (0.003)	0.147 (0.003)	0.153 **a** (0.003)	0.157 **a** (0.003)
		PIT	0.073 (0.023)	0.080 (0.025)	0.083 (0.028)	0.093 (0.019)	0.103 (0.028)	0.113 (0.023)
	150-300	CON	0.090 **a** (0.001)	0.090 **a** (0.001)	0.103 (0.003)	0.103 (0.003)	0.120 **a** (0.001)	0.120 (0.001)
		EFB	0.087 **a** (0.003)	0.097 **a** (0.003)	0.117 **a** (0.003)	0.113 (0.003)	0.127 (0.003)	0.137 **a** (0.003)
		ECO	0.103 (0.003)	0.110 (0.003)	0.123 **a** (0.003)	0.133 (0.003)	0.137 **a** (0.003)	0.140 **a** (0.001)
		PIT	0.067 (0.012)	0.063 (0.019)	0.077 (0.022)	0.083 (0.019)	0.097 (0.017)	0.107 (0.017)

Table 2 (Continued)

C, %	0-150	CON	1.160 **a** (0.010)	1.142 **a** (0.018)	1.177 **a** (0.013)	1.168 **a** (0.016)	1.145 (0.013)	1.154 (0.015)
T × M × D **		EFB	1.178 **a** (0.009)	1.196 **bc** (0.012)	1.351 (0.036)	1.441 (0.009)	1.572 (0.027)	1.725 (0.027)
(F = 11.386, df = 15)		ECO	1.109 **b** (0.009)	1.183 **ab** (0.005)	1.267 (0.054)	1.235 (0.041)	1.289 **a** (0.041)	1.420 (0.037)
		PIT	1.121 **ab** (0.017)	1.155 **ac** (0.018)	1.169 **a** (0.018)	1.183 **a** (0.020)	1.244 **a** (0.006)	1.296 (0.019)
	150-300	CON	1.026 **a** (0.006)	1.036 **a** (0.013)	1.039 (0.005)	1.028 (0.010)	1.062 (0.009)	1.068 (0.013)
		EFB	1.068 **a** (0.005)	1.058 **a** (0.003)	1.143 **a** (0.009)	1.151 **a** (0.023)	1.198 **ab** (0.020)	1.253 **a** (0.023)
		ECO	1.064 **a** (0.015)	1.040 **a** (0.009)	1.116 **a** (0.009)	1.176 **a** (0.026)	1.236 **a** (0.020)	1.261 **a** (0.016)
		PIT	1.042 **a** (0.004)	1.060 **a** (0.015)	1.102 **a** (0.022)	1.140 **a** (0.021)	1.180 **b** (0.028)	1.219 **a** (0.048)
C:N	0-150	CON	12.021 (0.356)	11.067 **a** (0.192)	9.811 **a** (0.108)	10.984 **a** (0.502)	8.810 **a** (0.103)	8.673 **a** (0.317)
T ´ M ´ D *		EFB	10.116 (0.335)	9.709 **ab** (0.174)	10.140 **a** (0.262)	11.128 **a** (0.523)	10.039 **a** (0.252)	11.258 **b** (0.252)
(F = 2.179, df = 15)		ECO	8.126 (0.247)	8.883 **b** (0.252)	9.283 **a** (0.180)	8.410 (0.177)	8.406 **a** (0.177)	9.076 **a** (0.389)
		PIT	18.108 (4.452)	17.053 (4.259)	17.140 (4.652)	13.670 (2.530)	13.712 (3.041)	12.345 **b** (2.214)

Table 2 (Continued)

	150-300	CON	11.396 **a** (0.063)	11.507 **a** (0.147)	10.072 **a** (0.274)	9.970 **a** (0.348)	8.850 **a** (0.072)	8.897 **a** (0.107)
		EFB	12.360 **a** (0.432)	10.968 **ab** (0.384)	9.815 **a** (0.274)	10.181 **a** (0.480)	9.467 **a** (0.222)	9.174 **a** (0.234)
		ECO	10.315 **a** (0.314)	9.455 **b** (0.217)	9.057 **a** (0.217)	8.825 **a** (0.192)	9.058 **a** (0.359)	9.010 **a** (0.115)
		PIT	16.572 (2.653)	19.312 (4.477)	16.388 (3.576)	14.837 (2.609)	12.806 (1.761)	11.922 (1.659)
pH	0-150	CON	4.847 **a** (0.084)	4.960 **a** (0.494)	4.833 **a** (0.090)	4.593 **a** (0.022)	4.850 **a** (0.146)	4.733 **a** (0.022)
T × D **		EFB	5.153 **a** (0.084)	5.330 **a** (0.257)	6.610 (0.197)	6.033 (0.496)	5.850 (0.195)	6.160 (0.195)
(F = 10.081, df = 3)		ECO	4.777 **a** (0.076)	4.557 **ab** (0.258)	4.957 **a** (0.226)	4.573 **a** (0.133)	4.650 **a** (0.133)	4.880 **a** (0.111)
T × M *		PIT	5.010 **a** (0.307)	4.390 **b** (0.125)	4.517 **a** (0.280)	4.080 **a** (0.106)	4.463 **a** (0.102)	4.547 **a** (0.063)
(F = 2.467, df = 15)	150-300	CON	4.950 **a** (0.178)	4.133 **a** (0.169)	4.643 **a** (0.113)	4.267 **a** (0.048)	4.787 **a** (0.081)	4.803 **a** (0.075)
		EFB	4.940 **a** (0.107)	4.800 (0.050)	5.037 **a** (0.529)	5.163 (0.432)	5.490 (0.215)	5.687 (0.209)
		ECO	4.570 **a** (0.040)	4.173 **a** (0.099)	4.513 **a** (0.099)	4.330 **a** (0.095)	4.457 **a** (0.063)	4.610 **a** (0.060)
		PIT	4.870 **a** (0.164)	4.277 **a** (0.054)	4.440 **a** (0.270)	4.393 **a** (0.438)	4.627 **a** (0.184)	4.570 **a** (0.140)

Table 2 (Continued)

Bulk density, Mg m^{-3}	0-150	CON	1.543 – (0.064)	1.596 – (0.067)	1.741 – (0.013)	1.630 – (0.024)	1.632 – (0.035)	1.616 – (0.008)
D *		EFB	1.581 – (0.051)	1.521 – (0.040)	1.554 – (0.007)	1.584 – (0.017)	1.555 – (0.002)	1.532 – (0.002)
(F = 61.813, df = 1)		ECO	1.652 – (0.023)	1.585 – (0.029)	1.592 – (0.048)	1.631 – (0.042)	1.592 – (0.042)	1.587 – (0.011)
		PIT	1.625 – (0.082)	1.607 – (0.073)	1.685 – (0.014)	1.603 – (0.045)	1.660 – (0.003)	1.623 – (0.077)
	150-300	CON	1.689 – (0.041)	1.653 – (0.067)	1.712 – (0.006)	1.671 – (0.059)	1.672 – (0.019)	1.621 – (0.027)
		EFB	1.626 – (0.027)	1.612 – (0.025)	1.633 – (0.084)	1.633 – (0.023)	1.625 – (0.014)	1.512 – (0.016)
		ECO	1.622 – (0.039)	1.598 – (0.089)	1.595 – (0.089)	1.637 – (0.047)	1.581 – (0.026)	1.588 – (0.028)
		PIT	1.635 – (0.061)	1.631 – (0.055)	1.704 – (0.053)	1.712 – (0.015)	1.656 – (0.039)	1.659 – (0.082)
Aggregation, mm	0-150	CON	3.036 **a** (0.074)	3.462 **a** (0.163)	2.891 **a** (0.266)	2.740 **a** (0.071)	2.646 **a** (0.073)	2.600 **a** (0.017)
T × M *		EFB	3.091 **a** (0.107)	3.447 **a** (0.231)	2.336 **b** (0.067)	2.272 (0.142)	2.640 **a** (0.017)	2.660 **a** (0.017)
(F = 2.596, df = 15)		ECO	2.859 **a** (0.239)	3.299 **a** (0.270)	2.678 **ab** (0.121)	2.822 **a** (0.080)	2.757 **a** (0.080)	2.830 **a** (0.017)
		PIT	2.750 **a** (0.099)	3.164 **a** (0.181)	2.588 **ab** (0.066)	2.702 **a** (0.149)	2.440 **a** (0.152)	2.510 **a** (0.017)

Table 2 (Continued)

	150-300	CON	3.316 **a** (0.073)	3.503 **ab** (0.157)	2.984 (0.219)	2.929 **a** (0.145)	3.032 **a** (0.056)	2.970 **a** (0.017)
		EFB	3.159 **a** (0.030)	3.765 **b** (0.235)	2.535 (0.047)	2.454 **b** (0.111)	2.685 **ab** (0.157)	2.700 **a** (0.017)
		ECO	3.139 **a** (0.148)	3.482 **ab** (0.220)	2.814 (0.220)	2.903 **a** (0.139)	2.869 **ab** (0.149)	2.760 **a** (0.017)
		PIT	2.979 **a** (0.127)	3.330 **a** (0.077)	2.862 (0.090)	2.732 **ab** (0.157)	2.645 **b** (0.110)	2.560 **a** (0.017)
Aggregate stability, mm	0-150	CON	1.210 **a** (0.017)	1.227 (0.015)	1.253 (0.015)	1.290 (0.017)	1.330 (0.017)	1.380 **a** (0.017)
T × M × D **		EFB	1.290 **b** (0.017)	1.280 **a** (0.017)	1.350 **ab** (0.017)	1.433 (0.020)	1.490 **a** (0.017)	1.560 **b** (0.017)
(F = 4.567, df = 15)		ECO	1.260 **ab** (0.017)	1.360 (0.017)	1.400 **a** (0.017)	1.380 **a** (0.017)	1.400 **b** (0.017)	1.430 **a** (0.017)
		PIT	1.240 **ab** (0.017)	1.300 **a** (0.017)	1.340 **b** (0.017)	1.370 **a** (0.017)	1.440 **ab** (0.017)	1.530 **b** (0.010)
	150-300	CON	1.220 **a** (0.017)	1.250 (0.017)	1.280 (0.017)	1.320 (0.017)	1.370 **a** (0.017)	1.430 **a** (0.017)
		EFB	1.360 (0.017)	1.380 **a** (0.017)	1.410 **ab** (0.017)	1.490 **a** (0.017)	1.530 (0.017)	1.680 (0.017)
		ECO	1.300 **b** (0.017)	1.380 **a** (0.020)	1.433 **a** (0.020)	1.260 (0.017)	1.467 (0.020)	1.450 **a** (0.017)
		PIT	1.270 **ab** (0.017)	1.340 **a** (0.017)	1.370 **b** (0.017)	1.440 **a** (0.017)	1.390 **a** (0.017)	1.420 **a** (0.017)

Table 2 (Continued)

Available water content, %	0-150	CON	11.213 **a** (1.891)	12.369 **a** (1.514)	15.193 **a** (0.109)	12.065 **a** (0.642)	12.425 **a** (0.130)	13.175 **a** (0.130)
T × M × D **		EFB	14.473 **b** (0.094)	15.204 **bc** (0.347)	16.918 **b** (0.035)	17.685 (1.301)	19.641 **b** (0.121)	20.391 **b** (0.121)
(F = 5.651, df = 15)		ECO	15.411 **b** (0.048)	16.062 **b** (0.073)	15.524 **ab** (0.463)	14.890 (0.138)	18.510 **b** (0.513)	19.260 **b** (0.513)
		PIT	12.689 **a** (0.493)	13.699 **ac** (0.385)	13.677 **a** (0.707)	12.874 **a** (0.338)	13.731 **a** (0.038)	14.481 **a** (0.038)
	150-300	CON	13.957 **a** (2.354)	13.258 **a** (1.623)	14.404 **a** (0.103)	13.608 **a** (0.724)	13.627 **a** (0.143)	12.637 **a** (0.125)
		EFB	12.734 **b** (0.083)	12.462 **bc** (0.284)	12.870 **b** (0.027)	12.870 **b** (0.947)	12.715 (0.078)	10.520 **b** (0.062)
		ECO	12.656 **b** (0.039)	12.190 **b** (0.055)	12.132 **b** (0.362)	12.948 **b** (0.120)	11.860 (0.329)	11.996 **b** (0.320)
		PIT	12.909 **a** (0.502)	12.831 **ac** (0.361)	14.249 **a** (0.737)	14.404 **a** (0.378)	13.317 **a** (0.037)	13.375 **a** (0.035)
Slope of soil water retention curve, % bar^{-1}	0-150	CON	3.227 – (0.434)	3.512 – (0.239)	2.805 – (0.113)	3.370 – (0.005)	2.930 – (0.002)	3.252 – (0.100)
		EFB	3.462 – (0.064)	3.552 – (0.083)	3.078 – (0.067)	3.180 – (0.050)	3.080 – (0.215)	2.990 – (0.178)
All factors are not significant		ECO	2.945 – (0.003)	3.075 – (0.013)	2.997 – (0.021)	3.245 – (0.206)	2.841 – (0.002)	3.181 – (0.162)
		PIT	3.059 – (0.028)	3.145 – (0.030)	3.021 – (0.074)	3.034 – (0.195)	2.832 – (0.047)	3.362 – (0.147)

Table 2 (Continued)

	150-300	CON	3.034 – (0.408)	3.120 – (0.212)	2.979 – (0.120)	3.077 – (0.005)	3.074 – (0.002)	3.196 – (0.098)
		EFB	3.184 – (0.059)	3.217 – (0.075)	3.167 – (0.069)	3.167 – (0.050)	3.186 – (0.222)	3.456 – (0.206)
		ECO	3.193 – (0.003)	3.251 – (0.014)	3.258 – (0.023)	3.158 – (0.200)	3.291 – (0.002)	3.274 – (0.167)
		PIT	3.163 – (0.029)	3.172 – (0.030)	2.998 – (0.073)	2.979 – (0.191)	3.112 – (0.052)	3.105 – (0.136)

[1] The significant interaction effects, if any, according to the ANOVA results; * $p < 0.05$, ** $p < 0.01$

[2] CON, EFB, ECO and PIT denote the control, empty fruit bunches, Ecomat, and silt pit treatment, respectively.

[3] Values in the brackets denote standard errors; for the same soil depth and month, the treatment means with the same letter are not significantly different from each other, based on the LSD method at the 5% level of significance, and for the bulk density and slope of the soil water retention curve, LSD was not performed because the ANOVA showed an insignificant effect involving the treatment factor (T) (a dash is shown instead).

the soil. The idea of constructing silt pits as a soil and water conservation method was to trap runoff water and nutrients which would later be redistributed after the rainfall event. In this study, however, the silt pit walls were found to be easily collapsible, particularly after a heavy rainfall period. Thus, over time, the silt pits became increasingly shallow, and this in turn would reduce their effectiveness to trap runoff water and sediments. Thus, the relative poor performance of the silt pits to improve the soil chemical properties (as well as the high C:N ratio in the sit pits) in this study was due to the increasingly ineffective silt pits over time to prevent the loss of soils and organic materials by erosion.

Changes in the Soil Physical Properties

The ANOVA revealed that the interaction effect T × M × D was significant at 1% level for only the aggregate stability property (Table 2). Meanwhile, the interaction effect T × M was significant at least at 5% level for the soil physical properties; aggregation and available water content (AWC). For the bulk density, only the depth (D) effect was significant at 5% level. This showed that bulk density was not significantly affected by any of the treatments (where as sole as interaction effect). In addition, the slope of water retention curve was not significantly affected by any of the factors (T, M, D, or their interactions with one another).

The mean separation test by the LSD method at 5% level showed that for a given month and soil depth, the EFB, Ecomat, and silt pit treatments generally had similar aggregate stability with one other, with the control treatment usually having the lowest aggregate stability.

There were, however, lesser significant differences between the effects of the four treatments on soil aggregation. Moreover, there was a trend of slow decline with time in the aggregation in all the treatments (nearly 2% mean reduction per month in aggregation). Soil aggregation is strongly affected by the cycles of wetting-and-drying of the soil (Wagner *et al*., 2007). Throughout this study, the experimental site experienced a mean daily rainfall of about 9 mm, without any long continuous periods of dry weather. In the long periods of wet weather, soil aggregation might decline over time without any distinct wetting-and-drying cycles.

The LSD method revealed that the available water content (AWC) for the soils under the EFB and Ecomat mulches were generally similar to each other for a given soil depth and month. The soils under these mulches had higher AWC than those in both the silt and control plots. Both silt pit and control treatments gave a similar AWC to each other for the given soil depth and month.

The slope of the soil water retention curve measures the ability of a soil to keep the water it has during soil drying. The larger the slope, the steeper the gradient of the curve and the less capable the soil keeps its water. In other words, the larger the slope, the faster the soil dries. The ANOVA, however, revealed that there was no significant effect at 5% level by the four treatments on the drying rate of the soil.

Fig. 2 shows the soil water profile up to 1 m of soil depth in all the treatments. Meanwhile, the mean volumetric soil water content at saturation point, field capacity point and permanent wilting point were measured at 0.33, 0.13 and 0.01 m^3 m^{-3}, respectively. All the treatments showed that the soil water content below the 0.8 m soil depth was very wet and at times, it was over the saturation point. This was probably because of the rise of water from the ground water table (i.e. below the 1 m depth). In addition, the soil water content in all the treatments was consistently above the field capacity point, whereas all the treatments showed a general trend of increasing total amount of soil water content as the experiment progressed (*Fig. 3*). As stated earlier, this increasing trend was due to the heavy rainfall received throughout the study, as well as the absence of a long spell of dry weather. As expected, after any period of rainfall, there was an increase in soil water content throughout the soil depth (*Fig. 2*).

More importantly, *Fig. 2* shows that each of the four treatments had a distinct soil water profile. The distribution of water in the Ecomat

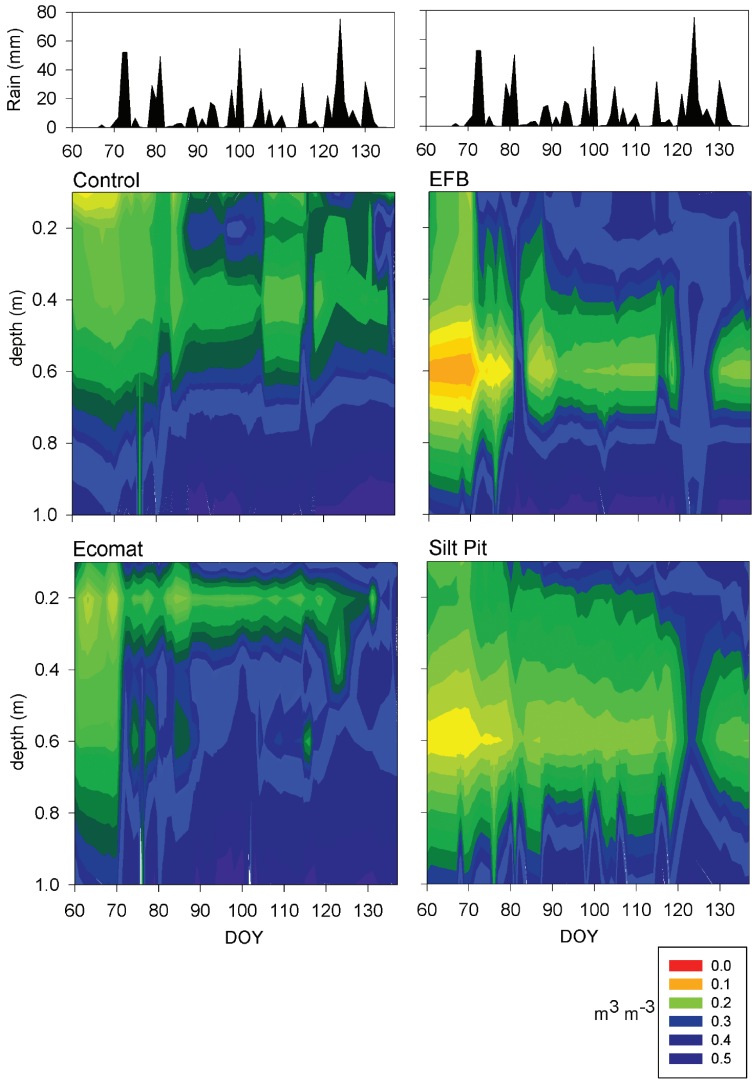

Fig. 2: Soil water profile in all the treatments, expressed as the average volumetric water content of three replicates

treatment was rather uniform throughout the soil profile, in particular, beginning from a depth below 0.3 m. In the EFB treatment, the soil water content tended to decrease with depth up to 0.6 m, after which the soil water content would increase. In more specific, the soil in the 0-0.5 m in the EFB treatment was generally the wettest, and the soil in the region of about 0.6 m was the driest. As compared to Ecomat, EFB concentrated water in the upper soil layers, whereas Ecomat tended to distribute the water more uniformly throughout the profile. Silt pit, like EFB, also concentrated water in the upper soil layers, but its water concentration was found to be restricted to a shallower depth compared to either EFB or Ecomat. In the control plots, the concentration of water occurred mostly in the lower soil layers, i.e. below 0.5 m. It was only during the wet weather periods (i.e. after DOY 90) that water would also be concentrated

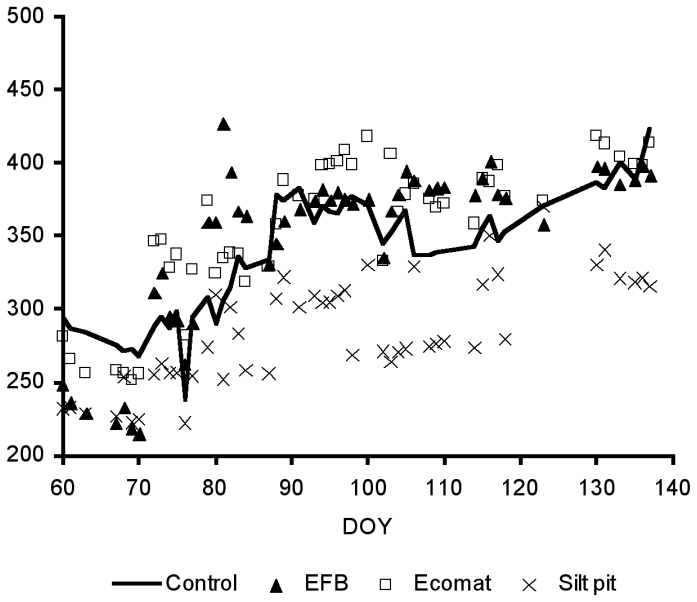

Fig. 3: Mean daily total soil water content (mm) up to 1.0 m soil depth in all the treatments. Solid line represents the control

in the upper soil layers as the soil increasingly received more rain.

The average daily total soil water contents up to 1 m of soil depth for the control, EFB, Ecomat, and silt pit treatment plots were calculated to be 338, 347, 356, and 285 mm, respectively. The ANOVA showed that there were significant differences at 5% level in the daily total soil water contents between the treatments. The mean separation test by the LSD method at 5% level, however, showed that there was no significant difference between the control, EFB, and Ecomat for the daily total soil water contents. Only silt pit had the significantly lowest mean daily soil water content compared to the other treatments. On average, the mean daily total soil water content in the silt pit plots were lower by nearly 18% compared to the control, EFB, and Ecomat plots.

The Ecomat mulches used in this study were only 20 mm in their thickness compared to the mean thickness of EFB, i.e. 130 mm. However, this study showed that the total soil water under the Ecomat mulches (though thinner than EFB) was statistically similar to that under the EFB mulches and the control plots which had pruned fronds as mulches. Surprisingly, silt pit, a conservation method supposedly to trap and redistribute runoff water, was shown to be the least effective method to conserve water, even when it was compared to control. The Department of Agriculture of Peninsular Malaysia recommends the use of silt pits for perennial crops on hill slopes between 6° to 25° (Eco-Factor Consulting, 2008). However, this study observed that even at 6° hill slope, the silt pit walls were easily collapsible, especially after heavy rainfalls. In some plots, the silt pits were observed to be half filled with soil in just one month and some completely filled two months later. Thus, over time and without rebuilding the walls, the silt pits became increasingly shallower and increasingly ineffective to trap runoff water. The observations carried out in this study suggest that silt pits require frequent maintenance so as to rebuild their walls and excavate the silt pits if they are to be effective as a soil and water conservation method, particularly for areas with high rainfalls and with even steeper hill gradients (> 6°).

CONCLUSIONS

Even in a short period of six months, there were significant effects of the four treatments (control EFB, Ecomat, and silt pit) on the chemical and physical properties of soil. Overall, the EFB was found to be the best treatment to improve the properties of soil, followed by the Ecomat treatment. In general, the silt pit and control treatments had similar effects on the properties of soil.

Although the intention of silt pit was to trap running water and return the water to the field, this study found that the plots with the silt pit treatment had the significantly lowest daily total soil water content as compared to the other three treatments (less by an average of almost 18%). Meanwhile, the mean daily water contents in the control, EFB, and Ecomat were not significantly different from one another at 5% level of significance.

This study in on-going and it will only end after three years of field experimentation. Nevertheless, the results have so far suggested that the EFB, followed by Ecomat, was the best soil and water conservation method, particularly to improve the chemical properties of soil. Silt pits are seen to be high-maintenance as their walls require frequent repairs and pit excavations if they are to be effective to trap runoff.

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Protein Profiling during Mesocarp Development in Oil Palm Fruit

Khalid Ghazi Fandi[1*], Farida Habib Shah[2] and Hasanah M. Ghazali[3]
[1]Department of Biological Sciences, Faculty of Science,
Al-Hussein Bin Talal University, P.O. Box 20, 71111, Ma'an, Jordan
[2]Novel plants Sdn. Bhd., 18, Jalan RH1, Taman Rembia, Melaka, Malaysia
[3]Faculty of Food Science and Biotechnology, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: fandikh@yahoo.com

ABSTRACT

This study was aimed at investigating the overall protein profiles of oil palm fruit during the mesocarp tissue development by means of isoelectric focusing (IEF) and two-dimensional gel electrophoresis (2-DE). Total protein was extracted from different stages of fruit development (namely, 5, 12, 15, 17, and 20 weeks after anthesis [WAA]) from *Elaeis guineensis* Jacq. Tenera and *E. oleifera* (17 WAA). The IEF separation was carried out on pH values ranging from 4.0-8.0. Changes in the patterns of protein after IEF were observed during mesocarp development and between the two species. The analysis of oil palm mesocarp gave rise to a protein map, comprising approximately 150 spots that were detectable by silver staining following high resolution 2-DE, with a pH range of 4.5-8.0 and a mass range of 8-100 kDa. Meanwhile, twenty five spots of protein showing variations in their intensity during the development of the mesocarp, with their p*I* ranging from 4.5-7.8 and M*r* 20-85 kDa, were analyzed. Continuous but non-uniform disappearance of some proteins and formation of new proteins were observed at the early stages of mesocarp development and during certain periods of oil synthesis and fruit ripening. The results of this study indicate that developing mesocarp revealed significant changes in the protein profiles during fruit development. However, further studies are still required to identify the proteins that are differentially expressed during fruit development.

Keywords: Two-dimensional gel electrophoresis (2-DE), mesocarp, *Elaeis guineensis, Elaeis oleifera*, oil palm

ABBREVIATIONS

WAA	:	Week after anthesis
IEF	:	Isoelectric focusing
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

INTRODUCTION

Palm oil is the second largest source of edible oil in the world and it has been recognized or accepted as one of the most usable consumer oil compared to other plant oils (Basiron, 2007; Basri *et al.*, 2005). In oil palm, there are two important species in the genus *Elaeis*, *E. guineensis* that has its centre of origin in Africa and the South American species, *E. oleifera.* During the fruit development in oil palm, oil

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[*]Corresponding Author

synthesizes and accumulates predominantly in the mesocarp tissue. Although the exact time of the beginning of oil accumulation in palms is still uncertain, published results have suggested that the period of active oil synthesis starts at 12 weeks after anthesis (WAA), and the period of active oil synthesis is usually around 15 WAA and it ends when the fruit ripen at about 20 WAA (Aziz *et al.*, 1986; Oo *et al.*, 1986). In the mesocarp, the level of unsaturated oil in *E. oleifera* is 56%, whereas this is around 39% in *E. guineensis* Jacq. (Tenera) oil. The iodine value is also much higher in *E. oleifera.* However, the oil yield of pure *E. oleifera* is much lower, with oil to bunch ratio of 5%, as compared to the *E. guineensis* (Tenera) with oil to bunch ratio of more than 25% (Rajanaidu *et al.*, 1997; Rajanaidu *et al.*, 2000). It is likely that in the oil palm mesocarp, certain enzymes involved in fatty acid biosynthesis are present or abundantly present during the oil synthesis period. Regulatory proteins which are involved in switching on or increasing the level of expression of the genes coding for these enzymes may be present at the start or just before the period of active oil synthesis. Meanwhile, the variation in the gene expression has been shown by analyzing proteins synthesized *in vitro* from translated mRNA of mesocarp *E. guineensis* at different stages of oil synthesis (Abdullah *et al*., 1994; Cha and Shah, 2005). The results showed the presence of two proteins (namely molecular weight 68 kDa and 32 kDa) in greater abundance than the rest during 15 WAA and 20 WAA, respectively (Oo *et al.*, 1986). Budiani *et al.* (2002) reported that the expression of the two proteins with M*r* 31.0 kDa and M*r* 34.3 kDa increased sharply at the beginning and just before the period of active oil biosynthesis, respectively. Another study using the SDS-PAGE analysis of total proteins (Shah, unpublished results) showed that proteins of different sizes (namely, M*r* 68, 42, 37, 34, and 32 kDa) were differentially expressed during the development of mesocarp in *E. guineensis* (Tenera), while a protein with M*r* 29 kDa was expressed in *E. oleifera*, but not in *E. guineensis* (Tenera).

In this study, the two-dimensional gel electrophoresis (2-DE) was utilized to give a better resolution in the separation of the total protein during the mesocarp development in *E. guineensis* (Tenera), and it was also used to detect different proteins expressed in the mesocarp of *E. oleifera* and *E. guineensis* (Tenera) during active oil synthesis, as this might suggest a differential gene expression of oil synthesis between the two species. Thus, the present study provides an overview of the main oil palm *E. guineensis* and *E. oleifera* proteome variations during the precise stages of mesocarp development (oil synthesis) and ripening. The protein profile, using 2-DE, shall be helpful in understanding the biochemical and the molecular changes at different stages of development of oil palm fruit and during the oil synthesis.

MATERIALS AND METHODS

Plant Material

Oil palm of two species (*Elaeis oleifera*) and (*Elaeis guineensis,* Jacq.) of Tenera type inflorescences were tagged at anthesis and fresh fruit bunches were collected at different stages of development (5, 12, 15, 17 and 20 WAA). After the collection, the exocarp and kernel were removed from the fruits. The remaining mesocarp tissues were immediately frozen in liquid nitrogen upon collection and then stored at -80°C until further use.

Protein Extraction

Proteins were extracted essentially following the method proposed by Des Francs *et al.* (1985) with slight modifications. Frozen mesocarp from different WAA were homogenized in liquid nitrogen with a mortar and pestle, and the powder was resuspended in an extraction buffer (300 mM NaCl, 1 mM EDTA, 2% ampholyte pH 3.5-10 and 5-7 in the ratio of 1:4 and 10 µg ml^{-1} leupeptin), at the ratio of 1 to 2 (v/w). This mixture was incubated at room temperature (~ 27°C) for 15 min and then centrifuged at 13 000 × *g* for 5 min. Solid urea was added to

the supernatant to a final concentration of 9 M. Protein concentrations were estimated using the method proposed by Bradford (1976).

First-dimensional Gel Electrophoretic Analysis

Polyacrylamide-gel isoelectric focusing (IEF) analysis was performed as described by O'Farrell (1975). The IEF was carried out in 100 mm × 5 mm (ID) cylindrical tubes on a stand-alone casting device. The components of the gel tubes were 9 M urea, 2% ampholines Bio-lyte, 4% N-P40, 3.2% T, and 2.5% C acrylamide. The IEF linear pH gradient (about 4.0 to 8.0) was performed in the rod gels, with a 2% (v/v) mixture of Bio-lyte Ampholines of pH 5-7 and 3.5-10 (Bio-Rad, Richmond, Calif.) at a proportion of 4:1. When the polymerization of IEF gels was completed, 80 µg of protein sample in a volume up to 100 µl were loaded onto separate pre-focused tube gel. The first dimensional separation was performed at a constant voltage of 400V for 12 hr and of 800V for the final hour.

Coomassie Blue Staining

Gels were extruded from cylindrical tubes and placed in large test tubes containing a fixing solution (4% sulfosalicyclic acid, 12% trichloroacetic acid) and shaken gently for several hours, after which the fixative was poured off and replaced with staining solution (0.04% Coomassie Blue R-250, 0.5% $CuSO_4$, 27% isopropanol, and 10% acetic acid) and shaken gently over-night. Excess dye was removed with destaining I solution (12% isopropanol, 7% acetic acid, 0.5% $CuSO_4$). The gel was then incubated in destaining II solution (7% acetic acid, 5% methanol). After staining with Coomassie Blue, the total protein was expressed at each individual WAA in *E. guineensis* and at 17 WAA in *E. oleifera*.

2D-PAGE

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with IEF in the first dimension was performed using SE-600 system (Hoefer, CA, USA) in accordance with the laboratory manual adapted by Anderson (1991) with minor modifications. Briefly, the first-dimension, isoelectric focusing (IEF), based on the method of O'Farrell *et al.* (1977), was carried out as above, except that 160 mm × 1.5 mm rod gels were used. The samples containing 30 µg of protein in 25 µl were loaded after pre-focusing the gels for 1 hr at 400V. Electrophoresis was carried out at 400V for 12 hr followed by 1 hr at 800V. After the IEF, the gel rods were extracted and then equilibrated for 30 min in sodium dodecyl sulphate (SDS) sample buffer (Laemmli, 1970), and were either stored at –70°C or immediately loaded onto second-dimension SDS-PAGE. The second-dimension was performed in a vertical-slab gel electrophoresis (16 × 18 cm) using a 12% acrylamide resolving gel, without stacking gel, run at 30 mA per gel for about 4 hr. A high-molecular-mass marker (Pharmacia Biotech) that produced bands at 97, 66, 45, 30, 20.1, and 14.4 kDa were used. After the electrophoresis, the analytical gels were fixed overnight in ethanol:acetic acid:water solution (5:1:4, by vol.). The protein profiles were visualized by silver staining method according to Oakley *et al*. (1980), and modified by Hochstrasser *et al*. (1988). The experiments were performed in duplicate, and the representative gels were shown.

Gel Drying and Analysis

Fresh gels were soaked in 3% glycerol for 30 min and then placed between 2 dry sheets (Gel drying film, Promega) over-night. The dried gels were scanned with a GS-800 calibrated densitometer (Bio-Rad) and the gel images were analyzed using PDQuest 2-D software quantification (Bio-Rad). The gels were standardized by calculating the intensity of each spot as the percentage of the total intensity of the spots visualized on a gel, after which the differences of expression (induction or repression) of the spots between gels were statistically meaningful according to the PDQuest software.

RESULTS AND DISCUSSION

Protein Patterns in IEF during Mesocarp Development

Comparison of the IEF profiles of proteins during the development of mesocarp showed changes in the patterns of the total protein accumulated. The results of the IEF of proteins, extracted from mesocarp at different WAA from *E. guineensis* (Tenera) (5, 12, 15, 17 and 20) and at 17 WAA for *E. oleifera*, are shown in *Fig. 1*. The findings obtained indicate that the variations in the intensity of the expression patterns in the same p*I* region are due either to the synthesis or degradation of protein(s). However, at different p*I* regions, the variations in the intensity during the development of mesocarp in *E. guineensis* (Tenera) were observed. For example, a group of acidic proteins with *p*I ranging between 5.0 and 6.1 were abundantly expressed at 5, 12 and 15 WAA, while the expression level for these proteins at the different weeks remained unaffected. Four proteins, namely b, c, f and h (*Fig. 1*) with p*I* values of 4.5, 5.0, 6.5 and 6.8, respectively, were found to be expressed only at 12 WAA. A similar observation was made for Protein I (p*I* 6.9) but this occurred at 15 WAA. Two proteins, namely e and g (with p*I* values of 6.82 and 7.85, respectively), were only expressed in 15 and 17 WAA, while Protein g was observed to be expressed at 20 WAA. Protein j (p*I* 7.1) was expressed at a higher level at 15 WAA compared to the other weeks of development. Three major proteins with p*I* values of 6.9, 7.2 and 7.5 were highly expressed only at 17 WAA in *E. oleifera*. For a better resolution, these proteins were then separated by 2-DE.

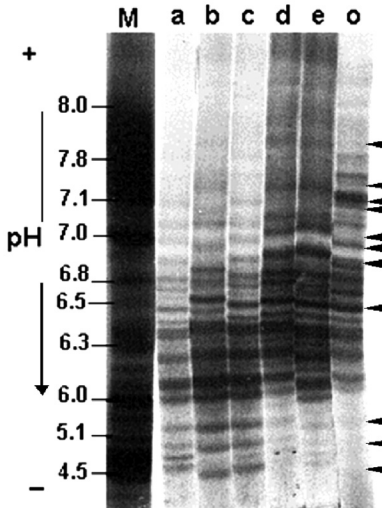

Fig. 1: IEF of the total proteins during the development of mesocarp. IEF was performed in pH 4.0-8.0 gradient using Ampholines mixture pH 3.5-10/5-7 in ratio of 4:1. A 80 µg protein loading was used for this purpose. Rod gels were stained with Coomassie Blue. Lanes a, b, c, d, e and o indicate 5, 12, 15, 17 and 20 WAA of E. guineensis *(Tenera) and 17 WAA of* E. oliefera*, respectively. Meanwhile, Lane M contained the IEF markers. Arrows show the observed variation of the proteins of different pI*

The earlier results by Abdullah *et al*. (1994), with the use of PAGE of *in vitro* translation products of mRNA, indicated that proteins with M*r* of 68 and 32 kDa had showed differential expression during the development of the oil palm mesocarp. The protein with M*r* 68 kDa molecular weight was observed at the start of oil synthesis, with the highest level seen at 15 WAA (Abdullah *et al.*, 1994). Extending this further, the researchers investigated the differential synthesis of total proteins during mesocarp and kernel development in the oil palm *E. guineensis* (Tenera) using the one-dimensional gel electrophoresis (Shah, unpublished data). Electrophoresis indicates the differences in the levels of proteins, as shown by the presence or absence of bands or the presence of bands with different intensities, showing that different proteins are synthesized at different week of mesocarp development corresponding to the different stages of oil synthesis. These results prompted the researchers to investigate the proteins using 2D-PAGE to give a higher resolution of the proteins of interest.

2-DE of Protein Analysis

In this study, the 2-DE patterns of the total and newly synthesized proteins during the development of mesocarp were also analyzed

at different weeks after anthesis. *Fig. 2* (*A, B, C, D, E*) show the overall patterns of the protein expression at the different stages of mesocarp development of *E. guineensis* (Tenera). Several proteins with molecular masses between 25 to 90 kDa showed changes in the level of expression, synthesized or disappeared during the five selected week of development.

The gels were divided into two squares (I) and (II) (4 × 2.5 cm) and amplified by computer scanning for mapping the total protein in the range of M*r* 25-90 kDa (*Fig. 3*). As observed, there were significant protein changes during mesocarp development in the Tenera. Meanwhile, twenty-five proteins showed variations during the development (*Figs. 3a, b, c, d, e*). The major protein changes noted during the development included the disappearance of some proteins, synthesis of new proteins, or an enhanced synthesis of preexisting protein (Table 1). For example, three proteins (1, 2, and 3) of M*r* 60, p*I* 6.0: M*r* 47, p*I* .6.0 and M*r* 47, p*I*

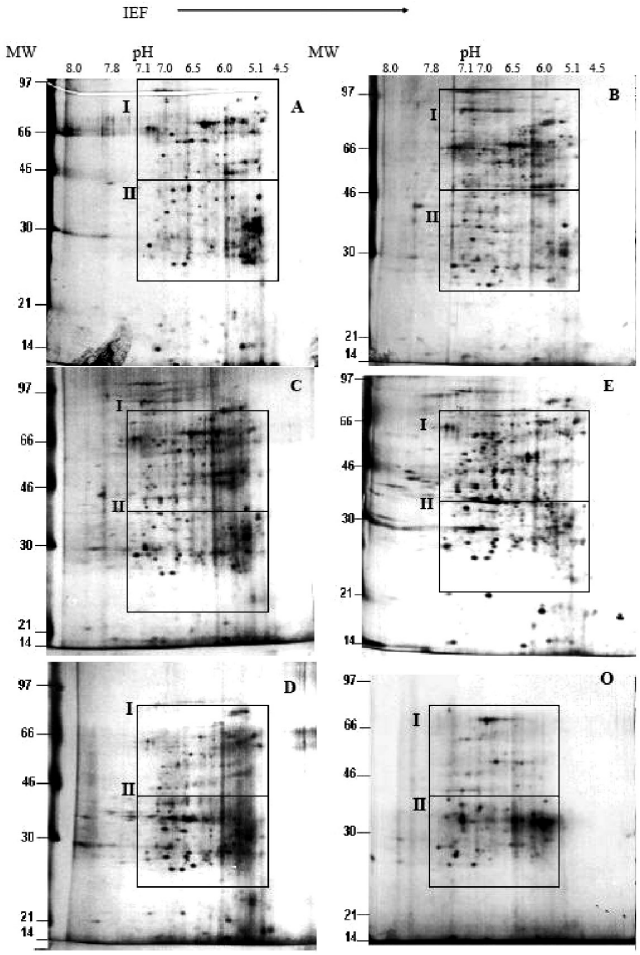

Fig. 2: Silver-stained 2-D gels of the total and newly synthesized proteins during mesocarp development. 2-DE patterns in E. guineensis *(Tenera) (A, 5 WAA; B, 12 WAA; C, 15 WAA; D, 20 WAA; E, 17 WAA) and 17 WAA for* E. oliefera *(O). The IEF was performed in pH 4.0-8.0 gradient. Mr standards are indicated for the analysis of gels which were divided into two squares I and II showing the variation in the protein patterns*

6.1 were observed only at 5 WAA, and were absent during the other stages of development. Four proteins (5, 6, 7 and 24) of M*r* 66, p*I* 7.3: M*r* 65, p*I* 7.0: M*r* 65, p*I* 7.1 and M*r* 28, p*I* 7.1, respectively, were highly expressed at 12 and 15 WAA, but were not detected at 17 WAA and they were found to be lower in their amounts at 5 and 20 WAA. One protein (4) of M*r* 71, p*I* 6.5 was abundantly present at 5, 12, 15 WAA, but it was not detected at 17 and 20 WAA. Three proteins (8, 11, 23) of M*r* 45, p*I* 6.2, M*r* 36, p*I* 5.1, and M*r* 29, p*I* 7.0 were observed to appear only at 12 WAA and were highly expressed at 15 WAA. These proteins were found to be either absent or present at low concentrations, at 17 and 20 WAA. Three other proteins (15, 16, 17) of

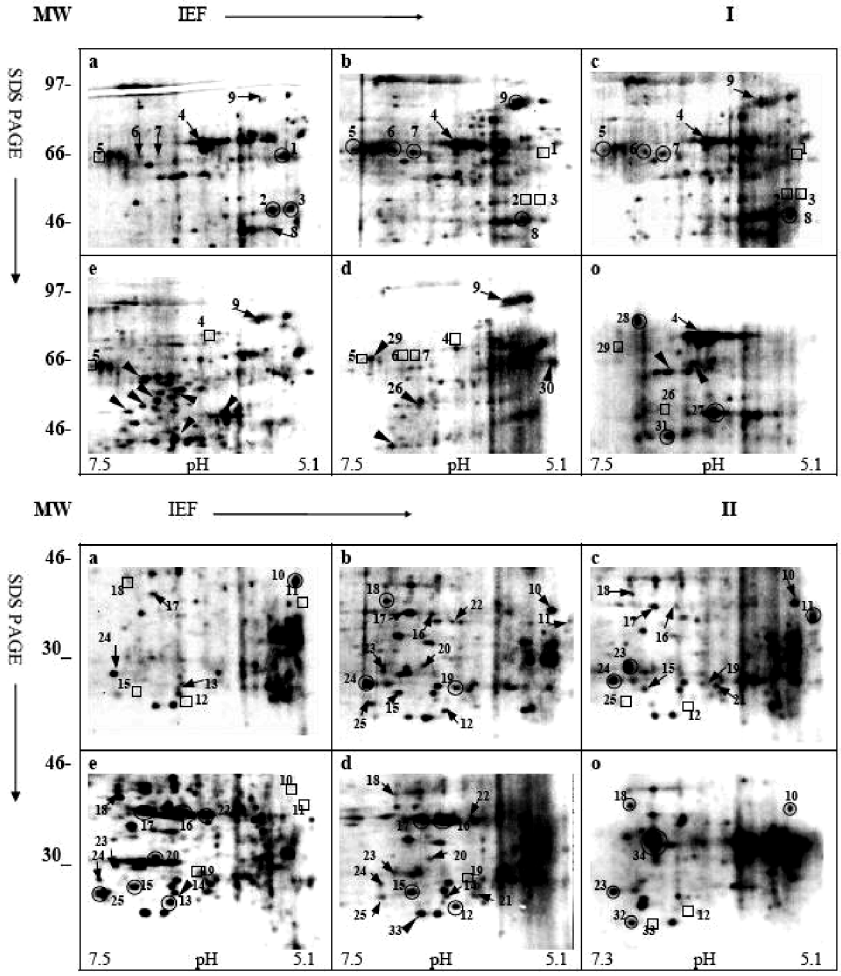

Fig. 3: Amplified 2-DE patterns of Squire I and II. The figure shows the locations of proteins in Square I and II from Fig. 2. The spots that disappeared or appeared (newly synthesized) in high amounts during the fruit development are indicated by the squares and circles, respectively. Arrows (►) point to the presence with enhanced expression during the fruit development protein; Arrow heads (—►) points to the proteins expressed during the fruit ripening stage. For E. oleifera *and* E. guineensis *(Tenera) at 17 WAA, the presence of highly expressed proteins is indicated by the arrowheads followed by the numbers and open circles, respectively*

TABLE 1
Protein changes during the development of mesocarp in *E. guineensis* (Tenera)

Protein	P*I*	Mr	5 WAA	12WAA	15WAA	17WAA	20WAA
1	6.0	60	H	N	N	N	N
2	6.1	47	H	N	N	N	N
3	6.0	47	H	N	N	N	N
4	6.5	71	H	H	H	N	N
5	7.3	66	N	H	H	N	P
6	7.0	65	L	H	H	N	N
7	6.9	65	L	H	H	N	N
8	6.2	45	N	P	H	N	N
9	6.2	82	L	H	P	P	P
10	5.6	38	H	P	P	N	N
11	5.1	36	N	L	H	N	N
12	6.6	26	N	P	N	H	N
13	6.7	26	P	N	N	N	H
14	6.7	27	N	N	N	L	H
15	6.8	27	N	P	P	H	H
16	6.7	37	N	P	L	H	H
17	7.0	37	L	P	P	H	H
18	7.2	38	L	H	P	L	P
19	6.6	28	L	H	L	N	N
20	6.9	32	N	L	N	P	H
21	6.4	27	N	N	P	P	L
22	6.5	37	N	P	N	P	H
23	7.0	29	N	L	H	L	N
24	7.1	28	P	H	H	L	L
25	7.1	27	N	L	N	L	H

Refer to *Fig. 2*, *Fig. 3* (I) and *3* (II) for the location of the different proteins on the two-dimensional protein map. M*r* data are the means of two gels. Protein intensity levels are expressed as H; high expression L; low expression P; present N; not present. Data were obtained through computer scanning of photos of the dried 2-DE gels at different stages during fruit development.

