Journal of Tropical Agricultural Science

About the Journal

Overview

Pertanika Journal of Tropical Agricultural Science (JTAS) is the official journal of Universiti Putra Malaysia published by UPM Press. It is an open-access online scientific journal which is free of charge. It publishes the scientific outputs. It neither accepts nor commissions third party content.

Recognized internationally as the leading peer-reviewed interdisciplinary journal devoted to the publication of original papers, it serves as a forum for practical approaches to improving quality in issues pertaining to tropical agriculture and its related fields.

JTAS is a **quarterly** (*February, May, August and November*) periodical that considers for publication original articles as per its scope. The journal publishes in **English** and it is open to authors around the world regardless of the nationality.

The Journal is available world-wide.

Aims and scope

Pertanika Journal of Tropical Agricultural Science aims to provide a forum for high quality research related to tropical agricultural research. Areas relevant to the scope of the journal include: agricultural biotechnology, biochemistry, biology, ecology, fisheries, forestry, food sciences, genetics, microbiology, pathology and management, physiology, plant and animal sciences, production of plants and animals of economic importance, and veterinary medicine.

History

Pertanika was founded in 1978. A decision was made in 1992 to streamline Pertanika into three journals as Journal of Tropical Agricultural Science, Journal of Science & Technology, and Journal of Social Sciences & Humanities to meet the need for specialised journals in areas of study aligned with the interdisciplinary strengths of the university.

After 37 years, as an interdisciplinary journal of Agriculture, the revamped Journal, a leading agricultural journal in Malaysia now focuses on tropical agricultural research and its related fields.

Goal of *Pertanika*

Our goal is to bring the highest quality research to the widest possible audience.

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 14 weeks. The elapsed time from submission to publication for the articles averages 5-6 months.

Abstracting and indexing of *Pertanika*

Pertanika is almost 38 years old; this accumulated knowledge has resulted in Pertanika JTAS being abstracted and indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO & EBSCOhost, DOAJ, Agricola, Cabell's Directories, Google Scholar, MyAIS, ISC & Rubriq (Journal Guide).

Future vision

We are continuously improving access to our journal archives, content, and research services. We have the drive to realise exciting new horizons that will benefit not only the academic community, but society itself.

Citing journal articles

The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

Publication policy

Pertanika policy prohibits an author from submitting the same manuscript for concurrent consideration by two or more publications. It prohibits as well publication of any manuscript that has already been published either in whole or substantial part elsewhere. It also does not permit publication of manuscript that has been published in full in Proceedings.

Code of Ethics

The Pertanika Journals and Universiti Putra Malaysia takes seriously the responsibility of all of its journal publications to reflect the highest in publication ethics. Thus all journals and journal editors are expected to abide by the Journal's codes of ethics. Refer to Pertanika's **Code of Ethics** for full details, or visit the Journal's web link at http://www.pertanika.upm.edu.my/code_of_ethics.php

International Standard Serial Number (ISSN)

An ISSN is an 8-digit code used to identify periodicals such as journals of all kinds and on all media–print and electronic. All Pertanika journals have ISSN as well as an e-ISSN.

Journal of Tropical Agricultural Science: ISSN 1511-3701 *(Print)*; ISSN 2231-8542 *(Online)*.

Lag time

A decision on acceptance or rejection of a manuscript is reached in 3 to 4 months (average 14 weeks). The elapsed time from submission to publication for the articles averages 5-6 months.

Authorship

Authors are not permitted to add or remove any names from the authorship provided at the time of initial submission without the consent of the Journal's Chief Executive Editor.

Manuscript preparation

Refer to Pertanika's **Instructions to Authors** at the back of this journal.

Most scientific papers are prepared according to a format called IMRAD. The term represents the first letters of the words **I**ntroduction, **M**aterials and Methods, **R**esults, **A**nd, **D**iscussion. IMRAD is simply a more 'defined' version of the "IBC" [Introduction, Body, Conclusion] format used for all academic writing. IMRAD indicates a pattern or format rather than a complete list of headings or components of research papers; the missing parts of a paper are: *Title, Authors, Keywords, Abstract, Conclusions*, and *References*. Additionally, some papers include Acknowledgments and Appendices.

The *Introduction* explains the scope and objective of the study in the light of current knowledge on the subject; the *Materials and Methods* describes how the study was conducted; the *Results* section reports what was found in the study; and the *Discussion* section explains meaning and significance of the results and provides suggestions for future directions of research. The manuscript must be prepared according to the Journal's **Instructions to Authors**.

Editorial process

Authors are notified with an acknowledgement containing a *Manuscript ID* on receipt of a manuscript, and upon the editorial decision regarding publication.

Pertanika follows a **double-blind peer-review** process. Manuscripts deemed suitable for publication are usually sent to reviewers. Authors are encouraged to suggest names of at least three potential reviewers at the time of submission of their manuscript to Pertanika, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

Journal of Tropical Agricultural Science

Notification of the editorial decision is usually provided within ten to fourteen weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed only on page 2 as described in the first-4 page format in Pertanika's **Instructions to Authors** given at the back of this journal.

The Journal's peer-review

In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts.

Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

Operating and review process

What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are seven steps to the editorial review process:

1. The Journal's chief executive editor and the editorial board examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The chief executive editor sends the article-identifying information having been removed, to three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The chief executive editor asks them to complete the review in three weeks.

   Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the literature.

3. The chief executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Edito-in-Chief, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors return a revised version of the paper to the chief executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor, usually in a tabular form. The author(s) may also submit a rebuttal if there is a need especially when the author disagrees with certain comments provided by reviewer(s).

5. The chief executive editor sends the revised paper out for re-review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the chief executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, an acceptance letter is sent to all the author(s), the paper is sent to the Press. The article should appear in print in approximately three months.

The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any minor queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, **only essential changes are accepted**. Finally, the article appears in the pages of the Journal and is posted on-line.

ABSTRACTING/INDEXING

Pertanika is now over 38 years old; this accumulated knowledge has resulted the journals being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO, DOAJ, Google Scholar, AGRICOLA, ISC, Citefactor, Rubriq and MyAIS.

Contents

Foreword

Welcome to the **Fourth Issue 2016** of the Journal of Tropical Agricultural Science (JTAS)!

The JTAS is an open-access journal on Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university and runs on a non-profit basis for the benefit of the scientific community.

This issue contains **thirteen (13) articles** of which three are review articles, one a short communication while nine are regular papers. The authors of these articles hail from different countries, namely **Malaysia**, **Nigeria**, **India**, **Iraq**, **Iran**, **Pakistan, Japan** and **Indonesia**.

The first review paper briefly discusses on the major components available in fish immunity, such as the adaptive and innate immunity (*M. Firdaus-Nawi* and *M. Zamri-Saad*). The second review paper focuses on the ichthyofauna of the peat swamp forests (PSFs) of Malaysia. It provides a checklist of blackwater fish species in Malaysia from published literature, and addresses their importance to the economy, their conservation status and problems of PSFs (*Sule, H. A., Ismail, A.* and *Amal, M. N. A.*). The third review paper discusses impact of heat stress on Immune responses of livestock as well as the impact of climate change on the immune function of the animal (*Sophia Inbaraj, Veerasamy Sejian, Madiajagan Bagath* and *Raghavendra Bhatta*). In the short communication, the authors provide a report, comparing the apparent metabolisable energy (AME) of three oil sources, namely palm oil (PO), soybean oil (SO), linseed oil (LO) and blend oil (BO), and their combination in broiler chickens diet (*N. R. Abdulla, T. C. Loh, H. Akit, A. Q. Sazili* and *H. L. Foo*).