M*r* 27, p*I* 7.0, M*r* 37, p*I* 6.7, as well as M*r* 38, p*I* 7.2 at 12 and 15 WAA continued to be highly expressed even at 17 and 20 WAA. Meanwhile, two proteins (18, 19) of M*r* 37, p*I* 7.0, and M*r* 28, p*I* 6.6 were present in the low level at 5 WAA initially and were also abundantly present in high expression at 12 WAA, which later declined to undetectable levels at 15, 17, and 20 WAA. On the other hand, the protein (9) of M*r* 82, p*I* 6.2 was shown to be highly expressed at 12 WAA, and it continued to be present until 20 WAA. Three proteins (20, 22, 25) M*r* 32, p*I* 6.9, M*r* 37, p*I* 6.5, and M*r* 27, p*I* 7.1 were present at 12 WAA but absent at 15 WAA, which were expressed at 17 WAA and shown to be highly expressed at 20 WAA. The only protein that was expressed at the ripening stage (20 WAA) was protein 13 with a M*r* 26 and p*I* of 6.7.

A comparison of the 2-D protein patterns of *E. oleifera* and *E. guineensis* (Tenera) at 17 WAA was done by analyzing silver staining (*Fig. 2*) (O and D), respectively. The major differences between the two species, as shown in *Fig. 3* (I) and (II) (O and D), are tabulated in Table 2. Four highly expressed proteins (4, 27, 28, 32) of Mr 71, p*I* 6.5, M*r* 50, p*I* 6.4, M*r* 75, p*I* 7.0, and M*r* 25, p*I* 6.9, and one protein (10) of M*r* 38, p*I* 5.6 were found to be present in *E. oleifera* but not in *E. guineenses* (Tenera). Meanwhile, three proteins (18, 23, 31) of M*r* 38, p*I* 7.2, M*r* 29, p*I* 7.0, and M*r* 45, p*I* 6.8 were highly abundant in *E. oleifera* but these were found to be less in

TABLE 2
Comparison between the 2-D pattern of total proteins from 17 WAA of *E. guineensis* (Tenera) and *E. oleifera* mesocarp

Protein	P*I*	Mr	17 WAA *Oleifera*	17 WAA Tenara
4	6.5	71	H	N
9	6.2	82	N	H
10	5.6	38	P	N
12	6.6	26	N	H
18	7.2	38	H	L
23	7.0	29	H	L
26	6.8	53	N	P
27	6.4	50	H	N
28	7.0	75	H	N
29	7.2	66	N	P
30	5.5	64	N	H
31	6.8	45	H	L
32	6.9	25	H	N
33	6.8	25	N	H
34	6.8	37	H	P

Refer to *Fig. 2*, *Fig. 3* (I) and *3* (II), D and O, for the location of the different proteins on the two-dimensional protein map. M*r* data are the means of two gels. Protein intensity levels are expressed as H; high expression L; low expression P; present N; not present. Data were obtained through computer scanning of photos of the dried 2-D gels from active oil synthesis 17 week *E. guineensis* (Tenera) and *E. oleifera.*

E. guineenses (Tenera). Similarly, four highly abundant proteins (9, 12, 30, 33) of M*r* 82, p*I* 6.2, M*r* 26, p*I* 6.6, M*r* 64, p*I* 5.5, and M*r* 25, p*I* 6.8, and two proteins (26, 29) of M*r* 53, p*I* 6.8, and M*r* 66, p*I* 7.2, were present in *E. guineensis* (Tenera), but they were not found in *E. oleifera*.

The analysis by 2-DE confirmed the differential expression of the total proteins observed in the IEF during the mesocarp development and between *E. guineensis* and *E. oleifera* at the week of active oil synthesis (17 WAA). The changes in the lipid class and fatty acid compositions during the development of oil palm mesocarp and the variation in the lipid metabolism between *E. guineensis* and *E. oleifera* were reported by Oo *et al.* (1985) and Sambanthamurthi *et al.* (1987), respectively. Therefore, the differences observed between the total protein patterns during the period of active oil synthesis (17 WAA) may suggest the possibility of a different regulatory mechanism of oil synthesis between the two species. A previous work on the differential gene expression in different tissues and species, using mRNA differential display, revealed that one of the mesocarp-specific genes was specific for *E. oleifera* (Shah and Cha, 2000). Meanwhile, sequence homology showed that it codes for enzyme sesquiterpene synthase, which has a molecular weight ranging from 68-72 kDa. Therefore, it would be very interesting to analyze, in future studies, the protein obtained in this study from *E. oleifera* with molecular weight of 75 kDa, whether it could be similar to the protein observed in the other work or being involved in oil synthesis.

Development is characterized by the differential synthesis of gene products (transcripts) in time and space; few plant genes whose expression directly influences development have been identified. While these transcripts are produced in response to developmental signals (Davies and Robinson 2000), some proteins themselves associated with fruit maturity and in the development process (Abdi *et al*., 2002; Barraclough *et al.*,

2004). In the recent years, proteome analysis has been successfully applied to a range of plant tissues, such as grape skin ripening (Deytieux *et al.*, 2007), rip grape mesocarp (Sarry *et al.*, 2004), germinating tomato seeds (Sheoran *et al.*, 2005), and leaves, shoots, and roots of grapevine plantlets (Castro *et al.*, 2005). Enlargement of mesocarpic cells and differentiation of endocarp may not require very marked qualitative or quantitative changes at the molecular level. The former process, which is responsible for rapid increase of size and weight of the oil palm, may not involve the production of a different set of proteins but a more rapid synthesis of the existing ones. Meanwhile, developmentally regulated genes can be either codes for proteins with regulatory function or codes for proteins involved in tissue-specific function (Gasser *et al.*, 1988). A previous report on N-terminal amino acid sequences of proteins expressed during the period of oil synthesis in mesocarp tissues with p*I* range between 4.5 and 4.95 revealed a similarity to acetyl-CoA carboxylase (ACCase), enoyl-ACP reductase, and glyceraldehyde-3-phosphate dehydrogenase that are involved in oil biosynthesis (Budiani *et al.*, 2002; Harwood and Page, 1994). Therefore, proteins which were synthesized during or just before the active oil synthesis [12 WAA] might play significant roles in oil synthesis or regulatory mechanisms involved in oil synthesis. However, this would have to await further analysis on the eluted proteins. From the eluted proteins, DNA probes shall be designed according to the information from the amino acid sequence. The genes coding of the proteins of interest could be isolated and characterized in order to find out whether they have any important regulatory function/s or whether they code for functionally important proteins in oil synthesis.

CONCLUSIONS

To date, apart from the work published by Shah and Cha (2000), no other work has been done to investigate the production of different metabolites during mesocarp development and oil production in oil palm. The present study provides basic information on proteins map using 2-DE which is of utmost importance for an understanding of molecular and biochemical changes that happen at different stages of oil palm fruit development. The electrophoretic patterns/spots, obtained through the IEF and 2-DE of the developing mesocarp of *E. guineensis* proteins showed the variations in their intensity during precise stages of mesocarp development (oil synthesis) and ripening stage. Meanwhile, the disappearance of protein patterns/spots and loss of staining intensity appeared to be more abrupt during the early stages of fruit (5 WAA). Other proteins were observed to start synthesis at approximately 12 WAA (oil deposition in the mesocarp) and continue until fruit maturity at about 20 WAA. The findings of the present study also indicate that there are differences in the protein profiles during mesocarp development in oil palm between *E. guineensis* and *E. oleifera* at the accumulating oil stage (17 WAA). However, further analyses have to be done using MALDI-MS to identify the spots of the proteins of interest and to elucidate whether they contribute in any direct way to the mechanisms of oil synthesis or involve in repining process.

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Accumulation and Depuration of Cu and Zn in the Blood Cockle *Anadara granosa* (Linnaeus) under Laboratory Conditions

Yap, C.K.[1*], Muhamad Azlan, A.G.[1], Cheng, W.H. and S.G. Tan[2]
[1]Department of Biology, Faculty of Science,
[2]Department of Cell and Molecular Biology,
Faculty of Biotechnology and Biomolecular Sciences,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: yapckong@hotmail.com

ABSTRACT

Studies on the accumulation (4 days of single metal exposure) and depuration (6 days in natural seawater) of Cu and Zn were conducted in the blood cockle *Anadara granosa* under laboratory conditions. Different rates of accumulation and depuration between the soft and hard tissues probably reflect the different mechanisms of binding and regulation of Cu and Zn between the soft tissues and shells of cockles. At the end of depuration, the concentrations of Cu and Zn in the soft tissues were only 1.71 and 1.75 times higher than prior to the exposure, respectively. Thus, no significant difference was found in the depuration level between Cu and Zn. Hard tissues showed that the levels of Cu and Zn are similar to those before the exposure. This indicated the slow rates of accumulation and depuration in the shells as compared to the soft tissues of *A. granosa*. The condition index of *A. granosa* could be used as a potential physiological indicator of metal pollution. The capabilities to accumulate Cu and Zn and to depurate both metals in the soft tissues indicate that *A. granosa* is a potential biomonitoring organism for its health assessment using the condition index.

Keywords: ***Anadara granosa*, Cu and Zn exposure, laboratory studies, accumulation and depuration of Cu and Zn in the cockles**

INTRODUCTION

Cu and Zn have attracted a lot of attention in ecotoxicological studies in the literature. For example, Yap *et al*. (2003b: 2004a) proposed the use of mussel shell as a biomonitoring material for Zn, while Yap *et al*. (2003c) suggested the use of different soft tissues of *Perna viridis* as biomonitoring agents for the Cu pollution in coastal waters.

The cockle, *Anadara granosa* (Order: Arcoida; Family: Arcidae), exhibits characteristics as a good biomonitor of heavy metal pollution. They have a sessile life style, have a wide geographical distribution in the tropical intertidal muddy sediments (Lowe and Kendall, 1990; Dame, 1996), and are filter feeders ingesting both zooplankton and phytoplankton, in addition to being easily collected. They are important to local commercial fisheries and are easy to maintain in the laboratory (Ong and Din, 2001). Cockles thrive best under calm conditions, especially in shallow inlet bays, with soft and flocculent mud with salinity ranging from 18 to 30 ppt (part per thousand) (Ng, 1984; Panthasali and Soong, 1985).

Some previous studies have shown that *A. granosa* are able to accumulate Cd and Cu to a significant level in their tissues (Mat, 1994;

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[*]Corresponding Author

Mat *et al*., 1994a, b; Noorddin, 1995). The exposure to Cd causes significant increases in the concentrations of Cd in the gills, hepatopancreas and the total soft tissues of cockles as compared to the controls (Chan *et al*., 2002). However, little is known about the distributions of Cu and Zn in the soft and hard tissues of *A. granosa*. Therefore, the objective of this study was to determine the accumulation and depuration of Cu and Zn in the soft and hard tissues of *A. granosa* under laboratory conditions.

MATERIALS AND METHODS

Sampling and Acclimatization of the Cockles

Individual *A. granosa* were purchased from the wet market in Port Dickson, Negeri Sembilan, Malaysia (collected from Teluk Intan, Perak). For acclimatization purpose, the cockles were then transplanted to the mudflat areas in Pasir Panjang, Negeri Sembilan for 7 days. The cockles were checked everyday and they were considered dead if they did not show any tactile stimulus and gape their shell valves wide. Similarly, salinity and temperature were also checked every day. The Pasir Panjang area has a salinity range of 26 to 30 ppt and a temperature range of about 25 to 30°C. Later, all of the cockles were transported to Hatchery 1, at the Center of Marine Science Research, Universiti Putra Malaysia, Teluk Kemang, Port Dickson, Negeri Sembilan, Malaysia.

All the experimental seawater was sand filtered. Healthy acclimatized cockles were selected for the exposure study. Sixty cockles were exposed in each plastic aquarium. All the experimental treatments were based on a single metal exposure study. Prior to the metal exposure, 20 cockles were collected to analyse the background metal concentrations. The cockles were exposed to sublethal concentrations of Cu (nominal: 0.10 mg/L; measured 0.133 mg/L) and Zn (nominal; 1.00 mg/L; measured 1.323 mg/L) for 6 days. A control aquarium, with cockles but without the addition of metal solution to sand filtered seawater, was also simultaneously set up. The measured concentrations were close to the nominal concentrations, as shown in Table 1. The test solutions (10 L) were constantly changed once every two days to new seawater spiked with standard solutions of Cu and Zn on Days 0, 2 and 4. On Day 6, the cockles were rinsed with clean seawater and were transferred into a clean seawater aquarium for the depuration study. Samplings (12 cockles at each sampling) were conducted on Days 2, 4 and 6 during the metal accumulation period and on Days 8 and 10 during the depuration period. All the samples were stored at -10°C until further analysis. The test seawater was constantly aerated and held at room temperature (26-30°C) and salinity 28-30 ppt.

Sample Preparation

The samples of cockles were then thawed at room temperature (27°C) on a clean tissue paper to drain away the excess water before being analyzed, dried for 72 h at 105ºC in an oven

TABLE 1
The nominal and measured test concentration (mg/L) of Cu and Zn during exposure periods

Metals	Nominal	Day of exposure	Measured Mean ± SE
Cu	0.1	0	0.137 ± 0.004
		2	0.162 ± 0.015
		4	0.122 ± 0.020
Zn	1.0	0	1.320 ± 0.033
		2	1.262 ± 0.024
		4	0.987 ± 0.040

to constant dry weight. After that, the samples were digested in concentrated nitric acid (Ajax Chemicals, HNO_3 65 %, Australia) in a hot-block digester, first at a low temperature (40°C) for 1 hour and then at a high temperature (140°C) for at least 3 hours. The samples were assumed to be digested after the acidic solutions had became clear (Yap *et al*., 2003b). The digested samples were then diluted with double distilled water.

Determination of Cu and Zn in Cockles

Cu and Zn were determined using the air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin Elmer model AAnalyst 800. The data were calculated on μg/g dry weight basis. All the glassware and equipment used were acid-washed to avoid possible contamination, while procedural blanks were analysed in every five sample to check for contamination. Finally, the quality control samples (made from the standard solutions of Cu and Zn) were analyzed in every five sample to check for their recoveries (Yap *et al*., 2002).

Data Analysis

The bioconcentration factor (BCF) was calculated in relation to the metal concentration in seawater according to Taylor (1983) and Yap *et al*. (2003a: 2004b):

$$BCF = \frac{Ce - Ci}{Cs}$$

Ce = he metal concentration in the cockle tissue during metal exposure (g/g wet weight)

Ci = the initial metal concentration in the cockle tissue before metal exposure (g/g wet weight)

Cs = the experimental metal concentration in the aquarium test seawater

The concentration factor (CF) was calculated at the end of depuration (Day 6) in comparison with the level of metal before exposure, as follows (Yap *et al*., 2004b):

$$CF = \frac{\text{Metal level}_{\text{end of metal depuration}}}{\text{Metal level}_{\text{pre-exposure of metal}}}$$

In order to measure the percentage of metal reduction, metal concentrations in the soft tissues and shells of *A. granosa* were taken as the end of exposure (Day 4), i.e. (after 4 days of the exposure period. At the end of the depuration period, the percentage of metal reduction in the soft tissues and shells of *A. granosa* (Yap *et al*., 2003a) was characterized based on the following equation:

Percentages of metal reduction =

$$\frac{\text{Metal level}_{\text{end of metal exposure}} - \text{Metal level}_{\text{end of metal depuration}}}{\text{Metal level}_{\text{end of metal exposure}} - \text{Metal level}_{\text{pre-exposure of metal}}} \times 100\%$$

The rate of metal accumulation was calculated according to the following formula (Yap *et al*., 2003a):

Rate of metal accumulation =

$$\frac{\text{Metal level}_{\text{exposed}} - \text{Metal level}_{\text{control}}}{\text{Day(s) of metal exposure}}$$

The rate of metal depuration was calculated according to the following formula (Yap *et al*., 2003a):

Rate of metal depuration =

$$\frac{\text{Metal level}_{\text{end of metal exposure}} - \text{Metal level}_{\text{end of metal depuration}}}{\text{Day(s) of metal depuration}}$$

T-test of metal levels between the end of accumulation (Day 6) and control treatment, and between the end of depuration (Day 10) and the control treatments were done by using STATISTICA.

RESULTS AND DISCUSSION

Accumulation and Depuration in the Soft Tissues of Cockles

Table 2 shows the accumulation and depuration of Cu and Zn in the soft tissues of cockles. For both metals, the metal levels were found to increase during the accumulation period but they decreased during the depuration period. Basically, there were only slightly differences in the accumulation and depuration patterns of these two metals. Both metals were found in significantly ($P< 0.05$) higher levels in the soft tissues at the end of accumulation (Day 6) and at the end of depuration (Day 10) as compared to those in the control treatments (Table 3).

Table 4 shows the rates of accumulation and depuration of Cu and Zn. The rates of metal accumulation in the soft tissues were faster at Day 2 than at Days 4 and 6. In general, the accumulation rates in the heavy metal exposure were higher than in the control exposure. Exposures to Cu and Zn singly showed the highest BCF values at the end of the accumulation period (i.e. Day 6), as shown in Table 4. However, the levels of Cu and Zn in the soft tissues of cockles, exposed to both the metals at the end of depuration, were close to the levels prior to the exposure (CF: < 2 times).

During the depuration period, their levels were found to decrease (Table 2). According to the accumulation rate, the cockles have a higher capability to accumulate Zn (highest rate = 37.98) than Cu (highest rate = 2.91) from ambient water. This conclusion was also supported by the BCF values (Table 4).

The high accumulations of Cu and Zn in the soft tissues of *A. granosa* were able to

TABLE 2
Concentrations (mean μg/g dry weight ± standard error) of Cu and Zn during the accumulation and depuration in the soft and hard tissues (shell) of *Anadara granosa* (N=3)

Metal/Tissue		During accumulation period				During depuration period	
		Day 0	Day 2	Day 4	Day 6	Day 8	Day 10
Cu	Soft tissue	6.92	10.99 ± 0.20	14.27 ± 0.42	18.20 ± 0.16	14.59 ± 0.05	11.83 ± 0.15
	Shells	6.56	6.69 ± 0.09	6.85 ± 0.06	6.94 ± 0.06	6.70 ± 0.03	6.63 ± 0.03
Control	Soft tissue	6.92	5.17 ±0.09	4.60 ± 0.03	5.30 ± 0.01	5.75 ± 0.10	5.93 ± 0.10
	Shells	6.56	6.59 ± 0.04	6.52 ± 0.11	6.51 ± 0.07	6.45 ± 0.14	6.39 ± 0.05
Zn	Soft tissue	106.9	182.04 ±2.32	258.3 ± 0.68	264.6 ± 1.25	201.8 ± 1.72	187.0 ± 0.87
	Shells	4.17	5.05 ±0.08	5.22 ± 0.06	5.57 ± 0.05	5.40 ± 0.05	5.30 ± 0.06
Control	Soft tissue	106.9	106.08 ± 0.39	107.9 ± 0.46	106.1 ± 0.37	109.2 ± 0.50	107.5 ± 0.51
	Shells	4.17	4.16 ± 0.03	4.22 ± 0.04	4.43 ± 0.07	4.20 ± 0.09	4.12 ± 0.04

TABLE 3
T-test results of the metals between the end of accumulation and the control treatment, and between end of depuration and control treatment

		End of accumulation (Day 6)	End of depuration (Day 10)
Cu	Soft tissues	$P< 0.05$	$P< 0.05$
	Shells	$P> 0.05$	$P> 0.05$
Zn	Soft tissues	$P< 0.05$	$P< 0.05$
	Shells	$P> 0.05$	$P> 0.05$

TABLE 4
Bioconcentration factor (BCF) and concentration factor (CF) and the accumulation and depuration rates (µg/g per day) of Cu and Zn in the cockles' soft and hard tissues exposed to 0.1 mg/L of Cu and 1.0 mg/L of Zn

	Metal	BCF	CF	Rate of accumulation			Rate of depuration	
				Day 2	Day 4	Day 6	Day 8	Day 10
Zn	Soft tissue	157.99	1.75	37.98	37.58	26.42	31.43	19.4
	Hard tissue	1.41	1.27	0.45	0.25	0.19	0.09	0.07
Cu	Soft tissue	112.80	1.71	2.91	2.42	2.15	1.81	1.60
	Hard tissue	3.40	1.01	0.05	0.08	0.07	0.12	0.08

induce metallothionein-like protein in high metal concentrations (Viarengo *et al*., 1985; Chan *et al*., 2002). Secretion of metallothionein in high metal concentrations could be a mechanism to counteract metal toxicity (Phillips and Rainbow, 1993). Although the function of this protein is still not fully understood, MT is generally accepted to involve in the regulation of essential metals, such as Cu and Zn for cell growth and development (Mackay *et al*., 1993). Meanwhile, Viarengo *et al*. (1985) reported that higher levels of accumulated metals in soft tissues were correlated to the metals binding to metallothionein and they could therefore be stored in detoxified forms.

The rate of accumulation and BCF showed the ability of cockles to accumulate metals to certain levels. Their accumulations over time in the bodies of organisms may cause toxicity (Gorell *et al*., 1997). In more specific, all heavy metals are potentially harmful to most organisms at some levels of exposure and absorption (Yilmaz, 2003). In order to survive in this metallic stress environment, cockles have to counteract metal toxicity. This could be done by regulating the intracellular concentrations of the metal and removing it to prevent deleterious effects on the functions of cell (Phillips, 1985). Results showing the depuration rate of metals during the depuration period are presented in Table 4. During the depuration period, a more rapid loss of Zn than Cu was observed. The CF values showed the ability of cockles to retain the accumulated metals during the depuration period (Table 4). Based on the CF values (Zn: 1.75 and Cu: 1.71), the soft tissues of cockles have almost similar abilities to retain the accumulated Cu and Zn.

Meanwhile, Zn and Cu are known as essential metals for metabolic functions (Mackay *et al*., 1993) and they can be regulated in bivalves (Yap *et al*., 2003a: 2004b). However, since the CF values for Zn (1.75) was almost similar to Cu (1.71), a rapid loss of Zn reported for the green-lipped mussel, *Perna viridis* (Watson *et al*., 1995), was not observed for cockles. Therefore, whether Zn was bound to the easily mobilized compartment was not evidently shown based on the present findings. Yap *et al*. (2003a) showed that in *P. virirdis,* Zn was possibly bound to the easily mobilized compartment in comparison to the non-essential Cd which was clearly shown to be not easily lost possibly due to its metallothionein binding. Since the present work focused only on Zn and Cu, such a comparison with a non-essential metal is not possible. In addition, although Zn was reported to be regulated in the soft tissues of many bivalves in the literature, this is not shown in the present study.

Accumulation and Depuration in the Shells of Cockles

Table 2 shows the levels of metals accumulated in the shells during the accumulation period. However, the increases in the Cu and Zn levels in the shells at the end of accumulation (Day 6)

and at the end of depuration (Day 10) are not significantly higher (P>0.05) when compared to the control treatment (Table 3). Based on the metal accumulation rates, the shells of cockles were found to have a slightly higher capability to accumulate Zn (i.e. highest rate = 0.45) than Cu (highest rate = 0.08) from the ambient seawater, as depicted in Table 4. This result is also similar to that in the soft tissues of *A. granosa* and was supported by the BCF values of shells (Table 4).

Table 4 shows the different patterns of metals depuration in the shell and soft tissues. A rapid depuration was observed in the soft tissues, but there was a slow depuration of metals in the shells. Meanwhile, a biodeposition in the crystalline lattices of the shells would cause the metals to permanently stay there (Yap *et al*., 2003b). This also caused the ability of the shell to act as a sink for metals (Yap *et al*., 2004a).

Based on the CF values (Zn: 1.27 and Cu: 1.01), the shells of cockles have higher ability to retain the accumulated Zn than Cu. Both the rates of accumulation and depuration in the shells were significantly (P< 0.05) lower than in the soft tissues of *A. granosa*. However, in long-term exposure, shells might accumulate higher levels of metal as compared to the soft tissues.

Based on the findings of the present study, the shells could possibly be good biomonitoring materials for Cu and Zn. Moreover, most previous studies have shown that shells (hard tissues) could be a potential biomonitoring material of heavy metals due to some of their characteristics (Yap *et al*., 2003b: 2004b). Firstly, different mineralogies and chemistries in the shells are the characteristics that cause them to have the ability to accumulate a wide range of metals (Yap *et al*., 2003b). Secondly, some trace metals have been incorporated into the shells through the substitution of the calcium ions in the crystalline lattices of the shell. They would associate with the organic matrix during shell growth (Watson *et al*., 1995).

Condition Index

Based on the data presented in Table 5, the CI values of *A. granosa* were found to be directly proportional to exposure days. After the experimental period (6 days for the accumulation period and 4 days for the depuration period), the CI values of *A. granosa* were decreased as compared to those in the controls. During the depuration period, the CI values were slightly decreased as compared to the accumulation period. For the single metal exposure after 2 days of the accumulation period, the CI value of cockles in the Cu exposure was 18.48 g/cm^3 and this was 19.23 g/cm^3 for the Zn exposure. At the end of the depuration period (i.e. at Day 10), the CI values were reduced to 15.23 g/cm^3 for Zn and 14.59 g/cm^3 for Cu.

TABLE 5
Condition index (CI) and CI reduction in *Anadara granosa* between the control and exposed cockles

Day	CI (g/cm3)			CI reduction (%)	
	Control	Zn (1.0 mg/L) exposed	Cu (0.1mg/L) exposed	Zn (1.0mg/L) exposed	Cu (0.1 mg/L) exposed
Accumulation					
2	20.56	19.23	18.48	6.47	10.12
4	20.33	18.13	17.26	10.82	15.10
6	18.76	15.64	14.77	16.63	21.27
Depuration					
8	17.84	15.35	14.65	13.96	17.88
10	17.34	15.23	14.59	12.17	15.86

According to Yap *et al*. (2002), there are two types of mechanisms usually used by organisms to cope with pollution. First, the mechanical responses and metabolic requirement. In order to cope with pollution, cockles decrease their filtration activity, and this leads to reduction of the amount of food consumed. In addition, the cockles may have to use more food than consumed to maintain the normal metabolic activities. Sequentially, the stored glycogen, carbohydrate, protein and lipid might be utilized to maintain those activities (Yap *et al*., 2002). This situation explained the decrease in their CI values when compared to the control exposure. This could also explain the low accumulated metals in their soft tissues in the initial exposure period. Second, the induction of metallothionein (MT) (Chan *et al*., 2002). MT is involved in the regulation of essential metals, such as Cu and Zn for cell growth and development (Mackay *et al*., 1993). Metallothionein reacted to bind the uptake metals and stored them in detoxified forms (Viarengo *et al*., 1985). Although the detoxification mechanisms would not cause acute mortality, they would impose energetic costs to organisms through increased rates of metallothionein synthesis and subsequent excretion (Yap *et al*., 2002). Cockles utilize stored energy to meet this metabolic requirement for detoxification process. As a result, the declining energy stored is likely to be measured in its lower CI value.

These results indicated the increase in the percentage of CI reduction as compared to those in the control treatment. Since it can reflect changes in the nutrient state of the bivalve for the stored energy reserves and the animal's metabolic response to environmental stress (Peddicord, 1977), the CI of *A. granosa* is therefore a simple physiological index to evaluate its response to Cu and Zn stress.

CONCLUSIONS

This laboratory experimental study on the accumulation and depuration of Cu and Zn in *A. granosa* indicated two phenomena. Firstly, *A. granosa* (especially the soft tissues) is a potential biomonitor of Cu and Zn. It is proposed that the shells, together with the soft tissues of *A. granosa*, should be analysed to provide a better interpretation on the bioavailabilities of Cu and Zn. Secondly, the CI of *A. granosa* is a potential physiological indicator of Cu and Zn toxicities under laboratory conditions. However, field-based experiments using *A. granosa* should be conducted to assess the response of transplanted cockles to heavy metal pollution in mudflat intertidal areas.

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ITS-PCR-RFLP Analysis of *Ganoderma* sp. Infecting Industrial Crops

Nusaibah, S.A.[1*], Latiffah, Z.[2] and Hassaan, A.R.[3]
[1]Institute of Tropical Agriculture, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[2]School of Biological Sciences, School of Pharmaceutical Sciences,
Universiti Sains Malaysia, 11800 Minden, Pulau Pinang, Malaysia
[*]E-mail: nus_ali@yahoo..com

ABSTRACT

Ganoderma is a disastrous pathogen that has been causing tremendous losses to economically important crops in many countries. Vast genetic variations have been observed among several *Ganoderma* species, even from the same host. In this study, genetic variation was assessed among 44 isolates of *Ganoderma* sp. isolated from the basidiocarps of four different hosts (oil palm, rubber, tea, and forest trees) collected from selected areas of Peninsular Malaysia. Restriction Fragment Length Polymorphism (RFLP) technique, using ITS1 and ITS4 primers, was used to amplify Internal Transcribed Spacer (ITS) regions. Amplified products were further digested using *Bsu* 151, *Hind* III and *Taq* I restriction enzymes. Cluster analysis with UPGMA using genetic distances clustered all the isolates studied into four main groups. Generally, *Ganoderma* isolates from the same host were clustered together. The isolates from tea and rubber were more closely related compared to oil palm and forest trees. Similarly, the *Ganoderma* isolates from the same host were also clustered together, and three species were identified, namely, *G. boninense* (from oil palm and coconut stumps), *G. philippii* (rubber) and *G. australe* (forest trees). The results obtained from the analysis showed that host preference was a possible factor in the differentiation of *Ganoderma* species.

Keywords: *Ganoderma*, ITS region, PCR-RFLP

INTRODUCTION

Ganoderma is a basidomycetous fungus that causes disease in many crops, such as rubber, tea, palms and forest trees. Among other, *Ganoderma* also causes basal stem rot (BSR) disease in oil palm (*Elaeis guineensis*), red rot in rubber (*Hevea brasiliensis*) and white rot in forest trees. The BSR disease in oil palm creates symptoms like large lesions at the early stage and at the foliar stage. One-half of the cross-sectional areas at the stem base necroses by the pathogen causing restriction of water supply and nutrients to the aerial part that lead external symptoms such as wilting and malnutrition (Turner, 1981). Meanwhile, wood or forest trees under decay by white rotters show symptoms where the wood appears stringy in later stages of decay and it also gets bleached, exhibiting lighter pigmentation than sound wood (Alexopoulos *et al*., 1979).

Genetic studies on the population and host range of *Ganoderma* species showed that inter- and intra-genetic variations occur specifically in this fungus (Miller, 1995; Idris, 1999; Moncalvo *et al*., 1995b; Utomo and Niepold, 2000; Latiffah, 2001; Pilotti *et al*., 2003; Nusaibah *et al*., 2007). Nonetheless, the identification of *Ganoderma* at the species level is difficult and a reliable identification key does not exist

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[*]Corresponding Author

for pre-adult stages based on the morphological characteristics. The use of restriction fragment length polymorphism (RFLP) has the advantage of combining highly conserved sequences in the Internal transcribed spacer (ITS) - 5.8S- ITS4 rDNA regions with variable sequences in the ITS regions at species level (Moritz *et al.*, 2000), whereby, the ITS shows a high inter-specific variability and an extremely low intra-specific variability. ITS-PCR-RFLP is a power tool that has been proven to facilitate in genetic variation studies among *Ganoderma*. A similar tool was used by Moncalvo *et al*. (1995b), Utomo and Niepold (2000), and Latiffah (2001) in their *Ganoderma* population work.

The aim of the present study was to collect the *Ganoderma* basidiocarps from different hosts (oil palm, rubber, tea and forest trees) from some selected geographical locations (states in Peninsular Malaysia), isolate the pure cultures of *Ganoderma* from its basidiocarps, as well as identify it at species level using the molecular approach, the PCR-RFLP on ITS - 5.8S- ITS4 rDNA regions.

MATERIALS AND METHODS

Sampling and Isolation of Ganoderma sp. *from Its Basidocarps*

The species name, isolates code, host and origin of the *Ganoderma sp*. used in this study are given in Table 1. Meanwhile, the pure cultures of *Ganoderma sp*. from all the hosts were isolated from its basidiocarps following the method proposed by Latiffah (2001) with a slight modification. These isolates were maintained at 27± 2°C on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI).

Genomic DNA Extraction Protocol

In this study, the *Ganoderma* mycelium, grown on the PDA plates, was used for DNA extraction. Four pieces of 2 × 2 cm^2 dialysis membrane were cut and sterilized. These pieces of membrane were placed on the PDA plates using sterile forceps and one drop of liquid PDA was subsequently placed on each membrane. The pure cultures of *Ganoderma* were then sub-cultured on each membrane on the PDA plates until the growth covered the whole plate, a process which took about seven to eight days. In order to prepare the samples for DNA extraction, mycelium which had grown on dialysis membrane was thorn using the forceps and placed in a mortar. Later, an adequate amount of liquid nitrogen was added before the mycelium was ground into fine powder. For DNA extraction, 20 to 25 mg of the powdered sample was weighed in a 1.5 ml Eppendorf tube. The DNA extraction was carried out using the phenol-chloroform method described by Reader and Broda (1985). This was followed by dissolving the DNA TE buffer and storing it at -20°C until further use. The extracted genomic DNA was checked for its concentration and purity using a spectrophotometer (Ultrospec 2000, UV/Visible Spectrophotometer, Pharmacia Biotech).

DNA Amplification

The ITS regions were amplified using primer ITS1 (5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1991). Amplification was performed in 25µl of reaction mixture containing 0.25 µl of DNA template, 2.5 µl 10X PCR buffer, 2.5 mM $MgCl_2$, 200 µM of each dNTP (dATP, dTTP, dGTP, and dCTP), 0.4 µM of both primers, 1 unit *Taq* polymerase and ddH_2O. Each PCR tube was overlaid with two drops of paraffin oil to prevent evaporation during the process. Meanwhile, DNA Engine™ Peltier Thermal Cycler Model PTC-100 was used to run the Polymerase Chain Reaction (PCR). The PCR started with denaturation for 2 min at 95°C. This was followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 30s at 63°C and extension for 2 min at 72°C. The final step of extension was then carried out for 10 min at 72°C, before it was maintained at 10°C. The PCR product was run on 1.7% agarose gel, stained with ethidium bromide (EtBr) and visualized under a UV transilluminator (BioRad).

TABLE 1
The isolate code, origin and host of *Ganoderma* sp. sampled from 2004 – 2006.

No.	Isolate code	Origin	Host
1	TPS	Pondok Tanjung, Perak	*Shorea* tree
2	BG2	Botanical Garden, Pulau Pinang	Kedondong tree
3	BG10	Botanical Garden, Pulau Pinang	Forest tree
4	BG12	Botanical Garden, Pulau Pinang	Forest tree
5	BG9	Botanical Garden, Pulau Pinang	Forest tree
6	BG21	Botanical Garden, Pulau Pinang	Forest tree
7	BG22	Botanical Garden, Pulau Pinang	Forest tree
8	BG23	Botanical Garden, Pulau Pinang	Forest tree
9	BW10	Bukit Wang, Kedah	Forest tree
10	BW3	Bukit Wang, Kedah	Forest tree
11	BW11	Bukit Wang, Kedah	Forest tree
12	BW5	Bukit Wang, Kedah	Forest tree
13	BW2	Bukit Wang, Kedah	Forest tree
14	BW1	Bukit Wang, Kedah	Forest tree
15	BW8	Bukit Wang, Kedah	Forest tree
16	FP589	FRIM- *Ganoderma philippii*	Unknown
17	FP152	FRIM- *Ganoderma lucidum*	Unknown
18	FP104	FRIM- *Ganoderma australe*	Unknown
19	O9BS3	Bukit Serampang Estate, Tangkak Johor	Oil palm
20	38PL2	Paya Lang Estate, Segamat, Johor	Oil palm
21	BKS1	Bertam Estate, Seberang Prai	Oil palm
22	BKS2	Bertam Estate, Seberang Prai	Oil palm
23	BKS5	Bertam Estate, Seberang Prai	Oil palm
24	BKS7	Bertam Estate, Seberang Prai	Oil palm
25	BKS10	Bertam Estate, Seberang Prai	Oil palm
26	BKS11	Bertam Estate, Seberang Prai	Oil palm
27	BKS12	Bertam Estate, Seberang Prai	Oil palm
28	BKS13	Bertam Estate, Seberang Prai	Oil palm
29	BKS14	Bertam Estate, Seberang Prai	Oil palm
30	BKS15	Bertam Estate, Seberang Prai	Oil palm
31	BKS16	Bertam Estate, Seberang Prai	Oil palm
32	TTKS26	Trong, Taiping, Perak	Oil palm
33	PRBKS27	Padang Rotan, Bruas, Perak	Oil palm
34	LPOP	Ladang Pelam, Perak	Oil palm
35	UPKS	Teluk Intan, Perak	Oil palm
36	BIO	Rumah Tumbuhan, USM	Oil palm
37	TRKS	Teluk Ramunia, Johor	Oil palm
38	SEL28	MPOB- *Ganoderma boninense*	Oil palm
39	PER71	MPOB- *Ganoderma boninense*	Oil palm
40	CTEA1	Rubber Research Institute Malaysia	Tea
41	CTEA2	Rubber Research Institute Malaysia	Tea
42	CTEA3	Rubber Research Institute Malaysia	Tea
43	JPG2	Kluang, Johor	Rubber
44	JPG1	Kluang, Johor	Rubber
45	TH1	Titi Hayun, Kedah	Rubber

Enzyme Digestions

The amplified products were digested for 2 hours at 37°C, using the following restriction enzymes, *Bsu* 151, *Taq* I, and *Hind* III. The digested DNA fragments were then separated on 1.7% agarose gel and stained with EtBr before visualizing them under UV transilluminator.

Data Analysis

The molecular size of each fragment was estimated using a 100bp ladder. Meanwhile, the software NTSYS-PC (Numerical Taxonomy System of Multivariable Program) version 2.0 (Rohlf, 2000) was used to analyze the electrophoretic data. The presence and absence of restriction bands were scored as 1 or 0. The similarity of Simple Matching Coefficient (SMC) was used to generate the similarity matrix and coefficient, as described by Romesburg (1984), using the following formula:

SMC = (a + d) / (a + b + c + d), where:
a = number of bands present in two isolates
b = total number of bands unique in isolate 1
c = total number of bands unique in isolate 2
d = number of bands absent in two isolates

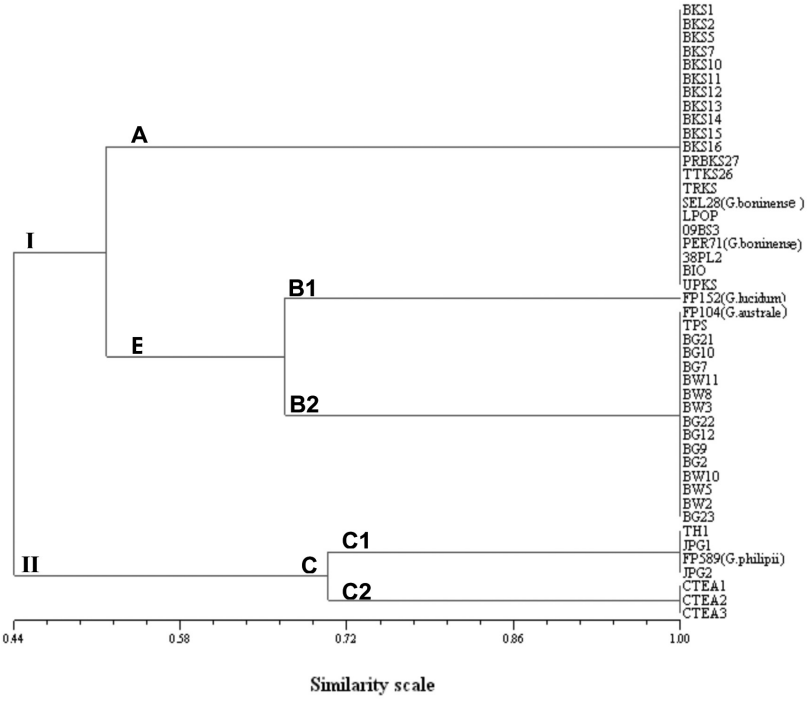

Fig. 1: Dendrogram from the UPGMA analysis using Simple Matching Coefficient based on the PCR-RFLP restriction patterns of ITS-5.85S-ITS2 region of Ganoderma *samples from various host*

Restriction Pattern of Hind *lll*

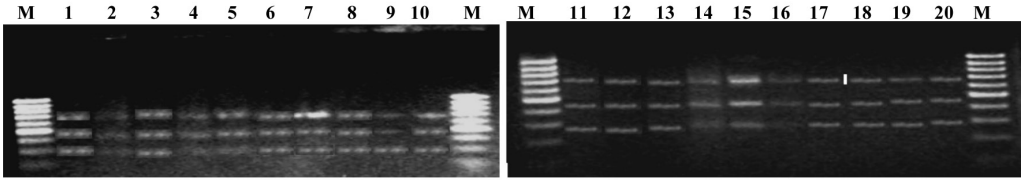

Fig. 2a: Restriction patterns of the amplified ITS regions + 5.8S gene of oil palm isolates digested using Hind *lll restriction enzyme Lane 1: BKS1; Lane 2: BKS2; Lane 3: BKS5; Lane 4: BKS7; Lane 5: BKS10; Lane 6: BKS11; Lane 7: BKS12; Lane 8: BKS13; Lane 9: BKS14; Lane 10: BKS16; Lane 11: PBRKS; Lane 12: TTKS; Lane 13: TRKS; Lane 14: SEL 28* (G. boninense)*; Lane 15: UPKS; Lane 16: PER 71* (G. boninense)*; Lane 17: 09BS3; Lane 18: 38PL2; Lane 19: BIO; Lane 20: LPOP; M=Marker 100 bp* (Fermentas)

Restriction Patterns of Bsu *151*

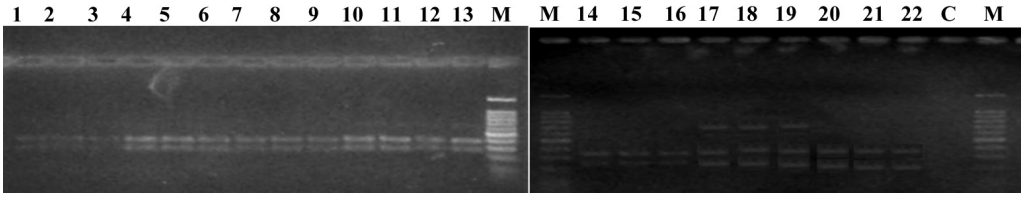

Fig. 2b: Restriction patterns of the amplified ITS regions + 5.8S gene of forest tree isolates digested using Bsu *151 restriction enzyme. Lane 1: BW2; Lane 2: BW3; Lane 3: BW5; Lane 4: BW8; Lane 5: BW10; Lane 6: BW11; Lane 7: BG2; Lane 8: BG7; Lane 9:BG9; Lane 10: BG10; Lane 11: BG12; Lane 12: BG21; Lane 13: BG22; Lane 14-16: isolates from rubber (1: PG1, 2: PG2, 3: PG3); Lane 17-19: isolates from tea (4: CTEA1, 5: CTEA2, 6: CTEA3); Lane 20: FP152* (G. lucidum)*; Lane 21: FP589* (G. philippii)*; Lane 22: FP104* (G. australe)*; Lane C: Control; M= Marker 100 bp* (Fermentas)

Restriction Patterns of Taq *I*

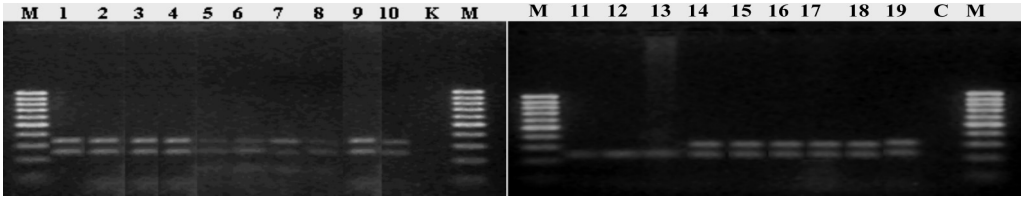

Fig. 2c: Restriction patterns of the amplified ITS regions + 5.8S gene of oil palm isolates digested using Taq *l restriction enzyme. Lane 1: PBRKS; Lane 2: TTKS; Lane 3: TRKS; Lane 4: SEL 28* (G. boninense)*; Lane 5: UPKS; Lane 6: PER71 (*G. boninense*); Lane 7: 09BS3; Lane 8: 38PL2; Lane 9: BIO; Lane 10: LPOP; Lane 11-13: isolates from rubber (1: PG1, 2: PG2, 3: PG3); Lane 14-16: isolates from tea (4: CTEA1, 5: CTEA2, 6: CTEA3); Lane 17: FP152* (G. lucidum)*; Lane 18: FP589* (G. philippii)*; Lane 19: FP104* (G. australe)*; Lane C: Control; M=Marker 100 bp* (Fermentas)

RESULTS AND DISCUSSION

Fig. 1 shows a dendrogram from the UPGMA cluster analysis of the ITS1-5.8S-ITS4 restriction fragments from all the samples analyzed. The dendrogram can be separated into two major clusters. Major cluster I comprises of sub-clusters A and B, while major cluster II is formed by only one sub-cluster that is labelled as sub-cluster C. Major Cluster II was linked to major cluster I with approximately 44% similarity. The sub-cluster A consisted of 21 isolates of *Ganoderma* from oil palm (BKS1, BKS2, BKS5, BKS7, BKS10, BKS11, BKS12, BKS13, BKS14, BKS 15, BKS16, PBRKS27, TTKS 26, TRKS, SEL28, LPOP, 09BS3, PER71, 38PL2, BIO, and UPKS), whereas sub-cluster B was divided into sub-sub cluster B1 and B2. The sub-sub cluster B1 contained only one isolate, FP152 (*G. lucidum*), whereas the sub-sub cluster B2 comprised all the isolates from the forest trees (TPS, BG21, BG10, BG7, BW11, BW8, BW3, BG22, BG12, BG9, BG2, BW10, BW5, BW2, and BG23) and FP104 (*G. australe*). In the major cluster II, sub-cluster c was divided to sub-sub cluster C1 and C2. Sub-sub cluster C1 comprised of all the isolates from rubber THI, JPG1, JPG2) and the isolate FP589 (*G. philippii*). Meanwhile, all the isolates from tea (CTEA1, CTEA2, and CTEA3) were clustered under the sub-sub cluster C2. The overall matrix similarity obtained among all the isolates, from the different hosts, was found to be between 44-100%. A 100% similarity was obtained from all the isolates within the same host. The highest similarity of 70% from the different hosts was observed between rubber and tea. A similarity of 52% was obtained between oil palm and both rubber and forest trees, followed by 49% of the matrix similarity between rubber and forest trees. Finally, the lowest similarity of 30% was observed among the *Ganoderma* isolates from oil palm and tea.

Table 2 indicates the estimated restriction fragment sizes of the *Ganoderma* isolates from oil palm, rubber, tea, and forest trees, FP152, FP589 and FP104 digested using *Bsu* l5l, *Taq* l and *Hind* lll restriction enzymes. The total PCR-RFLP size for all the *Ganoderma* isolates was about 600 bp. A similar amplification result was obtained by Bridge *et al*. (2000) in their *Ganoderma* phylogenetic study. Nonetheless, the total restriction fragment sizes recorded in Table 2 did not give 600 bp as a sum. A smaller sum of fragment sizes was observed in *Taq* l (*Fig. 2a*) restriction enzymes fragments for forest tree *Ganoderma* isolates. According to Gottlieb *et al*. (2000), this phenomenon might have occurred because the size of the developed fragments was too small (10 bp to 50 bp) and it could have been lost during the electrophoresis procedure. The other phenomenon that was detected was the larger sum of fragments from the total PCR-RFLP product. This was observed in the fragments developed by *Bsu* 151 (*Fig. 2b*) in all its restricted *Ganoderma* isolates from tea plant and by *Hind* lll (*Fig. 2a*) in all its *Ganoderma* isolates from oil palm. The explanation for this phenomenon was probably the presence of heterocaryon in the mycelium of *Ganoderma* that had been prepared for sexual reproduction (Hibbett, 1992). In this study, not all restriction enzymes managed to digest the PCR-RFLP amplified products. For instance, *Taq* l (*Fig. 2c*) restriction enzyme could digest all the PCR products of *Ganoderma* isolates from all the hosts. However, restriction enzyme *Hind* lll (*Fig. 2a*) only managed to digest the *Ganoderma* isolates from oil palm, SEL 28, and PER 71 (*G. boninense*). This was because these restriction enzymes did not find recognition sites on the ITS1-5.8S-ITS4 regions of the undigested isolates.

A previous study by Miller (1995) revealed that the use of PCR-RFLP had proven variability could be detected between the *Ganoderma* isolates in a population that differs in terms of the isolate field-related characters. Nusaibah *et al*. (2007) reported that by using the AFLP as a molecular marker on the *Ganoderma* isolates from oil palm could reveal both the inter- and intra-specific variations between the same species of *Ganoderma* which had made the fungus difficult to be characterized at the species level. Moreover, Nusaibah *et al*. (2007) have also managed to cluster the pathogenic *Ganoderma* in a cluster other than the non-

TABLE 2
Estimated restriction fragment sizes of *Ganoderma* isolates from oil palm, rubber, tea, and forest trees, FP152, FP589 and FP104 digested using *Bsu* l5l, *Taq* l and *Hind* lll restriction enzymes

Isolate	Estimated restriction fragment size (bp)								
	Bsu 151			*Taq* I			*Hind* III		
Oil palm									
O9BS3	150	200	350	150	250	350	200	350	600
38PL2	150	200	350	150	250	350	200	350	600
BKS1	150	200	350	150	250	350	200	350	600
BKS2	150	200	350	150	250	350	200	350	600
BKS5	150	200	350	150	250	350	200	350	600
BKS7	150	200	350	150	250	350	200	350	600
BKS10	150	200	350	150	250	350	200	350	600
BKS11	150	200	350	150	250	350	200	350	600
BKS12	150	200	350	150	250	350	200	350	600
BKS13	150	200	350	150	250	350	200	350	600
BKS14	150	200	350	150	250	350	200	350	600
BKS15	150	200	350	150	250	350	200	350	600
BKS16	150	200	350	150	250	350	200	350	600
TTKS26	150	200	350	150	250	350	200	350	600
PRBKS27	150	200	350	150	250	350	200	350	600
LPOP	150	200	350	150	250	350	200	350	600
BIO	150	200	350	150	250	350	200	350	600
TRKS	150	200	350	150	250	350	200	350	600
SEL28 (*G. boninense*)	150	200	350	150	250	350	200	350	600
PER71(*G. boninense*)	150	200	350	150	250	350	200	350	600
UPKS	150	200	350	150	250	350	200	350	600
Forest tree									
TPS		300	400		150	250			
BG2		300	400		150	250			
BG10		300	400		150	250			
BG12		300	400		150	250			
BG9		300	400		150	250			
BG21		300	400		150	250			
BGBG23		300	400		150	250			
BW10		300	400		150	250			
BW3		300	400		150	250			
BW11		300	400		150	250			
BW5		300	400		150	250			
BW2		300	400		150	250			
BW1		300	400		150	250			
BW8		300	400		150	250			
Tea									
CTEA1	250	300	600	100	250	300			
CTEA2	250	300	600	100	250	300			
CTEA3	250	300	600	100	250	300			
Rubber									
JPG2		250	350	100	250	350			
JPG1		250	350	100	250	350			
TH1		250	350	100	250	350			
FP152 (*G. lucidum*)		250	350		250	350			
FP589 (*G. philippii*)		250	350	100	250	350			
FP104 (*G. australe*)		300	400		150	250			

pathogenic ones. The ITS region was used by Bridge *et al.* (2000) to develop a genetic marker that could differentiate the pathogenic and non-pathogenic *Ganoderma* isolates from oil palm.

A cluster analysis carried out on the banding patterns, obtained from the amplified ITS1-5.8S-ITS4 regions digested by the restriction enzymes, showed that the isolates were clustered according to the host. A similar study by Gottlieb *et al.* (2000) also proved that the *Ganoderma* isolates which were collected from South America could be identified at the species level using the ITS1-5.8S-ITS4 regions as they managed to identify the *Ganoderma* isolates collected as *G. lucidum*, *G. zonatum*, *G. sessiliformen*, *G. platense*, and *G. praelongum.* Another study by Smith and Sivasithamparam (2000a, b) used ITS region for species level characterization of *Ganoderma* sp. in Australia. An internal transcribed spacer region analysis was also found to be a useful tool in a phylogenetic study for *Ganoderma sp*. characterization. Moncalvo *et al.* (1995c) stated that the ITS1 region of *Ganoderma* is small enough to be easily amplified by PCR and is flanked by highly conserved sequences. Meanwhile, a phylogenetic study by Moncalvo *et al*. (1995a, c) managed to characterize *Ganoderma* from the temperate and tropical locations to its species level.

CONCLUSIONS

Based on the restriction fragment banding pattern analysis on all the isolates used in this study, three different species of *Ganoderma*, namely *G. boninense, G. philippii* and *G. australe*, gave different banding patterns, which were grouped under different clusters. This finding indicates that the PCR-RFLP on ITS1-5.8S-ITS4 region is a reliable technique to discriminate the *Ganoderma* species from different hosts. Nonetheless, further research on genetic variation is important to understand how the disease caused by *Ganoderma* on industrial crops is spread.

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The Spatial Interconnection between Agro-Ecological Dissimilarities and Poverty in Bangladesh: A Case Study

Md. Jahan Boksh Moral[1*] and Ruslan Rainis[2]
[1]Department of Geography and Environmental Studies, University of Rajshahi, Rajshahi 6205, Bangladesh
[2]School of Humanities, Universiti Sains Malaysia, 11800 Penang, Malaysia
[*]E-mail: jahanboksh@gmail.com

ABSTRACT

The study explores spatial deviation of agro-ecology and its interconnection with poverty. Agro-ecology provides more than the resources need for material welfare and livelihoods, in addition to alleviating poverty. Bangladesh, especially the northwest region, remains high intensity of poverty with divergence of agro-environment. Heterogeneous agro-ecological features were found which affect poverty dissimilarity. Incidence of poverty seems to have linkage with agro-ecological variations. The areas having low productivity have higher incidence of poverty. Both the field and printed documents have been exploited to put in writing this manuscript.

Keywords: Agro-ecology, poverty, Rajshahi, variations

INTRODUCTION

In the Asian and Pacific region, land and water resource management has been acknowledged as one of the priority sectors for reaching sustainable food security throughout, increasing land productivity, reducing land degradation and water failure, and increasing biodiversity as well as improving the quality of environment. In particular, the Asian countries have also committed themselves to manage their natural resources. In the last three decades, the Land and Water Development Division of FAO has made considerable progress on the appraisal of land use planning and management, land degradation assessment, and land use mapping as well as the potential utilizations of land resources for better food and agriculture (FAO, 2005). This is particularly because the ecosystem provides more than the resources needed for material welfare and livelihood. In addition to supporting all life and regulating natural systems, they specifically provide health and cultural benefits to people (UNEP and IISD, 2005). Both the status and trend of the ecosystems, ecosystem services, and their linkages to poverty reduction in Uganda have been explored in the report by the Centre for Resource Analysis Limited (CRA). The report serves as a tool for raising awareness among policy makers and the general public about the linkages between the ecosystems and poverty reduction (CRA, 2006).

Agro-ecosystem is a medium for growing food crops for human beings. The cultivation of plants for food is dependent on natural factors, such as fertile soil, adequate soil moisture, suitable climatic conditions, and a rich source of plant and animal species (CRA, 2006). Thus, the agricultural sector is a significant means for

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[*]Corresponding Author

feeding the additional growing population, which also provides an important role for economic growth in a sustainable manner. Globally, 2.8 billion people earn not more than US$2 per day, whereas 1.2 billion of them receive just a single US$ per day (World Bank, 2001) and 70% of these poor people are living in rural areas. In a developing country like Bangladesh, where poverty is a great problem, around half of its total population (140 million) are deprived of the living standard, where agriculture is a fundamental sector.

Bangladesh is a predominantly agricultural country. For their living, more than half of the country's population are directly or indirectly related to agriculture (Alam and Moral, 1997). Agriculture occupies almost 9.6 million hectares of about 14.5 million hectares of the country's total area. Meanwhile, around 70 percent of the total land areas are developed primarily for agricultural purposes or activities with regional variations. In other words, the agricultural productivity varies from one region to another because of the bio-physical, socio-economic and operational factors which have linkages with poverty (*Fig. 2*).

AGRO-ECOLOGY

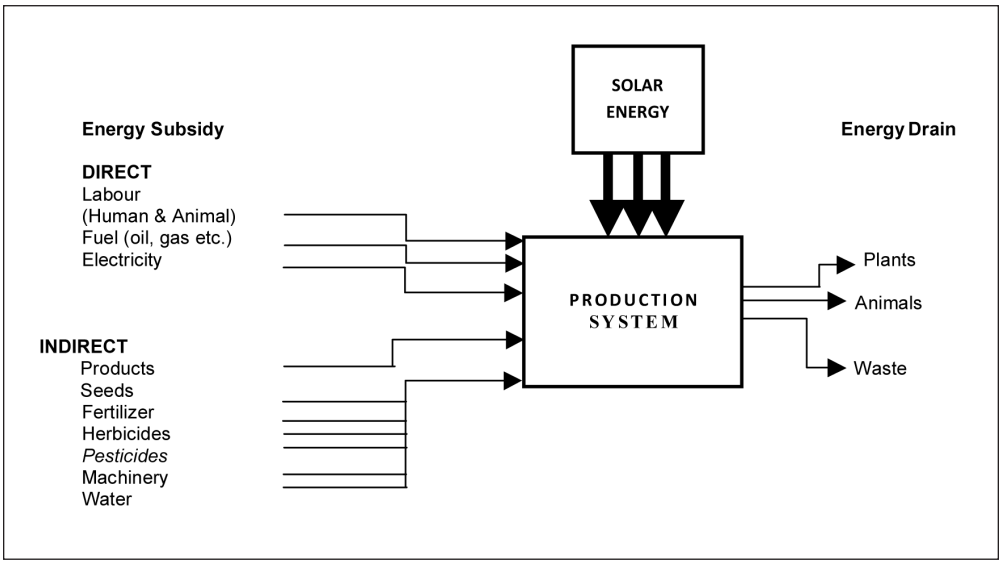
In the context of natural and social parameters, the system of agro-ecology is wide and diverse. The agro-ecology involves the interaction between agriculturally associated organisms and their physical habitats. The productions of food-grains, livestock, energy flow and nutrient cycle, etc. are therefore the fundamental elements of the agro-ecosystem. In addition, the system comprises communities of plants and animals which are interacting with their physical and chemical environments that are modified by people to produce their food, fire, fuel, and other products for human consumption and processing. Direct energy subsidy includes labour, fuel and electricity, whereas indirect energy subsidy includes seeds, fertilizer, herbicides, pesticides, machinery, and water (*Fig. 1*). By using solar energy, the energy subsidies produce various types of agricultural products and waste products in the system (Tivy, 1992).

THEORETICAL STRUCTURE

Poverty refers to not only the lack of income but also being short of resources. It also includes other dimensions of deprivation like physical weakness, isolation, vulnerability and powerlessness. In Bangladesh, poverty is not only a sense of deprivation but it also interconnected with vulnerability. Most frequently, regular vulnerability generates critical problems of income generation (BIDS, 1988). The risk of entitlement failure determines the level of vulnerability of a household to food insecurity. In more specific, the greater the share of resources denoted to food acquisition, the higher the vulnerability of the household to food insecurity. The routine vulnerability in food security leads to income erosion and perpetuates the problems of poverty. Food production and availability at the household level is one of the major dimensions of poverty and the exploration need to identify and analyze the biophysical and socioeconomic factors of food production and availability and their linkages to poverty. One approach to examine the pattern of food production is to understand the control of agricultural production that has both spatial and temporal dimensions. The productive capacity of land is set by the agro-ecological conditions of the area concerned. Agricultural, socio-economic and operational factors, together determine agricultural productions and their inter-linkage is known as agro-ecological system (*Fig. 2*).

AGRO-ECOLOGY AND POVERTY

Ahmad and Zaman (1997) stated that there is a close relationship between poverty, high population growth and agro-ecology with positive feedback, and they feed upon one another to generate a vicious circle (*Fig. 3*). Nonetheless, the nature and strength of this interrelation vary from one situation to another.



(*Source*: Tivy, 1992)

Fig. 1: A diagram of an agro-ecosystem

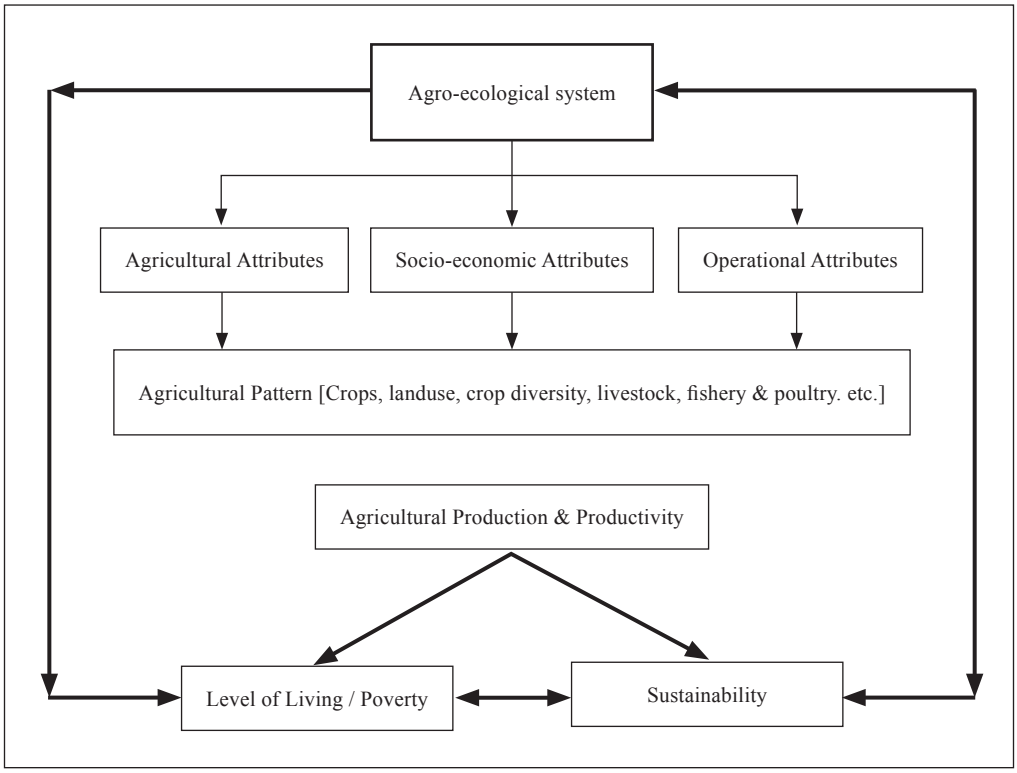
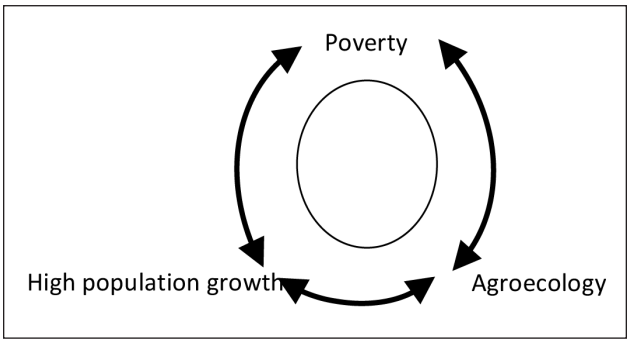


Fig. 2: Agricultural systems and poverty



(*Source*: Ahmad and Zaman, 1997)

Fig. 3: The interconnection between poverty and agro-ecology

The vicious circle of the population, poverty, and environmental poor quality can be extended to explicitly incorporate with the linkages of agro-ecology.

METHODOLOGY

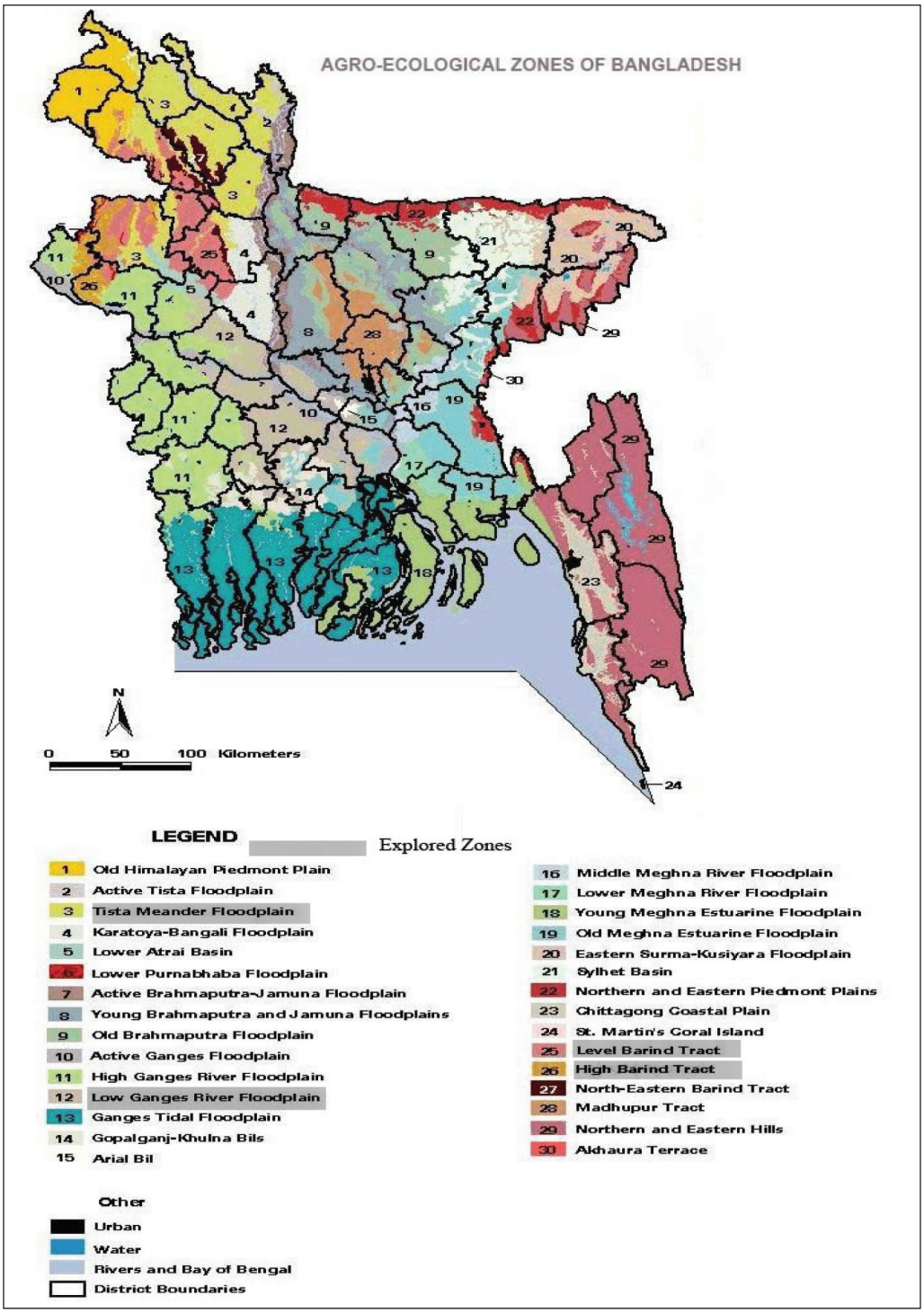
Both the primary and secondary data, which were respectively gathered from the field survey and published documents, were used in this study. Meanwhile, a structured questionnaire was used to acquire information from the fields of selected villages. In particular, the BARC/ FAO (Bangladesh Agriculture Research Project/ Food and Agricultural Organization) and AEZ (Agro-Ecological Zones) project reports were used as the key sources of the secondary data. The northwest region of Bangladesh comprises eleven agro-ecological zones, as illustrated in Map 1. Four zones out of eleven, known as Tista Meandering Floodplain, High Barind Region, Level Barind Region and Low Ganges Floodplains, were investigated. It is important to note that the studied regions also have wide agro-ecological variations and diverse poverty. In order to finding the micro-level ecological characteristics, four villages named Uttar Dhumitary, Uttar Chandipur, Krisna Pur, and Vhabanipur from each of the four investigated zones were studied. The total numbers of households (HHs) for those villages were 85, 190, 522 and 361, respectively. A structured questionnaire was used to collect the field data. Meanwhile, a stratified random sampling comprising of 30% of the samples of HHs were searched out, at 0.05 significant level.

STUDY REGIONS

The whole Bangladesh can be divided into 30 agro-ecological zones, and each of these zones has distinct characteristics (*see* Map 1). Eleven zones are located in the northwestern region of Bangladesh. Land types, such as low, high and other types, can be found in the northwest part of Bangladesh. Out of these, one is high piedmont plain, three terraced to level barind tract, three active, 3 low, 1 high and 2 other types of floodplains (UNDP/FAO, 1988). For the purpose of area selection, the different agro-ecological regions were grouped into three categories based on more or less similar physiographic characteristics, which include:

- High Barind
- Level Barind and Old Himalayan Piedmont Plain
- Floodplains

Among these regions, floodplains occupy a major proportion of the area. Four case studies have been investigated, which include two from the floodplain zones, one from a high barind region, and another one from the level barind tract (*see* Maps 1-5).



(*Source*: BARC/FAO/UNDP GIS Project: BGD/95/006, based on UNDP/GOB/FAO Project BGD/81/035)

Map 1: Agro-ecological zones in Bangladesh

Agro-ecology and Poverty in the Northwest of Bangladesh

UNDP/FAO identified 11 main agro-ecological zones in the northwest region of Bangladesh. Each zone indicates a wide range of agro-ecological variation. The main characteristics of each zone have been highlighted into subsequent Boxes 1-4.

The High Barind Region (HBR)

The High Barind Region is one of the fundamental agro-ecological zones in the northwest region. The total area occupies around 1600 km^2, whereby there are a wide range of ecological variations along with significant differences in physiography, soil, and slope. These diverse and complex physical conditions provide both the opportunities and limitations for land use and development. Land level of the region increasingly rises to the western side but gradually lowers to the level barind tract. Most of the land is free from flood with prevailing uniform climate all over the region and a mean annual rainfall of 1300-1400 mm. The soil is of grey, silty, puddle topsoil, and plough-pan. The topsoil, which has small moisture holding capacities with slow permeability, contains high acid with low organic matter. Limited surface waters are available in tanks. Groundwater is available in the eastern region. Transplanted Aman is a major crop. Meanwhile, Broad Cast Aus is cultivated before Aman through irrigation. HYV Boro is also farmed there. Rabi crops are cultivated by hand irrigation from the adjoining water tanks in field. The main constraints of agriculture include terrace soil and shortage of soil moisture during the dry season. The development strategies may exercise the ways of widespread irrigation facilities from deep tube wells to improve the crops.

The Level Barind Region (LBR)

Just like the HBR, the Level Barind Region (LBR) has also been developed over Madhupur clay which has ecological variations and great opportunities for development. The total area is around 5049 km^2, with flat landscape increasingly rises to the high barind region (HBR). In the relatively higher parts, rainwater is reserved in ponds for paddy cultivation. In lower sites, rainwater is accumulated up to about 60 cm deep in the rainy season. Flood occurs occasionally due to heavy rainfalls. In addition, the climatic condition is not uniform. The mean annual rainfall is 2000 mm in the northeast region and this is around 1300-1500 mm in the southwest region. The soil is strongly acidic with low organic matter, and there is limited surface water that can be used for irrigation during the dry season. Groundwater is available within 100 m depth from the surface but varies from one place to another. A higher proportion of the land is used for mixed Aus and Aman, deepwater Aman, capsularis jute and irrigated HYV Boro. Meanwhile, a lower proportion is devoted to transplanted Aman and cash crops, such as tobacco, cotton, potatoes, bananas, and spices.

Low Ganges Floodplain Region (LGFR)

The region comprises eastern half of the Ganges River with broad landscape of smooth ridges and basins (7968 km^2). Moderate to deep flash floods occur respectively during rainy and dry seasons. The mean annual rainfall increases from about 1600 mm in the northwest to about 2000 mm in the southwest. Soils are olive-brown, silt loams, and silty clay loams on the upper parts of floodplain ridges and soil are dark grey, with mottled brown clay soil on the lower ridge sites. Most of the ridge soil is calcareous and non-calcareous in the upper layers. Meanwhile, non-calcareous upper layers are slightly acidic or neutral. Ample groundwater apparently exists throughout the region with limited amounts of surface water. Aus is the main crop, while early Rabi cash crops (tobacco, potato, vegetables, and spices) are cultivated by hand irrigation.

Meandering Tista Floodplain Region (MTFR)

This region is one of the major parts of Tista Floodplain (9468 km^2) with broad almost level floodplain ridges. The ridge in the northwest

BOX 1
Agro- ecology and poverty of the HBR

Location
Rajshahi,
Chapinawabganj
Naogaon Districts
Physiography
Highest and most steeply sloping areas
Terraced valley sides
Deep valley to the south and west
Long valleys to the eastward
Shallower towards level Barind
Drainage
Well drained
Rapid run-off
Proportion of landscape
High land 93%
Medium high land 1%
Medium lowland<1%
Low land <1%
Very low land 0%
Homesteads, water 6%
Climate
High deviation of climate from year to year
Both winter and summer are the longest
Mean annual rainfall of 1300-1400
Soil
Poorly drained soils
Soil types and total percentage
Acid basin clays---<1%
Shallow red brown terrace soils-3%
Shallow grey terrace soils-----3%
Deep grey terrace soils-------72%
Gray valley soils---16%
Soil texture
Loamy---13%
Clayey--87%
Water resources
Limited surface water
Ground water poor in western region
but rich in eastern region
Present land use
Transplanted Aman grown dominantly
Development constraints
Uncertain rainfall
Inadequate surface and ground water
Low soil Fertility
Sloping relief
puddle silty topsoil
Big land ownership
Development possibilities
Crop production by increasing irrigation, soil and crop management
Poverty (%) *73*

(*Source*: UNDP/FAO, 1988 and field survey)

BOX 2
Agro-ecology and poverty of the LBR

Location
Denajpur, Gaibandha, Jaipurhat, Naogaon, Natore, & Sirajgang Districts
Physiography
Almost level, gradually elevated in the west side
Drainage
Seasonally flooded by heavy rainfalls
Proportion of the landscape
High land -30%
Medium high la--55%
Medium lowland--4%
Low land -2%
Very low land --0%
Homesteads, water --9%
Climate
High standard deviation of climate
Mean annual rainfall of -2000 mm in the north east region & 1300-1500 in the southwest
Soil
The maximum soils are Grey, Silty, puddle topsoil and plough pan
General soil types
Non-calcareous alluvium--<1%
Non-calcareous brown floodplain soil-----<1%
Non-calcareous dark Grey floodplain soil-----<1%
Acid basin clay----1%
Shallow red brown floodplain soil--<1%
Deep red brown floodplain soil--1%
Brown mottled terrace soil--1%
Shallow Grey terrace soil---46%
Deep Grey terrace soil-34%
Grey valley soil--6%
Made land-----<1%
Urban ----<1%
Homesteads + water----9%
Soil texture
Sandy---------<1
Loamy---------3%
Clayey--------97%
Water resources
Limited surface water i.e. rivers & tanks during dry season
Groundwater resources are good except for the border of High Barind Region
Present land use
T. Aman
HYV Aus
Aman varieties
Development constraints
Drought
Flood
Low soil fertility
Uncertain groundwater
Poor communication
Big land ownership
Development possibilities
Great possibilities for food-grain productivity
Poverty *64%*

(*Source*: UNDP/FAO, 1988 and field survey)

BOX 3
Agro-ecology and poverty of the LGFR

Location
Natore, Pabna, Goalundo, Faridpur, Madaripur, Gopalgong, And so on.
Physiography
Meandering floodplain landscape of broad ridges and basins
Drainage
Most of region flood except high region
Proportion of the landscape
High land ---13%
Medium high land 29%
Medium lowland 31%
Low land ----14%
Very low land -2%
Homesteads, water11%
Climate
Mean annual rainfall of 1600 mm in the north east region & 2000 mm in the southeast
Soil
General soil types
Calcarious alluvium--.-3%
Noncalcarious alluvium----<1%
Calcarious brown floodplain soil-----14%
Calcarious Grey floodplain soil....1%
Calcarious dark Grey floodplain soil--64%
Noncalcarious dark Grey floodplain soil<1%
General soil types
Peat------<1%
Made land-<1%
River-------2%
Urban -------<1%
Homesteads + water-.... 10%
Soil texture
Organic(peat) ---<1%
Sandy--------<1
Loamy-------48%
Clayey-------52%
Water resources
Limited surface water
Groundwater find within 100 m from the surface
Present land use
Aus
Aman
Deepwater Aman
HYV Boro
Development constraints
Widespread deep flooding
Doughtiness in north region
Heavy clay basin
Poor internal road communication
Big land ownership
Development possibilities
Large food-grain production
Poverty *53%*

(*Source*: UNDP/FAO, 1988 and field survey)

region is relatively higher other than elsewhere. Flash flood occurs in the Basin of Tista River. Deep floods hit in the south and south-east regions. Most of the soil is silty. Patches of grey sandy soil occurs erratically amongst the silty basin soil in several areas. Meanwhile, great climatic differences, with a mean annual rainfall of about 1500 mm in the southwest

BOX 4
Agro-ecology and poverty of the MTFR

Location
Rangpur, Nilphamari, Kurigram & Gaibandha District
Physiography
Low land with ridges and cut-off channels
Drainage
Well drained
Rapid run-off
Proportion of landscape
High land, 93%
Medium high land, 1%
Medium lowland, <1%
Low land, <1%
Very low land, 0%
Homesteads, water, 6%
Climate
High deviation of climate from year to year
Both winter and summer are longest
Mean annual rain fall of 1300-1400mm
Soil
Poorly drained soil
Soil types and percentage of total
Acid basin clay---<1%
Shallow red brown terrace soil----3%
Shallow grey terrace soil---3%
Deep grey terrace soil---72%
Gray valley soil---16%
Soil texture
Loamy--13%
Clayey--87%
Water resources
Limited surface water
Ground water poor in western region but rich in eastern region
Present land use
Transplanted Aman grown dominantly
Other varieties grown scarcely but poor yield
Development constraints
Uncertain rainfall
Inadequate surface and ground water
Low soil Fertility
Sloping relief
puddle silty topsoil
Big land ownership
Development possibilities
Crop production by increasing irrigation, soil and crop management
Poverty *85%*

(*Source*: UNDP/FAO, 1988 and field survey)

and about 2300 mm in the extreme north, are seen. Olive-brown soil with rapidly permeable and loamy soils are seen into the upper parts of high floodplain ridges, and grey or dark grey with slowly permeable and heavy silt loam or silty clayey loam soil can be found into the lower land. Clay soil is seen in limited basin areas. Silty Tista floodplain soil possesses a very high moisture holding capacity with low organic matter. Slight to strong acid of cultivated topsoil can also be seen there. The predominant soil is grey with puddled topsoil and ploughpan. During the dry season, surface water is unavailable in most of the rivers and tanks. Meanwhile, groundwater is available all over the region other than that adjacent to the HBR. During the rainy season, however, groundwater is close to the surface. T. Aman is the major crop, whereas HYV. Aus and Aman varieties are also widely cultivated. No irrigated land generally stays crop-free during the dry season.

Micro-level Agro-ecology and Poverty

Agro-ecology influences productivity of agriculture and it also leads to alleviation of poverty. The productivity varies from one region to another because of agro-environment disparity.

Uttar Chandipur

High intensity of poverty and wide variation of agro-environment are found in the investigated village of Uttar Chandipur. Most of the lands are owned by very few landlords. The rest of the people have limited lands and they live below

TABLE 1
Agro-ecological constraints and intensity of poverty

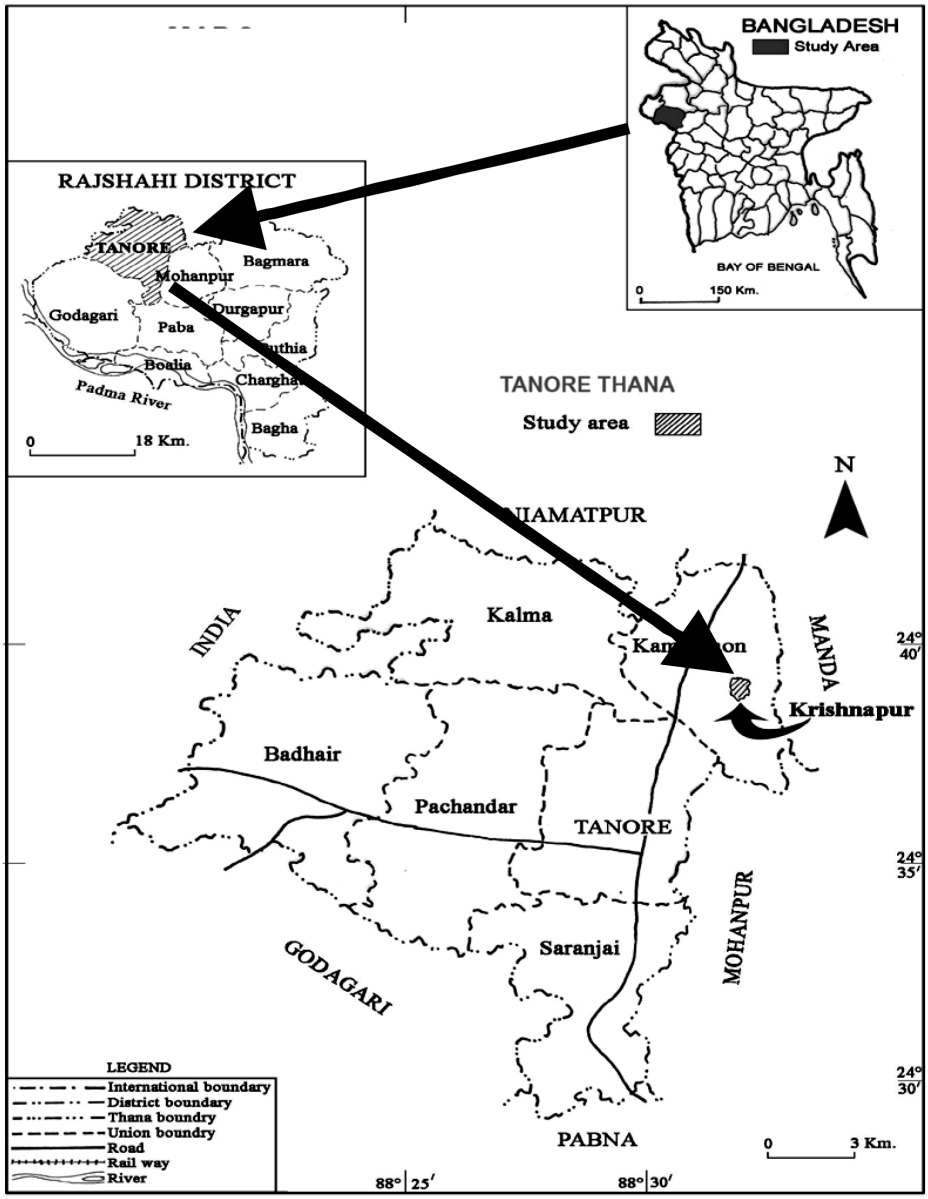
AEZ	Ecological constraints	Intensity of poverty (%)
HRB	• steep slope • terrace valley • high erosion, • rapid run-off • high land 93% • high deviation of climate • clayey soil texture • limited surface water	73
LBR	• plain land • low erosion, • medium land 55% • high standard deviation of climate • clayey soil texture (97%) • limited surface water	64
LGFR	• floodplain • seasonally flooded • less deviation of climate • loamy (48%) and clayey (52%) soil texture • limited surface water within 100m find ground water	53
MTFR	• floodplain • seasonally flooded • loamy soil texture • limited surface water and ample ground water	85

(*Source*: UNDP/FAO, 1988 and field survey)

the poverty line. Agriculture is the main sector of employment, where most of the poor work. Deep grey terrace soil and grey silty soil in the subsoil, with medium to strong acid, are the main features of the soil.

Krishnapur

This place has an almost homogeneous flat environment, where more than 50% of the households are poor. It has deep grey terrace soil with poorly drained and silty soil, where medium to strong acid soil can also be found.