The nine research articles cover a wide range of topics: A report on the stability analysis of panicle and grain traits of rainfed upland rice in two tropical ecologies of Nigeria (*Nassir, Adesola L.* and *Alawode, Yetunde O.*); the effect of cultivation techniques on the growth rate of marine microalgae species in the coastal waters of Kudat, Sabah, Malaysia (*Nurzafirah Mazlan* and *Ridzwan Hashim*); production and characterisation of cellulose from solid state fermentation of rice straw by *Trichoderma harzianum* SNRS3 (*Rahnama, N., Shah, U. K. M., Foo, H. L., Rahman, N. A. A.,* and *Ariff, A.*); effects of higher density planting on irrigation water use and yield of sugarcane (*D. Khodadadi Dehkordi*);

effects of feeding different levels of low crude protein diets with different levels of amino acids supplementation on layer hen performance (*M. Tenesa, T. C. Loh, H. L. Foo, A. A. Samsudin, R. Mohamad* and *A. R. Raha*); characterisation of plant growth-promoting bacteria from Kacip Fatimah (*Labisia pumila*) under natural tropical forest (*Yasmi, A., Radziah, O., Hawa, ZE. J., Zainal Abidin, M. A.* and *Qurban, A. P.*); impact of daily supplement of probiotic on the production performance of Akar Putra chickens (*Hasan, S. A. Jawad, Lokman, I. H., Saad, A. Naji, Zuki, A. B. Z.* and *Kassim, A. B.*); the effects of fermented feed on broiler production and intestinal morphology (*Saad A. Naji, I. F. B. Al-Zamili, Hasan, S. A. Jawad* and *J. K. M. Al-Gharawi*); and characteristics and potential usage of dissolved silica in rice cultivation in Sumani watershed, Sumatra, Indonesia (*Hiroaki Somura, Drmawan, Kuniaki Sato, Makoto Ueno, Husnain, Aflizar* and *Tsugiyuki Masunaga*).

You will find the discussions intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend this journal to your colleagues and students.

I would also like to express my gratitude to all the contributors, namely the authors, reviewers and editors, who have made this issue possible. Last but not least, we record our appreciation to the staff at the journal division for their editorial assistance.

The JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry.

Chief Executive Editor
Nayan Deep S. KANWAL, FRSA, ABIM, AMIS, Ph.D.
nayan@upm.my

Review Article

Major Components of Fish Immunity: A Review

M. Firdaus-Nawi and M. Zamri-Saad*

Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Fish are fascinating creatures with a certain degree of immunity comparable to those of mammals. The fish's immune system consists of two major components, innate and adaptive immunities. Innate immunity is non-specific and acts as the primary line of protection against pathogen invasion while adaptive immunity is more specific to a certain pathogen/ following adaptation. Innate immunity consists of the non-specific cellular and the non-specific humoral components. The non-specific cellular component consists of toll-like receptors (TLRs), macrophages, neutrophils, eosinophils and non-specific cytotoxic cell while the non-specific humoral component involves lysozyme, the complement, interferons, C-reactive proteins, transferrins and lectins. They work together at the initial stage to prevent pathogen invasion. On the other hand, the adaptive immune system consists of highly specilised systemic cells and processes that are separated into two main components: the humoral and cellular components. Three types of antibodies, the IgM, IgD and IgT, are the major constituents of the humoral immunity, which act on invaded extracellular pathogens. The cytotoxic T-lymphocyte cells are the major component of the cellular immunity that frequently kills virus-infected and intracellular bacterial or parasitic-infected cells. Both innate and adaptive immunities complement each other in the host's attempt to prevent infection.

Keywords: Immunity, component, mechanism, fish

INTRODUCTION

The fish is an organism that comes in various shapes, and the general picture of a fish is of a water creature with scales that coat the whole body and which has fins to swim. However, these are not always true

since some species of fish lack scales such as clingfish, while others lack fins, such as the eel. Furthermore, some fish like the lungfish can survive for several hours outside of water. Berra (2001) defined fish as a poikilothermic chordate with gills as the major respiratory organ. Scales and/or mucus protects the whole body.

Fish are classified in the paraphyletic group of creatures that comprises all gill-bearing aquatic vertebrates lacking limbs with digits. Thus, hagfish, lamprey and cartilaginous and bony fish and the various extinct related groups are included in this class. Generally, fish are cold-blooded or ectothermic i.e. its body temperature follows the ambient temperature, and some giant fish such as tuna and white shark are able to hold a higher core temperature (Carey & Lawson, 1973; Goldman, 1997). Fish live in various water bodies of the world and they have been discovered in almost all watery environments, from the streams of high mountains, where gudgeon and char may be found to the deepest oceans where anglerfish and gulpers live. Thus, fish demonstrate the greatest species diversity compared to other classes of vertebrates on earth. So far over 32,000 species of fish have been identified (Fish Base, 2011).

The immune system is a vital physiological mechanism that prevents infection and preserves internal homoeostasis. Therefore, the immune system acts as a shield to the fish and provides protection from attacks by a broad spectrum of invading microorganisms. The system has various specialised organs designed to detect and react against any microbe that enters the host by mobilising cells and molecules in the blood stream. Failure of the system to react leads to immunodeficiency while over-reacting against foreign microbes causes autoimmunity that can cause tissue damage. Principally, the immune system is regulated by sophisticated and complicated mechanisms (Lydyard et al., 2000) and any failure leads to infection, disease and death. This review describes the major components of the fish immune system as well as its working mechanism in protecting the fish from invasive pathogens.

THE FISH IMMUNE SYSTEM

Similar to mammals, the fish immune system is built with two major parts i.e. the innate and adaptive immunities. Innate immunity reacts to invading pathogens by recognising the germ's line-encoded molecules. TLRs and phagocytosis are the key components of innate immunity that protect the host against foreign invaders by recognising and finally destroying the phagocytised cells (Silva et al., 2002). Adaptive immunity, on the other hand, recognises pathogens via molecules that are generated by somatic mechanisms (Medzhitov & Janeway, 1997) followed by humoral and cellular responses via B- and T-lymphocytes (Dixon & Stet, 2001).

The immune organs vary with the type of fish (Zapata et al., 1996). Lamprey, hagfish and other jawless fish lack a true lymphoid organ. They depend only on the lymphoid tissues that are found within other non-lymphoid organs. Thus, plasma cells, macrophages and erythrocytes are

fabricated within the pronephros or anterior kidney and certain parts of the gut, where maturation of the granulocytes occurs. A more advanced immune system is observed in cartilaginous fish such as rays and sharks. Plasma cells, lymphocytes and granulocytes are housed in Leydig's organ, the spiral valve of the intestine and the epigonal organs. These lymphoid organs are unique to the chondrichthyes and do not exist in other types of fish. Thymus and spleen, the vital immune organs where lymphocytes, plasma cells and macrophages are stored and mature are also found in cartilaginous fish. However, for paddlefish, sturgeon and bichir of the chondrostean sub-class, the major site for fabrication of granulocytes is located within the meninges. Covering the heart of the chondrostean is a tissue that comprises reticular cells, lymphocytes and some macrophages. Nevertheless, the macrophages, granulocytes, erythrocytes and lymphocytes develop inside the kidney, an important hemopoietic organ of chondrostean.

Similarly, the vital hemopoietic organ for bony fish or teleost is the kidney, specifically, the anterior kidney where various types of immune cells develop (Anderson, 1977). The teleost fish also have lymphoid organs in the form of the spleen, thymus and the scattered lymphoid cells found within the mucosal tissues such as the gonads, gut, skin and gills. Lymphocytes are the major immune cells in the thymus as well as neutrophils, erythrocytes and granulocytes (Chilmonczyk, 1992). Zebrafish are one of the teleost species reported to have a similar

lymphatic system as mammals (Kutcler et al., 2006).

Innate Immunity

The innate immune system is the first layer of host defence against pathogenic organisms or invaders. It responds in a non-specific manner before the specific adaptive immune system is ready to take over the defence work (Holland & Lambris, 2002). Unlike the adaptive immune system, the innate immune system identifies and reacts to invaders in a general manner. Thus, the protection provided is shorter and weaker than that provided by adaptive immunity (Alberts et al., 2002).