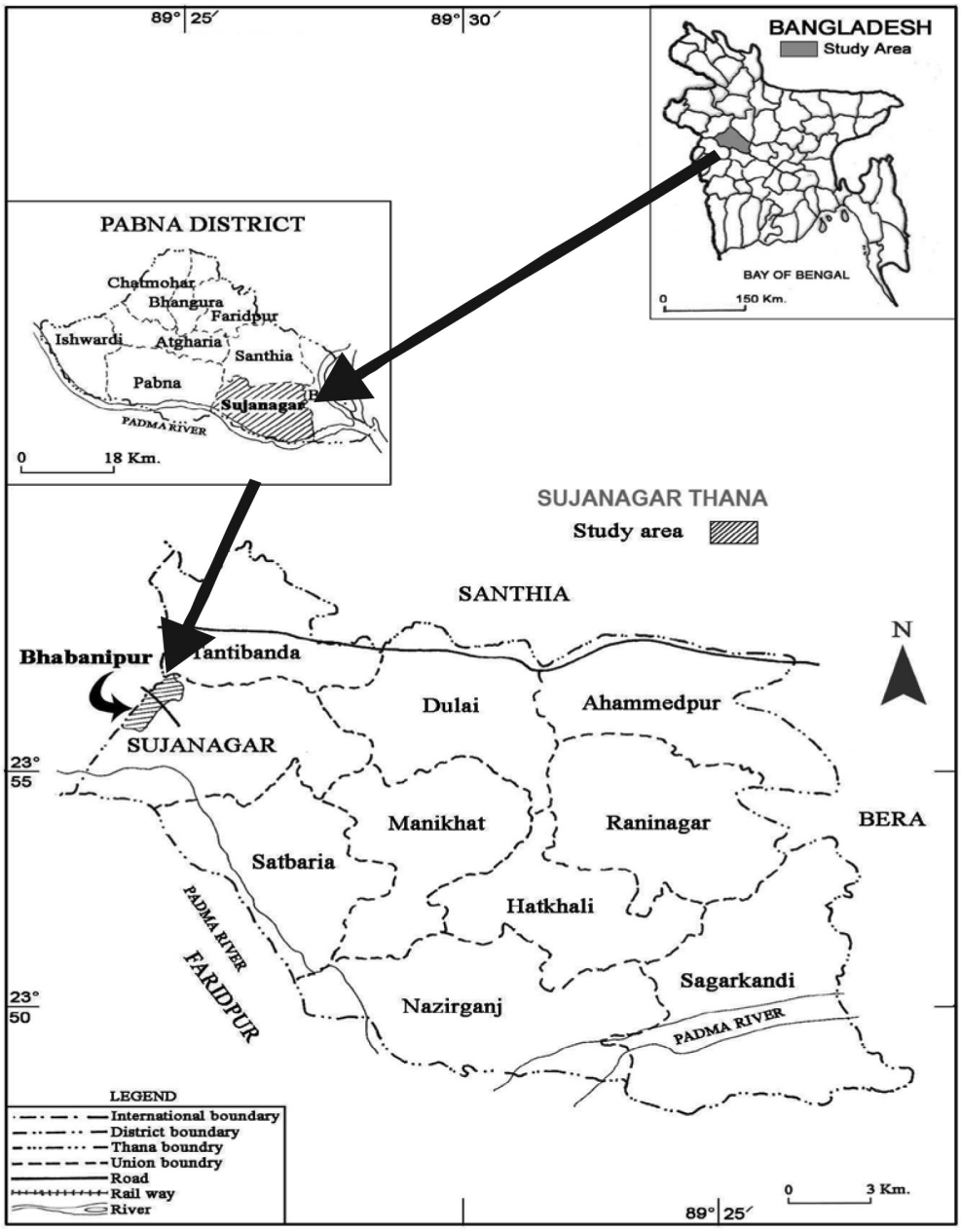


Source: BARC/FAO/UNDP GIS Project: BGD/95/006, based on UNDP/GOB/FAO Project BGD/81/035)

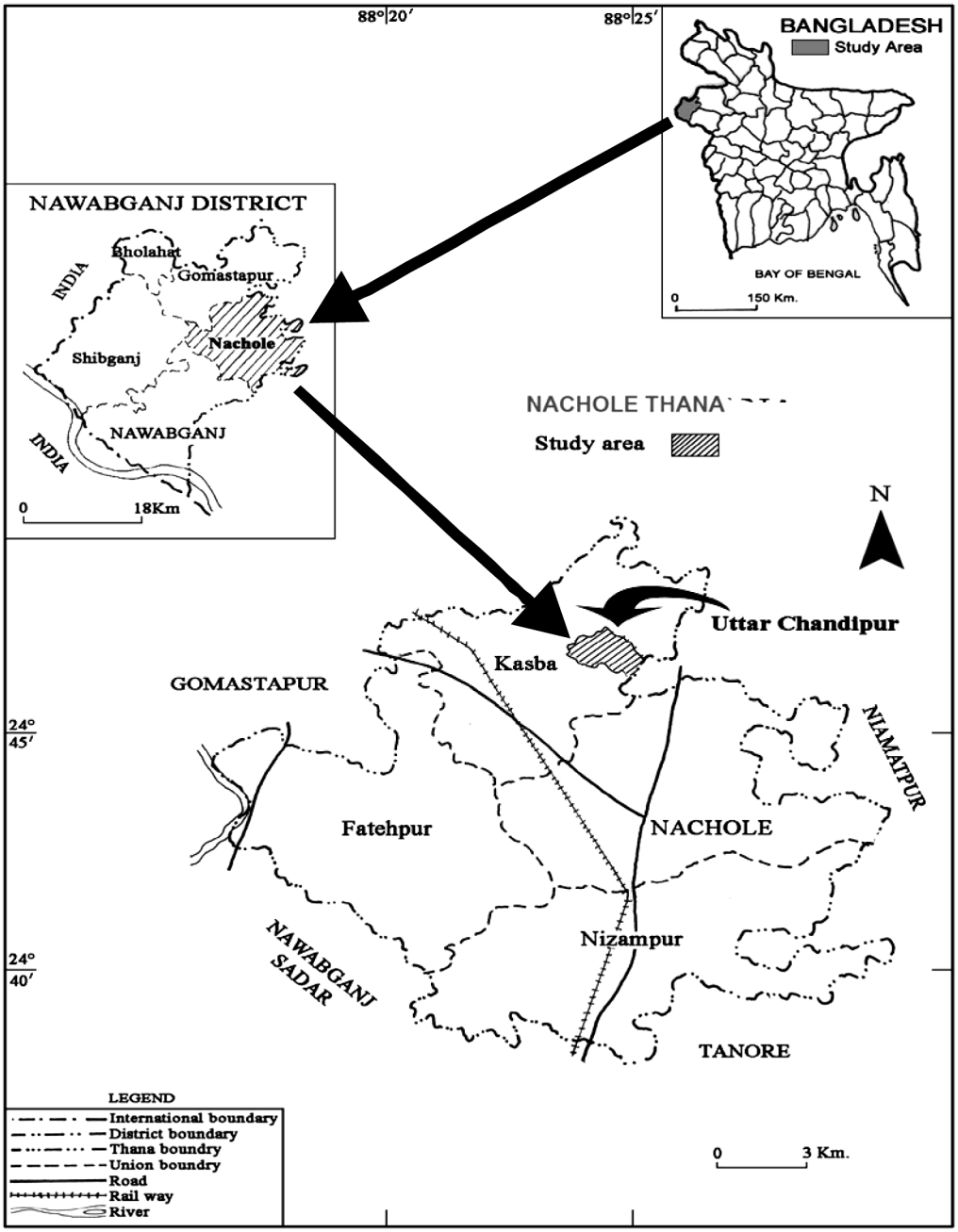
Map 2: Shows the location of Krishnapur

Vhabanipur

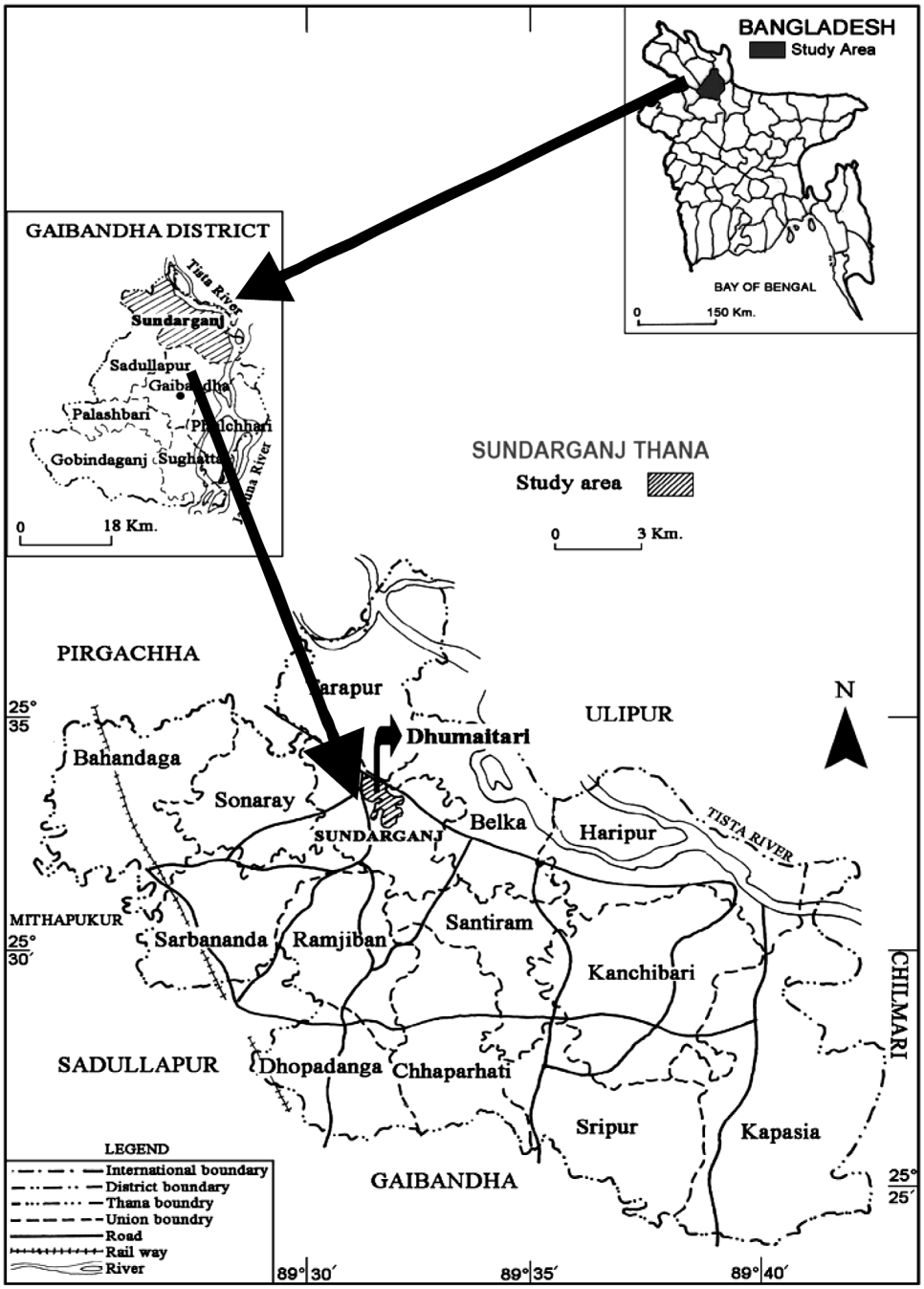
The environmental uniqueness includes calcareous dark grey floodplain soil, seasonally flooded soils with a cambic B horizon which is either dominantly dark grey or has a prominent dark grey or pressure faces, and calcareous within 125 cm from surface. Much of the basin soil has a neutral to acid topsoil and near-neutral subsoil over a calcareous substratum at 40-60 cm.



Map 3: The location of Bhabanipur



Map 4: The location of Uttar Chandipur



Map 5: The location of Uttar Dhumaitri

Uttar Dhumitury

The high intensity of poverty is seen at the village. Most of the poor live in floodplain basin, where frequent floods and river bank erosion are major disasters. Divergent agro-environment was also observed there. Meanwhile, non-calcareous dark grey floodplain soil, with a dark grey cambic B horizon, is also found at this place.

CONCLUSIONS

The heterogeneous agro-environment, which influences food-grain production and income of rural poor, was found in the investigated areas. In addition, the production systems, which are primarily influenced by physical, biological, climatologically and socio-economic factors, are highly complex and diverse. The present ranges of technologies are not uniformly distributed all over the agro-ecological zones. Instead, a wide gap of technologies could be seen at the sites. The environmental heterogeneities, which influence cropping intensity and productivity, are directly interconnected with poverty. The main causes of poverty found at the study areas include unequal landownership distributions of the respondents together with agro-ecological variations. Meanwhile, individual components of agro-environment, like climate and soil, and its interconnection with poverty, are suggested for further study.

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Impacts of Livestock Grazing on Selected Soil Chemical Properties in Intensively Managed Pastures of Peninsular Malaysia

Majid Ajorlo[1*], Ramdzani Abdullah[1], Ahmad Husni Mohd. Hanif[2], Ridzwan Abd. Halim[2] and Mohd Kamil Yusoff[1]
[1]Faculty of Environmental Studies,
[2]Faculty of Agriculture,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: Ajorlo_m54@yahoo.com

ABSTRACT

This study investigates the impacts of short-term (1.5-year) heavy (SHG) and long-term (33-year) moderate (LMG) grazing intensities on the chemical properties of soil in the tropical pasture ecosystems. Two pastures with different grazing intensities and two ungrazed pastures were sampled at the varied depths of 0-10 and 10-20 cm in the Livestock Section of Universiti Putra Malaysia (UPM), Selangor, Malaysia. The EC and pH values in both the moderately and heavily grazed pastures were higher than the ungrazed ones. Meanwhile, the total carbon (TC) in the surface soil of the grazed pastures was 63% and 57% higher than the ungrazed pastures in the LMG and SHG pastures, respectively. The concentration of total nitrogen (TN) was not affected ($P > 0.05$) by livestock grazing. The concentration of available phosphorus (AP) in the surface soil and grazed pasture was significantly greater than the sub-surface soil and ungrazed pastures in the LMG pastures. The AP concentration in the grazed pasture and surface soil was 62.2% and 68.4% less than the ungrazed pasture and subsurface soil in the SHG pasture, respectively. It was found that the concentration of exchangeable cations (Ca^{+2}, Mg^{+2}, and K^{+}) was affected by grazing. In particular, the concentration of exchangeable cations in both the moderately and heavily grazed pastures was also observed to be less than the ungrazed ones, except for Mg^{+2} in the LMG pasture. These results indicate that the impacts of livestock grazing on soil chemical properties were almost limited to the surface soil. After 33 years of moderate grazing, greater values were found for pH, EC, AP, and Mg^{+2} whereas lower values were stated for TN, TC, Ca^{+2}, and K^{+}. As compared to the ungrazed control, the concentrations of AP, TN, Ca^{+2}, Mg^{+2}, and K^{+} decreased after 1.5 years of heavy grazing.

Keywords: Long-term moderate grazing, short-term heavy grazing, exchangeable cations, total nitrogen, total carbon, available phosphorus

INTRODUCTION

In Malaysia, grazing reserves are tracts of land officially allocated by various state governments to be used by farmers to graze their animals. Grazing reserves consist of native and improved grasslands. Large areas of native grazing reserve are available in Sabah (96,479 ha), Sarawak, and Peninsular Malaysia (38,000 ha). Meanwhile, the total of the current areas of improved pastures was approximately 25,000 ha in Peninsular Malaysia, 5,000 ha in Sabah, and 20,000 ha in Sarawak (FAO, 2002). Native grasslands with low productivity and poor forage quality comprise basically a mixture of Hillo grass (*Paspalum conjugatum*), Carpet grass (*Axonopus compresus*), and Slender panic grass (*Ottochloa*

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[*]Corresponding Author

nodosa) that are used for communal livestock ranching by smallholders. Improved grasslands, which mainly consist of Guinea grass (*Panicum maximum*), Signal grass (*Brachiaria decumbens*), and Napier grass (*Pennisetum purpureum*), are used for commercial livestock ranching by both governmental and private sectors. In Malaysia, overgrazing is a common problem in the great majority of native grasslands. Low productivity of native grasslands, coupled with overgrazing, leads to a rapid degradation of soil and vegetation in such ecosystems (Arevalo *et al*., 1998; Chin, 1998). Overgrazing leads to significant changes in vegetal cover and to a complete absence of vegetation cover in some places (Xie and Wittig, 2004). In particular, livestock grazing has been found to significantly alter almost every aspect of soil structure and function, including its physical, chemical, biological, nutrient cycle, and productivity (Roberson, 1996). It is believed that animals alter soil chemical properties through direct defecation and urination (Bilotta *et al*., 2007). Livestock wastes are often a rich source of nutrients, such as N and P because a small percentage (3–30 %) of the nutrients in the food ingested by the livestock is actually utilized by the animal body, the remainder being excreted in faeces and urine (Bilotta *et al*., 2007). For instance, Tajuddin (1984) indicated that an adult sheep could produce about 186 g dry manure, containing 2.40 % N, 0.40 % P, 2.89 % K, 1.84 % Ca, 0.54 % Mg, and 0.051 % Na, every day. If added to the soil, there would be an increase in soil fertility and the supply of nutrients for vegetation growth could therefore be expected. The nutrient content of livestock excreta may be enhanced further when the animals are fed with concentrated feeds (Tamminga, 1992). Smet and Ward (2006) believe that a more likely source of high nutrients around the watering points in the commercial cattle pastures is the supplementary feed that is given to cattle at watering points.

Recently, Malaysia has placed special emphasis on the development of agriculture, specifically in livestock production, to meet the increasing demands for ruminant products. Thus, it is important to know whether grazing affects soil properties in the tropical pastures. Although the effects of animal grazing on pasture soils have been well documented in developed countries, there is still limited understanding on the extent and intensity of the impacts of animal grazing on the chemical properties of soil in the tropical pastures in Malaysia. The objective of this study was to determine the effects of short-term (1.5-year) heavy and long-term (33-year) moderate rotational grazing intensities on the chemical properties of the soil in the tropical native and improved permanent pastures.

MATERIALS AND METHODS

Site Description

The study was carried out in the catchment at Taman Pertanian Universiti (TPU) as improved pasture with long-term moderate grazing intensity and Ladang 2 farm as native pasture with short-term heavy grazing intensity. The catchments lie between 2° 58' 53" and 2° 59' 57" N latitude, and 101° 43' 38" and 101° 44' 03" E longitude in the main campus of Universiti Putra Malaysia. The coordinates of the centre of the farm are 3° 00' 28" N latitude and 101° 42' 10" E longitude. The areas experience a humid tropical climate with seasonality in rainfall distribution. Meanwhile, the mean annual rainfall is about 2141 mm, whereas the mean annual temperature is 26°C (Taman Pertanian Universiti, 2008). It is important to highlight that the textures of the soil vary from sandy clay to silty clay and clayey loam. The soils are generally well drained in both the areas. The TPU catchment extends over a total area of 317 ha. With the exception of the 37% of the TPU catchment, which are directly covered by oil palm plantations, settlements, roads and paths, the rest of the catchment is devoted to improved pastures and livestock grazing. The soil type has been classified as Munchong soil series (Typic Hapludox) representing the Oxisols. The soil is generally well-drained in the catchment. Within the catchment, two field sites having similar slope, topography, soil, and vegetation were selected. These were designated as grazed and ungrazed sites. The vegetation at both sites is homogenous with a dominant cover of tropical

grasses, such as Signal grass (*Brachiaria decumbens* Stapf.) and Guinea grass (*Panicum maximum* Jacq.). Meanwhile, the grazed site, with an area of 180 ha, has experienced a history of regular moderate rotational grazing by cattle in the order of 2.7 livestock unit/ha/yr under a year-round rotational grazing system since 1975. Over the past 33 years, grazing intensity has remained consistent within the low to moderate range. A fenced, ungrazed exclosure of 20 hectares with topography, soil, and vegetation that was similar to the grazed area was installed in the catchment in 1975 and it has never been grazed to date.

Ladang 2 farm with an area of 2 ha was established at the beginning of 2007. Ladang 2 is an ex-tin mining land which was abandoned a long time ago. This area is a sandy clay texture. The soil is well drained in the farm. There is an ungrazed site beside the farm, with the same area, but without any anthropogenic manipulation during the recent years. The farm, both grazed and ungrazed sites, was useless natural grassland before the establishment. Both the grazed and ungrazed sites were predominantly covered by Carpet grass (*Axonopus compressus* (Sw.) Beauv.), Hillo grass (*Paspalum conjugatum* Berg.), and Slender panicgrass (*Ottochloa nodosa* Kunth). The farm has experienced regular grazing by cattle in the order of five livestock unit/ha/yr under a year-round heavy rotational grazing system since its establishment. Management practices in the study areas were typical for a controlled rotational grazing system in intensively managed pastures, where managers carry out several activities including inputs of chemicals (fertilizers, pesticides, and herbicides), offering supplementary minerals and concentrated feed to the cattle, and reducing biodiversity to enhance the productivity of the pasture beyond the natural level. Sites that have been grazed by cattle and those that remain ungrazed constitute the actual subject matter of this research.

Apart from free grazing on pasture, cattle were supplied with concentrated feed in the form of Palm Kernel Cake (PKC), in the quantities of 1 kg/head/day in a feeding trough on the pastures. Furthermore, the cattle had free intake of mineral supplement blocks every day. The chemical constituents of the Malaysian PKC and mineral lick block are summarized in Table 1.

Pastures of the TPU catchment have been fertilized with NPK fertilizers since the year of establishment. For this purpose, urea-N has added to the grazed and ungrazed pastures over 33 years at the rates of 150-200 and 200-300 kg/ha/year, respectively. Furthermore, triple super phosphate (TSP, 200 g P/kg) has been applied in the rates of 40-60 kg P/ha/year over the same period. Grazed and ungrazed pastures received 50-100 kg and 100-150 kg of potassium (K)/ha as muriate of potash (MOP, 50% K), respectively. On the contrary, native pastures of Ladang 2 farm has never been fertilized since its establishment.

TABLE 1
Mineral contents of Malaysian Palm Kernel Cake (PKC)* and mineral lick blocks**

Contents	PKC	Mineral block
Calcium (%)	0.21 – 0.34	8.50
Magnesium (%)	0.16 – 0.33	0.50
Potassium (%)	0.76 – 0.93	-
Phosphorus (%)	0.48 – 0.71	22.8
Sulphur (%)	0.19 – 0.23	-
Total nitrogen (%)	2.32 – 3.13	40
Salt (%)	-	50

* *Source*: Alimon (2004)
** *Source*: Yeong *et al*. (1983)

Sampling Procedure

Soil sampling was carried out in June 2008. In order to facilitate the sampling in the TPU catchment, four typical areas with four sampling plots (0.5 ha) in each area were identified in both the grazed and ungrazed sites. In Ladang 2 farm, four typical areas, with two sampling plots in each area, were established as well. All the samples were taken at least 100 m away from any roads in the study catchment to minimise contamination from vehicle emissions and road dust. Meanwhile, the litter layer was removed from the surface of the soil before taking the samples. The soil samples were collected at randomly selected points, i.e. at 0-10 and 10-20 cm depths in both the grazed and ungrazed sites of the study areas. Ten samples were randomly taken from the selected points at the same depth in each sampling plot and they were then pooled together and thoroughly mixed in order to get one composite sample. The pooled samples were dried at room temperature (25 ºC) and these were gently ground in a porcelain mortar with a pestle after that. The crushed soil was passed through a 2 mm stainless steel sieve to obtain the < 2-mm fraction. Stones, litter and roots were collected from the samples, before and during the grinding. Fine roots passing through the sieve were removed with forceps as far as possible. Air-dried and 2 mm sieved soil was transferred into an airtight plastic bag and stored in an air-conditioned room for chemical analysis.

Chemical Analyses

The chemical characteristics of the soil analyzed include pH, electrical conductivity (EC), total carbon (TC), total nitrogen (TN), available phosphorus (AP), and exchangeable cations (Ca^{++}, K^{+} and Mg^{++}). Meanwhile, soil pH was measured potentiometrically in the supernatant suspension of a 1:1 (soil to distilled water ratio on a volume basis) using a meter (Orion 3-star Portable pH meter, Thermo Electron Corporation, USA), following a 30 min's equilibration period. The EC was measured in the soil-water mixture (1:1 soil/deionised water ratio on a volume basis) using a meter (Orion 3-star Portable Conductivity meter, Thermo Electron Corporation, USA). Carbon was measured by the Leco CR-412 Carbon Determinator (Leco Corporation, USA). Total nitrogen was determined using the Kjeldahl method (Bremner and Mulvaney, 1982). Extractable phosphorus was determined using Bray and Kurtz No. 2 method (Bray and Kurtz, 1945). The exchangeable cations were extracted from the soil with an extracting solution of 1.0 M NH_4OAc (buffered at pH 7.0) (Rhoades, 1982) and were determined in the filtered extract using the Atomic Absorption Spectrophotometry.

Statistical Analysis

The tests were carried out to determine the main effects of grazing intensities and the GLM Repeated Measures Analysis of Variance (ANOVA). For this purpose, soil depths were included as repeated measures to account for the potential effects of grazing at various depths. Meanwhile, an independent *t*-test was applied to compare the two management regimes (grazing versus no-grazing) and determine the differences between soil depths with regard to soil chemical elements. All the tests were run using statistical software (SPSS Inc., 2007). A $P \leq 0.05$ level for testing significance was used in this study.

These long-term and short-term grazed pastures were not replicated. For statistical purposes, each of the sampling locations per treatment was designated as replicates, as the grazing treatments were not replicated. This approach has also been reported by Bauer and Black (1981), Bauar *et al*. (1987), Frank *et al*. (1995), Liebig *et al*. (2006), and Wienhold *et al*. (2001). The use of pseudo-replications has a role in certain situations. Their uses are justified from the standpoint of "space for time" substitution, given the treatments were 33 and 1.5 years old when the study was conducted. They were also used in on-farm research, where treatments were often not replicated (Liebig, Personal Communication, 10 May 2009). Although not ideal, the justification for using this approach hinges upon the value of the long-term status of the grazing treatments (Frank *et al*., 1995;

Wienhold *et al*., 2001) and the big size of the study areas.

RESULTS AND DISCUSSION

The results derived from the multivariate analysis showed that there was a significant difference between the management regimes (namely, grazing vs. no-grazing) in relation to soil chemical nutrients altogether in the short-term heavily grazed pastures of Ladang 2 farm (Wilks' $\lambda = 0.043$, $P < 0.05$) and long-term moderately grazed pastures of the TPU catchment (Wilks' $\lambda = 0.071$, $P < 0.05$). The concentration of all the soil nutrient contents was found to have been affected by soil depth (0-10 and 10-20 cm) in Ladang 2 farm (Wilks' $\lambda = 0.072$, $P < 0.05$), the TPU catchment (Wilks' $\lambda = 0.033$, $P < 0.05$) and by the interaction between soil depth and grazing treatment in Ladang 2 farm (Wilks' $\lambda = 0.069$, $P < 0.05$), and the TPU catchment (Wilks' $\lambda = 0.107$, $P < 0.05$) (Table 2).

Soil pH and EC

Naturally, the soil types in both study areas are strongly acidic. Although soil pH value was found to be not affected ($P > 0.05$) by grazing, it was affected either by soil depth ($P < 0.05$) (Table 3a) or the interactions between grazing treatment and soil depth ($P < 0.05$) in the long-term moderately grazed pastures (Table 5a). The pH values varied from 4.68 to 4.53 between the management regimes (grazing versus non-grazing) (Table 3a). The average pH value (4.68) in the surface soil (0–10 cm) was higher ($P < 0.05$) than the value (4.53) in the subsurface soil (10–20 cm) (Table 3b).

Meanwhile, the depth-by-depth comparison between grazed and ungrazed treatment (interaction between the treatment and soil depth) showed that there was a significant effect ($P < 0.05$) of the long-term moderate grazing on the soil pH level with regard to the soil depths and grazing treatment. The value of soil pH (4.82) in the top soil of the grazed pasture was greater than ($P < 0.05$) the pH (4.54) of top soil of the ungrazed pasture in the long-term moderately grazed pasture. However, the subsoil of the grazed pasture had lower pH value than the ungrazed pasture (Table 5a). Overall, in relation to both soil depths, the two pastures were significantly differed in terms of their soil pH ($P < 0.05$) (Table 5a).

Soil pH was affected ($P < 0.05$) by short-term heavy grazing (Table 4a) and the interaction between the grazing treatment and soil depth ($P < 0.05$) (Table 5b). In more specific, the soil pH ranged from 4.41 to 4.93 and from 4.28 to 5.40 respectively in the grazed and ungrazed pastures of Ladang 2 farm, respectively. The mean pH value varied from 4.81 in the grazed pasture to 4.64 in the ungrazed pasture (Table 4a). At the same time, the depth-by-depth comparison between the grazed and ungrazed treatment (interaction between treatment and soil depth) showed the soil pH value (4.69) in the top soil of

TABLE 2
The effects of cattle grazing on the chemical properties of the tropical pasture soil

Study area	Effect	Wilks' l	F	P	η
Short-term heavily grazed pasture (Ladang 2 farm)	Grazing treatment	0.043	19.41	00	0.957
	Soil depth	0.072	11.23	0.002	0.928
	Treatment × depth	0.069	11.76	0.002	0.931
Long-term moderately grazed pasture (TPU catchment)	Grazing treatment	0.071	34.56	00	0.929
	Soil depth	0.033	76.92	00	0.967
	Treatment × depth	0.107	21.96	00	0.893

η: effect size

TABLE 3
Soil chemical properties in response to the long-term moderate grazing treatment in the pastures of the TPU catchment

Chemical properties	(a)				(b)			
	Treatment		SE††	F	Soil depth (cm)		SE	F
	Ungrazed	Grazed			0-10	10-20		
pH (-log[H^+])	4.53	4.68	0.09	2.94	4.68a	4.53b	0.03	25.09
EC (S cm^{-1})	50.21a†	69.54b	4.14	21.76	73.39a	46.35b	2.35	132.17
TC (%)	1.86	1.80	0.09	0.42	2.32a	1.42b	0.61	173.66
AP (mg kg^{-1})	2.71a	5.88b	1.08	8.65	5.63a	3.03b	0.87	8.48
TN (%)	0.30	0.22	0.04	3.36	0.25	0.27	0.06	0.141
K^+ (cmol(+)kg^{-1}))	0.049 a	0.037 b	0.004	12.88	0.058 a	0.028 b	0.003	154.09
Ca^{+2} (cmol(+)kg^{-1})	2.133 a	0.501 b	0.136	135.65	2.371a	0.264 b	0.133	250.79
Mg^{+2} (cmol(+)kg^{-1})	0.167a	0.275 b	0.034	14.14	0.345 a	0.097 b	0.025	105.05

†† Standard error
† Means in a row with unlike lower case letters significantly differ at $P < 0.05$

TABLE 4
The soil chemical properties in response to the short-term heavy grazing in the pastures of Ladang 2 Farm

Chemical properties	(a)				(b)			
	Treatment		SE††	F	Soil depth (cm)		SE	F
	Ungrazed	Grazed			0-10	10-20		
pH (-log[H^+])	4.64 a†	4.81 b	0.07	5.63	4.68	4.77	0.076	1.54
EC (S/cm)	50.72 a	70.09 b	3.51	30.32	72.55a	48.26b	4.07	35.64
TC (%)	1.43 a	2.25 b	0.18	18.94	2.25 a	1.43 b	0.18	18.90
AP (mg/kg)	54.98 a	32.08 b	14.23	13.82	28.05a	89.00 b	14.43	17.84
TN (%)	0.32	0.23	0.08	1.17	0.19	0.35	0.08	3.80
K^+ (cmol(+)kg^{-1})	0.030	0.029	0.003	0.071	0.034	0.025	0.005	2.82
Ca^{+2} (cmol(+)kg^{-1})	2.38 a	0.983 b	0.263	28.28	0.963	1.081	0.245	34.60
Mg^{+2}(cmol(+)kg^{-1})	0.114 a	0.045 b	0.014	25.34	0.058a	0.101 b	0.014	9.41

†† Standard error
† Means in a row with unlike lower case letters significantly differ at $P < 0.05$

the grazed pasture tended to be greater than the pH (4.67) of the top soil of the ungrazed pasture. However, the subsoil of the grazed pasture had a lower pH value than the ungrazed pasture (Table 5b). In total, the two pastures were significantly differed in terms of soil pH ($P < 0.05$) (Table 5b) with regard to both soil depths.

In Malaysia, Kamaruzaman (1988) found that in the soil was significantly less acidic for the pastures that were grazed by sheep than that of the non-grazed pastures. Meanwhile, Smet and Ward (2006) indicated that long-term grazed commercial cattle ranches had higher soil pH value than the communal livestock ranches, i.e.

by around water-point in a semi-arid savannah of South Africa. In contrast, Xie and Wittig (2004) reported that there was no correlation between soil pH value and moderate and heavy grazing intensities in the grassland steppes of Northern China. Similarly, no consistent trend was observed for the effects of livestock grazing on the soil pH value in a universal grazing comparison (Milchunas and Lauenroth, 1993). The large amount of ammonia produced from the hydrolysis of urea in animal urine and the cations deposited from the manure have been attributed by Kamaruzaman (1988) as the reasons to the high pH value in the top soil of the grazed pasture. At the same time, herbivore grazing, trampling, defecation, and urination have been reported to have increased the soil pH level (Killham, 1994). The pH increase of 0.28 units in the top soil of the long-term grazed pasture could be related to Al complexing with organic matter, and it was also possibly due to the recycling of Ca and Mg to the soil surface through livestock grazing and excretion (Arevalo *et al*., 1998). It could be concluded that cattle grazing could decrease the acidity of the tropical pasture soil in the long- and short-term. Low pH level at the non-grazed sites could be explained by two-fold annual applications of N fertilizer (urea) at the mentioned site as compared to the grazed site. Meanwhile, greater acidification of fertilized crested wheatgrass (*Agropyron desertorum*) pasture is driven by the annual application of N fertilizer (Liebig *et al*., 2006).

The results indicated that both the moderate and heavy grazing by cattle had caused the soil to become less acidic. This result was also supported by the findings of Liebig *et al*. (2006) and Kamaruzaman (1988). The variation of soil pH, with respect to soil depth and grazing treatment interaction, was similar in both Ladang 2 farm and the TPU catchment. The livestock grazing impacts on soil pH were noticeable in the top soil. In particular, the non-grazed pastures showed low pH level and greater acidification.

The soil EC was affected ($P < 0.05$) by grazing treatment and soil depth in both pastures (Tables 3ab and 4ab), and the interaction between them was found to be significant only in the long-term moderate grazing (Table 5a). The EC value in the grazed pasture was higher ($P < 0.05$) than the ungrazed pasture in both pastures. The means soil EC of the grazed pastures were 38 % greater than the ungrazed pastures in both the areas studied (Tables 3a and 4a). Meanwhile, the EC values in the top soil were higher ($P < 0.05$) than the subsoil in both the pastures. The means EC of the top soils were around 58 and 50 % higher than the subsoil in the long-term moderately and short-term heavily grazed pastures, respectively (Tables 3b and 4b).

The depth-by-depth comparison between the grazed and ungrazed treatment (i.e. the interaction between the treatment and soil depth) showed that the EC of the top and sub-soil of grazed pastures were greater ($P < 0.05$) than the adjacent ungrazed exclosure in both the study areas (Table 5ab). The grazing treatment and soil depth had significant interaction in the long-term moderately grazed pastures (Table 5a). Although the EC values of the grazed surface (81.66 S/cm) and the subsurface soil (58.52 S/cm) were significantly higher ($P < 0.05$) than the ungrazed surface (63.45 S/cm) and subsurface soil (38.00 S/cm) in the short-term heavily grazed pasture, there was no significant interaction between the grazing treatment and soil depths ($P > 0.05$) (Table 5b).

The soil EC values were lower in the ungrazed pastures of both Ladang 2 farm and the TPU catchment. This result agrees with the findings of Chaneton and Lavado (1996) who reported that the topsoil EC was significantly lower within the long-term ungrazed grasslands. Nonetheless, the finding contrasts with the lower soil EC found to have been caused by grazing in the tropical pasture of Costa Rica (Reiners *et al*., 1994). Liebig *et al*. (2006) and Li *et al*. (2008) observed that the soil EC did not vary between the grazing treatments. The low EC values in the soils indicated that the area was not under risk of salinization (Li *et al*., 2008). Continuous grazing increases salt content by reducing aerial plant and litter cover, which lead to higher soil temperatures and evaporation rates. There are some salt in cattle excreta due to unrestricted daily access to salt and mineral

TABLE 5
Soil chemical properties in response to the interaction between grazing treatment and soil depth in the studied pastures

Chemical properties	Long-term moderately grazed pastures of the TPU catchment (a)								Short-term heavily grazed pastures of the Ladang 2 farm (b)							
	Grazing treatment × soil depth								Grazing treatment × soil depth							
	0-10 cm		SE††	10-20 cm		SE	F	P	0-10 cm		SE	10-20 cm		SE	F	P
	Ungrazed	Grazed		Ungrazed	Grazed				Ungrazed	Grazed		Ungrazed	Grazed			
pH (-log[H^+])	4.54 a†	4.82 b	0.002	4.97 a	4.59 b	0.002	22.28	00	4.67	4.69	00	4.97 a	4.59 b	0.374	6.90	0.02
EC (S cm^{-1})	60.00 a	86.79 b	0.132	40.41 a	52.29b	0.108	9.36	0.004	63.45 a	81.66b	00	38.00a	58.52b	00	0.08	0.78
TC (%)	2.10 a	2.40 b	0.003	1.53 a	1.32 b	0.002	22.00	00	1.78 a	2.72 b	00	1.08 a	1.77 b	00	0.44	0.51
AP (mg kg^{-1})	7.53 a	3.60 b	0.027	4.29	1.81	0.042	0.22	0.396	19.85	36.25	00	150.00a	28.00b	00	23.07	00
TN (%)	0.25	0.24	0.001	0.19	0.36	0.002	2.15	0.153	0.16 a	0.23 b	00	0.48	0.23	00	3.61	0.07
K^+ (cmol(+)kg^{-1})	0.045 a	0.072 b	00	0.028	0.027	00	31.43	00	0.027	0.039	00	0.032 a	0.018 b	00	8.09	0.01
Ca^{+2} (cmol(+)kg^{-1})	0.719 a	4.02 b	0.006	0.284	0.245	00	161.15	00	0.640 a	1.286 b	00	1.48 a	0.681 b	00	69.81	00
Mg^{+2} (cmol(+)kg^{-1})	0.285 a	0.41 b	0.002	0.146 a	0.048 b	00	0.40	0.528	0.035 a	0.076 b	00	0.188 a	0.013 b	00	57.10	00

†† Standard error

† Means in a row with unlike lower case letters significantly differ at $P < 0.05$

blocks in the pastures. Salt moves upward from lower soil horizons and reaches the soil surface in the periods of high atmospheric demand (dry season). Topsoil salinization peaks are followed by salt leaching caused by rainfall and soil water-logging events in rainy season (Chaneton and Lavado, 1996).

Soil Exchangeable Cations

The Ca^{+2}, Mg^{+2}, and K^{+} concentrations in the soil were affected ($P < 0.05$) by grazing treatment, soil depth (Table 3ab) and the interaction (except Mg^{+2}) between them (Table 5a) in the long-term moderately grazed pasture of the TPU catchment. Meanwhile, the soil Ca^{+2} and K^{+} contents varied significantly between the grazed and ungrazed pastures. In particular, the grazed pasture had about four-fold and 1.32 times lower Ca^{+2} and K^{+} contents than ungrazed pasture in the catchment. Conversely, Mg^{+2} content of the grazed pasture soil was found to be significantly higher (65 %) than the ungrazed exclosure (Table 3a). The concentrations of all the measured exchangeable cations in the top soils were significantly higher than the sub-soil in the long-term grazed pastures (Table 3b).

The depth-by-depth comparison between the grazed and ungrazed treatments (i.e. the interaction between treatment and soil depth) showed that Ca^{+2}, K^{+} and Mg^{+2} concentrations in the surface soil (0-10 cm) of the grazed pastures were greater ($P < 0.05$) than the same depth of the ungrazed pastures of the catchment. Ca^{+2} and K^{+} had relatively similar concentrations in the subsurface soils (10-20 cm) of the grazed and ungrazed pastures. Overall, the interaction between the grazing treatment and soil depth was significant for Ca^{+2} and K^{+} (Table 5a).

The concentrations of Ca^{+2} and Mg^{+2} were affected ($P < 0.05$) by heavy grazing treatment in Ladang 2 farm (Table 4a). The divalent cations (Ca^{+2} and Mg^{+2}) concentration in the short-term heavily grazed (SHG) pasture was significantly lower than the adjacent ungrazed pasture (Table 4a). The content of K^{+} was not affected ($P > 0.05$) by the grazing treatment or by the soil depth (Table 4b) in the farm. This is consistent with the results of Xie and Wittig (2004) and Liebig *et al*. (2006) who reported that K^{+} was not influenced by heavy grazing intensities.

Only the concentration of Mg^{+2} in the top soil was significantly lower than the sub-soil in the short-term heavily grazed pastures. The concentration of Ca^{+2} in the top soil of the farm seemed to be lower than the sub-soil (Table 4b). Although only the concentration of Mg^{+2} was affected ($P < 0.05$) by soil depth (Table 4b), there was a significant interaction between the grazing treatment and soil depth in pastures of Ladang 2 farm for the concentrations of all the measured cations (Table 5b). The depth-by-depth comparison between the grazed and ungrazed treatments (i.e. the interaction between grazing treatment and soil depth) showed that only the K^{+} content of surface soil (0-10 cm) did not vary significantly between the heavily grazed and ungrazed pastures of Ladang 2 farm. Overall, the Ca^{+2}, K^{+} and Mg^{+2} concentrations in the surface soil (0-10 cm) of grazed pastures were greater than at the same depth of the ungrazed pastures in the farm, with disregard to statistical significant. However, the concentrations of K^{+}, Ca^{+2}, and Mg^{+2} in the subsurface soil (10-20 cm) of the heavily grazed pasture were lower than ($P < 0.05$) the ungrazed control. On the whole, significant interactions ($P < 0.05$) between grazing and soil depth were detected for all the measured exchangeable cations in Ladang 2 farm (Table 5b).

In general, the exchangeable K^{+}, Ca^{+2}, and Mg^{+2} concentrations were affected ($P < 0.05$) in the surface soil by grazing treatment in both the short-term and long-term grazed pastures. The soluble cation concentrations in the grazed surface soil (0-10 cm) were greater than ($p < 0.0$) the ungrazed surface soils in both the pastures, except for K^{+} in the heavily grazed pasture (Table 5ab). The results showed that grazing treatment seemed to affect soluble cations at 0-10 cm soil depth.

Meanwhile, the grazing treatments, which included both long-term moderate and heavy grazing, tended to affect divalent cations (Ca^{+2} and Mg^{+2}) at 0–10 cm, whereas grazing was found to affect monovalent cations (K^{+}) below

30 cm in Missouri Plateau, USA (Liebig *et al*., 2006). In Peru, Arevalo *et al*. (1998) reported that the concentration of K^+ remained constant until 42 months after grazing began in the tropical pastures. The same result was also observed in the short-term (18-month) grazed pasture in this study. Meanwhile, elevated levels of exchangeable cations in the surface soils (0-10 cm) might be caused by a stocking effect, resulting in a greater deposition of waste through faeces and urine, followed by subsequent decomposition and distribution throughout the soil profile. A low concentration of potassium in the short-term heavily grazed pasture might be related to the reduction in the water-soluble K due to high salinity as a result of the addition of animal manure to the soil. It is also likely that K, being highly mobile, is lost by leaching, since heavy rain is usually experienced in Malaysia (Kamaruzaman, 1988). The increase of potassium amount in the surface soil of long-term grazed pastures might be related to the recycling of nutrients in plant dead material and litter, root biomass turnover, and through the recycling of cattle excreta in long-term (Arevalo *et al*., 1998).

Total carbon, total nitrogen and available phosphorus

Nonetheless, total carbon (TC) did not differ ($P > 0.05$) between the grazing treatments (Table 3a) but it was significantly affected ($P < 0.05$) by soil depth (Table 3b) and the interaction between them (Table 5a) in the LMG pasture. The TC in the surface soil layer was 63% higher than the subsurface soil in this pasture (Table 3b). The depth-by-depth comparison of the grazing treatments (grazing treatment and soil depth interaction) showed that the TC of the surface soil (0-10 cm) of the grazed pasture was greater ($P < 0.05$) than the ungrazed pasture. However, this was reverse in the subsurface soil (Table 5a). Overall, a significant interaction was found between grazing treatments and soil depth.