In fish, the innate immune response is a crucial initial component in preventing infection due to slow lymphocyte proliferation and a limited antibody repertoire that leads to a delay in the adaptive immune response (Magnadottir, 1998). Therefore, the innate immune response acts as an alarm that allows the adaptive immune system time to mount a response (Fearon & Locksley, 1996).

The innate immunity is subdivided into cellular and humoral immune responses. The cellular immune response provides a physical barrier in the form of mucus and epithelial cells that line the skin, gills and stomach, responsible for preventing invasion of microorganisms into the body. If the pathogen passes these barriers, specialised cells like granulocytes, monocytes or macrophages and the non-specific cytotoxic cells are ready to kill and digest the pathogens. Non-specific immune cells are

recruited into the site of infection primarily by inflammatory cytokines. The humoral component of the innate immune system employs a wide variety of proteins and glycoproteins that are capable of destroying or inhibiting the growth of microorganisms (Aoki et al., 2008).

Non-specific cellular immunity. Many types of leukocyte are involved in the innate, non-specific cellular immunity of fish. They include toll-like receptors (TLRs), granulocytes, macrophages and non-specific cytotoxic cells (NCCs). TLRs are the small protein molecules that have ability to recognise the conserved molecules of microbes. Granulocytes and macrophages are mobile phagocytic cells found circulating in the blood and within the secondary lymphoid tissues. Both cells play vital roles in inflammatory reaction, which actually is the cellular immune response to any invaders or tissue injuries. Eosinophilic granular cells (EGCs) are less mobile granulocytes that target parasites. EGCs are the host's innate cellular immune response against helminth infestation at the mucosal sites such as the gut and gills. Similarly, protozoa and virus-infected host cells are the targets for NCCs, making them appear in mucosal sites, blood circulation and lymphoid tissues. They are able to spontaneously kill the affected cells through apoptotic and necrotic mechanisms (Secombes, 1996).

Innate immunity lacks specificity to the pathogen, thus making innate immunity cells to mobilise quickly in large numbers. Unlike the specific immune system, there is no memory component in non-specific innate immunity. Therefore, exposure to a similar pathogen does not lead to better and quicker secondary immune response. However, the cells that are involved in the non-specific cellular immunity may interact with the cells of the adaptive immunity system and can be recruited by them or their products (Secombes, 1996).

Toll-like receptors (TLRs). Toll-like receptors (TLRs) are one of the vital components of innate immunity. They are able to recognise the pathogen's unique molecules. The word 'toll' originated from vernacular German and means fantastic or super (Chtarbanova & Imbler, 2011). Nu¨sslein-Volhard first discovered TLRs in the early 1980s following his mutagenesis studies of the fruit fly *Drosophila melanogaster* (Anderson et al., 1985). However, some years later in 1996 Lemaitre et al. (1996) discovered that this receptor also played major roles in adult-fly immunity as well as a key role in the mammal's innate immune system (Chtarbanova & Imbler, 2011).

The first report of TLRs in fish was by Stafford et al. (2003) in goldfish, *Carassius auratus auratus*, followed by Oshiumi et al. (2003) in pufferfish, Jault et al. (2004) in zebrafish while Takano et al. (2011) identified 11 types of TLR homologues in Japanese flounder (*Paralichthys olivaceus*). As in mammals, TLRs of fish work by recognising the unique conserved molecules of the microbes, known as pathogen-associated molecular patterns (PAMPs). This recognition stimulates an inflammatory response that initiates the innate immunity (Akira et al., 2006).

Macrophages. Macrophages are mononuclear, non-specific esterase positive and peroxidase-negative leukocytes. They are avidly phagocytic and emit nitrogen-free radicals and oxygen that kill various pathogens (Secombes, 1990). Macrophages have both complement and antibody (Fc) receptors (Secombes & Fletcher, 1992) and express the class II MHC molecules (Secombes, 1994). Since they belong to cellular innate immunity, macrophage-specific antibodies are not being made in fish, although antibodies to a related cell in the brain (the glial cells) exist (Dowding et al., 1991). Their actions usually rely on another immune component, the antibody (Thuvander et al., 1992).

Production of macrophages in fish occurs during primary hematopoiesis. The resident populations are self-maintained with contribution from monocytes that are circulating in blood to mature as tissue macrophage (Hodgkinson et al., 2015). Macrophages are commonly found in both layers of fish thymus, the cortex and medulla. Three kinds of macrophages have been observed in the thymus of the teleost, the melanomacrophages (Gorgollon, 1983; Pulsford et al., 1991), the monocytes (Castillo et al., 1990) and the multinucleated giant cells (Pulsford et al., 1991). Macrophages are strongly positive for non-specific esterase, acid phosphatase and 5'-nucleotidase (Castillo et al., 1990). Other than in the thymus, macrophages are also found scattered throughout the area in between the inner and outer zones of the pharyngeal epithelium and in the lymphoid organs, blood and peritoneal cavity (Secombes, 1990).

Granulocytes. Fish granulocytes have a distinctive structure and are sometimes referred to as the polymorphonuclear (PMN) leukocytes. Their cytoplasm contains numerous fine granules that give rise to the three types of granulocytes, the neutrophils, basophils and eosinophils. The neutrophil is stainable by neutral dyes but has no affinity for acidic or basic dyes. It is the most abundant granulocyte that migrates from blood into the affected tissues to engulf bacteria. On the other hand, acid dyes like eosin are suitable for staining the eosinophil that plays a primary role in allergic inflammatory reactions as well as destruction of internal parasites. Finally, basophil can be stained with basic dyes and is found only in low numbers (Secombes, 1996).

The granulocytes are distributed differently in the different parts of fish. Fletcher (1986) concluded that many factors influence the distribution of granulocytes in blood, tissues and other body fluids. These factors include season of the year, disease, environmental pollutants and the various stressors. Lowe-Jinde (1986) and Steinhagen et al. (1990) supported this and revealed an increased numbers of leukocytes, especially the granulocytes following infection. Lamas and Ellis (1994) reported that the numbers of granulocytes in the blood were greatly increased within 24 hours of stressing the fish. As macrophages, the granulocytes can also be isolated from the lymphoid tissues, blood and the peritoneal cavity (Lamas & Ellis, 1994).

Pertanika J. Trop. Agric. Sci. 39 (4): 393 - 420 (2016)

397

Non-specific cytotoxic cells. Non-specific cytotoxic cells (NCC) of fish are considered to be similar to that of mammalian natural killer (NK) cells. They share several similarities, particularly the competent lytic cycle, the target cells for lysis, recognition of target cell and the effecters to lyse the infectious microorganisms (Jaso-Friedmann et al., 1993). However, there are also differences, which include the kinetics of killing and the morphology and specificity of the target cells (Evans & Jaso-Friedmann, 1992). Studies on NCCs of teleosts found that they tend to target various cells including tumor cells, virus-transformed cells and some protozoa (Whyte, 2007). NCCs are reported to be most active in the head of kidney of teleosts but spleen and peripheral blood leukocytes (PBL) also demonstrate the cytolytic abilities (Evans et al., 1984). In sharks, however, macrophages are the cells that are responsible for spontaneous cytotoxicity (McKinkey et al., 1986).

Non-specific humoral immunity. Teleost fish have been shown to have substances of non-specific humoral defence. These substances include the lysozyme, alkaline phosphatase, complement, interferon, C-reactive protein, transferrin, lectin and several other substances. They are extremely important for fish (Ingram, 1980) and play significant roles in maintaining homoeostasis (Saurabh & Sahoo, 2008).

Lysozyme. Lysozyme involves in mediating defence against invasion by pathogens. It is one of the major substances in the saliva, mucus and blood of vertebrate.

Lysozymes are also broadly distributed in invertebrates, bacteriophages, microbes and plants (Jollès & Jollès, 1984). Lysozyme is a leukocyte-released enzyme and has a broader activity in fish compared to in mammals (Demers & Bayne, 1997). It has an antibiotic effect and is normally used as an indicator of non-specific immune functions.