There was a significant difference ($P < 0.05$) between the grazing treatments (Table 4a) and soil depth (Table 4b) but not by the interaction between them ($P > 0.05$) (Table 5b) in the SHG pasture in relation to soil carbon. Meanwhile, the TC percentage in soil in heavy grazing treatment and surface soil was 57 % greater than the ungrazed pasture and subsoil. Xie and Wittig (2004) found a significant difference between heavily grazed and ungrazed grassland communities with regard to soil organic substances. The total carbon percentages in the upper and lower soil layers in ungrazed pasture of SHG pasture were significantly lower than the adjacent grazed pasture (Table 5b).

The mean soil total C in heavily grazed pasture (2.25) was greater for the pasture than the moderately grazed pasture (1.80). This result is in agreement with the findings of Liebig *et al*. (2006) and Frank *et al*. (1995). Greater TC in the SHG pasture might be caused by the dominance of mat-forming grasses, including *Axonopus compresus* and *Ottochloa nodosa* in this pasture, which transfer most of their photosynthetic products belowground to root mass. Furthermore, root death in the pastures under heavy grazing was also found to be high. The limited increase in the soil total carbon under long-term moderate grazing could be attributable to a decrease in the amount of plant litter and an increase in soil compaction due to cattle treading, which apparently causes unfavourable living conditions for those organisms that are vital for decomposition of organic matter and incorporation of the humus into the soil (Xie and Wittig, 2004).

Nonetheless, the influence of long-term moderate grazing on the content of soil TC was limited to topsoil (0-10 cm). The effect of short-term heavy grazing on the content of soil TC in the subsoil was lower than the top soil. Xie and Wittig (2004) indicated that with the increasing depth of soil, the influence of grazing intensity on soil organic substances was found to significantly reduce. These results indicate that grazing cattle increases soil total carbon in the short-term, but there is no a consistent trend in the increase of soil total carbon over time, as the effect of long-term grazing on soil TC amount was not statistically difference in the grazed areas versus adjacent ungrazed areas.

The higher concentrations of the total carbon in the upper soil layer of the grazed pastures could be explained by deposition of organic matter by cattle faeces, greater detrital inputs of grass litter into the soils and concentration of grass roots in surface soil at 0-10 cm. Based on the results of dead material and litter measurements in these pastures (unpublished data), detrital input of grass litter into soil in grazed area is therefore greater than the ungrazed area. The higher the concentration of the total C in the subsoil of the ungrazed pasture in long-term grazed pasture might be attributable to the high grass root turnover rate in this layer. According to Dahlgren *et al*. (1997), another source of organic matter in soil B horizon is from the retention of dissolved organic matter (DOC) leaching from the litter decomposing at the soil surface and to lower soil layers.

The concentration of soil TN was not affected ($P > 0.05$) by the grazing treatments, soil depth (Tables 3 and 4) and the interaction between them (Table 5ab). Meanwhile, the concentration of TN ranged from 0.22 to 0.30 and 23 to 0.32 in the grazed and ungrazed sites of the LMG and SHG pastures, respectively.

The concentration of AP was affected ($P < 0.05$) by the grazing treatments (Table 3a) and soil depth (Table 3b), but not ($P > 0.05$) by the interaction between them in the LMG pasture (Table 5a). The mean soil AP in the pasture under the long-term moderate grazing treatment was about two-fold greater than the ungrazed control pasture. The soil AP content of the surface soil was about 46% greater than the subsurface soil in the TPU catchment.

Soil AP content was affected ($P < 0.05$) by the grazing treatments (Table 4a) and soil depth (Table 4b), but not ($P > 0.05$) by the interaction between them in the SHG pasture (Table 5b). The concentration of soil AP in the grazed pasture and surface soil (0-10 cm) was significantly ($P < 0.05$) lower than ungrazed pasture and subsurface soil (10-20) in SHG pasture. The AP concentrations in the grazed pasture and surface soil were 62.2% and 68.4% lower than the ungrazed pasture and subsurface soil in this pasture. Xie and Wittig (2004) stated that the concentration of TN and AP in grasslands, under moderate and heavy grazing, was less than the non-grazed areas. Dahlgren *et al*. (1997) observed no significant difference in the soil N concentration between the grazed and ungrazed treatments. Meanwhile, Tiedemann *et al*. (1986) reported a net loss of 3.2 kg N/acre under a moderate grazing compared to the ungrazed area in the Pacific Northwest. The depth-by-depth comparison of the grazing treatments (grazing treatment and soil depth interaction) showed that the surface soil AP and TN contents in the SHG pasture were higher than the ungrazed exclosure, but not were significant for the AP. However, this was reverse in the subsurface soils (Table 5b).

The Available Phosphorus (AP) concentration in soil was not increased after 33 years of moderate grazing (Chaneton and Lavado, 1996; Smet and Ward, 2006). Meanwhile, the total N in the surface soil of heavily grazed pasture was greater than the moderately grazed pasture (Liebig *et al*., 2006). Similar to the results retrieved from the tropical pastures in Malaysia (Kamaruzaman, 1988), the increases in the total N and the available P were observed in the surface soils of the short-term grazed pasture. The effects of grazing management on the total N were however limited to the surface soil, i.e. at 5 cm depth (Liebig *et al*., 2006). The increased N in the surface soil of the short-term grazed pasture is probably due to the increased input of organic matter from cattle manure. The increase of plant available P could be attributed to the organic part of manure retarding P fixation by mechanically separating soluble P from the mineral part of the soil (Kamaruzaman, 1988). The nutrient content of cattle excreta might be increased when the cattle have been fed with concentrated feeds like PKC (Biolotta *et al*., 2007). Since carbon, nitrogen, and phosphorus are major components of organic matter, it is assumed that the strong relationship between these components is associated with organic matter concentrations (Dahlgren *et al*., 1997). Nutrients are lost through increased erosion in the grazed pastures, leaching and consuming of plants by animals in the pasture ecosystems.

Grazing animals profoundly affect the horizontal distribution of nutrients in pasture soil. Grazing animals remove plant biomass and redeposit it in other areas like bedding ground, under shade, around feeding trough and watering points in the forms of urine and faeces.

CONCLUSIONS

The long-term moderate and short-term heavy grazing have been found to have effects on the soil chemical properties in improved and native intensively managed pastures. After 33 years of moderate grazing by cattle, greater soil pH, EC, AP and Mg^{+2} and lower TN, TC, Ca^{+2} and K^{+} concentrations were found at the improved pastures of the TPU catchment. For the native pastures of Ladang 2 farm, higher pH, EC, TC and lower AP, TN, Ca^{+2}, Mg^{+2} concentrations were observed after 1.5-year of heavy grazing by cattle. Exchangeable cation concentrations in heavily and moderately grazed pastures were lower than the ungrazed pastures, except for Mg^{+2} in moderately grazed pastures. The heavily grazed pasture had a greater total C than the moderately grazed pasture. Heavy grazing by cattle led to a decrease in the TN and AP contents of soil. Variations of the topsoil chemical properties were noticeable as a consequence of cattle grazing. Knowledge of soil nutrient properties provides quick and useful clues to help pasture managers to make accurate decision about fertilizers application practice. Based on the findings of this study, it can be recommended that the intensity of grazing should not exceed a moderate (30-50%) grazing in order to avoid damages to pastures, such as vegetation degradation, soil compaction, and erosion. Stocking rate should ensure sustainability of the pasture ecosystem to support livestock production in the long-term.

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Genotyping of Sarawak Rice Cultivars Using Microsatellite Markers

Lee, H.H., Neoh, P.P.N., Bong, W.S.T., Puvaneswaran, J., Wong, S.C., Yiu, P.H. and Rajan, A.*
Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia, Bintulu Campus, Nyabau Road, 97000 Bintulu, Sarawak, Malaysia
**E-mail: rajan@btu.upm.edu.my*

ABSTRACT

Genetic diversity of 53 Sarawak rice cultivars, originating from Southern Sarawak, was assessed using 54 microsatellite markers. Initial polymorphism detection was conducted using 54 primer pairs distributed on 12 rice chromosomes. Polymorphic markers were chosen from the initial screening results in order to obtain microsatellite marker panels that can differentiate the rice cultivars undertaken in the study. The chosen microsatellite marker panel consisted of RM1, RM240, RM489, RM252, RM413, RM204, RM11, RM404, RM316, RM271, RM206, and RM19, with one representative from each chromosome. A total of 43 alleles were detected with an average of 3.58 alleles per locus. The polymorphism information content (PIC) values obtained from the microsatellite marker panels ranged from 0.306 to 0.730, with an average of 0.622. The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram (r = 0.789) revealed 2 major groups with 6 sub-clusters and the wide range of similarity values (0.24-1.0) obtained showed a high degree of diversity among the cultivars. The results suggest microsatellite markers as a useful tool for the estimation of genetic diversity and cultivar differentiation and present invaluable genetic information for future breeding and association mapping efforts.

Keywords: Rice, microsatellite markers, polymorphism, genetic diversity, Sarawak

INTRODUCTION

Rice is a major food source of humans and the second major global calories contributor after wheat (FAO, 2008). It belongs to the genus *Oryza* that has modest size genomes with 20 wild species and 2 advanced cultigens, known as *Oryza sativa* and *Oryza glammerima* (Chang, 2003). *Oryza sativa*, the Asian cultivated rice, consists of two major subspecies, namely *Indica* and *Japonica*, which are believed to have been spread from China towards the Southeast Asia, particularly Malaysia (Morishima, 2000; Chang, 2003; Subudhi *et al.*, 2006).

Rice genetic resources are important for crop improvement and its sustainable production. Preservation and conservation of genetic resources are therefore useful since genetic diversity provides information to monitor germplasm and prediction of potential genetic gains (Chakravarthi and Naravaneni, 2006). As rice is a staple cereal crop consumed by humans, high degree of similarity between different cereal genomes in terms of gene content and gene order facilitates crop improvement and breeding for other cereal crops as well (Garris *et al*., 2005). Rice genetic resources can be utilized to improve crop performance through

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*Corresponding Author

breeding with objectives of improving rice quality and yield (Bao *et al*., 2000; IRRI, 2007), disease resistance (Brar and Khush, 1997), insect resistance (Sharma *et al*., 2007), and lodging resistance (Yue *et al*., 2005).

Molecular marker technology provides a powerful tool in the assessment of genetic relationships within and among species, in which differences among accessions can be revealed at the DNA level (Ni *et al*., 2002; Chakravarthi and Naravaneni, 2006). Simple sequence repeats (SSRs) or microsatellites are tandemly arranged repeats of short DNA motifs ranging from 1 to 6 base pairs, which exhibit variations in the number of repeats at a locus (Tautz, 1989; McCouch *et al*., 1997; Mackill, 1999; Temnykh *et al*., 2000). The codominant characteristics of the microsatellite markers and their well known map positions on the rice genome reveal high polymorphisms among different plants (Chen *et al*., 1997; Temnykh *et al*., 2000; Garcia *et al*., 2004).

In particular, microsatellite markers have been widely applied in rice genetic studies as they are able to detect high levels of allelic diversity (McCouch *et al*., 1997). The applications of microsatellite markers in rice research include studies on genetic diversity of Yunnan rice germplasm by Tu *et al*. (2007), genetic diversity analysis of traditional and improved Indonesian rice germplasms by Thomson *et al*. (2007), and other general rice genetic diversity studies by Ni *et al*. (2002), Ravi *et al*. (2003) as well as Chakravarthi and Naravaneni (2006).

Sarawak is a rich biodiversity centre, with diverse types of rice that rang from commercial high yielding varieties to indigenous traditional cultivars. However, genetic studies on local rice cultivars in Sarawak were only initiated in 2006, in which three Bario varieties were studied using 50 microsatellite markers for the purpose of establishing Bario certification scheme (Tan *et al*., 2006). More recently, an unpublished report on genotyping of 25 Sarawak rice cultivars, using 12 microsatellite markers suggested microsatellite markers, can be useful in cultivar identification and differentiation among some Sarawak rice cultivars (Valentine, 2008).

As Sarawak is rich in rice biodiversity, the hidden potential of the local rice cultivars is yet to be discovered. Nonetheless, the characterization of genetic information on the rice cultivars in Sarawak is still lacking. Therefore, this study was aimed at identifying the microsatellite marker panels so as to differentiate the Sarawak rice cultivars, analyze genetic diversity, and enlarge their genetic base of these rice cultivars.

MATERIALS AND METHODS

Plant Material and DNA Extraction

A total of 53 rice cultivars listed in Table 1 were collected from the areas shown in *Fig. 1* and evaluated in the present study. Rice seeds were sterilized with 5% Clorox solution (v/v) for 10 minutes, rinsed with distilled water and germinated in Petri-dishes in the laboratory. The germinated seeds, with approximately 1 cm root length, were transplanted into pots and maintained for 8 months. The leaf samples, about 4-5 cm in length from individual rice plants of each cultivar, were collected and immediately frozen in –80 °C. Thirty milligrams samples of each cultivar were weighed, and the DNA extraction was conducted according to the protocol by GF-1 Plant DNA Extraction Kit (Catalog No. GF-PT-50; Vivantis Technologies).

Selection of the Microsatellite Markers

Fifty-four published microsatellite markers from the regional research papers were selected based on the degree of the polymorphism shown. The polymorphic information content (PIC) was compared in order to select potential markers to differentiate the cultivars studied. The details for these markers can be obtained from RiceGenes database (www.gramene.org). The selected primers were synthesized by First BASE Laboratories, Malaysia.

PCR Amplification and Band Detection

The PCR amplification was performed according to the recommended protocol provided by the DNA Amplification Kit (Catalog No. PL1202-

TABLE 1
Rice cultivars

No	Cultivars	Source	Divisions
1	Bajong Wangi	Lubok Nibong (LN)	Betong
2	Biris	DOA Semenggok (D)	Kuching
3	Biris	Stumbin (ST)	Sri Aman
4	Buntal Pulut	Roban (RO)	Betong
5	Kuduk	DOA Semenggok (D)	Kuching
6	Kuning	Betong (B)	Betong
7	MR219	Stumbin (ST)	Sri Aman
8	MR219	Kpg. Bunan, Serian (KBS)	Kuching
9	Rotan	DOA Semenggok (D)	Kuching
10	Rotan	Betong (B)	Betong
11	Sabak	Betong (B)	Betong
12	Suratani	DOA Semenggok (D)	Kuching
13	Tajol	Kpg. Bunan, Serian (KBS)	Kuching
14	Selasih	Tatau (T)	Bintulu
15	Serendah Kuning	UPMKB (U)	Bintulu
16	Bario Halus	UPMKB (U)	Bintulu
17	Adan Sederhana	UPMKB (U)	Bintulu
18	Bukit Wangi	Lubok Nibong (LN)	Betong
19	Buntar-B	DOA Semenggok (D)	Kuching
20	Empawah	Stumbin (ST)	Sri Aman
21	Empawah Merah	DOA Semenggok (D)	Kuching
22	Lasak	DOA Semenggok (D)	Kuching
23	Lemak	Meradong (M)	Sarikei
24	Lemak	Roban (RO)	Betong
25	Mamut	Stumbin (ST)	Sri Aman
26	Mamut-2	Stumbin (ST)	Sri Aman
27	Muyun	Tatau (T)	Bintulu
28	Rotan Wangi	Lubok Nibong (LN)	Betong
29	Sampangan B	DOA Semenggok (D)	Kuching
30	Sia	Lubok Nibong (LN)	Betong
31	Tulang	Tatau (T)	Bintulu
32	Wangi	Stumbin (ST)	Sri Aman
33	Bubuk Wangi	Stumbin (ST)	Sri Aman
34	Silah	Roban (RO)	Betong
35	Silah	Betong (B)	Betong
36	Raden	Meradong (M)	Sarikei
37	Secasan Putih	DOA Semenggok (D)	Kuching
38	Ukong	DOA Semenggok (D)	Kuching
39	ARC-II	DOA Semenggok (D)	Kuching

Table 1 (Continued)

40	Kanowit Merah	DOA Semenggok (D)	Kuching
41	Selimbau Merah	DOA Semenggok (D)	Kuching
42	Selambau Merah	DOA Semenggok (D)	Kuching
43	Lebat	Kpg. Bunan, Serian (KBS)	Kuching
44	Lebat	Lubok Nibong (LN)	Betong
45	Chelum	Betong (B)	Betong
46	Chelum Halus	Stumbin (ST)	Sri Aman
47	Boria	Betong (B)	Betong
48	Hitam	Stumbin (ST)	Sri Aman
49	Palang	Meradong (M)	Sarikei
50	Sebuti	Roban (RO)	Betong
51	Tembakau	Betong (B)	Betong
52	Selasih	Roban (RO)	Betong
53	Kanowit	Tatau (T)	Bintulu

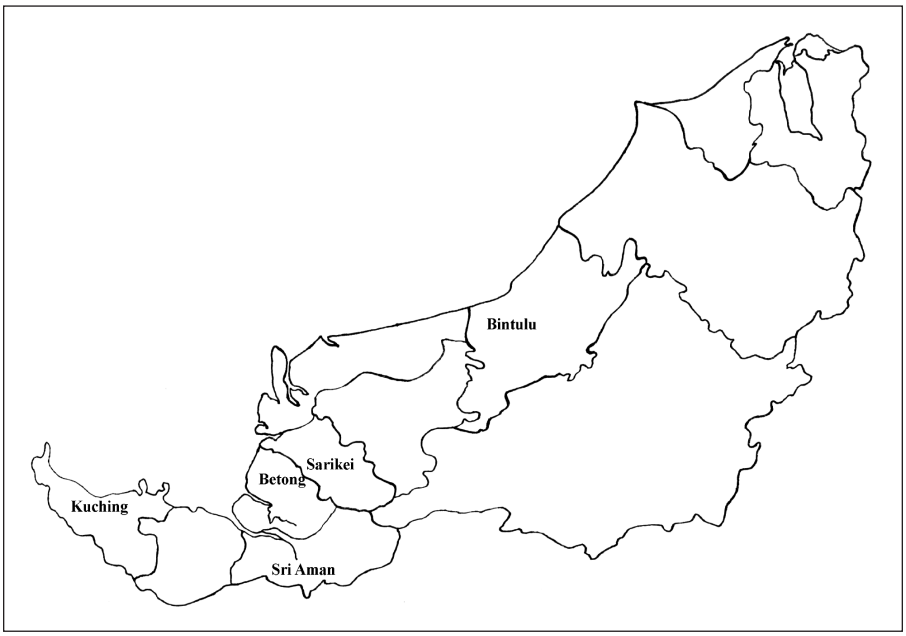

Fig. 1: Sarawak map showing the sampling areas

GF; Vivantis Technologies, Malaysia). An initial screening of the microsatellite markers was carried out in the electrophoresis systems (Model: FB300; Fisher Scientific) using 2.0% of agarose gel (Genetic Analysis Grade; Fisher Scientific) at 100 V for 90 minutes in 0.5x TBE buffer. Later, the PCR-products from the selected microsatellite markers were subjected to band detection using polyacrylamide gel electrophoresis (Mini-PROTEAN 3 Cell; Bio-

Rad Laboratories). Meanwhile, polyacrylamide gel (5%) was prepared according to the procedure described by Sambrook and Russell (2001) and run at 80 V, where the running time was dependent on the size of the PCR products, i.e. from 45 to 60 minutes, for larger products. The gel obtained was stained using ethidium bromide and visualized under UV light (302 nm) using FluorChem 5500 gel imaging system and analyzed by AlphaEaseFC Version 3.3.2 software.

Selection of the Microsatellite Marker Panels

The microsatellite marker panels, with one representative marker from each chromosome, were selected from the initial screening of 54 microsatellite primers. Banding pattern of microsatellite markers was compared to identify polymorphic primers. The microsatellite primers with the highest levels of polymorphism and unambiguous banding patterns were therefore chosen.

Data Analysis

The banding patterns obtained were scored in binary format as present (1) or absent (0) to analyze the genetic relationship between cultivars, as well as the number of alleles per loci to determine the polymorphism information content (PIC) values. The PIC value of each microsatellite marker was calculated using the formula $PIC = 1 - \sum_{j=1}^{n} P_{ij}^2$ where P_{ij} represents frequency of the *j*th allele for the *i*th marker, while n represents the total number of alleles to identify the polymorphic and monomorphic markers. The binary matrix was used to calculate the similarity as DICE coefficient using 'SIMQUAL' sub-routine in NTSYSpc version 2.20r N software package (Rohlf, 2005). The resultant similarity matrix was employed to construct dendrograms using the 'Sequential Agglomerative Hierarchical Nesting' (SAHN) based on the 'Unweighted Pair Group Method with Arithmetic Means' (UPGMA) to infer genetic relationships. Meanwhile, the Mantel matrix correspondence test was carried out using the MXCOMP procedure in NTSYSpc Version 2.20r N to define the degree of congruence in the estimation of genetic relationships by the SSR markers.

RESULTS

SSR Analysis

Fifty-four SSR primer pairs on 12 rice chromosomes were used to genotype the rice cultivars. The results showed that 46 primer pairs provided a clear DNA amplification, while 8 others revealed a low yield PCR amplification which required further optimization of PCR conditions. In more specific, the primers with low yield amplification results were RM514, RM55, RM161, RM334, RM162, RM118, RM171, and RM144. Among the primers used in the initial screening, 36 primer pairs (66.7%) produced polymorphic banding patterns, while the rest of the primers (33.3%) showed monomorphic banding patterns. Examples of these polymorphic banding patterns are shown in *Fig. 2*.

The polymorphic primers identified from 54 primer pairs screened are shown in Table 2. The results obtained show that the majority (69.4%) of the polymorphic primers produced bands which consist of dinucleotide repeat region, while seven primers produced bands having trinucleotide repeats (namely RM452, RM489, RM125, RM152, RM144, RM519, and RM19) and one tetranucleotide (i.e. RM421).

The polymorphic primers, with compound repeat region, were also obtained as shown in RM316, RM228, and RM333. Meanwhile, the large number of the monomorphic amplification products obtained during the screening was unexpected as the PIC values reported in the previous literature were relatively high.

Microsatellite Marker Panel

A total of 43 alleles were amplified from 53 cultivars studied, ranging from 2 to 5 alleles per locus with average of 3.58 alleles per locus (Table 3). The microsatellite panel chosen

TABLE 2
Microsatellite markers for the initial screening of the genetic relationship of 53 Sarawak rice cultivars based on the agrarose gel electrophoresis

Primers	Chromosome	Position	Repeat motifs	Expected product size (bp)	Polymorphism
RM1	1	29.7	$(AG)_{26}$	113	Polymorphic
RM243	1	57.3	$(CT)_{18}$	116	Polymorphic
RM237	1	115.2	$(CT)_{18}$	130	Polymorphic
RM226	1	154.8	$(AT)_{38}$	274	Monomorphic
RM154	2	4.8	$(GA)_{21}$	183	Polymorphic
RM452	2	58.4	$(GTC)_{9}$	209	Polymorphic
RM240	2	158.0	$(CT)_{21}$	132	Polymorphic
RM207	2	191.2	$(CT)_{25}$	118	Polymorphic
RM22	3	13.0	$(GA)_{22}$	194	Polymorphic
RM338	3	108.4	$(CTT)_{6}$	183	Monomorphic
RM55	3	168.2	$(GA)_{17}$	226	Monomorphic
RM514	3	216.4	$(AC)_{12}$	259	Polymorphic
RM489	3	29.2	$(ATA)_{8}$	271	Polymorphic
RM81B	3	77.5	$(TCT)_{10}$	110	Monomorphic
RM307	4	0	$(AT)_{14}(GT)_{21}$	174	Monomorphic
RM335	4	21.5	$(CTT)_{25}$	104	Monomorphic
RM252	4	98.0	$(CT)_{19}$	216	Polymorphic
RM124	4	150.1	$(TC)_{10}$	271	Monomorphic
RM413	5	26.7	(AG)11	79	Polymorphic
RM161	5	96.9	(AG)20	187	Polymorphic
RM421	5	111.2	(AGAT)6	234	Polymorphic
RM334	5	141.8	(CTT)20	182	Monomorphic
RM133	6	0	(CT)8	230	Monomorphic
RM204	6	25.1	(CT)44	169	Polymorphic
RM541	6	75.5	(TC)16	158	Polymorphic
RM162	6	108.3	(AC)20	229	Polymorphic
RM125	7	24.8	(GCT)8	127	Polymorphic
RM11	7	47.0	$(GA)_{17}$	140	Polymorphic
RM118	7	96.9	$(GA)_{8}$	156	Monomorphic
RM138	7	196.8	$(GT)_{14}$	233	Polymorphic
RM152	8	9.4	$(GGC)_{10}$	151	Polymorphic
RM404	8	60.9	$(GA)_{33}$	236	Polymorphic
RM284	8	83.7	$(GA)_{8}$	241	Monomorphic
RM149	8	103.7	$(AT)_{10}$	253	Polymorphic
RM316	9	1.8	$(GT)_{8}$-$(TG)_{9}$ $(TTTG)_{4}$-$(TG)_{4}$	192	Polymorphic
RM105	9	32.1	$(CCT)_{6}$	134	Monomorphic

Table 2 (Continued)

RM278	9	77.5	$(GA)_{17}$	141	Polymorphic
RM215	9	99.4	$(CT)_{16}$	148	Polymorphic
RM474	10	0.0	$(AT)_{13}$	252	Monomorphic
RM271	10	59.4	$(GA)_{15}$	101	Polymorphic
RM171	10	92.8	$(GATG)_5$	328	Monomorphic
RM484	10	97.3	$(AT)_9$	299	Monomorphic
RM216	10	17.6	$(CT)_{18}$	146	Polymorphic
RM228	10	130.3	$(CA)_6(GA)_{36}$	130	Polymorphic
RM333	10	110.4	$(TAT)_{19}$-$(CTT)_{19}$	191	Polymorphic
RM202	11	54.0	$(CT)_{30}$	189	Polymorphic
RM287	11	68.6	$(GA)_{21}$	118	Polymorphic
RM206	11	102.9	$(CT)_{21}$	147	Polymorphic
RM144	11	123.2	$(ATT)_{11}$	237	Polymorphic
RM19	12	20.9	$(ATC)_{10}$	226	Polymorphic
RM277	12	57.2	$(GA)_{11}$	124	Monomorphic
RM463	12	75.5	$(TTAT)_5$	192	Monomorphic
RM519	12	62.6	$(AAG)_8$	122	Polymorphic
RM235	12	101.8	$(CT)_{24}$	124	Monomorphic

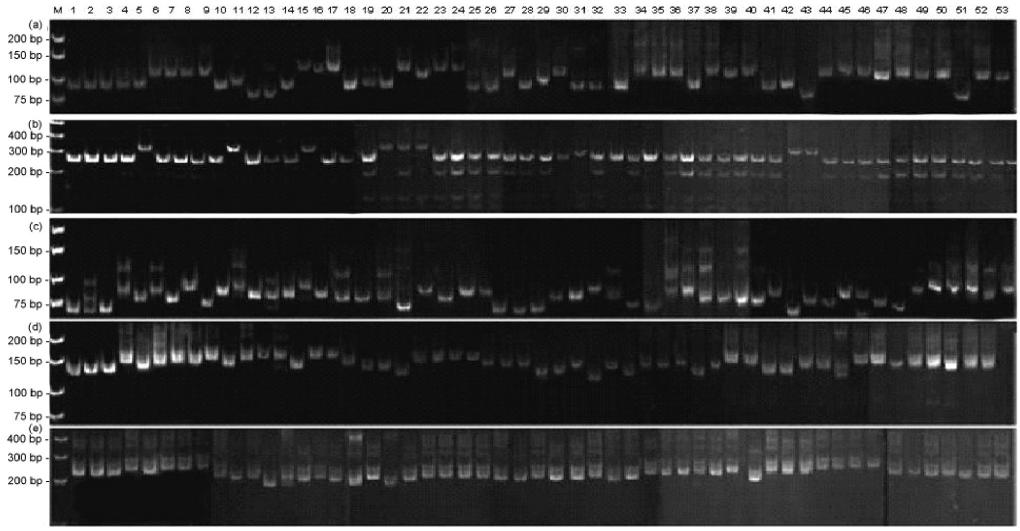

Fig. 2: The microsatellite banding patterns among 53 Sarawak rice cultivars shown by (a) RM1, (b) RM489, (c) RM413, (d) RM11 and (e) RM404 using polyacrylamide gel electrophoresis (PAGE)

showed the PIC values ranging from 0.306 (RM489) to 0.730 (RM413). The average PIC value obtained for the microsatellite panels in this study was 0.622. However, a combination of 12 primers, as the microsatellite marker panels described above, gave an overall PIC value of 0.969, indicating a high polymorphism for cultivar differentiation. Rare alleles were obtained from two markers, namely RM1 and RM404. A rare allele in RM1, as detected with an estimated size of 105 bp, was observed in Sabak and Sampangan B, while a rare allele in RM404 was detected with estimated size of 210 bp, as observed in Suratami and Tajol. Further details on the microsatellite marker panels chosen are shown in Table 3.

Genetic Relationship among Sarawak Rice Cultivars

The UPGMA cluster analysis, based on the genetic similarity values for the SSR alleles, was constructed. The correlation (r) between the similarity index and the cophenetic value was estimated at 0.789, indicating a high level of congruence. The UPGMA dendrogram constructed showed two main groups among the cultivars studied, where 39.62% of the cultivars studied were grouped in cluster A and the remaining 60.38 % were grouped in cluster B (*Fig. 3*).

Cluster A, with the similarity values ranging from 0.3356 to 1.000, consisted of two sub-clusters and one individual cultivar. The cultivar Chelum (B) diverged individually from the other cultivars at 0.3356, and this was followed by the branching into two sub-clusters at 0.4261. The two sub-clusters formed include eight cultivars, which were clustered together in sub-cluster I, while another twelve cultivars fall within sub-cluster II. Bajong Wangi (Lubok Nibong) and Biris (Stumbin) were closely related with a similarity coefficient of 1.00. Two Biris cultivars from different sources (DOA Sarawak and Stumbin) and Mamut and Mamut 2 cultivars were found to be slightly different with a similarity coefficient of 0.750.

TABLE 3
A summary of the information on the microsatellite marker panel chosen based on the polyacrylamide gel electrophoresis (PAGE)

Chromosome	Primers	Annealing temperature (°C)	Estimated alleles range (bp)	No. of multiple allele genotype	No. of rare allele	No. of alleles amplified	PIC
1	RM1	55	85-120	0	1	5	0.689
2	RM240	55	115-135	1	0	2	0.456
3	RM489	55	190-300	0	0	2	0.306
4	RM252	55	120-260	0	0	4	0.639
5	RM413	55	65-85	1	0	4	0.730
6	RM204	55	110-150	2	0	4	0.717
7	RM11	55	130-150	1	0	3	0.645
8	RM404	55	210-230	0	1	5	0.692
9	RM316	55	160-200	3	0	3	0.612
10	RM271	55	90-110	0	0	4	0.726
11	RM206	55	130-170	2	0	4	0.674
12	RM19	55	220-255	2	0	3	0.579
					Total:	43	7.465
					Average:	3.58	0.622

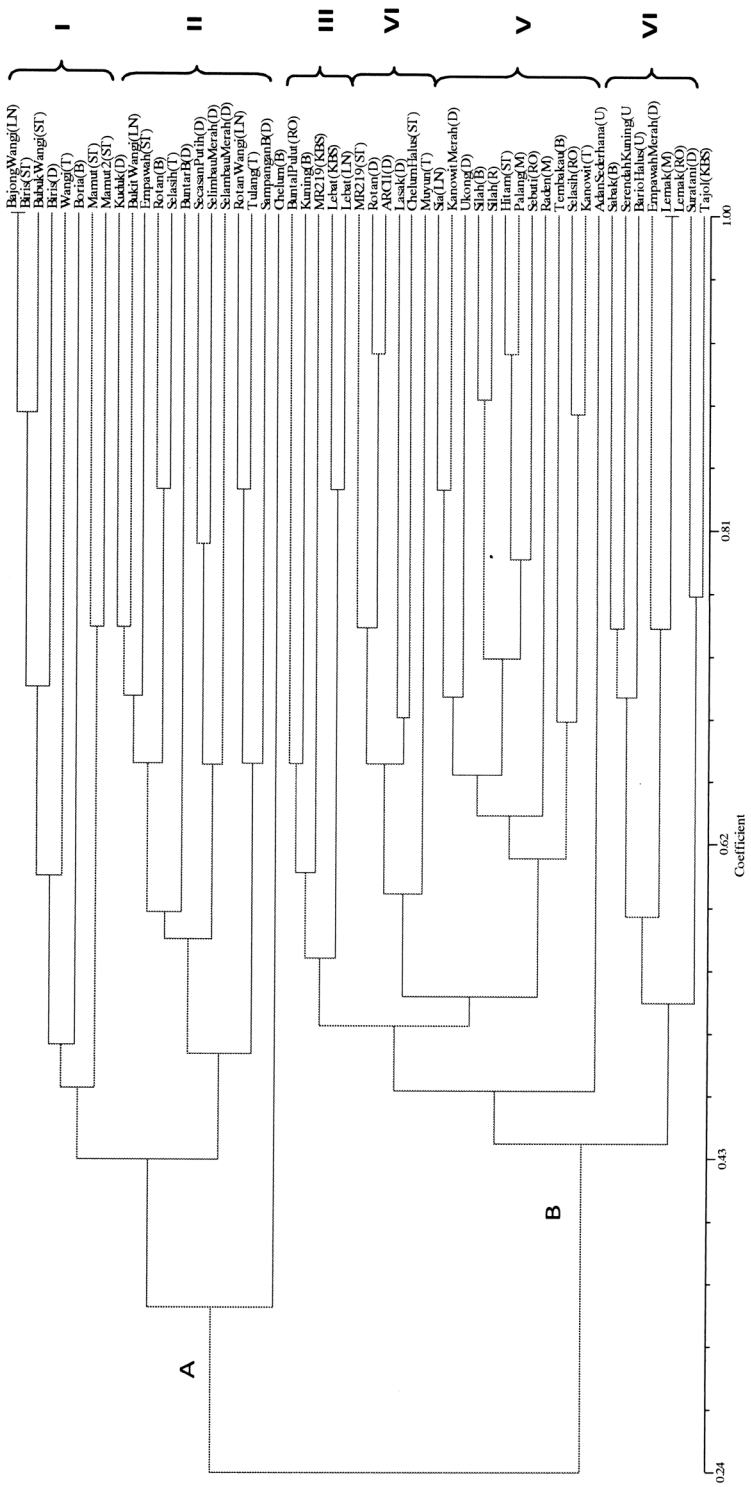

Fig. 3: Dendrogram showing genetic relationships among 53 Sarawak rice cultivars revealed by the cluster analysis based on 12 representative microsatellite markers (UPGMA, NTSYS-PC)

Cluster B contained four main clusters and one individual cultivar. Five cultivars were clustered in sub-cluster III, six cultivars in sub-cluster IV, twelve cultivars in sub-cluster V, followed by one individual cultivar (i.e. *Adan Sederhana*), and finally sub-cluster VI which consisted of eight cultivars. The cultivars with similar names that were found to be closely related were *Lebat, Silah* and *Lemak*, while MR219 and *Rotan* from the two different sources were shown to be different in terms of their genotypes.

DISCUSSION

SSR Analysis

The polymorphic markers obtained did not show any equivalent polymorphism information content as revealed in the previous literature. As for marker RM1, the present study showed a higher PIC value of 0.702 compared to 0.620 reported by Garland *et al.* (1999). Meanwhile, RM19 was reported to be polymorphic with the PIC value of 0.820 (Thomson *et al.*, 2007) but the PIC value of 0.678 was recorded in the present study. Meanwhile, the results for the monomorphic primer pairs were unexpected when compared to the published literature, which showed a relatively high polymorphism. For example, RM226, RM338, RM421, and RM 235 appeared to be monomorphic compared to 5, 6, 6, and 7 amplified alleles, as reported by Sundaram *et al.* (2008). Besides, RM335, RM215, and RM474 have been used by different groups of researchers and appeared to be monomorphic as opposed to studies by Ravi *et al.* (2003), Saini *et al*. (2004), Yuan *et al.* (2007), Sundaram *et al.* (2008), Thomson *et al.* (2007), Tu *et al.* (2007), and Lapitan *et al.* (2007). Generally, the PIC values reported in the literature were above 0.70, indicating high polymorphisms.

The difference in polymorphism shown above could probably be due to the different rice cultivars, as well as the number of cultivars screened. The large numbers of cultivars screened would increase the number of the alleles amplified and contribute to the PIC values. Furthermore, the diversity of the cultivars studied might affect the PIC value that serves as the scale of the polymorphism content. In the study conducted by Tu *et al.* (2007), their sample size covered 60 rice varieties from Yunnan province, with diverse geographical regions. Meanwhile, Coburn *et al.* (2002) reported the samples that covered two subspecies of rice, namely *Oryza sativa* ssp. *Indica* and *Japonica,* which might contribute to the PIC values of the markers as there were obvious variations in the varieties. Therefore, the relatively low PIC values in individual markers could be explained by the Sarawak cultivars studied being closely related and the small sample size.

The Microsatellite Marker Panels

The 12 selected marker panels in the present study showed an average of 3.58 alleles per locus and the average PIC value of 0.622. As compared to the previous studies (i.e. with the average number of alleles ranging from 4.86 to 13 alleles), the average number of the alleles obtained in this study was relatively low (Ni *et al.*, 2002; Saker *et al.*, 2005; Thomson *et al.*, 2007; Jayamani *et al.*, 2007; Sundaram *et al.*, 2008). The average PIC values obtained was in agreement to the average PIC values ranging from 0.62 to 0.68 by Ni *et al.* (2002), Akkaya and Buyukunal-Bal (2004), Thomson *et al.* (2007) and Jayamani *et al.* (2007), but was lower compared to those by Sundaram *et al.* (2008) who reported an average PIC value of 0.707.

The differences observed were expected as different loci and different rice varieties were used in the study. Meanwhile, the different primers used for the different loci screening contributed to the variation of the average number of alleles, as shown by Sundaram *et al.* (2008) who studied 35 rice genotypes with 25 microsatellite markers, Thomson *et al.* (2007) who studied 330 rice accessions with 30 microsatellite markers, and Ni *et al.* (2002) who investigated 38 cultivars with 111 microsatellite markers. As previously reported, a relatively higher number of loci screened and different primers used did contribute to the variation.

Although a relatively smaller sample size and fewer primers were used in the present study as compared to the previous studies, the selected markers were able to show an equivalent polymorphism content as reported in the earlier studies.

The microsatellite panel chosen consisted of perfect repeat motifs of dinucleotides and trinucleotides, except for RM316 which possessed a compound repeat motif. As a direct correlation was often observed between the number of perfect repeats and the level of polymorphism exhibited by PCR amplification, the marker panels chosen were observed to possess higher number of repeats compared to the other primers at the initial screening. This was also observed by Saghai-Maroof *et al.* (1994) and Russell *et al.* (1997) in their studies on barley genetics. For example, RM204 with the repeat motif of $(CT)_{44}$ having the highest number of perfect repeats, was more polymorphic compared to RM133 with the repeat motif of $(CT)_8$, RM541 with the repeat motif of $(TC)_{16}$ or RM162 with the repeat motif of $(AC)_{20}$ on chromosome 6. However, not all the loci in the selected microsatellite marker panel possessed the highest number of perfect repeats, while some primers required further optimization of PCR conditions.

The polymorphic primers producing clear amplification bands were selected to construct a microsatellite marker panel for use in further analysis. The number of alleles amplified was indirectly related to the polymorphism level of the marker. Luan *et al.* (2007) suggested that an effective allele number be assessed to estimate the level of genetic variation and select the markers with a relatively high number of alleles amplified. Besides, large allele size differences, which facilitate detection of alleles on gel, are necessary to obtain reproducible polymorphic banding patterns (Akkaya and Buyukunal-Bal, 2004). On the contrary, the primers that gave low intensities of amplification products and complicated banding patterns with stutter or contaminating bands were avoided. Optimization is required for these primers but it is rather laborious as alterations in the PCR conditions may only reduce but not necessarily eliminate the problems (Dograr and Akkaya, 2001; Akkaya and Buyukunal-Bal, 2004; Olejniczak and Krzyzosiak, 2006).

Genetic Relationships among the Sarawak Rice Cultivars

The clustering of the cultivars showed Mantel correspondence coefficient of 0.79 compared to Ravi *et al.* (2003) and Jayamani *et al.* (2007) who reported the values of 0.53 and 0.87, respectively. The findings of the present study showed that the information from the SSR analysis with the selected microsatellite panels was well conserved at 79% in this cluster analysis. Meanwhile, some cultivars with similar names but from different sources were found to be genetically different as shown by the cultivars *Rotan* and *MR219*. This might be attributed to geographic conditions or local traditional practices. As noted by Bajracharya *et al.* (2006), landraces displayed variability for the trait they gained their name from, but farmers did not maintain strict standards of landrace purity. Meanwhile, according to Brondani *et al.* (2006), any variety could, after years of successive cultivation, generate populations with a genetic constitution different from the original genotype due to adaptations to different environments. Variations in the geographic and climate conditions, diverse farming practices and diversified utilization of rice may also result in the loss of identity of the varieties grown (Tu *et al.*, 2007).

CONCLUSIONS

In the present study, a microsatellite marker panel with 12 polymorphic markers was identified, producing an overall PIC value of 0.969. The allelic diversity, determined by the microsatellite marker panel, had enabled the detection of differences among the Sarawak rice cultivars studied. However, the microsatellite marker panel could still be improved upon through further screening of polymorphic markers to enhance its discrimination power. The results

obtained in this study have provided information for the future development of a genetic database of the Sarawak rice cultivars.

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Short Communications

The Effects of Oral Vaccination of *Streptococcus agalactiae* on Stimulating Gut-associated Lymphoid Tissues (GALTs) in Tilapia (*Oreochromis* spp.)

M. Firdaus-Nawi[1], O. Noraini[1], M.Y. Sabri[1*], A. Siti-Zahrah[2], M. Zamri-Saad[1] and H. Latifah[1]

[1]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

[2]National Fish Health Research Centre, 11960 Batu Maung, Penang, Malaysia

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

ABSTRACT

Vaccination of fish by intraperitoneal (i.p.) injection and bath immersion against bacterial infections has been proven to be a commercial success. However, those routes of vaccination are not economical in practice due to several reasons such as high labour cost, highly time consuming, and causing stress to the fish. Meanwhile, oral vaccination is considered as the best route to vaccinate the fish due to less stress to the fish, ability to treat large batch at one time, and easy and practical to administer booster vaccination. In this study, effect of oral vaccination with various regimes in stimulating gut-associated lymphoid tissues (GALTs) against *Streptococcus agalactiae* infection was observed. In this vaccination experiments, four groups of fish with four replicates consisting of 15 tilapias each were used; four groups per treatment received antigen incorporated vaccine in different regimes. Group 1 was fed with vaccine once per week, Group 2 was fed three consecutive days per week, and Group 3 was fed five consecutive days per week, while Group 4 (control) was fed with standard commercial feed. Booster dose was administered at day-14 after the first administration, and humanely killed at day-28 post-booster vaccination. Ten fish from each group were collected for gut sampling and subjected for histological analysis using Olympus FIVE Image Analyzer. Aggregations of GALTs were observed in lamina propria of the gut. The sizes of GALTs were measured and the numbers of lymphoid cells were also counted. The diameter of GALTs showed no significant ($p>0.05$) difference between Groups 1 to Group 2 and Group 2 to Group 3, but a significant difference ($p<0.05$) was observed between Groups 1 and 3. In terms of the numbers of lymphoid cells, no significant differences ($p>0.05$) were found between Group 1 to Group 2 and Group 2 to Group 3; however, a significant difference ($p<0.05$) was observed between Groups 1 and Group 3. As a conclusion, the frequencies of administration play a role in stimulating the size of GALT which is correlated with the number of aggregated lymphoid cells in the gastrointestinal tract of tilapia.

Keywords: *Streptococcus agalactiae,* oral, gut associated lymphoid tissue (GALT)

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[*]Corresponding Author

INTRODUCTION

Among fresh water fish, tilapia is the most cultured food fish in the world (Anon, 2004). Wide aquaculture systems are used in cultivating tilapia, either in ponds or tanks or cage-cultured. However, tilapia has been found to be susceptible to bacterial disease, particularly streptococcal disease. Moreover, the infection is usually transmitted from fish to fish, in which the bacteria is released from dead and dying fish that is considered as the most important source of infection (Kitao, 1993).