Neutrophils are the major producers of lysozyme in fish (Ellis, 2001) but monocytes can also produce lysozyme (Fletcher & White, 1973). Therefore, fish lysozyme is mainly distributed in the leukocyte-rich organs, especially the head kidney and at sites of antigenic invasion such as the gills, skin, gastro-intestinal tract and eggs (Murray & Fletcher 1976; Lie et al., 1989). Lysozyme is also detected in the body mucus, peripheral blood and various tissues of both freshwater and marine fishes (Fletcher & Grant, 1968; Ebran et al., 2000; Fagan et al., 2003).

Lysozyme works by further disrupting the bacterial cell wall after an earlier disruption of the outer wall by the complement and other enzymes (Yano, 1996; Saurabh & Sahoo, 2008). Therefore, fish lysozyme attacks the lipopolysaccharide layer leading to damage of the outer cell membrane, allowing additional lysozymes to reach and injure deeper structures (Day et al., 1978; Iacono et al., 1980), increasing permeability that results in the loss of cell viability without lysis. Therefore, fish lysozyme has substantial antibacterial activity over the mammalian lysozymes against both Gram-positive and Gram-

negative bacteria (Itami et al., 1992). Furthermore, lysozyme plays an important role in preventing vertical transmission of some bacterial pathogens of fish (Yousif et al., 1994).

Alkaline phosphatase. Alongside with lysozyme, alkaline phosphatase (AP) is also an important enzyme in fish, especially in their innate immune system. It is a lysosomal enzyme and can be found in various body secretions such as body mucus, intestinal mucus and blood serum (Nigam et al., 2012). Concentration of AP increases when the host in stress making it as a potential stress indicator (Ross et al., 2000). Fast et al. (2002) in their study of Atlantic salmon found that the activity of mucus AP increases following parasitic infections and suggested that AP is one of the important enzymes in the innate immune system. Another study on catfish indicates high activity of AP during skin regeneration due to wound healing, demonstrating the role of AP as a protective enzyme (Rai & Mittal, 1983).

The complement. The complement is one of the major mechanisms of the humoral component of the immune system. It is involved in both initiation of the innate immune response and mounting of an adaptive immune response (Alvarez-Pellitero, 2008; Nakao et al., 2011) using its more than 35 soluble proteins (Sunyer & Lambris, 1999; Gasque, 2004). The complement works via a combination of three pathways: the alternative, the lectin and the classic pathways. The alternative pathway is active in the serum of fish than in that of mammals (Yano, 1996), and is important in the defence mechanism of fish (Ellis, 2001; Holland & Lambris, 2002). The classic pathway is more common in mammals, involving the formation of a complex blend of antigen and antibody (Gasque, 2004). This pathway is activated by the binding of the Fc portion of the IgG to the C1q component of the C1 complex (Muller-Eberhard, 1986; Kishore & Reid, 2000; Pangburn & Rawal, 2002). The lectin pathway requires interaction between lectins of the complement with sugar moieties found on the surface of microbes (Turner, 2003; Fujita et al., 2004), activating lectin-associated enzymes, the MBL-associated serine proteases (MASPs) that enhance the complement activation (Chen & Wallis, 2004). Microbes that are fixed with the complement are readily phagocytosed and lysed by the macrophages or the cytotoxic cells.

Interferons. Interferons (IFNs) are potent cytokines that act as key effectors of antiviral activity in the vertebrates (Castro et al., 2008). They are secreted proteins or glycoproteins that induce antiviral capability in cells and defends against virus infection by inhibiting viral replication (Yano, 1996; Samuel, 2001). IFN-like activity was first detected in fish in 1965 and has since been detected in cells and organs of many fish species infected with virus (Robertsen, 2006). The first IFN gene of fish was cloned in 2003 (Robertsen et al., 2003). Nevertheless, IFNs production has been confirmed in bony but not cartilaginous fish (Yano, 1996).

It is now established that fish cells secrete IFN-α and IFN-β molecules in response to virus infection (Kelly & Loh, 1973; Rio et al., 1973; Okamoto et al., 1983; Snegaroff, 1993). Type I IFNs are involved in the first line of defence against virus infection (Robertsen, 2006). They have five exon and four intron genes that are not found in the classic type I IFNs of birds and mammals (Lutfalla et al., 2003; Robertsen et al., 2003). Now, fish type I IFNs has been shown to have the same exon/intron structure as the IL-10 and IFN-λ gene families (Lutfalla et al., 2003).

C-reactive protein. C-reactive protein (CRP) is the first protein to exist in the blood plasma of humans and most animals as a response to tissue damage, infection and inflammation. It was first found reacted with the C-polysaccharide (CPS) of *Pneumococcus* bacterium in the serum of patients with acute inflammation, and was thus named C-reactive protein.

The liver, in response to factors released by fat cells, synthesises CRP. It is a member of the pentraxin family of proteins (Pepys & Hirschfield, 2003) and was the first pattern recognition receptor (PRR) to be identified (Mantovani et al., 2008). Since the first discovery in 1930, CRP has been found in many animal species, horseshoe crab and mollusk, *Achatina fulica* (Yano, 1996). Baldo and Fletcher (1973) reported CRP that binds to pneumococcal CPS in plaice serum. CRP has also been isolated from the smooth dogfish, *Mustelus canis* (Robey & Liu, 1983), Japanese eel (Nunomora, 1991), channel catfish (Szalai et al., 1994),

rainbow trout (Winkelhake & Chang, 1982; Murai et al., 1990), lumpsucker, *Cyclopterus lumpus* (Fletcher & Baldo, 1976; White et al., 1978), tilapia, *Tilapia mossambica* (Ramos & Smith, 1978) and murrel fish (Mitra & Bhattacharya, 1992). However, there are fish species that lack CRP such as flounder, *Platichthys flesus* while bacterial endotoxin (LPS) was found to be able to stimulate the production of CRP following exposure to fish (White et al., 1981; White & Fletcher, 1985).

Transferrin. Transferrin (Tf) is a multi-functional protein or bi-lobed monomeric iron-binding glycoprotein actively involved in iron metabolism that is associated with innate immune response (Garcia-Fernandez et al., 2011). The primary role of Tf is transporting iron in a safe state from absorption, utilisation or storage sites around the body (Gomme & McCann, 2005). Although iron is a vital element for growth and survival, excess free iron is toxic to the cells (Kohgo et al., 2008). Therefore, tight regulation of iron metabolism maintains a balance between beneficial and toxic effects and this is accomplished by the interactions of several genes, such as the iron transporter transferrin, that are also involved in the response to infection (Neves et al., 2009).

Transferrin is synthesised in the liver and secreted into the blood but also found in the brain and central nervous system, testes, ovary, spleen, mammary gland and the kidney (Lambert et al., 2005). Transferrin contributes to the immune system through binding to iron, creating a low iron environment where few microorganisms

can survive and the infectivity of pathogenic microorganisms becomes limited (Suzumoto et al., 1977; Chen et al., 2009; Jurecka et al., 2009a, b).

Tf has been detected in almost all fish species (Yano, 1996), including the Pacific hagfish (Aisen et al., 1972) and the lamprey (Boffa et al., 1967; Macey et al., 1982). For cartilaginous fishes, Tf has been detected in the cat shark, *Scyllium stellare* (Got et al., 1967) and the lemon shark (Clem & Small, 1967). In bony fish, Tf has been detected in more than 100 species of fish (Turner & Jamieson, 1987; Jamieson, 1990).

Lectins. Lectins are primordial molecules that have multiple functions. They have existed in fish and other animals for decades and were initially identified as hemagglutinins (Russell & Lumsden, 2005) as they bind carbohydrate and agglutinate cells (Ewart et al., 2001). Lectins comprise at least two sugar-binding sites but the monosaccharide or glycosaccharide that inhibits lectin-induced agglutination or precipitation provides lectins' specificity (Goldstein et al., 1980). Lectins have been divided into several types, which include the C- and S-type lectins (Yano, 1996). The C-type is calcium-dependent.