Teleost fish also possesses primary and secondary lymphoid organs; however, there are major structural and morphological differences between fish and mammalian immune systems (Salinas *et al*., 2007). The thymus is divided into a cortex and medulla that are composed of epithelial cell and thymocytes. Kidney is a major lymphoid organ and the foremost part lacks excretory tissue, frequently referred to as the head kidney (Press and Evensen, 1999). The head kidney is a predominantly lympho-myeloid compartment, which is also an important haematopoietic organ (Fange, 1986), and it has morphological similarity with bone marrow in higher vertebrates (Meseguer *et al*., 1995). The mucosa-associated lymphoid tissues (MALT) in teleost include skin, gills and gut-associated lymphoid tissues (GALT) (Salinas *et al*., 2007). Their layer of mucus and an array of non-specific immune defences are exposed to the external environment and form initial barrier to invasion by pathogens (Dalmo *et al*., 1997). In teleosts, GALT is obtained as individual leukocytes or as a lymphoid accumulation, including macrophages, lymphocytes, granulocytes, and plasma cells (Nakamura *et al*., 2000). Intra-epithelial leukocytes are present in the gut but most leucocytes are found in the lamina propria of the gut folds and luminal to the stratum compactum (McMillan and Secombes, 1997). Endo *et al*. (2002) found that self-fed tilapia had significantly lower blood cortisol, higher antibody production and a higher number of blood lymphocytes. Allison *et al*. (1979) found that sinking pellets were better utilized than unpelleted feed by blue tilapia. This report describes the stimulation of the GALT following oral exposures to killed whole-cell *Streptococcus agalactiae* that was incorporated into the feed.

MATERIALS AND METHODS

Fish Samples

A total of 240 tilapias (*Oreochromis* spp.) were selected from Aquaculture Extension Centre (AEC) in Jitra, Kedah, before they were transferred and conditioned at the National Fish Health Research Centre (NaFisH), Batu Maung, Penang. The mean weight was 150±10 g. Prior to experimentation, all the fish were screened for *S. agalactiae* to ensure that they were free of streptococcosis.

The tilapias were randomly assigned to sixteen 200-L tanks. Light cycle was held constantly at 12 h light per day. Feeding was *ad libitum* with local commercial feed. The water was continuously aerated while its temperature was checked on a daily basis. The water temperature, pH and dissolved oxygen were measured using HQ40d Meter (Hach Company, Loveland, CO). Meanwhile, ammonia, sulphate and nitrites were determined daily using a DR 2800 Portable Spectrophotometer (Hach Company, Loveland, CO).

Preparation of the Antigen

S. agalactiae, isolated from outbreaks of streptococcosis in tilapia, was cultured on blood agar at 37°C before the colonies were further sub-cultured into the BBL Brain Heart Infusion broth (BHIB; BD, USA) and incubated in a shaker incubator at 37°C for 18 h. Following the incubation, the bacterial concentrations were determined using the standard plate count technique. The bacteria were then killed by introducing 0.5% buffered formalin and incubated overnight at 4°C. After that, the bacteria were washed five times with Phosphate-Buffered Saline (PBS). Finally, the formalin-killed bacteria (FKB) were added homogenously into the feed mixture prior to pellet preparation.

Experimental Design

At the start of the experiment, the tilapias of Group 1 were fed with the FKB-incorporated feed at the rate of one day per week, whereas Group 2 was fed three consecutive days per week, Group 3 was fed five consecutive days per week and Group 4 served as the control that was fed with normal commercial pellet. Booster doses were applied two weeks after the primary oral exposure for the respective groups.

All the fish from each group were killed on day-28 post-booster vaccination before the entire gut was separated and fixed into 10% buffered formalin. The portion of the gut, that was located at 10 cm from the stomach, was sampled, routinely processed and stained with haematoxylin-eosin (HE) for histological examination.

Serology

The ELISA was performed as previously described by Shelby *et al*. (2001) and Grabowski *et al*. (2004) with slight modification by analyzing the mucus immunoglobulin M (IgM). The mucus was collected at weeks 0, 1, 2, 3, 4, 5, and 6 from all the fish. The body mucus was collected from anaesthetized fish via swabbing on one side of the fish 10 times from head to tail with sterile cotton bud and placed in micro-centrifuge tubes containing 0.9 mL of PBS supplemented with 0.02% (w/v) sodium azide. Briefly, mucus IgM levels were detected using 100 L of goat anti-tilapia immunoglobulin serum, and diluted at 1: 5000. Then, 100 L of conjugated rabbit anti-goat IgM-horseradish peroxidase (Nordic, the Netherlands), which were diluted at 1: 5000, were added. After the final three-wash step with PBST, bound conjugate was detected using 100 L of TMB One Solution substrate (Promega, USA), before stop reaction with 0.2 mol/L sulphuric acid, and the plates were read at 450 nm wavelengths (Anthos Zenyth 340st, Austria).

GALT Determination

The selected tissue samples were embedded in paraffin and sectioned at 4 m for slide preparation. Sections were allowed to dry overnight at 40°C. All the samples were processed and subjected to haematoxylin and eosin (HE) staining method. A total of 10 microscopic fields were examined for the presence of GALT. Once identified, the size of GALT and the number of lymphoid cells in each GALT were determined using FIVE Image Analyzer (Olympus, Japan).

Statistical Analysis

The relationship between the size and number of GALTs data were analyzed using a single linear regression (SLR). In highlight the significance of the results, the one-way analysis of variance was employed further using Tukey HSD in Statistix 9 (Analytical Software, USA). The results were considered as significant at $p<0.05$.

RESULTS AND DISCUSSION

The water quality parameters (mean±SD) were 4.97±0.3 mg/L dissolved oxygen, 32.6±0.8°C, 7.47±0.1 pH, 2.37 mg/L ammonia and 0.023 mg/L nitrate concentrations. These parameters were within the normal range of water quality (El-Sayed, 2006). All the guts of tilapias, exposed to oral vaccination, showed the presence of aggregations of lymphoid cells both within the epithelium and the lamina propria. Nonetheless, there was no observation of lymphoid cell aggregation in the gut of tilapias of the unexposed Group 4.

Table 1 shows the average size of GALT and the average number of lymphoid cells in the GALT of tilapias exposed to the killed *S. agalactiae* incorporated in feed. The GALT was observed in the exposed tilapias of Groups 1, 2 and 3 (*Figs. 2, 3,* and *4*), but not in the unexposed tilapias of Group 4 (*Fig. 5*). All the vaccinated groups were found to be significantly different when compared to the unexposed tilapias of Group 4. In the vaccinated tilapia, no significant difference ($p>0.05$) was observed in the size of

TABLE 1
Average size of the GALT and the number of lymphoid cells in tilapias, following different frequencies of oral exposures to killed *S. agalactiae*

Group	GALT	
	Diameter (µm)	No. of lymphoid cells
1	112.6±0.05[a]	458±5[x]
2	140.0±0.5[a]	763±5[x]
3	205.0±0.5[b]	1098±5[y]
4	0[c]	0[z]

[a,b,c,x,y,z] Values with different superscripts are significantly (p<0.05) different within the column.
Group 1: orally exposed once in a week.
Group 2: orally exposed for three continuously days.
Group 3: orally for five continuously days.
Group 4: unexposed control.

GALT between Groups 1 and 2, but significantly (p<0.05) larger size was observed in Group 3 when it was compared to Groups 1 and 2.

Similarly, in vaccinated tilapias, there were no significant differences (p>0.05) in the number of lymphoid cells between Groups 1 and 2, but (p<0.05) more numbers of lymphoid cells were significantly observed in Group 3 than Groups 1 and 2. Therefore, the frequency of antigen administration seemed to play an important role in the stimulation the size of GALT and the number of lymphoid cells within the GALT.

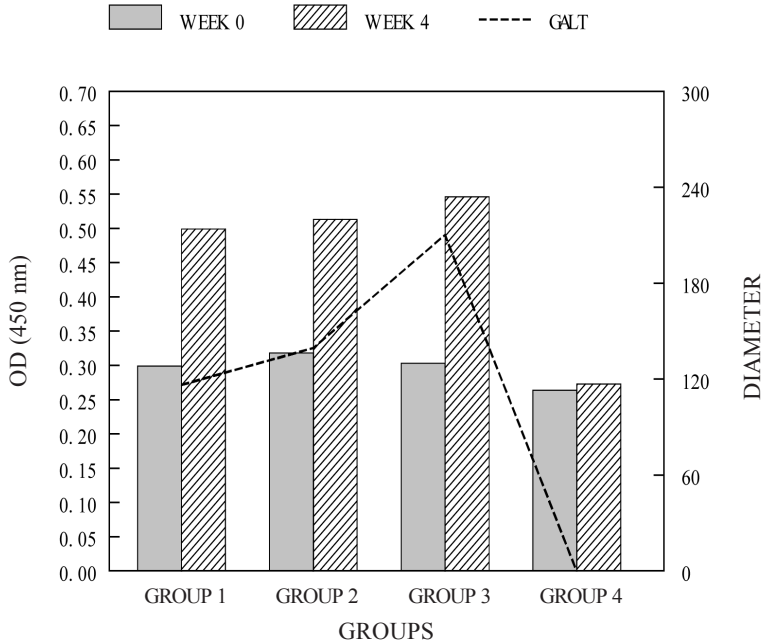

Fig. 1: Mucus antibody responses correlated to GALTs diameter in the exposed and unexposed tilapias

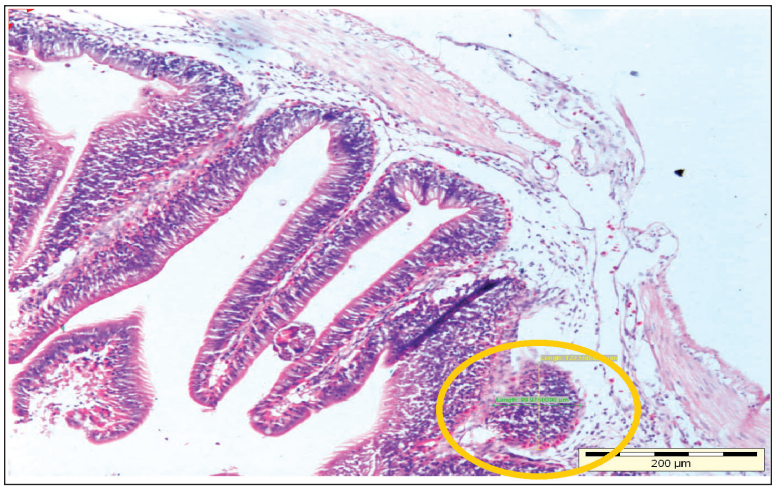

Fig. 2: A cross-section of the gut of fish from Group 1. The aggregation of lymphoid cells or GALT formed in the lamina propria is marked in yellow circle (HE × 200)

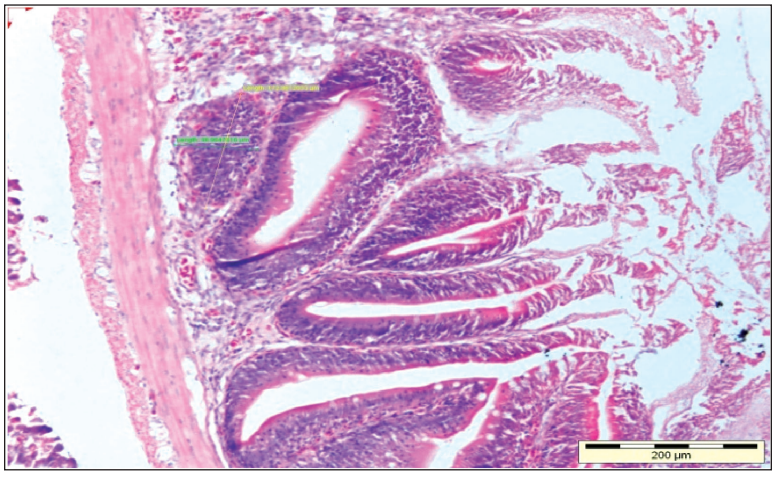

Fig. 3: A cross-section of the gut of fish from Group 2. The aggregation of lymphoid cells or GALT formed in the lamina propria is marked in yellow circle (HE × 200)

Gut is one of the organs of the mucosal immune system organ and it is the site where there is only a thin barrier between internal and external milieu (MacDonald and Miller, 2005). Consequently, this makes gut very sensitive to foreign antigens such as bacteria and reacted by stimulating the GALT by producing antigen-specific antibodies. The GALT consists of leukocytes, macrophages, lymphocytes, granulocytes, and plasma cells (Nakamura *et al*., 2000). Previous studies carried out on GALT indicate its importance to the function of the local immune system (Rombout *et al*., 1986). Meanwhile, an administration of antigens into the gut can lead to increasing of the numbers of intraepithelial leukocytes (Davina *et al*., 1982) and induce the production of specific antibodies in the mucosa and bile (Hart *et al*., 1987). *Fig. 1*

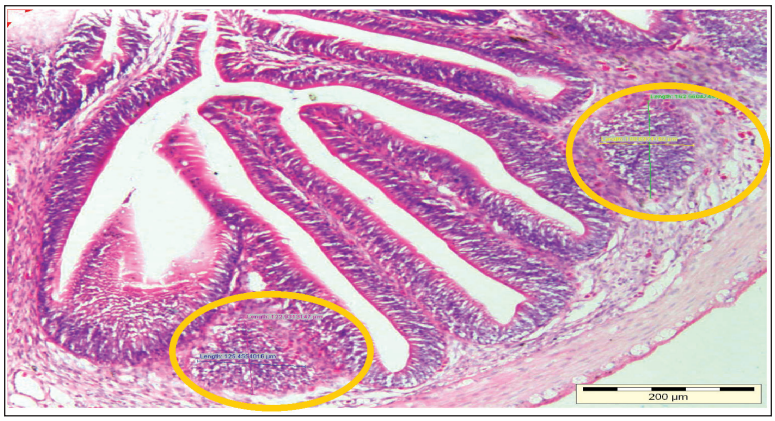

Fig. 4: A cross-section of the gut of fish from Group 3. The aggregation of lymphoid cells or GALT formed in the lamina propria is marked in yellow circles (HE × 200)

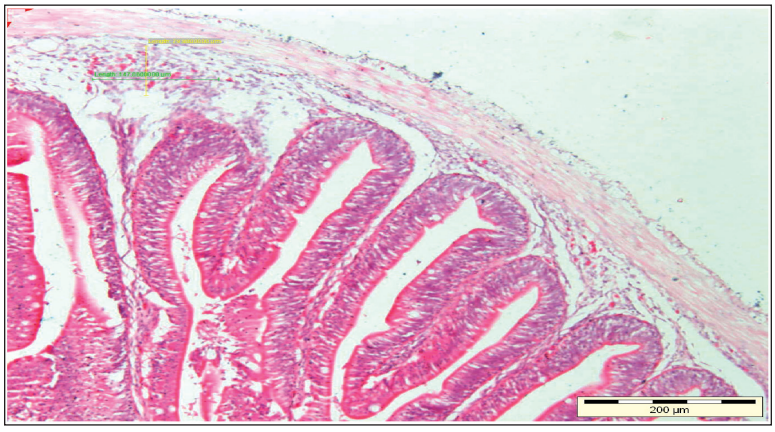

Fig. 5: A cross-section of the gut of fish from Group 4. No aggregation of lymphoid cells or GALT was formed in the lamina propria (HE × 200)

shows the correlation between mucus antibody responses and average size of GALTs in the fish exposed to the killed *S. agalactiae.* The graph shows a linear correlation between the average size of GALT and the mucus antibody responses level, indicating an oral exposure to the killed *S. agalactiae* that was incorporated in the feed could have also stimulated response in skin mucus.

This study has revealed that exposure at the rate of once a week to killed *S. agalactiae* incorporated in feed was sufficient enough to stimulate the GALT and skin mucus antibody responses. However, the data showed that frequent exposures could stimulate better GALT responses, as observed in the tilapias of Groups 2 and 3. This is an early indication that oral exposures to killed antigen that was incorporated in feed may be an alternative vaccination procedure against infection by *S. agalactiae*.

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Pathological Changes in the Organs of Mice Model Inoculated with *Corynebacterium pseudotuberculosis* Organism

Jesse, F.F.A.*, Sang, S.L., Saharee, A.A. and Shahirudin, S.
Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
**E-mail: Faez_Jesse@yahoo.com.my*

ABSTRACT

Corynebacterium pseudotuberculosis is a facultative, gram positive intracellular small club-shaped rod which produces lesions similar to those of tuberculosis. It is known worldwide to cause caseous lymphadenitis (CLA) in sheep and goats. CLA is characterized by the abscess formation in lymph nodes and/or visceral organs. In the recent outbreak of CLA in the TPU farm in UPM, it was reported that the CLA lesions were found in the visceral organs. In the present investigation, attempts were made to study the ethiopathogenesis of CLA in mice models which involved a comparison of clinical signs, haemogram and biochemistry, and histopathological changes in visceral organs between the diseased and non-diseased group. As an overall summary of this project, CLA in mice resulted in clinical signs, such as huddling together, dejection, anorexia, pasty faeces and accompanied by a rapid and shallow respiration pattern. For the haemogram and serum biochemistry profile, it showed a significant difference in the mean value between the diseased group and non-diseased group which include lymphocyte, plasma protein, monocyte, eosinophil, total bilirubin, total protein and potassium. Finally, the most pronounced histopathological changes in the visceral organs were septicaemia, with severe congestion and increased vascularization, together with the presence of capsulated abscess, micro-abscesses formation, infiltration of neutrophils and macrophages, tubercule granulomas, necrosis and early signs of degeneration in majority of the infected mice.

Keywords: ***Corynebactrium pseudotuberculosis,*** **caseous lymphadenitis (CLA), ethiopathogenesis, septicaemia, tubercule granuloma**

INTRODUCTION

In Malaysia, the small ruminant population has been steadily increasing over the past ten years. According to the livestock statistics provided by the Ministry of Agriculture, the total population of sheep and goats in Malaysia was 469,620 heads in 2008 (http://www.moa.gov.my). However, this livestock industry is plagued by diseases, which are the main causes of morbidity and mortality, reducing productivity and incurring losses for farmers. One such important disease is Caseous Lymphadenitis (CLA), which is caused by *Corynebacterium pseudotuberculosis.*

Pseudotuberculosis (formerly known as *c.ovis*) is a facultative, gram positive intracellular small club-shaped rod which produces lesions similar to those of tuberculosis. It is known worldwide to have caused CLA in sheep and goats. *C. pseudotuberculosis* is a very hardy organism that survives well in the environment and can infect a variety of animal species,

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*Corresponding Author

including humans. CLA is characterized by the abscess formation in lymph nodes and/or visceral organs. The affected sheep or goats typically have abscesses in the parotid or retropharyngeal lymph nodes, and the disease can be diagnosed through bacteriological culture of pus from such abscesses. However, a proportion of the infected sheep or goats may have only internal abscesses, often in the lungs or mediastinal lymph nodes and show no overt clinical signs of infection. The recent outbreak of CLA at the TPU farm in UPM reported that the CLA lesions were also found in the visceral organs (Jesse *et al.*, 2008). The disease is distributed worldwide, with cases being reported in Europe, Australia, North and South America, Africa and the Middle East (Dorella *et al.*, 2006). CLA causes economic losses for pedigree sheep/goat breeders and concerns have been raised that the disease may spread to commercial and small holder flocks and lead to a decrease in quality and increased condemnation of carcass at slaughter. In countries with large numbers of sheep, such as Australia, the disease causes considerable financial losses through condemnation and down-grading of affected carcasses at meat inspection (Paton, 1990). Nonetheless, information on the pathogenesis and clinical signs in mice model is scarce.

Therefore, in the present investigation, attempts were done to study the ethiopathogenesis of CLA in mice models which involve comparing the clinical signs, haemogram, and serum biochemistry profile, as well as histopathological changes in visceral organs between the diseased and non-diseased groups.

MATERIALS AND METHODS

Animals

120 apparently healthy mice, about 7 weeks of age, were used in this study. They were kept in the stocking density of 10 mice/cage in an air conditioned room, fed with commercial mice pellets and drinking water, which were freely available for an acclimatization period of 1 week before the beginning of the study.

Bacteria

Blood agar culture made from a lymph node that was naturally infected with caseous lymphadenitis was previously culturally and biochemically identified as *C. pseudotuberculosis*. This was sub-cultured in Brain Heart Infusion (BHI) broth for 24h and concentration were estimated to the standard dose of 1×10^9 CFU/ml using the Mac Farland technique.

Experimental Design

The experiment was carried out in two separate sets (Set A and Set B), in order to study the different objectives. In both the sets, the mice were divided into 2 groups. The diseased group comprised of 30 mice, each of which was injected intraperitoneally with 0.2 ml (approx. 1×10^9 CFU/ml) of the infective inoculums. Meanwhile, the non-diseased group also comprised of 30 mice, served as the control group which was not infected. After the inoculation, clinical signs were observed in both the experimental mice sets. For Set A, 15 post-inoculation, the blood samples were collected and processed for haematology and biochemistry profile. In Set B, an immediate post-mortem examination was performed on mice which died throughout the experiment.

Histopathology

The collected organs were placed in 10% buffered formalin. Paraffin-embedded sections were then routinely stained with haematoxylin-eosin (H&E).

RESULTS AND DISCUSSION

The pictures show the histopathological changes that took place after the *Corynebacterium pseudotuberculosis* organism inoculated after 15 hours.

Meanwhile, the histopathological changes in the diseased group of mice were generally those of septicaemia, in which there were generalised congestion of the organs in all the infected mice. The changes were most

pronounced in the liver and kidney, whereby the presence of tuberculous granuloma (caseating tubercule), giant multinucleated cells, infiltration of neutrophils and macrophages, degeneration, vacuolation (necrosis), haemorrhage, and formation of microabscesses, were detected. For the lungs, the most pronounced lesion was congestion and increased vascularisation, whereas for the heart, besides the common finding of congestion, there was one sample from the infected mice that showed the formation of the abscess with signs of calcification and infiltration of macrophages.

The histological findings from the visceral organs of the infected mice in this study are similar to the histopathological changes in the *C. pseudotuberculosis* infected sheep. In addition, there was a formation of caseous abscesses found in the sample of liver and the development of abscesses present in multiple visceral organs. Therefore, the pathogenesis of the lesion could be explained using the sample from the study carried out on the sheep. In CLA, once a lymph node has been colonized by *C. pseudotuberculosis,* it will undergo a short period of generalized inflammation. Phospholipase D, the soluble exotoxin produced by *C. pseudotuberculosis*, is the probable initiator of this lymphadenitis. Pepin *et al*. (1991) reported that within 24h of the subcutaneous inoculation of lambs, a

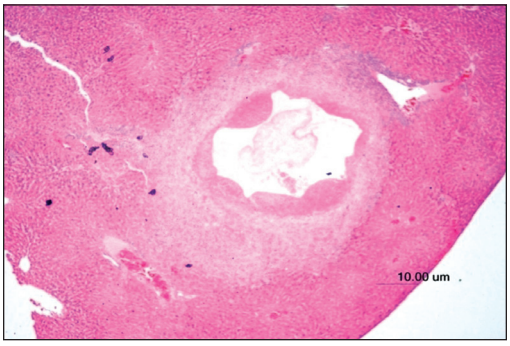

Fig. 1: Liver. Presence of capsulated abscess. Several distinct concentric layers are discernible within the lesion. Several distinct concentric layers are discernible within the lesion. Centrally, there is liquefactive necrosis (liquid pus; A). (HE, x40)

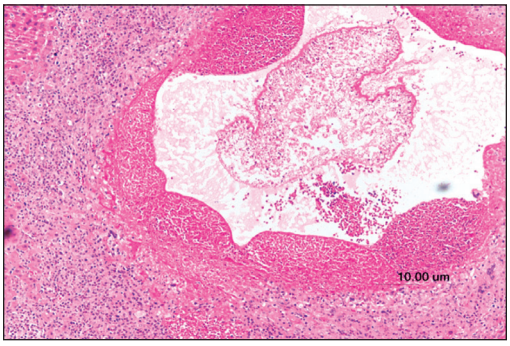

Fig. 2: Liver. Showing the border between the layers of caseous necrosis and active immature fibrosis containing mononuclear inflammatory cells (C). A thick layer of mature fibrosis (B) delineates the extent of the lesion (HE, x100)

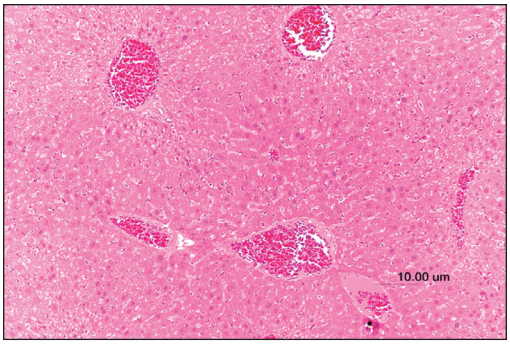

Fig. 3: Liver. Markedly congested (A) and vacuolation indicating necrosis (B) (HE, x40)

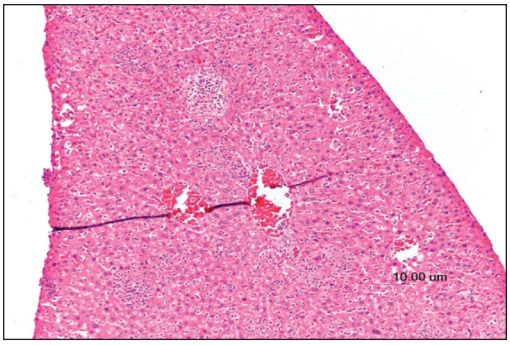

Fig.4: Liver. Micro abscesses formation (A); congestion (B) (HE, x40)

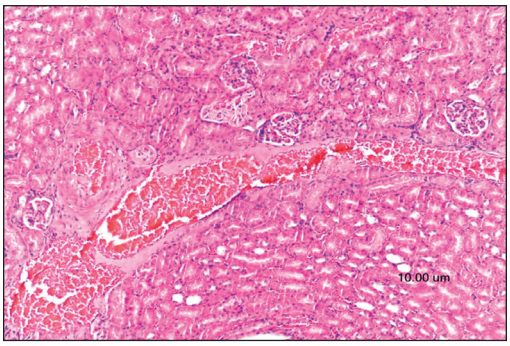

Fig. 5: Kidney. Congestion (A), intertubular hemorrhage (B) and signs of early degeneration (C). (HE, x40)

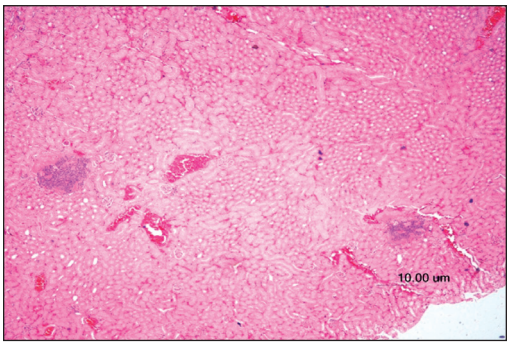

Fig. 6: Kidney. Severe intertubular hemorrhage (A) and early stage of abscess formation (B) (HE, x40)

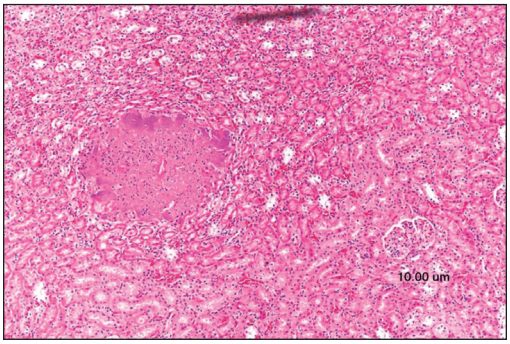

Fig. 7: Kidney. Severe interlobular hemorrhage (A) and tuberculous granuloma (B) (HE, x40)

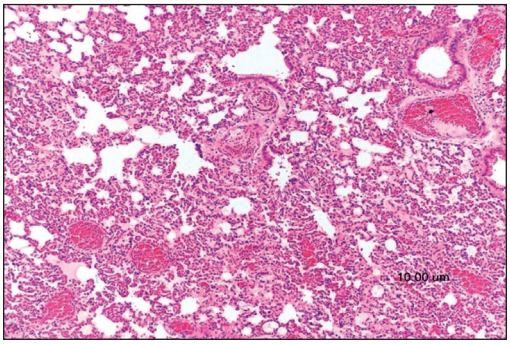

Fig. 8: Lung. Severe congestion (A) and massive influx of inflammatory cells (HE, x40)

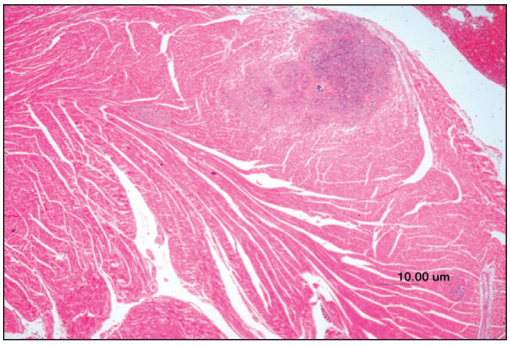

Fig. 9: Heart. Abscess formation with sign of calcification (HE, x40)

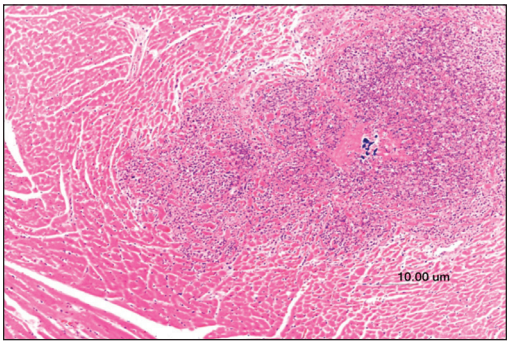

Fig. 10: Heart. Presence of macrophages infiltration and neutrophils (HE, x100)

number of micro abscesses were observed within the cortical region of the lymph node draining the site of inoculation. By day six of post-inoculation, these micro abscesses had become more numerous and began to expand, with coalesce forming larger purulent foci. The early pyogranulomas contained clumps of bacteria and cellular debris. At the same time, and in parallel with the cellular events at the point of entry, the infiltration of neutrophils diminished and monocytes or macrophages became the predominant cell type within the lesion (Pepin *et al*., 1994). A process during which the lesion was encapsulated followed shortly thereafter, leading to a diminution of the inflammatory reaction in the parenchyma of the node. In the early stages, the purulent contents of the abscess were soft and semi-fluid; as time progressed, however, the pus within the lesion took on a more plastic or solid form, in which scattered clumps of bacteria were sometimes noted. Meanwhile, the most pronounced histopathological changes in the visceral organs were septicemia with severe congestion and increased vascularization, together with the presence of capsulated abscess, micro-abscesses formation, infiltration of neutrophils and macrophages, tubercule granulomas, necrosis, and early signs of degeneration in majority of the infected mice.

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Effects of Exercise and Dietary Polyunsaturated Fatty Acid on Blood Lipid Profiles of Streptozotocin-induced Diabetes in Rats

Hasliza, A.H.*, Noordin, M.M. and Goh, Y.M.
Department of Veterinary Preclinical Sciences,
Faculty of Veterinary Medicine, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
**E-mail: haslizaabu@putra.upm.edu.my*

ABSTRACT

The efficacy of exercise and dietary polyunsaturated fatty acid (PUFA) on streptozotocin-induced diabetes in rats was investigated based on blood lipid profiles, with respect to triglycerides, total cholesterol, high-density lipoprotein (HDL)-cholesterol, and low-density lipoprotein (LDL)-cholesterol. A total of 32 Sprague-Dawley male rats were used and divided into eight groups. Four groups were exercised daily for 8 weeks, while the other four were sedentary. Treatment diets were defined as follows: rat chow diet only (Control diet), rat chow added with 2:1, menhaden oil:soybean oil (Diet 1), rat chow added with 1:2, menhaden oil:soybean oil (Diet 2), and rat chow added with 10% (w/w) butter (Diet 3). Blood plasma was collected at the end of 8 weeks for blood lipid profiles determination. The results showed that a combination of exercise and dietary PUFA significantly improved lipid abnormalities by lowering the triglycerides, total cholesterol, and LDL-cholesterol levels.

Keywords: Blood lipids, diabetes, exercise, polyunsaturated fatty acid, rat

INTRODUCTION

Diabetes mellitus is a serious chronic metabolic disorder which is becoming a major global health problem nowadays. It has significant impacts on the health, quality of life and life expectancy of patients, as well as on the health care system (Dey *et al*., 2002). The incidence and prevalence of diabetes are escalating, particularly in developing countries. The risk for diabetes mellitus results from a combination of genetic predisposition and lifestyle changes. The most important lifestyle changes are related to the changes in dietary habits and physical activities (Cockram, 2000).

Diabetic patients showed clinical characteristics, such as hyperglycaemia, elevated low-density lipoprotein (LDL) cholesterol and reduced high-density lipoprotein (HDL) cholesterol and high triglyceride levels. These hyperglycaemia and lipid abnormalities significantly contribute complications of diabetes mellitus, as well as increase cardiovascular risk (O'Keefe and Bell, 2007). Previous studies have shown that diets containing omega-3 and omega-6 may play a role in preventing or delaying the complication of diabetes mellitus. Meanwhile, beneficial effects of polyunsaturated fatty acid (PUFA) supplementation are probably mediated by their lowering blood lipid and preserves pancreas from some later complications of diabetes mellitus (Gvozdjáková *et al*., 2008). There are also studies that have indicated that low fitness increases the risk of diabetes and increased physical activity is effective in

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*Corresponding Author

preventing diabetes (Helmrich *et al*., 1994). The benefits of exercise performed by the diabetic patients include increased insulin sensitivity, improved glycaemic control, weight loss, lower blood pressure, and improved blood lipid profile (Wheeler, 1999). Thus, attention to diet and weight management, combined with physical activity such as exercise, may help to improve glycaemic control and lipid profiles (Wolever *et al*., 1999). This study was carried out to determine the efficacy of exercise and dietary PUFA intervention on blood lipid profiles, with respect to triglycerides, total cholesterol, HDL-cholesterol, and LDL-cholesterol.

MATERIALS AND METHODS

Experimental Animals

Thirty-two, 6 weeks old, male Sprague-Dawley rats with an average weight of between 250-300 grams were used in this study. Two rats were housed in stainless steel mesh cages in an air-conditioned room, with temperature-control of 23°C–25°C and maintained on a 12:12 h of light-dark cycle (Krinke, 2000). All rats were acclimatized for one week and the baseline values were taken as a control group of the normal, non-diabetic rats prior the commencement of the experiment. Standard rat chow pellet and water were provided *ad libitum* throughout the acclimatization period. Diabetes mellitus was induced by giving a single intraperitoneal injection of streptozotocin (STZ) at the dosage of 40 mg STZ/kg body weight. Prior to the injection, STZ was freshly prepared in a 0.10 M citrate buffer solution (pH 5.0). Forty eight hours following the injection, the blood glucose level was determined and rats exhibiting hyperglycaemia (fasting blood glucose level > 13.0 mmol/L) were considered as diabetic and used for the experiment.

Diet and Exercise Regime

The three treatment diets were formulated by adding 10% of specific fat sources, either in mixtures of oil (menhaden oil and soybean oil, omega-3 and omega-6, respectively) or butter only. The treatment groups were defined as follows: rat chow added with 2:1, menhaden oil:soybean oil, with exercise (D1E) and sedentary (D1XE); rat chow added with 1:2, menhaden oil:soybean oil, with exercise (D2E) and sedentary (D2XE); rat chow added with 10% (w/w) butter, with exercise (D3E) and sedentary (D3XE). DCE and DCXE were used as a control for standard diet with exercise and sedentary, respectively. The control diet used in this experiment was the standard rat chow diet. The rats were fed with daily prepared diet at the rate of 0.05 mg/kg of body weight.

Swimming was the exercise regimen used in this study. The swimming protocol was adapted from Molly and Catherine (2002), with a slight modification. In more specific, rats were trained to swim in a pool with a depth of 60 cm. Water and room temperature were maintained at 34°C to 35°C to eliminate cold-induced stress. Before the start of the experiment, rats were acclimatized to swimming for 10 minutes daily for 3 days. Training was accomplished in 8 weeks, starting with 5 minutes of swimming in the first week and this was gradually increased to 30 minutes in weeks 6, 7, and 8. Rats were continuously monitored during swimming so as to prevent drowning. At the end of each daily swimming session, each rat was dried using hair dryer and towel-dried. The sedentary rats remained in their cages during this exercise period.

Biochemical Analysis

Blood was collected at the end of the experiments. Blood plasma were assayed for their total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides levels using a commercial diagnostic kit (Pointe Scientific, Inc, Michigan, USA) and their values were colorimetrically determined in a Roche Cobes Mira Plus chemistry analyzer (Roche Diagnostic Systems, Basel, Switzerland).

Statistical Analysis

In this study, the collected data were analyzed using SPSS version 15.0. The blood lipid

profiles were analyzed using one-way ANOVA. The level of significance was set at 0.05 and only differences of $P<0.05$ were considered to be significant.

RESULTS AND DISCUSSION

The selected blood lipid profiles of triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol of rats at week 8 of the experiments are tabulated in Table 1. The highest level of triglyceride was seen in the D1XE, D2XE, D3XE and DCXE, whereas the rats in D1E, D2E, D3E had the lowest level with a significant higher reduction in triglycerides observed from the D1E group which had received more omega-3 diet and D2E group that had received more omega-6. This finding is similar to that described by Harris (1999) who reported a significant reduction in serum triglycerides by ingestion of omega-3 and omega-6 fatty acids. The possible mechanism for this phenomenon was the reducing effect of dietary omega-3 fatty acids on plasma lipids in plasma triglycerides by 20–50%. Furthermore, the triglycerides-lowering effect has been attributed mostly to a suppressed hepatic lipogenesis and partially to increased β-oxidation (Harris and Bulchandani, 2006).

The lowest ($P<0.05$) cholesterol concentration in the D2E group was similar to that of the DCE group only. The cholesterol concentration in the D3E, D2XE, D3XE, and DCXE groups were the highest ($P<0.05$), whereas the cholesterol concentration of rats in the D1E group was similar to that of the D1XE group. Some of the possible reasons of the lower concentration of the total cholesterol in the exercise group may be attributed to the increased muscular exercise or improvement of cholesterol catabolism. Agte and Tarwadi (2004) suggested a promising potential for exercise as a complementary treatment for patients with diabetes after they had observed a significant reduction by exercise on the total cholesterol and triglycerides. The hypocholesterolemic effect of PUFA-enriched diets on cholesterol levels (Sirtori and Galli, 2002) could also be due to the capability of dietary PUFA to attenuate hyperlipidaemia and reduced oxidative stress. Furthermore, the dietary PUFA may improve hypercholesterolemia by modifying lipoprotein metabolism, which then enhanced the uptake of LDL-cholesterol by increasing LDL-cholesterol receptors and increasing the LCAT activity which may contribute to the regulation of blood lipids (Khanna *et al*., 2002).

The lowest ($P<0.05$) LDL-cholesterol level seen in the D3XE group was comparable to those of the D1E, D2E, DCE, D1XE, and D2XE groups and the rats from the exercise and dietary PUFA intervention groups showed a significant decrease in the LDL-cholesterol levels after eight week of the study as compared to the control. It

TABLE 1
Blood lipid profiles (mmol/L) in exercise and non-exercise rats during the experimental period (Mean ± SE)

Group	Triglyceride	Cholesterole	LDL-chole	HDL-chole
D1E	0.52 ± 0.09^{a}	1.23 ± 0.12^{b}	0.36 ± 0.06^{ab}	0.84 ± 0.11^{ab}
D2E	0.35 ± 0.05^{a}	0.85 ± 0.19^{a}	0.39 ± 0.14^{ab}	0.57 ± 0.13^{a}
D3E	0.44 ± 0.01^{a}	1.86 ± 0.05^{c}	0.51 ± 0.02^{b}	1.33 ± 0.06^{c}
DCE	0.82 ± 0.06^{b}	0.92 ± 0.03^{ab}	0.37 ± 0.02^{ab}	1.03 ± 0.07^{bc}
D1XE	1.25 ± 0.11^{c}	1.22 ± 0.16^{b}	0.43 ± 0.09^{ab}	0.92 ± 0.06^{b}
D2XE	1.42 ± 0.18^{cd}	1.56 ± 0.09^{c}	0.30 ± 0.04^{ab}	1.06 ± 0.09^{bc}
D3XE	1.58 ± 0.07^{d}	1.78 ± 0.08^{c}	0.28 ± 0.02^{a}	1.14 ± 0.16^{bc}
DCXE	1.41 ± 0.02^{cd}	1.81 ± 0.05^{c}	0.80 ± 0.05^{c}	1.87 ± 0.03^{d}

Note: a, b, c, and d: values between rows bearing the same superscript/s do not differ at $P<0.0$

is well recognized that the hypotriglyceridaemic effect of omega-3 may influence the LDL-cholesterol levels by improving the LDL-cholesterol catabolism. The LDL-cholesterol levels are not influenced by the diet only, but it could be enhanced by a combination of aerobic exercises. Previous study has shown that the combination of dietary PUFA and 12 week of exercise training was able to reduce LDL-cholesterol levels more than the dietary PUFA alone (Hill *et al*., 2007).

The level of HDL-cholesterol in the D2E group was the lowest ($P<0.05$) but it remained comparable to that of the D1E group, whereas that of the DCXE group was the highest ($P<0.05$). Surprisingly, the HDL-cholesterol of these intervention groups showed a decrement when they were expected to register an increment. Stone (2008) reported that although dietary fat is effective in reducing microvascular and macrovascular complications in diabetes mellitus, these may be less effective in improving mixed dyslipidaemia after three months of experiment, where they found only the total cholesterol and LDL-cholesterol were lower but no changes in HDL-cholesterol and triglycerides levels were observed. This report has annotatively explained the findings of the low HDL-cholesterol observed in the exercise and the dietary intervention groups of this study, whereby the experiment was only carried out for eight weeks.

CONCLUSIONS

In conclusion, this study has demonstrated the interactions between exercise and dietary PUFA in improving the blood lipid profiles. This would further give better effects in alleviating the detrimental effects of diabetes mellitus in relation to lipid abnormalities.

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Nigella sativa Meal Alleviates Injury against Benzo[a]Pyrene Exposures in Broilers

Latif, I.K.[1,2], Karim, A.J.[1], Zuki, A.B.[1], Zamri-Saad, M.[1] and Noordin, M.M.[1*]
[1]Department of Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[2]Department of Pathology and Avian Diseases, Faculty of Veterinary Medicine, University of Baghdad, 10071 Baghdad, Iraq
[*]E-mail: noordin@vet.upm.edu.my

ABSTRACT

This study was conducted to evaluate the effects of *Nigella sativa* (Ns) meal on such influences of Benzo[a] Pyrene (BaP) on the broilers performance, pulmonary index (PI), and histopathological changes. Chicks were assigned into four equal groups, namely as controls, Ns, BaP and BaP with Ns. Six birds were sacrificed at days 7, 14, 21, and 35. The results revealed that there were significant decreases ($p<0.05$) in the mean body weight (BW) values and body weight gain (BWG) in the BaP alone group. Pulmonary weight (PW) and index increased ($p<0.05$) in the same group compared to the control groups. Pulmonary morphology showed heavy infiltration and denudation of ciliated epithelium in the trachea, with infiltration by polymorphonuclear and mononuclear cells in the parabronchi of the lung, in the BaP exposed chickens. *Nigella sativa* (Ns) supplementation significantly alleviated these alterations, and thereby showing a potent anti-inflammatory effect after 14 days in the treated group. A key finding from this study is that BaP triggers inflammatory disorders and this transient effect is believed to be fatal if an infection occurs warranting a reassessment of the health status on exposure, and Ns was found to be helpful in alleviating such effects in broiler chickens.

Keywords: Benzo[a]pyrene, *Nigella sativa*, pulmonary morphological, broiler

INTRODUCTION

Poultry industry has continuously been hit by adverse respiratory conditions, which are economically and scientifically important (Toth, 2000) since the lung is a major target organ for numerous atmospheric pollutants and micro-organisms. Among the atmospheric pollutants, polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in distribution.

Benzo[a]pyrene (BaP) is a member of the PAH family and a potent mutagen, carcinogen, and/or developmental toxicant (Castellano *et al*., 2003). There is a dearth of information on the effects of BaP as environmental contaminants on a non-mammalian species. Birds have been shown to be a suitable indicator of environmental pollution (Brown *et al*., 1997) to which intra-tracheal (i.t) administration of 15 mg/kg BW BaP impairs the respiratory macrophage activity and induces hemato and hepatotoxicity in broilers (Latif *et al*., 2009: 2010).