A number of lectins have been reported in fish, but most have been characterised only in terms of agglutination activity and carbohydrate specificity (Ewart et al., 2001). In fish, C-type lectins, galectins and pentraxins have been identified from the earliest jawed vertebrate (sharks) to the more advanced teleost species such as salmon and carp (Vasta et al., 2004).

Ingram (1980), Ellis (1981) and Fletcher (1982) found many antipathogenic materials in the fish mucus, including lectins. Lectins have also been isolated from the skin mucus of scaleless hagfish, freshwater eel, moray eel, loach, sea catfish, ayu, cusk eel, dragonet and flounders (Yano, 1996), suggesting that lectin is produced by club cells (Al-Hassan et al., 1986). Furthermore, lectin was also isolated from the eggs of many species of fish such as lamprey, herring, carp, loach, Japanese catfish, smelts, ayu, salmonid fishes, sea bass, perch, porgy and flounder (Yano, 1996).

There are many studies on the function of fish lectins. Kamiya and Shimizu (1980) reported the ability of lectins from windowpane flounder skin mucus to agglutinate marine yeast, *Metschnikowia reukafii.* Kamiya et al. (1990) revealed the same ability of conger eel skin mucus lectins to agglutinate *Vibrio anguillarum*. Blue gourami lectins were reported to agglutinate fish pathogen *Aeromonas hydrophila* and at low concentrations (<1 ng/ml) promoted phagocytosis of the same bacterium (Fock et al., 2001). A mannan-binding lectin in the plasma of the Atlantic salmon was showed to bind to fish pathogens *Vibrio anguillarum* and *Aeromonas salmonicida* in a calcium-dependent manner (Ewart et al., 1999) and to increase phagocytosis and killing following incubation with *A. salmonicida* (Ottinger et al., 1999). Voss et al. (1978) reported Chinook salmon egg lectins inhibited the growth of pathogenic bacteria such as *Vibrio anguillarum, Yersina ruckeri, Aeromonas hydrophila* and *Edwardsiella tarda.* Fish

egg lectins were suggested to provide some protection to the developing egg and to prevent the transmission of pathogenic organisms from mothers to their offspring.

Adaptive Immunity

Adaptive immunity or specific immune system is the third line of the immune system that invaders face after surviving the physical barrier and the innate immunity. The adaptive immune system is composed of highly specialised, systemic cells and processes that eliminate or prevent pathogenic growth. The term adaptive refers to the differentiation of specific from non-specific and the tailoring of response to a particular foreign invader. Adaptive immunity is activated by the non-specific or innate immunity (Rubio-Godog, 2010).

Adaptive immunity consists of two major components: the antibodies and lymphocytes, or often called the humoral and the cell-mediated immune response, respectively (Uribe et al., 2011). Cells of the adaptive immunity are the lymphocytes, both B and T cells. The B cells, derived from the bone marrow, become the cells that produce antibodies. The T cells, which mature in the thymus, differentiate into cells that either participate in lymphocyte maturation or kill virus-infected cells. A key feature of adaptive immunity is 'memory', which differentiates it from innate immunity.

Adaptive immunity is highly adaptable due to the mechanisms of somatic hypermutation and V(D)J recombination. These mechanisms allow a small number of genes to generate a huge number of different antigen receptors that are uniquely expressed on each individual lymphocyte. This gene re-arrangement leads to an irreversible change in the DNA of each cell and all progenies of that cell inherit the genes that encode the same receptor specificity, including Memory B and Memory T cells, which are the key to long-lived specific immunity.

Humoral Immunity

Humoral immunity refers to antibody secretion and the accessory processes that accompany it. These include the Th2 activation and cytokine production, germinal centre formation and isotype switching, affinity maturation and memory cell generation. The humoral immunity involves substances found in the humours or body fluids, which include pathogen and toxin neutralisation, complement activation, opsonin promotion of phagocytosis and pathogen elimination (Janeway, 2001). Thus, humoral immune response is one of the branches of adaptive immunity that are mediated by secreted antibodies produced by B lymphocyte lineage or the B cells. The B cells transform into plasma cells, which produce and secrete antibodies. The CD4+ T-helper cells provide co-stimulation that aids this entire process, allowing the secreted antibodies to bind to the antigens located on the surface of the invading microorganisms and send them for destruction (Pier et al., 2004).

Humoral immunity in fish is variable and quite different from other animals. It depends on the external conditions and the species of fish (Lukjanenko, 1971).

However, the fish humoral immune response does share several basic characteristics with that of mammals. These include the basic immunoglobulin (Ig) structure, the cellular requirement for stimulation of antibodies and the functions of antibodies in neutralisation, complement fixation and opsonisation of antigen.

Antibodies. Antibodies, also known as immunoglobulins (Ig), are the primary humoral component of the adaptive immune system (Magnadottir et al., 2005). The Ig molecule has a dual functions i.e. as antigen receptor on the surface of B-cells and as an antibody secreted into blood and other body fluids. Thus, there are two forms of H-chains in the immunoglobulins, one with a hydrophobic C-terminal peptide that can bind to a cell membrane and the other with a hydrophilic N-terminal region that is secreted. The same gene encodes the two forms and processing of the pre-mRNA determines which form should be synthesised. The N-terminal on both H and L chains is called the variable (V) domain and is the structure of the antibody that binds to the antigen (Pilstrom & Bengten, 1996).

The most prevalent immunoglobulin in the serum of teleosts is the IgM tetramer with eight antigen-combining sites. It has been detected in many species of fish including chondrichthyean and osteichthyean. It consists of 70 kDa heavy chain and 22-25 kDa light chains (Tort et al., 2003). In general, fish Igs are of lower affinity and diversity than those of mammals and birds (Du Pasquier, 1982). Therefore, better understanding of the structure and function

of fish IgM becomes extremely important for effective prevention and control of various fish diseases (Magnadottir, 1998).

IgM is tetrameric in teleost (Acton et al., 1971) but pentametric in higher vertebrates and cartilaginous fish (Kobayashi et al., 1984). Since isotypic repertoire of Ig is limited in fish (Kaattari et al., 1998), the degree of similarity between mucus and serum derived Ig is unknown. However, there are reports that monoclonal antibodies developed against serum of carp, *Cyprinus carpio* L do not react with the mucus Ig (Rombout et al., 1993a) due likely to the existence of varied redox forms of Ig in some teleost species (Kaattari et al., 1998) or perhaps the existence of different glycosylation patterns (Kenneth et al., 2000). Kenneth et al. (2000) revealed the different protein band patterns between the mucus and the serum Ig where the mucus Ig possesses four primary bands, the 72, 68, 43 and 28 kDa, while the serum Ig possesses two primary bands; the 72 and 28 kDa.

Recently, scientists have discovered the existence of IgD and IgT isotypes in teleost but not as abundant as the IgM (Tian et al. 2009). Wilson et al. (1997) first discovered IgD that was homologous with the mammalian IgD. Then, Hanzen et al. (2005) discovered IgT, sometimes referred to as IgZ, in rainbow trout while Danilova et al. (2005) reported the occurrence in zebrafish. However, unlike IgM, the roles of these new Igs are still obscure. Nevertheless, IgD might be involved in innate immunity as Edholm et al. (2010) found that the IgD secreted by channel catfish lacked the

antigen-specific V domain and could bind to basophils to stimulate the pro-inflammatory cytokines. According to Zhang et al. (2010), IgT might involve in the interactions between the host intestinal mucosa and the microflora.