Nigella sativa (Ns) is a multipurpose medicinal plant used in folk medicine for the treatment and prevention of a wide number of diseases all over the world (Ramadan, 2007). Feeding growing chicks on diet containing

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*Corresponding Author

natural feed additives, such as Ns, has been reported to improve chicks' performance, digestibility and decreased abdominal fat (Ashayerizadeh *et al*., 2009). One of the potential properties of Ns seeds is to reduce inflammatory activity as a result of their antioxidant activities (Salem, 2005). The aim of the study was to assess the effects of i.t exposure of BaP on broiler performance, pulmonary index (PI) and morphological changes of the trachea and lung.

MATERIALS AND METHOD

A total of 96 newly hatched male broiler chicks were weighed and randomly divided into four equal groups of 24 chicks in cages and provided with recommended management protocols, including provision of feed and water *ad libitum*. The first group was chosen as a control group, and it was given i.t tricaprylin only for 5 consecutive days and fed on commercial broiler diet only or with additional Ns 20 g/kg diet. The BaP groups were instilled with BaP 15 mg/kg body weight (BW) that was initially dissolved in tricaprylin by the same route and period and fed either with commercial broiler diet only or in addition to Ns. All the chickens were individually weighed every week to determine their BW, body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). Six birds from each group were sacrificed at 7, 14, 21, and 35 days post instillation (p.i), while necropsy was performed immediately. Trachea and lungs were lightly blotted and collectively weighed (pulmonary weight [PW]). Hence, the pulmonary index was calculated as follows: {PWg/BWg} × 100. Meanwhile, the morphological changes were observed by light microscopy using routine processing methods. The statistical analysis was determined by the analysis of variance (ANOVA) and when the differences were significant, the Tukey's tests were used and the value of $p<0.05$ was considered as significant.

RESULTS AND DISCUSSION

All the groups recorded an increase in BW (Table 1) throughout the experiment. Commencing from day 7 until the end of the experiment, the BW of the broilers from the BaP alone group was almost always the lowest ($p<0.05$). However, that of the BaP+Ns was only significantly higher ($p<0.05$) than the BaP alone group at days 21 and 35.

Likewise, the BWG of the BaP was the lowest ($p<0.05$) than the other groups at days 1-21 and 1-35 (Table 2). The calculated FI and FCR yielded a similar trend albeit without being subjected to statistical test due to single sampling (Table 3) during the experimental trail.

The findings of the present study have indicated that the i.t administration of BaP significantly impairs broiler performance (BW, BWG, FI and FCR) which is synonymous with the findings that the BaP administered at any route produces a systemic toxic effect (Sun *et al*., 1982). Such phenomenon, if converted into economic terms (farm scale basis), is inevitably defined as a substantial loss. On the other hand oral supplementation of Ns reduced the adverse

TABLE 1
The body weight of broilers throughout the trail (mean ± SD)

Organs	Groups	Days p.i			
		7	14	21	35
Body weight (g)	Control	180 ± 7.50[a]	509 ± 18.0[a]	971 ± 19.4[a]	1981 ± 43[a]
	Ns	177 ± 6.50[a]	512 ± 44.4 [a]	981 ± 40.7[a]	1990 ± 24[a]
	BaP	150 ± 10.6[b]	429 ± 30.0 [b]	844 ± 36.4[b]	1853 ± 48[b]
	BaP+Ns	159 ± 7.32[b]	468 ± 24.0[ab]	922 ± 58.0[a]	1929 ± 34[a]

[a, b] Values bearing similar superscript/s within the column do not differ at $p< 0.05$

TABLE 2
The body weight gain of broilers throughout the experiment (mean ± SD)

Groups	Days p.i		
	1-21	21-35	1-35
Control	926 ± 18.8[a]	1010 ± 56[a]	1936 ± 42[a]
Ns	939 ± 41.0[a]	1008 ± 62[a]	1947 ± 26[a]
BaP	800 ± 36.2[b]	1009 ± 29[a]	1810 ± 48[b]
BaP+Ns	880 ± 58.4[a]	1004 ± 84[a]	1884 ± 38[a]

[a, b] Values bearing similar superscript/s in the same row do not differ at $p < 0.05$

TABLE 3
The FI, FCR of broilers throughout the experiment

Groups	Days p.i					
	1-21		21-35		1-35	
	FI	FCR	FI	FCR	FI	FCR
Control	1082	1.168	2125	2.103	3207	1.656
Ns	1085	1.155	2168	2.150	3253	1.670
BaP	1028	1.285	2230	2.210	3258	1.800
BaP+Ns	1073	1.219	2202	2.193	3257	1.738

effects of BaP shown by an improvement of the broiler performance (BW, BWG, FI, and FCR). This is in consonance with the finding that feeding growing chicks on diet containing natural feed additives, such as Ns improved chicks performance, digestibility and decreased abdominal fat (Ashayerizadeh *et al*., 2009).

A trend of increased of PW was seen in all the groups during the course of the experiment (Table 4), but this increments were always higher ($p<0.05$) in the BaP groups at days 7 and 14. Nonetheless, a decreasing pattern of the PI was seen in all the groups as time advanced. Such a decrement was almost always lower ($p<0.05$) only in the BaP group, although significant changes were observed at day 7 in the BaP+Ns group as compared to the BaP group alone.

Organ and its relative body weight are important criteria for the evaluation of organ toxicity (Sellers *et al*., 2007). The reduction in PI, despite an increase in PW, was due to a much higher weight gain. The higher the PI in the BaP group suggests that pulmonary pathology is arising from BaP toxicity (Bailey *et al*., 2004) and is due to the massive invasion of inflammatory cells, congestion and atelectasis (*Fig. 1b*, *2b*) invoked by the instilled BaP (Slauson, 1982; Kontoni *et al*., 1999).

In the BaP+Ns, it was found that Ns improved the broilers organ and its relative body weights via alleviation of BaP effects by reducing damages to cells as a result of their antioxidant activity (Ramadan, 2007), enhancing the immunity of the host (Soliman *et al*., 1999) or by their anti-inflammatory action (Ali and Blunden, 2003).

While normal histology was observed in the trachea of the control and Ns groups (*Fig. 1a*), that of the BaP group displayed degenerative and inflammatory lesions (*Fig. 1b*). This was composed of mononuclear cell infiltration, necrosis and sloughing of the epithelial mucosa.

Similarly, the parabronchial sections of the lung of the control group (*Fig. 2a*) were within normal limits, but that of the BaP group showed mononuclear cell infiltration, atelectasis,

TABLE 4
The pulmonary weight and indices of broilers throughout the trail (mean ± SD)

Parameter	Group	Days p.i			
		7	14	21	35
Pulmonary weight (g)	Control	1.596 ± 0.082[a]	4.122 ± 0.319[a]	7.556 ± 0.375[a]	12.53 ± 0.395[a]
	Ns	1.622 ± 0.061[a]	4.123 ± 0.420[a]	7.697 ± 0.535[a]	12.74 ± 0.470[a]
	BaP	2.095 ± 0.151[b]	5.316 ± 0.319[b]	8.436 ± 1.142[a]	13.11 ± 0.685[a]
	BaP+Ns	2.053 ± 0.170[b]	5.328 ± 0.508[b]	8.146 ± 0.803[a]	12.60 ± 0.965[a]
Pulmonary index* Mean $\times 10^2$	Control	0.884 ± 0.010[a]	0.808 ± 0.045[a]	0.777 ± 0.031[a]	0.622 ± 0.037[a]
	Ns	0.914 ± 0.023[a]	0.804 ± 0.027[a]	0.783 ± 0.023[a]	0.639 ± 0.015[ab]
	BaP	1.395 ± 0.048[c]	1.240 ± 0.084[b]	0.998 ± 0.121[b]	0.708 ± 0.051[b]
	BaP+Ns	1.287 ± 0.053[b]	1.136 ± 0.090[b]	0.882 ± 0.051[ab]	0.653 ± 0.040[ab]

[a, b, c] Values bearing similar superscript/s within the column do not differ at $p < 0.05$
* Pulmonary index: pulmonary weight (g)/body weight (g) (PWg/BWg) × 100

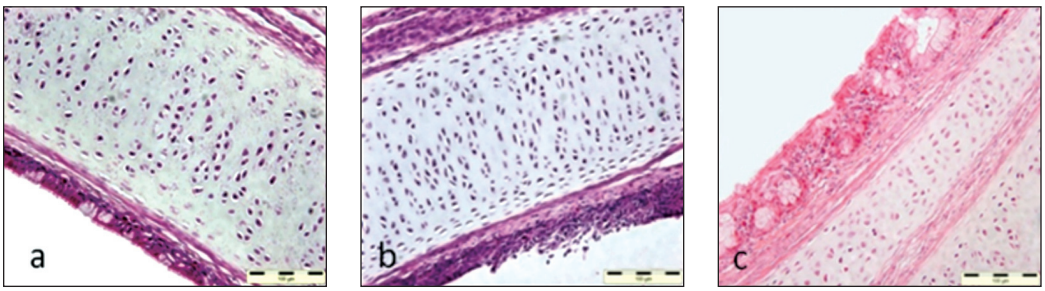

Fig. 1: Photomicrographs of the trachea of chickens necropsied at 21 days p.i (a) control group showing normal epithelium and lamina propria. (b) BaP group that is heavily infiltrated by mononuclear cells (predominantly lymphocytes and macrophages) in the lamina propria and exhibited denudation of ciliated epithelium. (c) BaP+Ns group showed reduction in severity as revealed by normal epithelium with slight thickening of the mucosa, and there is a mild infiltration by mononuclear cells in the lamina propria

whereas inter-parabronchial vascular congestion also revealed compactness with minimal air space (*Fig. 2b*). In that instance, the BaP+Ns was able to reduce these changes after 14 days (*Fig. 1c*, *2c*).

In the present study, the histopathological results revealed that the i.t. administration of BaP was able to trigger a network of inflammatory responses (Slauson, 1982; Kontoni *et al*., 1999) and induced migration of leukocytes into the respiratory tract. These leukocytes were probably mobilized in response to the damage which was inflicted either by the BaP itself or its reactive intermediate compounds onto the lung epithelial surface (Vrzal *et al*., 2004). The accumulation of leukocytes in the tissue induces tissue injury was through the production and release of reactive oxygen metabolites and cytotoxic proteins (e.g. proteases, myeloperoxidase, lactoferrin) into extracellular fluid (Kettle and Winterbourn, 1994).

Thus, the consumption of Ns leads to less damage seen in the treated group receiving Ns. The Ns group shows a better performance, less production of heterophils and macrophages compared to the BaP alone. Likewise, the

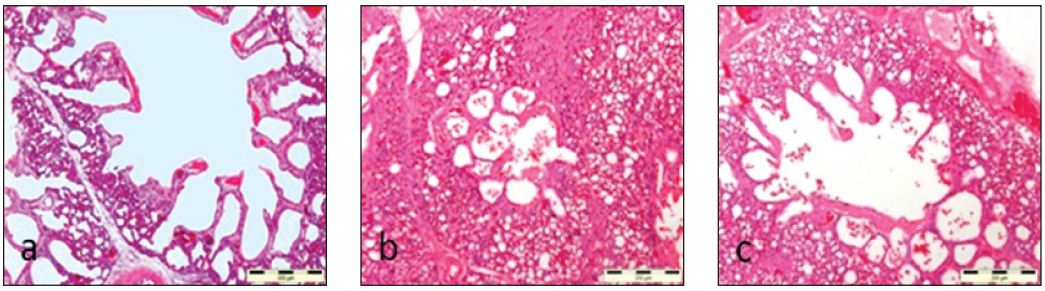

Fig. 2: Photomicrographs of the lung of chickens necropsied at 21 days p.i; (a) control group with normal atria, atrial muscles, infundibula, interparabronchial septae and inter-parabronchial vessels. Note that most areas contained ample air spaces that were devoid of inflammatory cells. (b) BaP group with infiltration by polymorphonuclear and mononuclear cells in the parabronchi of the lung, especially around the blood vessel also revealed compactness with minimal air space due to this heavy infiltration and interparabronchial vascular congestion. (c) BaP+Ns group showed reduction in severity revealed by more air space and milder mononuclear infiltration (H&E)

reversal or alleviation of toxic effects of BaP by Ns is attributed to the pharmacological properties existing in this particular herb.

In conclusion, these data, together with the histological findings suggests that i.t BaP induced oxidative inflammatory injuries in the tissues. Although the effect may be transient owing to the half-life of BaP, it is sufficient to lead to disastrous results if an infection sets in during this period. Attenuating the adverse effects of BaP can be achieved by treatment with Ns harnessing their potent antioxidant role, immune-modulation and their anti-inflammatory action.

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Apoptosis and Tumour Cell Death in Response to Pro-apoptotic Gene

Nik-Mohd-Afizan, N.A.R.[2], Zeenathul, N.A.[1,2*], Noordin, M.M.[1], Ruzila, I.[2], NorHidayah, M.[2] and Mohd-Azmi, M.L.[1,2]

[1]Faculty of Veterinary Medicine, Univesiti Putra Malaysia,
[2]Institute of Bioscience, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: zeenathul@putra.upm.edu.my

ABSTRACT

The process of cell death, or apoptosis, is commonly defined by its distinct morphological characteristic. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, the cellular antiviral response pathway, embryonic development and chemical-induced cell death. In this study, retroviral vector was utilized as the gene delivery vehicles and the tumour-specific viral death effector VP3 as pro-apoptotic agent for malignant colon cancer cells treatment. Here, pro-apoptotic gene inducing apoptosis in CT26 colon tumour cells is reported. In addition, the activation of a typical apoptotic programme was observed in response to this therapeutic agent. An increased number of colon tumour cells suffered apoptosis upon treatment with pro-apoptotic agent. As a result, these tumour cells displayed loss of membrane asymmetry and impermeability, cell shrinkage, nuclear condensation and DNA fragmentation under H&E images. In the presence of VP3, TEM also confirmed that VP3-treated CT26 tumour cells showed apoptotic features. These results suggested that VP3 was a potential pro-apoptotic agent and the apoptosis induced by VP3 was a key anti-tumour mechanism.

Keywords: CT26, mouse models, pro-apoptotic agent, retroviral vector, tumour, VP3

INTRODUCTION

Apoptosis is generally characterized by its distinct morphological characteristics such shrinkage of cells, segmentation of nucleus, condensation, and cleavage of DNA into domain-sized fragment in most cells followed by nucleolus degradation (Wagstaff and Jans, 2009). These characteristics of cell death may be identified easily in histological sections stained with haematoxylin and eosin (H&E) reagents. The identification and counting of apoptotic cells in the H&E staining have been extensively used to study apoptosis induced by radiation or other cytotoxic agents for many years (Matsuu-Matsuyama *et al*., 2008). This stain is also excellent for displaying tissue morphology, but insufficient to distinguish between the apoptotic and necrotic cells. Thus, powerful tools, such as transmission electron microscopy (TEM), are needed to solve this matter. In addition, TEM is especially used to distinguish between apoptotic and necrosis in oncolytic cell population, as these processes may share common features (Miller *et al.*, 2002). Normally, apoptotic cells have intact membranes and organelles, as well as condensed nuclear membrane fragments. Therefore, many references found on the subject of apoptosis were appropriately observed and recorded by using the TEM equipment (Wyllie *et al.*, 1999; Blankenberg *et al.*, 2000).

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[*]Corresponding Author

In the present study, a recombinant of retroviral vector containing pro-apoptotic gene (VP3 gene) was used. VP3 which is also known as apoptin is a 14-kDa basic protein derived from the chicken anemia virus (CAV) that can induce apoptosis in transformed and malignant cell lines (De Smit and Noteborn, 2009). This pro-apoptotic gene was predominantly found in the cytoplasm, whereas in transformed and tumour cells, it was located in the nucleus, suggesting that the location of apoptin is related to its activity (Los *et al.*, 2009).

The present study focused on determining VP3 induced apoptosis in light microscopic assessment and ultrastructure of tumour cells *in vivo* level. This particular study also concentrated on our studies with CT26 tumour-bearing mice. Apoptosis induced by VP3 in the treated tumour was analyzed using the H&E staining and TEM analysis. This combination of H&E staining and TEM application would provide us with a powerful approach to obtain useful information about the image of tumour cells, and to explain the results at the ultrastructural level.

MATERIALS AND METHODS

Cell Culture and Reagents

CT26 cells obtained from the ATCC were grown in high-glucose RPMI6410 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), penicillin (Gibco, USA) and streptomycin (Gibco, USA). Meanwhile, retrovirus packaging cell lines, PT67 cells from ATCC, were maintained in high-glucose DMEM medium supplemented with 10% FBS.

Transfection and Virus Production

The recombinant retrovirus of pMSCV-VP3 was transfected into PT67 packaging cells by Calcium Phosphate Transfection System (BD Biosciences Clontech, USA). The culture media were replaced after 12 h to remove the calcium phosphate and residual DNA. Stably transfected cells were selected in a medium containing 50 mg/mL neomycin (Sigma, USA). Pooled colonies of transfected cells were allowed to grow to confluency, and viral supernatant harvested, following overnight incubation in serum-free DMEM for infections. All the supernatants were filtered (0.45 μm) for further step.

In vivo Experiments in Mice Model

Balb/c mice at five weeks of age were kept at constant temperature in 12 h circadian cycles. They were provided with standard rodent chow and water *ad libitum*. For induction of tumour, 1.0×10^6 CT26 mouse colon carcinoma cells in 200 μl of phosphate buffered-saline were subcutaneously injected into the right flank of each mouse. The appearance time of palpable tumour was recorded, and the longest perpendicular diameters were measured twice weekly using callipers. Tumours were allowed to grow for 7-10 days prior to administration of recombinant retrovirus particles. A total of 250 μl (3×10^4) of the retroviral supernatants, produced as described above, were administrated to the tumour-bearing mice. Mice were sacrificed 48 h post-treatment, at which time small pieces of tumour tissues were then removed for histopatological processing and TEM analysis.

Histopathology

Subcutaneous tumour tissues were fixed overnight in fresh buffered 4% paraformaldehyde, and subsequently processed through a serial of alcohol dehydration, chlorate, and paraffinization. All of the tissues were embedded in paraffin and sectioned at 3 μm. All slides were stained with hematoxylin-eosin and examined microscopically.

Transmission Electron Microscopic (TEM)

The tissues were also particularly observed by the TEM in following procedure. They were cut into small pieces (2-4 mm) and fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2) at 4°C for 4 h. The tissues were then rinsed with cold PBS and post-fixed in 1% osmium tetroxide at room temperature for another 2 h. After dehydration with gradient ethanol and twice with

acetone for 15 min each time, the samples were embedded in resin and acetone (1:1) for 30 min, followed by 100% resin for 1 h. Finally, resin was solidified at 37°C for 24 h and at 60°C for another 48 h. Ultrathin sections were obtained and subsequently stained with uranyl acetate and lead citrate for examination by TEM.

RESULTS AND DISCUSSION

Histopathology

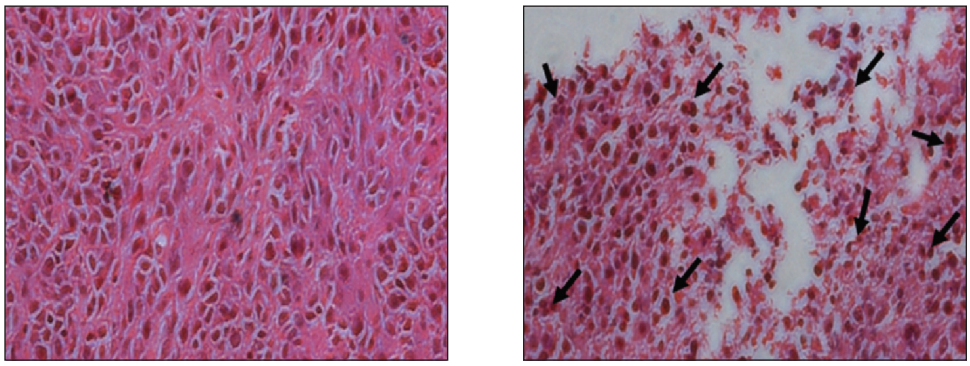
Apoptosis and tumour cell death in response to cytotoxic drug i.e., pro-apoptotic gene was normally assessed by monitoring the appearance of typical nuclear morphological changes and internucleosomal DNA cleavage. In this study, Balb/c mice were sacrificed after 48 h of the last injection of recombinant retroviral supernatants. The anti-tumour effect of VP3-expressing tissues in cellular biology was histophatologically observed using the H&E staining. In VP3-treated tumours, the induction of apoptosis by VP3 was confirmed by the cytomorphological features, which include nuclear condensation, rounded dark nucleic, presence of interstitial space and formation of apoptotic bodies (*Fig. 1*). Similar characteristics of the morphological changes of cells undergoing apoptosis have been reported (Pulkkanen *et al.*, 2002; Natesan *et al.*, 2006; Pietersen *et al.*, 1999).

TEM

For further determination of apoptosis response in subcutaneous tumour, the samples were evaluated using the TEM analysis, which is the best method for morphological observation through clearly differentiating nucleic and organelle. It is important to note that this approach is normally used to distinguish and confirm the presence of different alterations affecting tumour cells due to apoptosis or necrosis (Reid *et al*., 2003). The ultrastructures of the morphological changes during apoptosis were also seen, including the reduction in volume of the nucleus, condensed nuclear material and nuclear membrane blebbing, all of which could be found in VP3-treated tumours (*Fig. 2*). Supporting this was the presence of some intact organelles in the cytoplasm. In addition, these data showed that the administration of retroviral-mediated VP3 gene was able to induce apoptosis in tumour cells.

CONCLUSIONS

In conclusion, these data clearly demonstrated that cancer cells response against VP3 was predominantly found in nuclear membrane with distinctive morphological features. This investigation is also worthy for further consideration as a move approach in the treatment of solid tumours.



Control VP3

Fig. 1: Apoptotic effect of pro-apoptotic gene; VP3 on subcutaneous tumour at the cellular level (arrows). CT26 tumour tissue was isolated from mice treated with pro-apoptotic gene (right panel) or control (left-panel) (H&E, x 200)

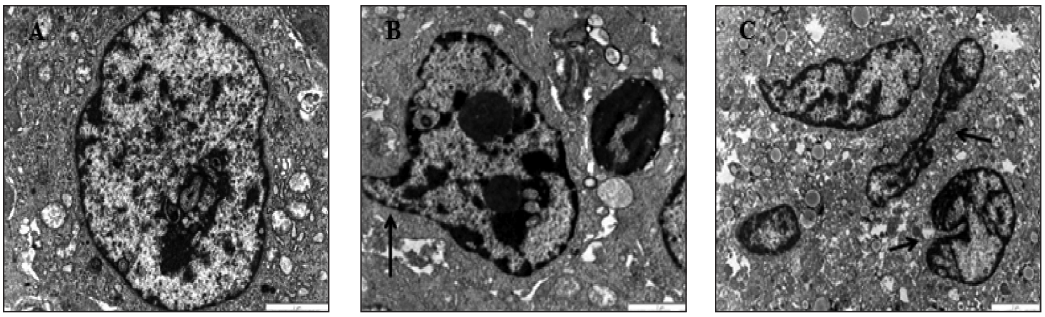


Fig. 2: TEM profiles showed control (A) and VP3-infected cancer cells (B&C). A: Ultrastructure of control tumour cells (× 5500); B: Note the demonstrated nuclear blebs (arrow) (x 12500); C: The features of apoptosis showed the shrinkage of nuclear membrane which had become smaller (arrows) (x 7700)

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Immunohistological Localisation of *Coxiella burnetii* in Various Organs of Naturally Q-Fever Infected Goats

Norina, L.[1], Sabri, Y.[1], Goh, M.Y.[1], Zamri-Saad, M.[1], Sarenasulastri, A.B.[2], Latifah, H.[1], Jamilah, J.[1] and Noordin, M.M.[1*]

[1]Department of Pathology and Microbiology,
Faculty of Veterinary Medicine, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[2]Regional Diagnostic Laboratory, Bukit Tengah,
14000 Bukit Mertajam, Penang, Malaysia
[*]E-mail: noordin@vet.upm.edu.my

ABSTRACT

The rather uncommonly reported Q-fever disease in Malaysia is currently demonstrating an increasing trend of outbreaks. A total of 197 goat carcasses during the period of July 2007 to December 2009 were submitted to Regional Diagnostic Laboratory (MVK), Bukit Tengah, Penang, for post-mortem examination. Morphological diagnosis of necrotic placentitis, interstitial pneumonia, hepatitis and nephritis were observed in majority of the cases. Likewise, Giemsa stained sections of selected tissues revealed *C.burnetii* trophoblast. Acute cases yielded lesions conforming to doughnut granuloma, while those of the chronic form exhibited chronic inflammation. In an attempt to further confirm the presence of the organism, these selected tissues were subjected to immunohistochemical confirmation. Out of the total suspected cases, 152 (77.2%) were confirmed as positive of Q-fever based on their IHC. Thus, this study demonstrated pertinent lesions of acute and chronic forms of Q-fever which might be beneficial to laboratories without IHC facilities.

Keywords: *Coxiella burnetii*, immunohistochemistry, goat, Peninsular Malaysia

INTRODUCTION

The incidences of Q-fever have been globally reported with the exception of New Zealand (Rodolakis, 2005). *Coxiella burnetii* is strictly an intracellular bacterium that inhabits monocytes and macrophages (Baca and Paretsky, 1983). The transmission to human originated from contaminated secretion or excreta from infected animals warranting importance of food safety when dealing with the consumption of contaminated raw milk and milk products.

Coxiellosis occurs during late pregnancy, i.e. about 15 days before term leading to abortion in small ruminants and stillbirth in cattle (Russo and Malo, 1981). The organism's *in-vivo* survival is largely dependent on its ability to with stand the acidic environment of macrophages (pH 4.0 to pH 5.5) without affecting the cell's viability (Seshadri *et al.*, 2003). The organism can be demonstrated within trophoblast and mononuclear cells in ruminant placenta (Bildfell, 2000). Janigan and Marrie (1983) discovered the presence of giants and plasma cells in a pulmonary pseudotumour induced by *C.burnetii*. Recently, Norina *et al*. (2008) reported the appearance of fibrin ring or

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[*]Corresponding Author

"doughnut granuloma" in infected lung, liver, and spleen in typical *C.burnetii* infection.

Several conventional staining methods have been used in demonstrating the organism in impression smears, cell culture, and formalin-fixed tissues samples (Russo, 1997). Likewise, egg inoculation, as well as immunohistochemistry (Anon, 2008) and PCR (Henning, 2002) have been used as the methods of isolating the organism. It is believed that cases or outbreaks of caprine Q-fever have been under-reported in Malaysia, owing the lack of database on lesions pertaining to the disease. Thus, the aims of the study were to demonstrate and develop a set of database on caprine Q-fever with the emphasis on tissue tropism and pertinent lesions.

MATERIALS AND METHODS

Tissue Samples

The study was performed retrospectively on formalin-fixed paraffin embedded tissue samples obtained from 197 Boer goats that had died from the period of July 2007 to December 2009. The tissues examined included the placenta, lung, heart, liver, spleen, and kidney, which were available for examination. Smears of the cut surface of placentome from every placenta received were stained by Giemsa and examined for *C. burnetii* trophoblast. Other organs were fixed in 10% buffered formalin and processed in the routine manner stained with Haematoxylin and Eosin (H&E). These tissues were examined microscopically for the evidence of necrotizing placentitis with cytoplasm of trophoblast cells, granulomatous lesion called as "doughnut granuloma" and infiltration of PMNs and macrophages cells.

Suspected C.burnetii *Positive H&E Samples*

A total of 152 cases for the last three year that were suggestive Q-fever through H&E staining were selected for immunohistochemical (IHC) confirmation.

Brief Description of the Immunohistochemical (IHC) Method

Briefly, the paraffin-embedded blocks were sectioned at a thickness of 2 µm. These sections were collected on coated silanized slides. The slides were placed in an oven at 60°C for 15 minutes. Then, they were deparrafinised using xylene and rehydrated. For antigen retrieval, rehydrated slides were immersed in commercially BIOCARE's peroxidized blocking buffer with goat serum for 10 minutes and washed in distilled water. The following steps were digested using the heat methods. The slides were placed in a pyrex beaker containing a Rodent Decloaker solution prepared by the manufacturer and heated to 80°C – 90°C for 30 minutes. After digestion and cooling down at room temperature for 15 minutes, they were washed by dipping them in TBS Tween 20, followed by immersing them in primary antibody using strongly positive goat serum at dilution 1:1000 and left at room temperature for 60 minutes. Then, they were washed again with TBS-Tween 20 and immersed for 15 more minutes in commercially secondary antibody Goat Probe as instructed by the manufacturer. It was washed in TBS wash buffer. Goat polymer HRP was applied for 15 minutes and later washed with TBS wash buffer. Later, it was immersed for 15 minutes in the DAB solution as recommended by the manufacturer. This was followed by washing with TBS wash buffer and rinsed again in distilled water. Counter-staining with Harris Hematoxylin solution for 10 seconds was later carried out and rinsed with distilled water, air-dried, and mounted with DPX and a cover slip.

Statistical Analysis

Statistical analyses were done using Cohen's Kappa test for reliability. The results were computed for HE positive and IHC positive samples. The associations between six organs samples for the HE positive and IHC positive in three year were assessed by comparing both the techniques, with the association being tested using the Cohen's Kappa test.

RESULTS

Tissue Samples

152 out of 197 samples were found to be positive for immunohistochemical test for the past three years (2007 – 2009), as illustrated in *Figs. 1-11* and Table 1.

On the HE stains, the histopahological lesions seen were:

- **Lung -** filled with interstitial edema and infiltrated with lymphocytes and macrophages. The alveolar spaces were filled with histiocytes, intra-alveolar focal necrosis, haemorrhages and necrotizing bronchitis. Giant cells and plasma cells were also observed to have been due to *C. burnetii,* syncthial-like cells in bronchiole, and severe interstitial pneumonia.
- **Liver** - hepatomegaly and speckled with dilated, congested central veins typical of nutmeg liver appearance indicative of right heart failure. Hepatic fatty degeneration, with accumulation of inflammatory cells and bizzarecells, granulomatous foci, were observed under a microscope. Granulomatous lesions containing the so-called doughnut granulomas consisting of dense fibrin rings or "doughnut" granulomas were also seen. These lesions were suggestive of acute Q-fever.
- **Spleen -** Congested and fragile spleen while palpating. Granulomatous spleenitis was observed at the early form of doughnut.
- **Heart -** hydropericardium with light yellow fluid accumulate in pericardium sac. Grossly looked normal. Myocarditis was revealed under histopathology.
- **Placenta -** with severe acute necrotizing placentitis and whitish 0.5 - 1.0 mm necrotic foci on cotyledons. Interestingly, numerous intracellular coccobacilli, that were indicatives of *C. burnetti*, were seen as bluish haze within cytoplasm of trophoblasts of *C. burnetii*. These organisms were detected as clearly revealed by green apple appearance seen under DIC microscopy in IFAT. Placenta vasculitis characterized by pleomorphic cellular infiltrations of mononuclear cells, neutrophils or eosinophils were also observed under microscopy.
- **Kidney -** tubulonephritis which leads to nephrosis. Miscroscopically, diffuse necrotic interstitial nephritis were shown with intra mononuclear cells that attempted to invade in the vessel, and inside they contained a lot of neurophils cells suggesting an acute inflammation.

DISCUSSION

Histopathology, Immunohistochemistry

Coxiella burnetii induces endemic abortion which results in necrotic placentitis in small ruminant. The most important route of infection is inhalation, especially when the environment is

TABLE 1
Comparison between H&E and IHC stained tissues in the detection of *C. burnetii*

Organs	H&E stain			IHC stain[a]		
	Positive (n)	Negative (n)	%	Positive (n)	Negative (n)	%
Placenta	11	0	100.0	11	0	100.0
Lung	67	41	62.0	58	9	86.6
Liver	57	51	52.8	45	12	78.9
Spleen	16	92	14.5	10	6	62.5
Kidney	26	82	24.0	16	10	61.5
Heart	23	85	21.3	12	11	52.2

[a]Only tissues that were positive on H&E were subjected to IHC staining

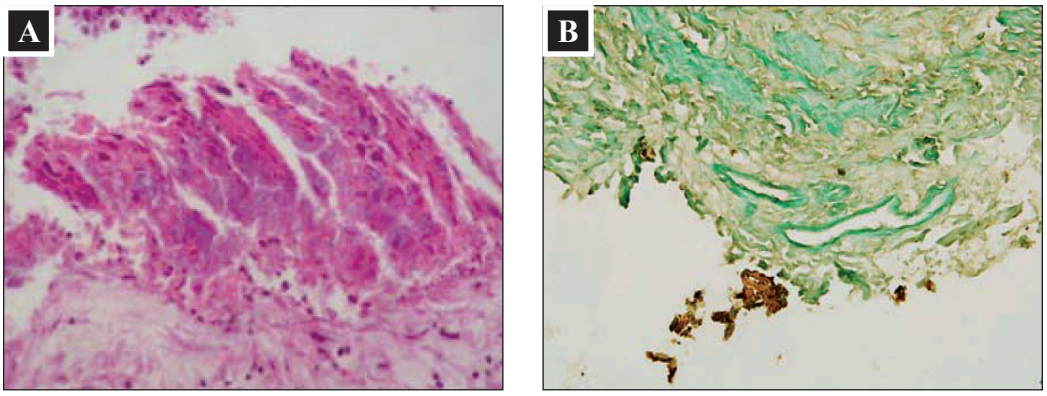

Fig. 1: (A) Photomicrograph demonstrating placentitis with trophoblast cell (H&E, X200); (B) which was later confirmed by to be C. burnetti *(IHC, X200)*

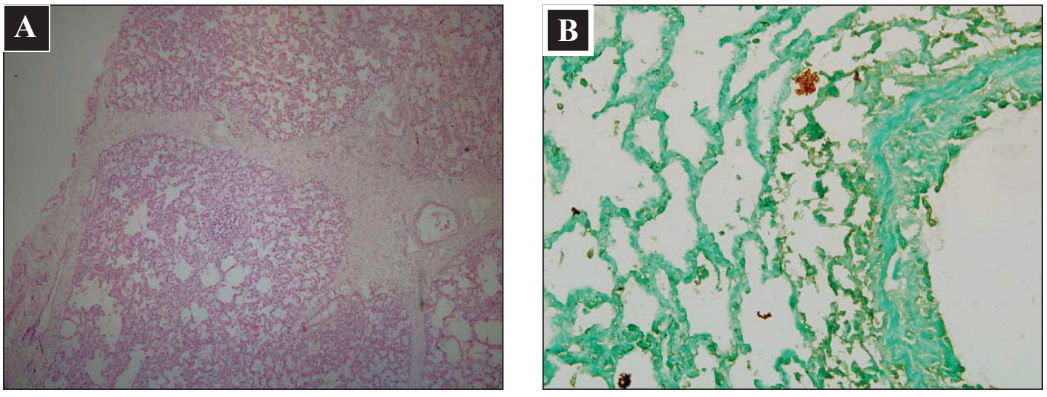

Fig. 2: (A) Photomicrograph of the lung demonstrating alveolar septa thickening and granuloma (H&E, X100); (B) and the presence of C. burnetii *(IHC, X200)*

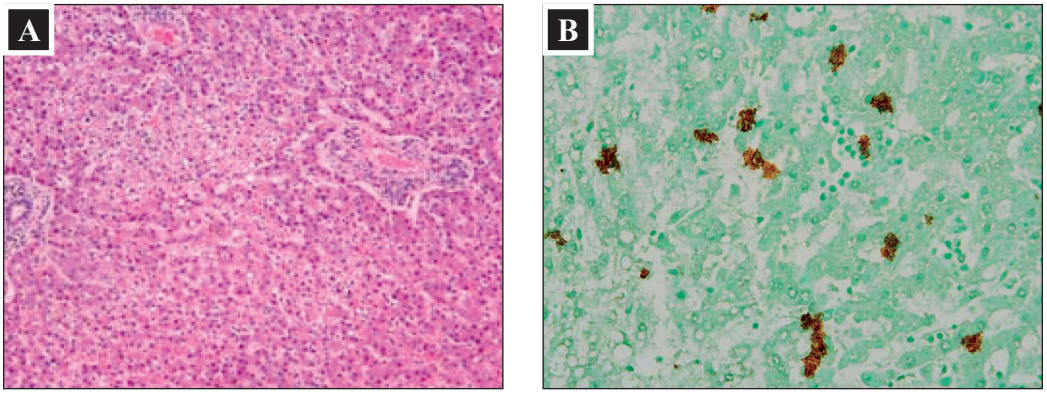

Fig. 3: (A) Photomicrograph of the liver showing fatty degeneration and early evidence of a doughnut granuloma (H&E, X100); (B) and the C. burnetii *positively-stained areas (IHC, X200)*

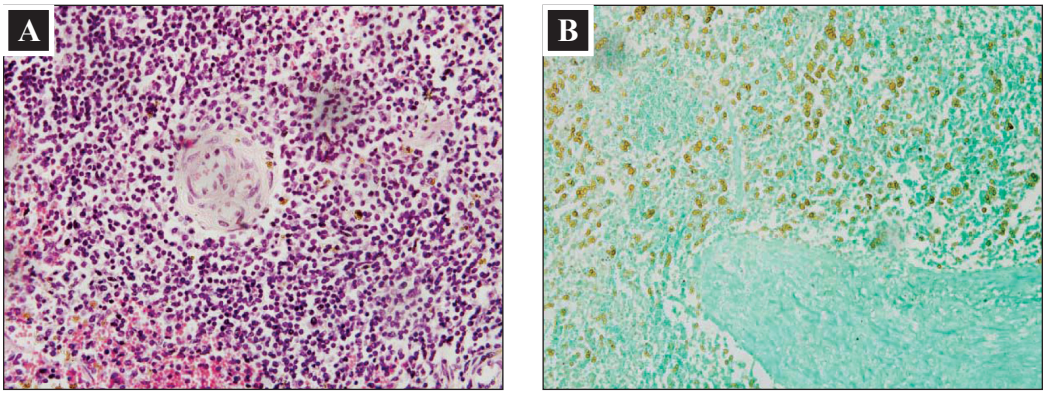

Fig. 4: (A) Photomicrograph of splenitis along with a doughnut granuloma (H&E, X100); (B) and strongly positive areas of C. burnetii *(IHC, X100)*

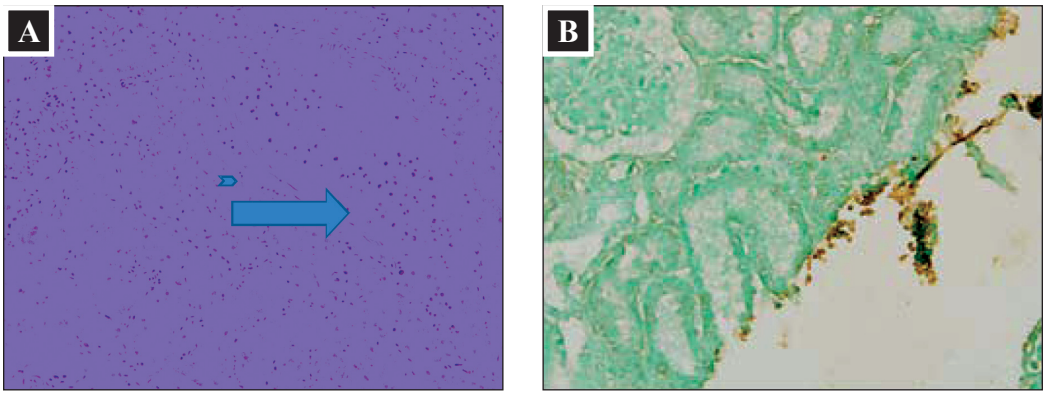

Fig. 5: (A) Photomicrograph of the kidney showing areas of interstitial nephritis, a vessel packed (arrow) with neutrophils (H&E, X200); (B) and borders of renal tubules being positive for C. burnetii *(IHC, X100)*

heavily contaminated by birth material (Marrie, 1990). *C. burnetii* is a eukaryotic cell with the characteristic of an intracellular pathogen that lives in an acidic vacuole (Raoult and Marrie, 1995). Reimer (1993) has reported that the pathogenesis of Q-fever, particularly in acute infection, is less understood because of the self-limiting nature of the illness and low mortality rates.

It is important to note that the histology studies of Q-fever have been based on pulmonary, hepatic, spleen, placenta, cardiac, and renal tissues. The immune response during Q-fever is associated with an inflammatory reaction that results in the information of granulomatous lesion which most commonly involves in the lung, liver, and spleen. Meanwhile, lymphocytes and macrophage are usually seen in infiltration and interstitial edema. Histiocytes fill the alveolar space and later form focal necrosis which will take place in intra-alveolar wall that leads to haemorrhages. This will also lead to the development of necrotizing bronchitis and bronchiolitis. In this study, less micro-organism were observed microscopically. Giant cell and plasma cells were also seen, and these could be due to *C.burnetii*. Syncitial-like cells in bronchiole indicated a severe

interstitial pneumonia. During autopsied, most cases resulted in hepatomegaly and speckled with dilated, congested central veins typical of nutmeg liver. This appearance is an indicative of the right heart failure. Meanwhile, hepatic fatty degeneration with the accumulation of inflammatory cells and bizzarecells, granulomatous foci were observed under the microscope. Lesions in the liver are different in acute and chronic Q- fever. In acute cases, granulomatous lesion containing the so-called doughnut granuloma is a pathognomonic finding which is noticeable of dense fibrin rings surrounding a central lipid vacuole (Srigley, 1985). In chronic cases, pathological findings are not restricted to the present of lymphocytic infiltration and foci of spotty necrosis (Janigan and Marrie, 1983). Grossly heart looked normal. The pericardium sac revealed hydropericardium with accumulated light yellow fluid. Under histopathology revealed myocarditis. Grossly spleen was observed congested, fragile, and palpating. Microscopically granuloma, i.e. an early form of doughnut spleenitis was also observed. Renal involvement in acute *C. burnetii* infection has been rarely reported (Tolosa-Vilella, 1995) but from this finding, tubulonephritis which has led to nephrosis is classified as a renal Q-fever. Renal microscopically showed diffuse necrotic interstitial nephritis with intra mononuclear cells attempting to invade the vessel, and containing a lot of neurophils cells which indicate on-going acute inflammation.

Abortion usually occurs during the third trimester of gestation due to *Coxiella burnetii* infection in small ruminant. The aborted foetuses are usually fresh with no lesion. The gross lesions are severe acute necrotizing placentitis with copious amounts of tan-brown exudate and whitish necrotic foci on placentome. Interestingly, numerous intracellular coccobacilli, indicative of *C. burnetii*, were seen as bluish haze within cytoplasm of trophoblasts of *C. burnetii* when stained with Giemsa. These organisms were detected, as clearly revealed by green apple appearance that was seen under the DIC microscopy in IFAT. Placenta vasculitis, characterized by pleomorphic cellular infiltrations of mononuclear cells, neutrophils or eosinophils, was rarely observed under microscopy. Necrotizing placentitis is a common lesion of *C.burnetii* with the appearance of numerous trophoblast containing intracytoplasmic coccobacilli colonies stain pale thin reddish coccobacilli in Modified Ziehl Neelsen. Although Giemsa staining of fresh placental impression smear is a good screening test, the diagnosis should be confirmed through IHC testing.

The statistical data presented in this study revealed that the organs of placenta, lung, and liver are significant samples to look for under histology for quick diagnosis purposes. The findings help pathologist to classify the infection into either acute or chronic Q-fever. This study provides evidence that in spite of placental, lung and liver are also significance for pathological diagnosis of *C.burnetii*. This being proved by Cohen's kappa analysis, i.e. placenta revealed greater than liver greater than lung ($0.95 \geq 0.780 \geq 0.772$) at $p < 0.0001$ indicate higher than ≥ 70.

CONCLUSIONS

Coxiella burnetii can be detected in various organ samples by using impression smear, histopathology and immunohistochemistry testing. In other words, immunohistochemical techniques are sufficiently sensitive to detect the antigen in various organ samples. The fact that *C.burnetii* causes Q-fever in man, safeguards against inhalation of infective material should be done when laboratory personnel work with infective material from abortion cases.