Immunoglobulins of fish are found in the skin mucus, gut, gill mucus, bile and systemically in the blood plasma (Morrison & Nowak, 2002). The presence of Ig on the skin and gill surface is important since these organs are consistently exposed to a wider natural environment. The systemic and mucosal immune responses are autonomous because specific antibodies against certain antigens can be elicited from the skin, gills and gut. However, intravenous injection of antigen stimulates little activity in the mucus (Lobb & Clem, 1981), indicating that the mucosal Ig is exclusive from the systemic plasma cells. Grabowski et al. (2004), on the other hand, showed the stimulation of the mucus antibody response following intraperitonium vaccination with sonicated formalin killed *Flavobacterium columnare*. Similarly, Firdaus-Nawi et al. (2011) demonstrated increasing mucus antibody following oral vaccination with killed *Streptococcus agalactiae*. This pattern was also observed with *Flavobacterium psychrophilum* (LaFrentz et al., 2002), indicating that the systemic antibodies may disseminate to mucosal sites from blood circulation (Di Conza & Halliday, 1971; St. Louis-Cormier et al., 1984; Cain et al., 2000).

B cells. B cells are a type of lymphocyte that plays an important role in the humoral immune response. The primary functions of B cells are to produce antibodies against antigens, to perform the role of antigen-presenting cells (APCs) and finally, to develop into memory B cells after activation by antigenic interaction. The head of kidney (HK) or pronephros is the source of B cells in teleost fish, making HK the primary lymphoid tissue (Zapata et al., 2006). The spleen is considered secondary lymphoid tissue in which plenty of B cells are found in teleost fish. Bromage et al. (2004) revealed that the spleen is a site for B cell activation, plasmablast formation and differentiation into plasma cells. Plasma cells then migrate to the HK, which explains the presence of few Ig-secreting cells in the spleen compared to HK.

Other than the lymphoid tissues, B cells are also found in various organs and tissues including the intestine, skin and gills. In the intestine, the distribution of B cells is low and variable among different species of fish. Studies in sea bass, carp and rainbow trout demonstrated between 2% and 12% of the leukocytes in the intestine were IgM-positive, mainly in the lamina propria of both anterior and posterior intestines. However, a small number of these cells were also detected in the epithelium (Salinas et al., 2011). B cells are also detectable in the skin of cartilaginous and teleost fish (Wolfle et al., 2009) and in the epithelium of carp skin (Rombout et al., 1993b). Another study in rainbow trout revealed the large numbers of B cells in the basement membrane area followed by the epithelial layer and the cells in the dermis or sub-epidermal layer (St.

Louis-Cormier et al., 1984). Furthermore, Zhao et al. (2008) reported that the skin of channel catfish contains B cells and antibody secreting cells (ASC), which most likely serve as the major source of mucosal antibody. On the other hand, Grove et al. (2006) reported a large number of IgM-positive cells in the stratified epithelium of the gill arch and filaments of Atlantic halibut fish, while Grøntvedt and Espelid (2003) reported an abundance of B cells in primary gill lamellae and filaments along the blood vessels of spotted wolfish.

Mucosal Immunity

Mucosal immunity is vital because it is the first line of adaptive humoral defense that effectively blocks or neutralises the pathogen. However, fish lack secretory IgA, Peyer's patches and tonsils that play important role in mammalian mucosal immunity (Kaattari & Piganelli, 1996). Instead, massive intraepithelial lymphocytic aggregations are observed in the central region of the spiral intestine of elasmobranch (Tomonaga et al., 1986). They are believed to play a similar role as the Peyer's patch of mammals. Furthermore, minor subepithelial lymphoid accumulations were reported in the intestine of roach and perch (Zapata & Solas, 1979; Rombout & van den Berg, 1989). A recent study by Firdaus-Nawi et al. (2011) demonstrated aggregations of lymphoid cells in the lamina propria of red tilapia following oral immunisation against *Streptoccoccus agalactiae*. Antibody-secreting cells (ASCs) were observed in the lamina propria of perch following

immunisation with sheep red blood cells (Pontius & Ambrosius, 1972). Fletcher and White (1973) reported increased antibody titers within the intestinal mucus of plaice upon oral immunisation with heat-killed *Vibrio anguillarum*. Similarly, Firdaus-Nawi et al. (2011) demonstrated increased antibody titers in the intestinal mucus of red tilapia following oral immunisation against *Streptoccoccus agalactiae*.

Mucosal immunity also gives protection against parasitic infestation. A study by Sitja-Bobadilla et al. (2006) using co-habitation challenge of turbot with *Enteromyxmum scophthalmi* resulted in leukocyte infiltration in the intestine. The infiltration consisted of lymphocytes but no specific IgM was detected in the serum. On the other hand, Zhang et al. (2010) demonstrated the unchanged numbers of IgM-positive cells in the gut of surviving trout that were naturally infected with parasite *Ceratomyxa shasta*. However, parasite-specific IgM were detected in the serum. This suggests that different fish species respond differently to different parasites.

Cellular Immunity

Cellular immunity, also referred to as cell-mediated immunity, is a specific immune response that involves macrophages, natural killer cells (NK), mast cells, basophils, eosinophils and neutrophils (Broere et al., 2011). Various cytokines are released in response to the antigen. Cellular immunity protects the body by activating the antigen-specific cytotoxic T-lymphocytes that induce apoptosis of cells that display epitopes of

foreign antigen on their surface. These include virus-infected cells, intracellular bacteria-infected cells and cancerous cells. Cell-mediated immunity is directed primarily at the pathogen that survived phagocytosis and the pathogens that infect non-phagocytic cells. It is most effective in removing virus-infected cells, but also participates in protection against fungi, protozoans, cancer cell and intracellular bacteria (Kerry & Hansen, 2011).

Cellular immunity also plays a major role in transplant rejection. Graft-versus-host reaction (GVHR) is a representative phenomenon of cell-mediated immunity involving CD4 and CD8 T-lymphocytes. Nakanishi and Ototake (1999) employed a model system of clonal triploid ginbuna and tetraploid ginbuna-goldfish, *Carassius auratus* hybrids to demonstrate the presence of GVHR in a teleost fish. The sensitised triploid cells were injected into tetraploid recipients and a typical GVHR was induced that led to the death of the recipients within one month. Post-mortem conducted during the course of the clinically apparent graft-versus-host disease (GVHD) showed several pathological changes including enlargement of the spleen, infiltration of mononuclear cells and focal necrosis particularly in the skin, liver and lymphoid tissues. Most features of acute GVHR are similar to those found in mammals and birds, providing evidence for the presence of allo-reactive cytotoxic T cells in teleosts (Manning & Nakanishi, 1996).

T cells. T cells are lymphocytes that play a vital role in cell-mediated immunity as well as the adaptive immune system (Nakanishi et al., 2015). The presence of an antigen-specific receptor or T-cell receptor (TCR) on the cell surface distinguishes them from other lymphocytes (Manning & Nakanishi, 1996). They are called T cells because they mature in the thymus. They are also sometimes called thymocytes.

There are several types of T cell, which include T helper cells (T_H cell), cytotoxic T cells (CTL), memory T cells, regulatory T cells (Treg cell) and gamma delta T cells. Naive $CD4^+$ T cells can differentiate into the five types of effector T cells (Th1, Th2, Th17, Th9 and Th22), three subsets of regulatory T cells (Treg, Th3, Tr-1) and memory T cells (Annunziato & Romagnani, 2009; Wan & Flavell, 2009). Thus, these $CD4^+$ T cells play vital roles in regulation of the immune system, immune pathogenesis and host defense mechanism. According to Zhu and Paul (2010), $CD4^+$ T cells are characterised by their plasticity in addition to heterogeneity. Fischer et al. (2006) detected T-cell-related genes such as TCR, CD3, CD4 and CD8 as well as MHC class I and class II genes in several fish species. Additionally, mRNA expression of T cell surface marker genes in alloantigen or virus-specific effector cells has been reported in several fish such as TCRβ and CD8α in ginbuna (Somamoto et al., 2006) and rainbow trout (Fischer et al., 2013) and TCR in channel catfish (Stuge et al., 2000). This suggests the presence of $CD4^+$ helper T cells and $CD8^+$ CTL in fish similar to their presence in higher vertebrates (Fischer et al., 2013).

Overall Working Mechanisms of Fish Immune System

In an attempt to prevent establishment of infection, both innate and adaptive immunities work in complement. Upon exposure to pathogenic organism, the innate immunity is activated to prevent the invasion through TLRs that recognise the pathogen-associated molecular patterns (PAMPs). The first hurdle faced by the microorganism is the efficient physical barrier in the form of mucus and epithelial cells of the mucosal organs such as the intestines, gills and skin (Figure 1). Should the pathogen successfully pass these barriers, invasion starts.

Successful invasion by pathogens stimulates two major mechanisms i.e. the innate cellular and humoral immunities, and the specific adaptive immunity. Innate immunity involves granulocytes, phagocytes and the non-specific cytotoxic cells that are employed by inflammatory cytokines to kill and digest the invading pathogens through a process known as phagocytosis (Secombes & Fletcher, 1992). At the same time, the humoral component of the innate immune system employs a wide variety of proteins and glycoproteins described earlier that are capable, either alone or in collaboration with the cellular innate immunity, of destroying or inhibiting the growth of the microorganisms (Aoki et al., 2008). The phagocytosis process especially by residence macrophages results in full elimination of an invading pathogen (Figure 2).

After the degradation process, selected small protein fragments from the pathogen are displayed by macrophages on the Class II MHC (MHC II). This turns macrophages into antigen presenting cells (APC) and activates the adaptive immunity where helper T-cells (T_h) attract and bind to the MHC II of APC by T-cell Receptors (TCR). Formation of APC-T_h complex releases signalling molecules known as cytokines by the activated T_h that triggers the proliferation and maturation of B cells as well as other immune mechanisms. Maturation of B cell leads to formation of two types of cell, the plasma cells that produce specific antibody and the memory B cells that remember the specific antigen for certain periods of time (Kum & Sekkin, 2011) (Figure 3). The released antibodies act to disable the pathogen through the mechanism of opsonisation or neutralisation before the disabled pathogen is destroyed by the complement system and cleared by macrophages via phagocytosis.

Intracellular antigens (Ag) such as virus undergo another effective mechanism of elimination by the body. The pathogen is phagocytised and processed before being displayed on Class I MHC (MHC I) of APC, which attracts the CD8$^+$ cells to bind to the MHC I of APC via CD8$^+$ receptors. Then the activated CD8$^+$ cells start to clone themselves into two types of cell, the memory T cell and the Cytotoxic T-Lymphocyte (CTL) cell that destroy the virus-infected cells using various enzymes and cell apoptosis (Figure 4).

Subsequent exposure to the same antigen predisposes the pathogen to the same physical barrier of the mucosal organs.
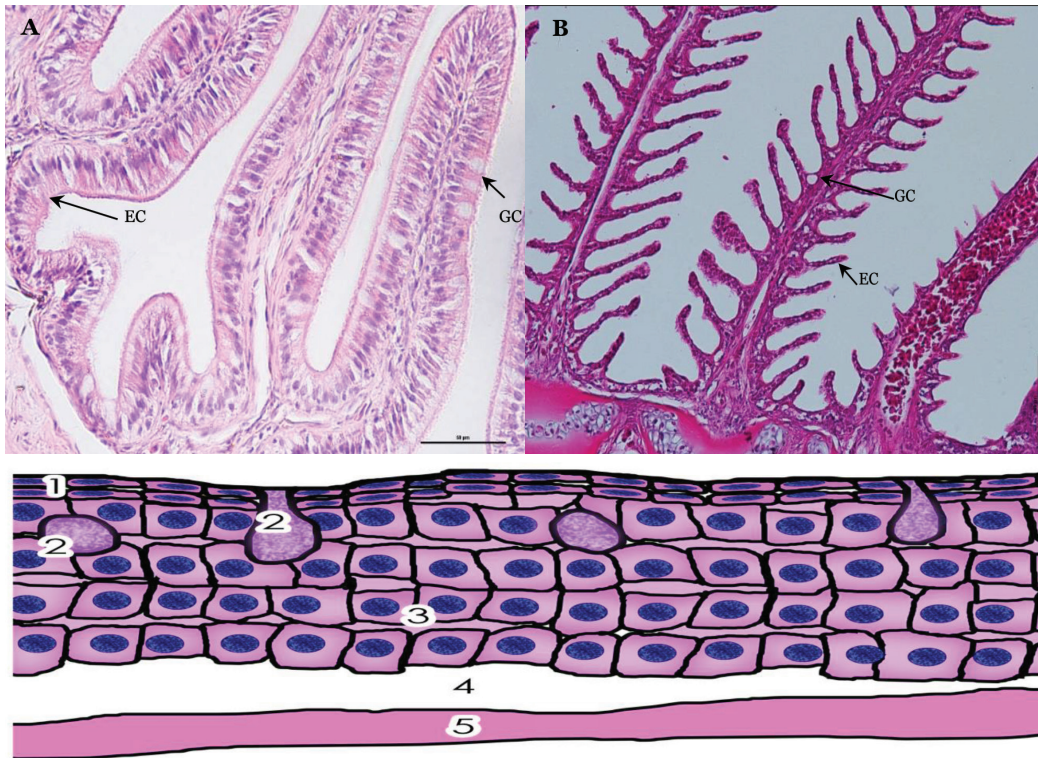
Figure 1. Intestine and gills of the fish are coated with mucus layer produced by goblet cells (GC) as primary innate protection, followed by the layer of epithelial cells (EC) (A & B). Similarly, the mucus also covers the skin of the fish and the epidermal layer is made up of epithelial cells (C) that provide both a physical and chemical barrier against invading pathogens.
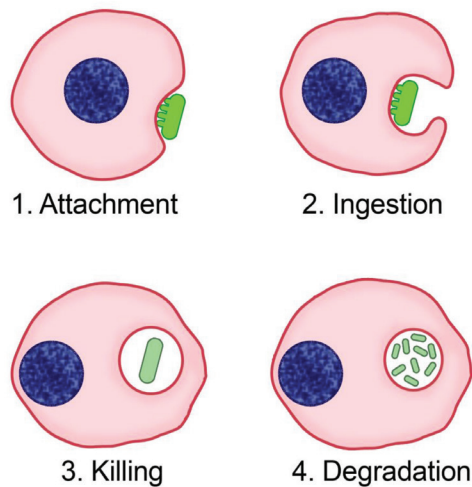
Figure 2. Process of phagocytosis by macrophages starting with attachment of pathogen such as bacterium followed by ingestion before it is killed and lysed into small fragments by the enzyme lysosome. Then the degradation process takes over.
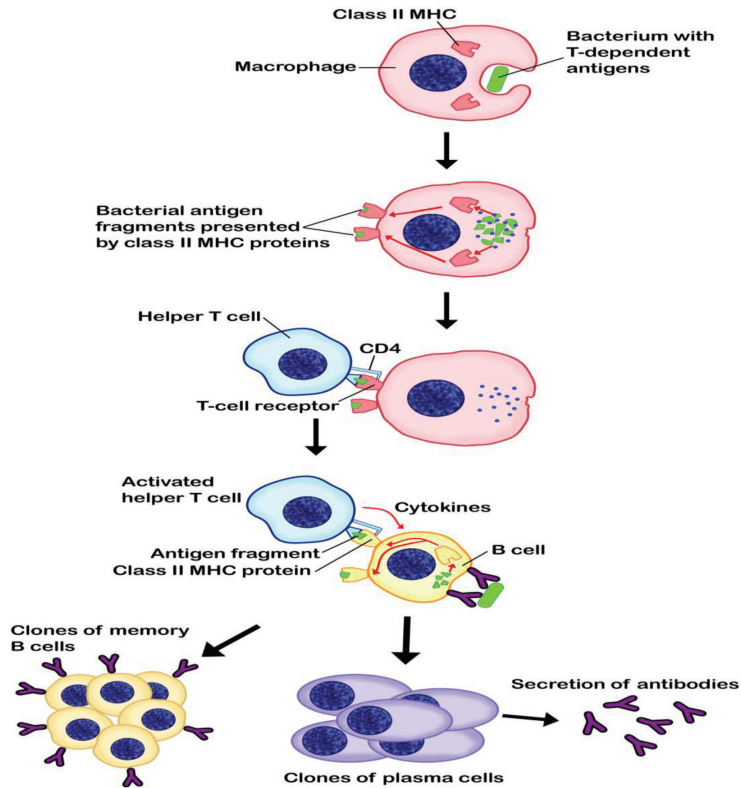
Figure 3. Mechanism of B cell activation and maturation by helper T-cell (Th) resulted in formation of specific antibody secreting plasma cells and memory B cells that have the ability to remember the infection.
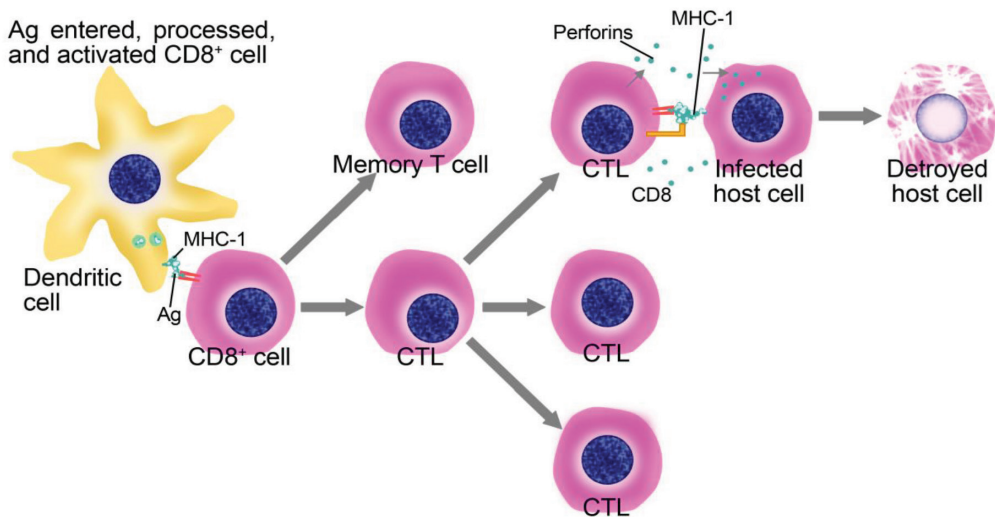
Figure 4. Elimination of virus-infected cell by the Cytotoxic T-Lymphocyte (CTL). This mechanism does not involve antibody production and is also known as Cell-Mediated Immunity (CMI).