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Melamine Toxicity in Pigs

Lee, C.H.[1], Ooi, P.T.[2*], Sheikh Omar, A.R.[2] and Lim, B.K.[3]
[1]Rhone Ma Malaysia (M) Sdn. Bhd., 46100 Petaling Jaya, Selangor, Malaysia
[2]Faculty of Veterinary Medicine, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[3]Vet Food Agro Diagnostics (M) Sdn. Bhd.,
46100 Petaling Jaya, Selangor, Malaysia
[*]E-mail: ooi@vet.upm.edu.my

ABSTRACT

Three farms in Penang, Malaysia, reported illness in grower-finisher pigs with discarded kidneys from abattoir. Affected animals were inappetant, anorexic, thin, dull, and depressed. The episode was closely related with the feed formulation changes, where soybean and fishmeal were replaced with yeast. No significant mycotoxin levels were detected in both the feed and yeasts. However, high levels of melamine were detected in yeast (30,064 ppm) and finished feed (750 to 1500 ppm), which exceeded the acceptance level set by WHO (<2.5 ppm). Nonetheless, prominent gross abnormalities were detected, except for the lesions in the kidneys during necropsy. The affected kidneys were enlarged and yellowish in colour, firm in consistency and the cortical surface was wrinkled and dimpled. Dilated cortical and medullary lesions with yellow gritty crystalline materials were also observed. A microscopic examination revealed the lesions of acute tubular nephrosis, interstitial nephritis with multiple cysts in the kidney cortex. After the removal of the yeast source in the feed, the illness and kidney lesions were in remission.

Keywords: Acute renal failure, kidney, melamine toxicity, pigs, yeast

INTRODUCTION

Melamine (1,3,5-triazine-2,4,6-triamine) is an industrial chemical which is used to produce melamine resins (polymers of melamine) such as plastic kitchen items, urea, and fire retardant. It contains high nitrogen content which when added to animal feed or milk, gives the false impression of higher protein content. Although melamine is not approved for usage in animal food, there were multiple cases of renal failures fatalities reported in dogs and cats in 2007 (Bhalla *et al.*, 2009). The cause was related with contaminated feed with high level of melamine that led to a massive recall of pet food in North America. The contamination was traced back to the wheat gluten imported from China which contained a high level of melamine. In addition, recent cases of nephrolithiasis and acute kidney injury among children in China have been linked to ingestion the milk-based infant formula contaminated with melamine (Bhalla *et al.*, 2009). The melamine contamination was not limited to pet food, adulteration also were detected in livestock which included chicken, fish, and swine feed (Reimschuessel *et al.*, 2008). A syndrome characterized by progressive weight loss, wasting, and jaundice, with high mortality has been reported in swine herds in Malaysia. This is the first melamine toxicity case in the country.

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[*]Corresponding Author

BACKGROUND OF THE STUDY

This case study involved three individual farms that are located in Penang, Malaysia. The farms were Farm X- Kampung Valdor, Farm Y- Kampung Selamat and Farm Z- Tasek. During the first quarter of 2008, meat inspector at the local abattoir condemned kidneys from pigs that originated from Farm X, and the rejected kidneys were from apparently healthy animals. Due to the complaint, an in-depth histopathologic and clinical investigation was initiated. At about the same time, abnormal illnesses were detected in grower-finishers in Farms Y and Z. During the investigation, feed samples and raw materials were dispatched for toxin analysis in the Vet Food Agro Diagnostics, Selangor and Pacific Laboratory, Singapore, respectively, whilst affected kidneys were delivered to Universiti Putra Malaysia for histopathological studies.

RESULTS

The clinical investigation in Farm X showed that grower-finisher pigs were inappetant, anorexic, thin, dull, and depressed. Meanwhile, the herd performance was poor and pigs were about 3-4 weeks behind their compatriots expected i.e. 80-95 kg instead of 100-120kg bodyweight. The episode started three weeks following the change in the feed formulation diet for grower-finisher pigs, where the protein source in soybean and fish meal was substituted with yeast at an inclusion rate of 5%. However, no increase in mortality was detected. The morbidity rate of the affected pigs gradually rose from 20% to 50% over a period of 2 weeks. Feed intake was decreased between 30% up to 50%. The farmers became concerned and reported the case to the investigating veterinarian when the state abattoir reported to the producer that a number of kidneys were condemned from clinically healthy pigs. Farm Y and Farm Z also reported similar clinical signs and abattoir findings.

History indicated that in all the three farms, where the disease was observed, feed formulation changes occurred within 4-5 weeks after introducing yeast source originated from China, and supplied by the same importer. Yeast was included in the diets at between 2.5 to 5%. In the investigation, no clinical or pathological disease was observed in grower-finisher pigs with yeast replacement at 1.6% in the diet (Table 1) at the fourth farm (Farm A). A tentative diagnosis of toxicity of an unknown origin was attributed with differential diagnosis of mycotoxicosis and heavy metals at that time.

During necropsy, a gross examination revealed no other prominent abnormalities, with the exception of kidney pathology and a general loss in body condition. The affected

TABLE 1
Clinical description of melamine toxicity in 4 farms

	Farm X Kg. Valdor	Farm Y Kg. Selamat	Farm Z Tasek	Farm A Kg. Selamat
Clinical signs	Thin, inappetence, dull, depressed	Minor episodes of sick pigs, kidney lesion in abattoir	None. kidney lesion detected in abattoir	No syndrome and no complaint from slaughter house
Affected age group	Grower to finisher	Grower to finisher	Grower to finisher	None observed
Morbidity (%)	20-50	2	2-3	0
Severity of kidney lesions	Severe	Moderate	Moderate	Not observed
Inclusion rate of contaminated yeast (%)	5	2.5	2.5	1.6
Feeding period	2-3 wks	4 wks	4-5 wks	4-5 wks

kidneys were yellowish, discoloured, enlarged, hard in consistency with wrinkling and dimpled cortical surface (*Fig. 1A*). In addition, dilated cortical and medullary lesions, with yellow gritty crystalline materials extending into the ureter, were also detected (*Fig. 1B*). In more severe cases, the kidneys were reduced in size, with clear pitting and dimpling, were dark red brown in colour and with the cortex and medulla atrophied resembling hydronephrosis (*Fig. 1C* and *1D*).

The microscopic examination revealed lesions of acute tubular nephrosis, and interstitial nephritis with multiple cysts in the kidney cortex (*Fig. 2A*). Interstitial nephritis may have been attributed to other infections, i.e. leptospirosis. Multiple stages of tubular neprhosis were also detected indicating involvement of acute toxicity. The collecting tubules remain intact and no casts were detected inside the tubules (*Fig. 2B*).

However, no significant mycotoxin levels were detected in both the feed and yeast samples. The chemical analysis of the yeast showed that the melamine levels were at as high as 30,064 ppm and crude protein of 60-75%. A typical yeast product contained about 45-55% of crude protein. The level of melamine contamination in the feed was between 750 and 1500 ppm, exceeding the acceptance level of <2.5 ppm, as

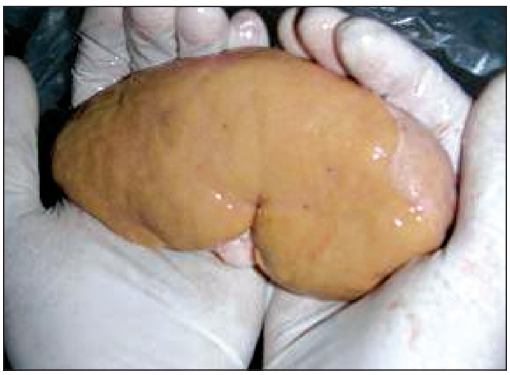


Fig. 1A: The affected kidney showing yellowish discolouration with dimpled cortical surface

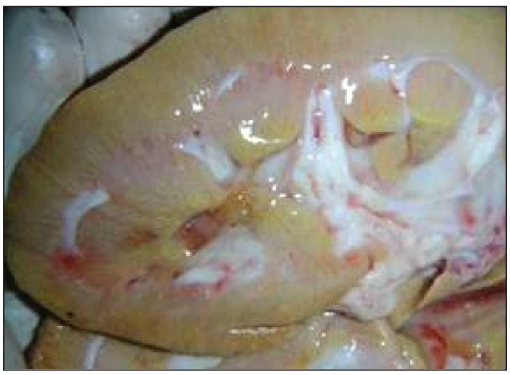
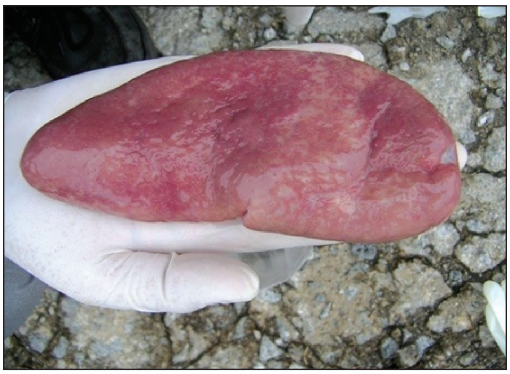


Fig. 1B: Yellowish crystalline material was detected at the medullar upon longitudinal dissection



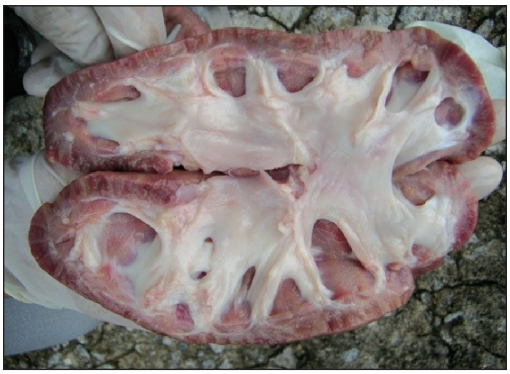


Fig. 1C and 1D: Dark red brown with medulla atrophied resemble grossly hydronephrosis was detected in suspected chronic cases

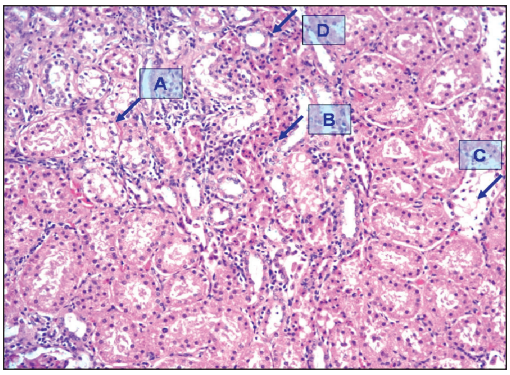


Fig. 2A: Kidney; cortex; pig. Acute tubular nephrosis, characterized by various stages of nephrosis; (A) cellular swelling, (B) esinophilic and pyknosis of the tubular epithelium, (C) karyorrhexia and karyolysis with sloughing of necrotic epithelium into the tubular lamina. (D) A healthy and normal tubular (H &E stain, X20)

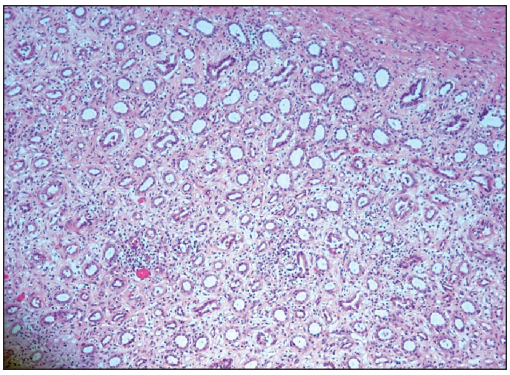


Fig. 2B: Kidney; medulla; pig. The collecting tubules remain intact, clean and no cast was observed, indicating there might be an obstruction in the upper pathway and at the early stage of lesion (H &E stain, X10)

set by the World Health Organization (WHO) during the WHO Expert Meeting to discuss on the Toxicological and Health Aspects of Melamine and Cyanuric Acid in Canada (Anonymous, 2008). On the suspicion of toxicity, the yeast was withdrawn from the diet. The disease syndrome was in remission clinically and a reduction of condemned kidneys in two weeks time at the affected farms.

DISCUSSION

Melamine is rapidly excreted in the urine (99% of the administrated dose would be excreted in 28 hour); however, it is precipitated in the distal renal tubules (Bhalla *et al.*, 2009). Meanwhile, degeneration of the proximal and distal tubules with crystalline material are common findings in melamine affected pigs (Nilubol *et al.*, 2009). In this study, the collecting tubules remained intact and no casts were detected (*Fig. 2B*). The lesion is primarily a consequence of the physical obstruction of the tubules causing reduced blood flow and glomerular filtration rate. Together with renal vasocondtriction and inflammatory mediators, acute renal failure is a consequence (Reimschuessel *et al.*, 2008). In chronic melamine toxicity, interstitial inflammation with large crystals and fibrous tissues would be detected (Bhalla *et al.*, 2009).

In this study, no crystals were detected via histology examination of kidney, as the uric/melamine crystals were dissolved in formalin and during tissue processing (Vernon, 2006). The urine of the affected animals was collected for further analysis, where higher level of blood urine nitrogen (BUN) and serum creatinine, together with crystalline material, were reported in the urine from the melamine affected pigs herd (Nilubol *et al.*, 2009).

In 2007, livestock feed was found to be supplemented with pet food scraps that were contaminated with melamine and melamine-related compounds such as cyanuric acid, ammelide and ameline (Anon., 2007) at 30-120 ppm. The risk of ingestion of meat products from animals with melamine residues has not been comprehensively assessed (Baynes *et al.*, 2008) and there was no meat withdrawal interval set for melamine in any food producing animal species (Buur *et al.*, 2008). The public is concerned about consuming meat from hogs and poultry exposed to melamine and/or its analogues. In this case study, the level of melamine toxicity

(750 to 1500 ppm) detected extremely surpasses the recommended level (<2.5 ppm) set by WHO. Thus, more comprehensive feed analysis and examination should be carried out to identify potent toxins before it is released into the livestock industry so as to help safeguard the public health. Meat withdrawal period in pigs exposed to contaminated melamine should be determined before it is safe for human consumption.

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The Effects of Oestrogen and Progesterone on Lymphocyte and Plasma Cell Population in the Oviduct and Uterine Mucosae during Follicular and Luteal Phases in Ewes

A.R. Intan-Shameha[1*], Zuki, A.B.Z[1]., Noordin, M.M.[2], H. Wahid[3] and Azmi, T.I.[1]
[1]Department of Veterinary Preclinical Sciences,
[2]Department of Veterinary Pathology and Microbiology,
[3]Department of Veterinary Clinical Studies,
Faculty of Veterinary Medicine, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: intan@vet.upm.edu.my

ABSTRACT

Hormonal changes during the oestrous cycle influence the immune cells pattern in ewe's reproductive tract, particularly in the uterus and oviduct. This study was conducted to quantify the number of lymphocytes and plasma cells of the uterus and oviduct under the influences of oestrogen or progesterone. Results showed that the number of lymphocytes in different parts of the uterus was significantly ($p<0.05$) higher during the follicular phase as compared to the luteal phase. Nevertheless, in the follicular phase group, the number of lymphocytes was not significantly different between the middle and anterior horn, while in the luteal phase group, the number of lymphocytes was not significantly different between the posterior and middle horns. Similarly, the number of plasma cells was significantly ($p<0.05$) higher in the follicular phase compared to the luteal phase for the different parts of the reproductive tract. In the luteal phase group, on the contrary, the number of plasma cells was not significantly different between the posterior and middle horns and between the anterior horn and oviduct. Thus, the results emphasize that the ewes are much more protected when they are in follicular phase since the number of lymphocytes and plasma cells are higher.

Keywords: Oestrogen, ewe, lymphocytes, plasma cells, progesterone, oviduct, uterine mucosae

INTRODUCTION

During oestrous cycle, the uterine endometrium undergoes proliferation and differentiation in response to the changes in the levels of sex hormones. The uterine endometrium contains cellular elements of the immune system, including lymphocytes, macrophages, plasma cells, and polymorphonuclear leukocytes (Gogolin-Ewens *et al*., 1989; Segerson *et al*., 1991; Gottshall and Hansen, 1992). In certain species, including the human's endometrium, lymphoid aggregates and scattered interstitial lymphocytes are constant features which are thought to be involved in maintaining the sterile environment of the uterine lumen (Cobb and Watson, 1995). However, the female reproductive system is exposed to infectious agents during mating, following copulation, and during parturition (Gogolin-Ewens *et al*., 1989).

Many previous studies have shown that in the normal uterus, leukocyte infiltration occurs at a certain stage of the oestrous cycle of various

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[*]Corresponding Author

species of animals, including sow, cattle, mice, ewes and human (Zamri-Saad, 1987; Lander-Chachin, 1990; Bischof *et al*., 1994; Kaeoket, 2001; Engelhardt *et al*., 2002; Trundley and Moffett, 2004). The qualitative estimation of T lymphocytes subsets in ovine endometrium during follicular and luteal phases has been reported (Lee *et al.*, 1988). However, the sample collection was done in the abattoir, where careful clinical examination of the animals was not conducted before slaughter. Hence, the ovarian hormones were not analysed and determination of luteal and follicular phases were solely based on the appearance of ovaries. Thus, the quantitative determination of lymphocytes subsets in ovine endometrium at peak levels of oestrogen and progesterone has never been done in both cyclic and pregnant ewes.

MATERIALS AND METHODS

Animals

Fourteen adult, cycling ewes were used in this study. They were randomly divided into two different groups, namely follicular phase and luteal phase groups, with 7 ewes allocated for each. The ewes were kept in raised slatted floor house with an open environment. They were fed with commercial pellets and water was provided *ad libitum*. The ewes were kept in the same house throughout the experimental period with the temperature ranging from 26.8ºC to 32.8ºC and 84.90% to 98.40% humidity.

The ewes were synchronized into oestrus by using the progesterone sponge (Chronogest®) containing 40 mg flugestone acetate intravaginally, for 12 days. At sponge removal, the ewes were given an intramuscular injection of 350-450 IU of pregnant mare serum gonadotropin (PMSG) (Folligon®). Blood samples were collected every alternate day in order to determine the hormonal profiles by using RIA technique. The ewes of luteal phase and follicular phase were slaughtered at the peak level of progesterone and estradiol, respectively.

Sample Collection and Processing

Immediately after slaughtering, both sides of the middle portion of the Fallopian tube (ampulla region), anterior, middle and posterior parts of the uterine horns samples were fixed, processed accordingly and stained with Haematoxylin and Eosin.

Morphometry Evaluation

Morphometry evaluation was done according to the modified method proposed by Segerson *et al*. (1991). Six arbitrarily chosen microscopic fields were analysed in each of the two sections obtained from different tissues of each ewe. The morphometry evaluation was done using a light microscope, with objective × 40 and eyepieces × 10 with 1mm^2 ocular micrometers. Cell counting was performed by using an ocular reticule (ocular micrometer, 10mm × 10 mm, with 100 squares) (Leitz Wetzlar, Germany) placed onto the left side of the eyepiece of the light microscope. Only the optimal sections (free of artefacts) and correct orientation were accepted for counting. For each section, the cell counts were performed at x400 magnifications by movement of the ocular micrometer across the entire epithelium area in a non-overlapping manner.

The number of lymphocytes and plasma cells of the mucosal area (within the surface and glandular epithelium of the endometrial glands), expressed as cells per mm^2 field, were counted and recorded. Similarly, the number of lymphocytes and plasma cells in the surface epithelium of the oviduct (ampulla) and within the stromal area were also recorded. The mean cell counts and the morphometric parameters were calculated.

Statistical Analysis

The cells counts were analyzed using 2-way ANOVA (SPSS version 17.0) to compare the differences between the groups and at different anatomical parts of the reproductive tract. This

was followed by the Duncan multiple comparison test in the event of significant ANOVA findings. All the statistical tests were conducted at 95% confidence level and the differences of $P<0.05$ were considered significant.

RESULTS AND DISCUSSION

Population of Lymphocytes in the Uterus and Oviduct

The lymphocytes were localized in the luminal epithelium, as well as in the glandular epithelium and in some areas of the stroma immediately beneath these epithelia in all parts of reproductive tract during both follicular and luteal phases (*Fig. 1A-1B*). In the oviduct, the lymphocytes were preferentially localized near the border between the propria-submucosa and the epithelium regardless the phases of the oestrous cycle (*Fig. 1C-1D*).

The numbers of lymphocytes were significantly higher ($P<0.05$) in all parts of the reproductive tract during the follicular phase as compared to the luteal phase (Table 1). The highest lymphocyte count was recorded in the posterior horn for both groups, while the least count was recorded in the oviduct of both groups. The increased neutrophils and lymphocyte counts during the follicular phase might be due to their active involvement in the phagocytosis of sperms following mating (Bischof *et al.*, 1994). Moderate to high densities of leukocyte infiltrations were observed in the uterus of the non-pregnant mice, and reached the minimum

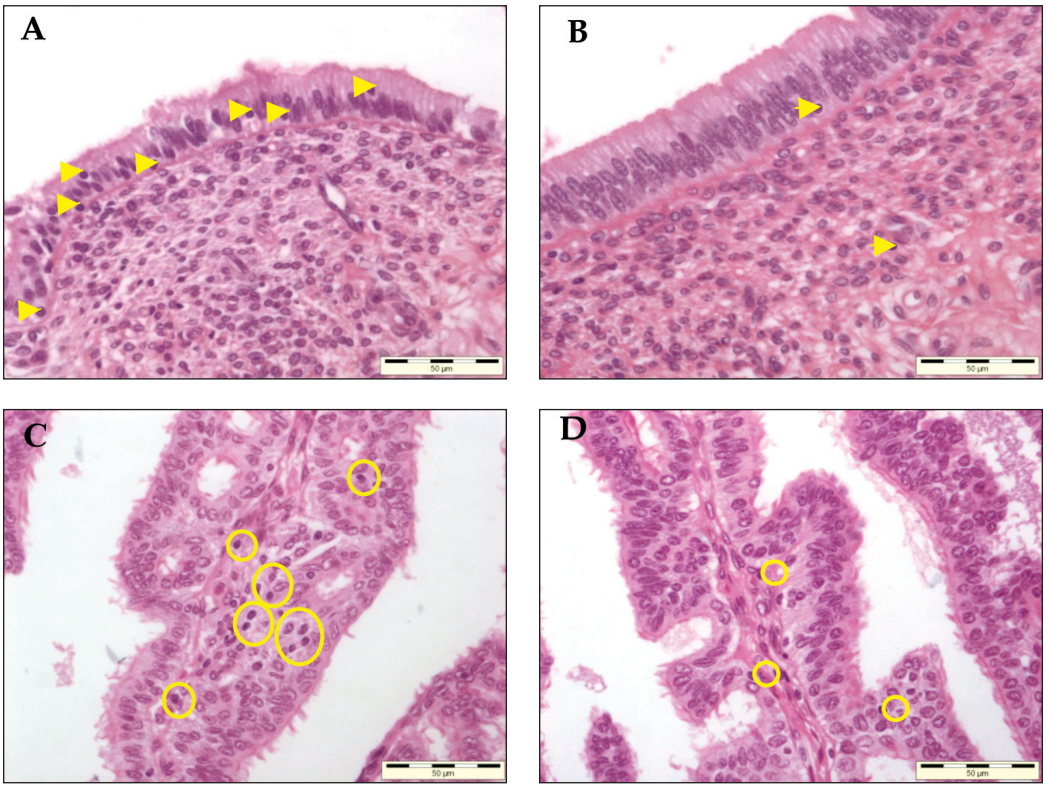

Fig. 1: Photomicrographs of the posterior horn of the uterus during follicular phase (A) luteal phase (B), and in ampulla of follicular (C) and luteal phase (D) showing the distribution of lymphocytes near the border between the propria-submucosa and the epithelium (H&E, X400). Note the remarkable difference in the lymphocyte population (H&E, X400)

TABLE 1
Total count of lymphocytes (number of cells per mm^2) at different parts of the reproductive tract during follicular and luteal phases of ewes

Different parts of the reproductive tract	Follicular phase group (mean ± S.E.)	Luteal phase group (mean ± S.E.)
Posterior horn	114.80 ± 20.00*	49.60 ± 2.44[b]
Middle horn	65.37 ± 2.62[a]	43.25 ± 3.32[b]
Anterior horn	59.00 ± 5.20[a]	24.25 ± 2.39*
Oviduct	31.10 ± 3.37*	22.50 ± 2.98*

[ab]Means with similar superscripts within the columns did not differ significantly at p=0.05
*Means with asterisks within columns differed significantly at p=0.05. (n=14)

during pregnancy. However, the infiltrations were abundant during post-parturient as it is important in the removal of the placental debris and bacterial contamination (Zamri-Saad, 1987).

The Population of Plasma Cells in the Uterus and Oviduct

Similar to the lymphocytes, the plasma cells were mostly distributed within the luminal epithelium (*Fig. 2A*). However, they were sparsely distributed and rarely found in the glandular epithelium. The plasma cells were localized mostly in the areas of the stroma, preferentially near to the glands beneath these epithelia (*Fig. 2B*). In contrast, the numbers of the plasma cell counts were much lower than lymphocytes, especially in the oviduct (ampulla region), as presented in Table 2.

There were significant differences (P<0.05) between the two phases in each part of the reproductive tracts. The number of the plasma cells was found to be highest in the posterior horn in both groups, followed by the middle, anterior horn and the least in the oviduct (P<0.05). Meanwhile, the mean number of the plasma cells was significantly different in all the parts of the reproductive tract during the follicular phase (P<0.05). During the luteal phase, however, the number was not significantly different between the posterior and middle horns, as well as between the anterior horns and the oviduct (P>0.05). The number of plasma cells was found to decrease from the posterior horns (highest) to the oviduct (lowest) for both the phases.

A gradual decrease in the population of these cells, from the posterior horn progressing

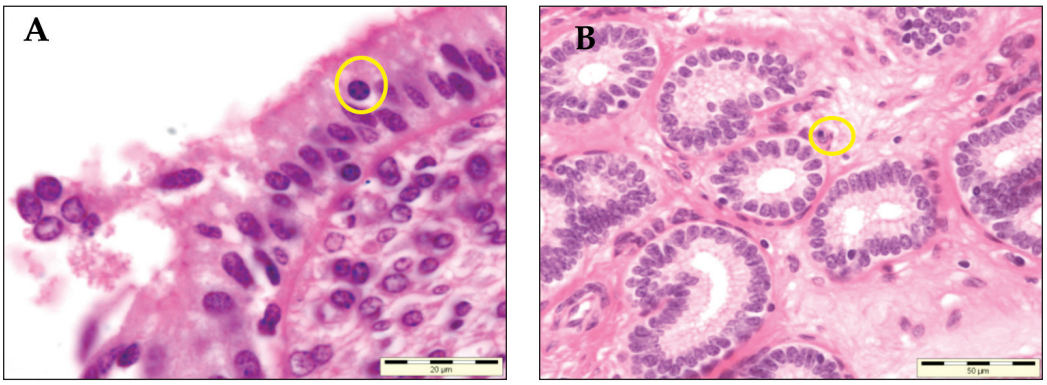

Fig. 2: Photomicrographs show the plasma cell at high magnification located in the luminal epithelium (A) (H&E X1000) and near the gland within the endometrium (H&E, X400)

TABLE 2
The average number of plasma cells (number of cells per mm^2) at the different parts of the reproductive tract during the follicular and luteal phases of ewes

Different parts of the reproductive tract	Follicular phase group (mean ± S.E.)	Luteal phase group (mean ± S.E.)
Posterior horn	5.70 ± 0.47*	2.00 ± 0.33[a]
Middle horn	3.00 ± 0.36*	1.70 ± 0.26[a]
Anterior horn	1.70 ± 1.11*	0.80 ± 0.24[b]
Oviduct	0.50 ± 0.12*	0.30 ± 0.15[b]

[ab]Means within the columns with same superscripts did not differ significantly at $p<0.05$
*Means with asterisks within columns differed significantly at $p=0.05$. (n=14)

towards the anterior horn, would probably signify the need for pathogen surveillance. Thus, such a phenomenon would have existed for two main reasons. First, infection usually stems from the exterior, i.e. via the vagina into deeper parts of the tract. Here, lies the need for a much more vigil inflammatory response and surveillance. However, the lesser number of the cells in the oviduct reflects the least contaminated area and prevention of unnecessary phagocytic activity especially during implantation. Alternatively, environmental modifications of leucocytes also have major pregnancy-associated functions that include facilitation of implantation, modulation of maternal uterine vasculature, supply of growth factors to the placenta, promotion of trophoblast differentiation and facilitation of parturition (Hunt *et al*., 2000). Furthermore, the fewer plasma cells in the Fallopian tube and endometrium than lower down the tract might be due to the lack of antigenic stimulation in the sterile environment of the upper tract (Brandtzaeg, 1997; Vaerman and Ferin, 1974).

Similar findings in the different parts of the sow oviduct were observed, whereby it was found to be significantly lower from the lower part to the upper portion. This finding indicates different immune functions within various parts of the oviduct (Jiwakanon *et al.*, 2005). Hussain *et al*. (1983) revealed that there is a cyclic variation in the distribution of plasma cells in the reproductive tract of the sow and possibly ewes, whereby oestrus is accompanied by a general increase in the number of plasma cells, whereas during dioestrus the cell counts were usually low. The reduction in the number of plasma cells during the luteal phase may reflect the amount of antibody production. When the production of antibody is lesser, the immune system will be impaired and the ability of the animals to combat infections will be lower.

Progesterone is responsible for slowing infiltration of leukocytes into the uterus at the luteal phase. This suggests that the difference in bactericidal activities between the follicular and luteal phases is closely related to the difference in the infiltrating rate of leukocytes into the uterus (Matsuda *et al*., 1985). Progesterone has been shown to affect activities of immune cell directly or indirectly. Directly, lymphoid tissue has receptors for progesterone and indirectly, progesterone induces the synthesis of uterine proteins that eventually inhibit the proliferation of lymphocytes (Staples *et al*., 1983; Low and Hansen, 1988) and neutrophil activity (Seals *et al*., 2003). Furthermore, progesterone induces the presence of γδ T cells which actually suppresses the lymphocytes (Majewski *et al*., 2001).

In contrast, oestrogen was found to increase uterine blood flow (Dickson *et al*., 1969), and this might contribute to the increasing numbers of these cells infiltrated on the uterine and adjacent tissues. In the endometrium, a higher level of oestrogen during the follicular phase would increase the permeability of blood capillaries (Keys, 1988) and this might contribute to the higher population of immune cells infiltrated to the adjacent tissues.

CONCLUSIONS

Hormonal changes during the oestrous cycle influence the immune cells pattern in the ewe's reproductive tract, particularly in the uterus and oviduct. In more specific, progesterone inhibits lymphocyte proliferation that resulted in the lesser number of lymphocytes population in the uterus during the luteal phase. These findings corresponded with the previous studies on other various species, such as porcine (Kaeoket *et al*., 2001; Jiwakanon *et al*., 2005; Hussain *et al*., 1983), bovine (Cobb and Watson, 1995), caprine (Perez-Martinez *et al*., 2002) and ovine (Segerson *et al*., 1991). During the oestrous cycle, the period of the luteal phase is longer than the follicular phase. This study has shown that the ewes were much more protected when they were in the follicular phase since the number of lymphocytes and plasma cells were found to be higher. Thus, the ewes would be more susceptible to infection during the luteal phase and this might have to be considered when diagnosing pathological cases.

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Immunolocalization of 3β-Hydroxysteroid Dehydrogenase (3β-HSD) in the Testis of Lesser Mouse Deer (*Tragulus javanicus*)

Sriyanto[1], Zamri-Saad, M.[1*], Agungpritono, S.[2], Zuki, A.B.S.[1] and Wahid, H.[1]
[1]Department of Veterinary Preclinical Sciences,
Faculty of Veterinary Medicine, Universiti Putra Malaysia,
43400 UPM, Serdang, Selangor, Malaysia
[2]Faculty of Veterinary Medicine, Bogor Agriculture University, Bogor, Indonesia
[*]E-mail: zamri@vet.upm.edu.my

ABSTRACT

The present study describes the location of 3β-hydroxysteroid dehydrogenase in the testis of lesser mouse deer (*Tragulus javanicus*) using immunoperoxidase technique. Testicular samples were obtained from 4 adult male lesser mouse deer; two samples were collected during the dry and the other 2 samples during the wet season. All the samples were subjected to immunoperoxidase staining technique to determine the localization of 3β-hydroxysteroid dehydrogenase (3β-HSD), an enzyme that plays a central role in biosynthesis of steroid hormones, including androgen and oestrogen and an indicator for spermatogenesis activity. The positive reactions were observed in the Leydig, Sertoli, and spermatogonia cells of all animals. The number of positive Leydig cells was significantly ($p<0.05$) higher in the testes sampled during the dry season while the number of positive Sertoli cells was significantly ($p<0.05$) higher in the testes of animals that were sampled during the wet season. However, the total number of cells showing positive reactions remained insignificantly ($p<0.05$) different in all the animals.

Keywords: 3β-Hydroxysteroid dehydrogenase (3β-HSD), lesser mouse deer

INTRODUCTION

Lesser mouse deer (*Tragulus javanicus*, family Tragulidae) is the smallest ruminant that inhabits the tropical rain forest of the Southeast Asia (Medway, 1969). Currently, its population is decreasing. In order to prevent its extinction, conservation and breeding strategies which include management and knowledge on the reproductive system are important. However, only limited information on the reproductive physiology of the male lesser mouse deer is currently available.

In male animals, the pattern of reproductive activity can be observed by measuring the testosterone levels (Payne and Youngblood, 1995). It is important to note that the synthesis of steroid hormones, such as androgen (testosterone) and oestrogen, requires the activity of steroidogenic enzymes like cholesterol side chain cleavage cytochrome (P450scc), 3ßhydroxysteroid dehydrogenase (3ßHSD), and cytochrome 17-a hydroxylase (P450c17) (Bhasin *et al*., 2003; Donnel, 2003).

The enzyme 3 β-hydroxysteroid dehydrogenase/isomerase (3β-HSD) catalyses an essential step in the biosynthesis of steroid hormones, which include androgen and oestrogen production, and it is widely distributed

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[*]Corresponding Author

in the peripheral steroid target organs (Vidal *et al.,* 2000; Prisco *et al*., 2007). Nonetheless, the distribution of 3β-HSD enzyme in mammals is not only expressed in the steroid-producing organs (Penning, 1997), it is also found in several peripheral tissues including the skin (Dumont *et al*., 1992), breast and liver (Zhao *et al*., 1990, Zhao *et al*., 1991), lung (Milewich *et al*., 1977), kidney (Bain *et al*., 1991), endometrium (Bonney *et al*., 1985), prostate (El-Alfy *et al*., 1999), and brain (Ukena *et al*., 1999).

Thus, investigation on the immunolocalization of 3β-hydroxysteroid dehydrogenase can be used to characterize the sites of biosynthesis of androgen intervening in spermatogenesis. This is because the knowledge on the distribution and appearance of steroidogenic enzymes in the testis will lead to a better understanding of the synthesis and proportion of steroid hormones in a species. This study investigated the locations of 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme, which is important in biosynthesis of androgens within the testis of male lesser mouse deer.

MATERIALS AND METHODS

Animals

Four sexually mature male lesser mouse deer (*Tragulus javanicus*), that were kept individually in single cages, were used in this study. Unilateral castration was made on each animal; two were during the wet season of October, while the remaining two were done during the dry season of July. All the experiments were conducted in accordance to the Ethical Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Immunohistochemistry

The testicular samples were fixed for 24 h in 8 ml of Bouin's fixative, followed by 70% ethanol until processing. The testicular samples were sectioned between 3 and 4 µm, de-waxed, rehydrated in graded ethanol, and washed in distilled water. The sections were subjected to antigen retrieval by microwaving in 0.01 M citrate buffer (pH 6) on a full power (boiling point) for 10 min. After that, the sections were allowed to cool at room temperature for 30 min before they were washed, three times for 5 min each with distilled water and subsequently incubated in 3% H_2O_2 in PBS for 10 min to block endogenous peroxidase. After 3 washes for 5 min in PBS, the sections were preincubated with blocking solution 3% normal goat serum in PBS for 60 min to prevent non-specific immunostainning with secondary antibody. The sections were washed in PBS (10 min) and incubated with primary antibody, the rabbit polyclonal to 3 β-HSD1 (Abcam Cambridge, UK #ab65156) 1:400 in 1% BSA. The reactions were detected with a biotin-conjugate secondary antibody (Biotinylated anti-rabbit IgG (H+L), Vector, USA # BA-1000) 10 ug/ml in 10 mM Phosphate, pH 7.8, 0.15M NaCl and ABC complex using DAB as chromogen. Counterstaining was performed with Mayer's hemalum. Negative controls were performed by omitting the primary antibody. On the contrary, the positive controls were performed on the paraffin sections of the rats' testis.

The samples were then examined under light microscopy. The numbers of Leydig, Sertoli, and spermatogonia cells found in five microscopic fields at 400 magnifications with positive reactions were counted.

Statistical Analysis

All the data were statistically analysed using student t-test.

RESULTS

Positive reactions were observed in the Leydig, Sertoli and spermatogonia cells (*Fig. 1*), with the most significant ($p<0.05$) positive reactions observed among the Leydig cells of the animals sampled during the dry season. In contrast, significantly ($p<0.05$) more positive reactions were observed in the Sertoli cells of the animals sampled during the wet season (*Fig. 2*). The numbers of spermatogonia, showing positive reactions, were insignificant ($p>0.05$) between the animals that were sampled during the dry

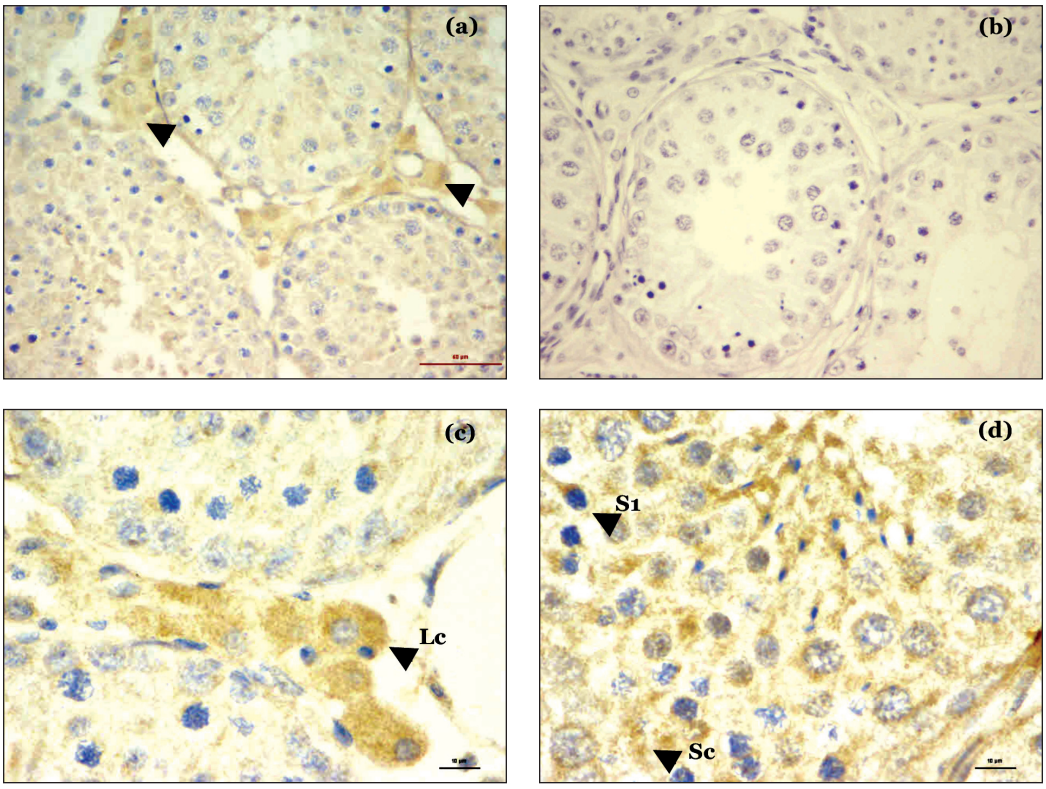

Fig. 1: Photomicrographs showing the immunohistochemistry staining for 3β-hydroxysteroid dehydrogenase (3βHSD) enzyme in the testis of lesser mouse deer. (a) Positive reaction against 3β-HSD and (b) negative sample. (c) The distribution of 3βHSD enzyme in Leydig cells, Lc and (d) in Sertoli cells Sc and spermatogonia S1

or wet season. However, the total numbers of cells showing positive reaction revealed insignificant ($p<0.05$) differences between all the four animals.

DISCUSSION

The present investigation showed that the Leydig, Sertoli and spermotogonia cells were positive to 3 beta-hydroxysteroid dehydrogenase (3β-HSD), a bifunctional enzyme that catalyzes the oxidative conversion of Delta (5)-ene-3-beta-hydroxy steroid and the oxidative conversion of ketosteroid, which also plays a crucial role in the biosynthesis of all classes of hormonal steroids (Kowalewski *et al*., 2006). Meanwhile, immunolocalization of 3β-HSD was observed in all the testicular samples but with different cell involvements during the dry (July) and wet (October) seasons. However, the overall number of the positive cells remained insignificantly different between all the four animals, regardless of the season of sampling. This is correlated with the spermatogenesis process in the testis of lesser mouse deer. In particular, the Sertoli and Leydig cells are differently involved in the hormonal control of spermatogenesis, whereby the Sertoli cells play a role before the beginning of meiosis and after spermiation. The Leydig cells involve in the meiosis phase (Prisco *et al*., 2007). The results of this study strongly suggest that both the Sertoli and Leydig cells are involved in the production of androgen hormone needed for spermatogenesis in the testis of lesser mouse

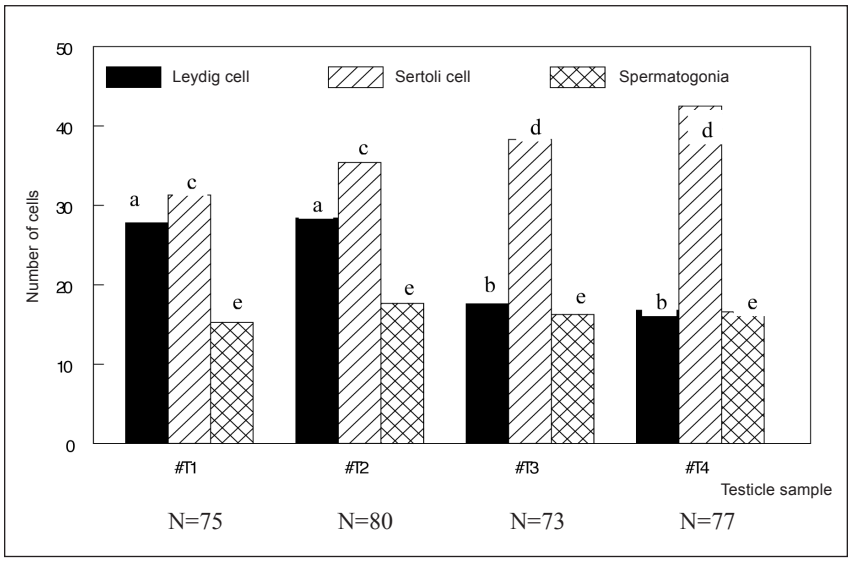

Fig. 2: The number of cells showing positive immunohistochemical reaction for 3β-HSD enzyme. Animals #T1 and #T2 were sampled during the dry season, while animals #T3 and #T4 were sampled during the wet season. Different superscripts indicate significant ($p<0.05$) difference. N=the total number of positive cells for the animals

deer. In addition, the findings also revealed that the Sertoli cells were more prominent than the Leydig cells in controlling and regulating spermatogenic cells, peritubular cells and Leydig cells (Skinner, 1991). Although positive reactions were observed in the spermatogonia, the number was rather low and therefore insignificant.

The findings of this study also suggest that during the dry season, the Leydig cells are more active in producing 3β-HSD enzyme compared to during the wet season. On the other hand, the Sertoli cells are active in producing the enzyme in both the dry and wet seasons. This is correlated well with the concentration of testosterone and mating. Hesterman *et al*. (2005) have reported that during the oestrus cycles, males demonstrate a high level of olfactory interest in their partner's ano-genital area and excreta, affecting the pituitary gland to release luteinizing hormone which regulates testosterone secretion. Subsequently, the relatively high level of testosterone stimulates spermatid maturation (Garner and Hafez, 2000).

In conclusion, this study revealed the presence of immunolocalization of 3β-HSD in the testis of lesser mouse deer in the Leydig cells, Sertoli cells and spermatogenia. Hence, they are considered as androgen-producing cells.

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