At the same time, the Memory B and T cells located in the mucosal layer of the exposed fish stimulate production and release of specific antibodies and/or cytotoxic cells against the pathogen onto the mucosal surface to prevent adhesion and invasion of the pathogen. This forms the basis for mucosal immunity.

Inability of the mucosal layer to efficiently prevent invasion leads to a second invasion of the pathogen into the host. As described earlier, this invasion stimulates the non-specific cellular and humoral innate immunities to kill and remove the invading pathogen. Simultaneously, this second invasion activates processing of the pathogen by phagocytes to be presented to the adaptive immune system for the Memory B cells to enhance production and release of antibodies specific to the pathogen (humoral immunity) or the Memory T cells to enhance the cytotoxic T-lymphocytes (cell-mediated immunity), depending on the type of invading pathogen. These form the basis for vaccination against diseases.

CONCLUSION

The immune system of fish is not as complex as that of mammals because of the absence of several components. However, the immune system of fish is adequate in providing protection against antagonistic pathogens from the surroundings. Recent findings in fish immunology and its components help to provide a better understanding of fish immunology as well as to pave the way for further research. This will help in efforts to improve the health and disease protection of fish.

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414

Pertanika J. Trop. Agric. Sci. 39 (4) 393 - 420 (2016)

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418

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Review Article

A Review of the Ichthyofauna of Malaysian Peat Swamp Forest

Sule, H. A.[1,2], Ismail, A.[1]* and Amal, M. N. A.[1]

[1]*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2]*Department of Integrated Science, School of Science, Kogi State College of Education, PMB 1033 Ankpa, Nigeria*

ABSTRACT

A review of literature showed that numerous intensive surveys have been carried out on the ichthyofauna of the peat swamp forests (PSFs) of Malaysia. This review aim to provide a checklist of blackwater fish species in Malaysia from available published literature, and address their economical importance, conservation status and problems of PSFs. A total of 198 peat swamp fish species from 32 families have been recorded in Malaysia. From this number, a total of 114 species from 23 families, representing about 40% of the known fish fauna in Peninsular Malaysia, were recorded from north Selangor PSF. Meanwhile, a total of 49 species belonging to 18 families, 13 species from seven families, 58 species belonging to 19 families, and nine species from five families were recorded from the peat swamps of Perak, Johor, Pahang and East Peninsular Malaysia (part of Pahang and Terengganu), respectively. Meanwhile, 31 species from 12 families and 40 species belonging to 13 families were recorded from Sabah and Sarawak, respectively. Family Cyprinidae has the highest recorded species, followed by Osphronemidae, Bagridae and Siluridae. The IUCN Red List revealed 12 threatened species facing risk of extinction. The importance of conserving PSFs was outlined and suggestions made in line with the objectives of conservation. Findings from literature revealed that Malaysia's PSFs are rich in fish diversity, contrary to previous belief, and should therefore be conserved and protected to ensure the richness of their fish diversity.

Keywords: Ichthyofauna, peat swamp forest, conservation, Malaysia

INTRODUCTION

Peat swamp forests (PSFs) are one of the most unusual and harsh ecosystems in the tropical rainforest biome. The PSFs of Peninsular Malaysia are one of the most threatened (illegal logging, irrigation of oil palm and paddy fields), yet one of the most poorly understood biotopes (Ng et al., 1994). These swamps derive their name from their substrate of peat consisting of plant detritus, which gradually release tannins and organic acids into poorly buffered water and contribute to its characteristically low pH (Ng et al., 1994; Yule, 2010; Posa et al., 2011; Wantzen et al., 2011). Peat swamps are typically deficient in oxygen, which is one consequence of plant decay (Beamish et al., 2003). Peat swamps are generally referred as 'black waters', while waters originating from PSFs are highly acidic with pH values ranging from 3.6 to 5.9, tea-coloured when seen against transmitted light, and black when seen en masse via reflected light (Johnson, 1967ab, 1968). Generally, the acidity of black waters is due to the high concentrations of humic acids and other phenolic acids (Goltenboth, 2006; Irvine et al., 2013).

Most of the black waters of Singapore and Peninsular Malaysia recorded by Wyatt-Smith (1959, 1964), Johnson (1967a, 1968), Anderson (1983) and Whitmore (1984, 1988) have been converted to agriculture, industrial states and residential areas (Ng et al., 1994). The peat swamp forests of Johor, which were of staggering vastness in Peninsular Malaysia, are almost completely degraded. Peat swamp forests of considerable size in Peninsular Malaysia are now restricted to north Selangor, central Terengganu and Pahang (Ng et al., 1994). In Peninsular Malaysia, the fish fauna of the PSF of north Selangor is comparably well studied and recorded.

The unique characteristics of black waters (dark colour, low dissolved oxygen and high acidity) led to an initial misinterpretation of 'habitat inhospitality', and the conclusion that such habitats will sustain very poor faunal diversity. One of the earliest surveys of the fish fauna of Malaysia peat swamps was by Johnson (1967ab, 1968) who recorded only 26 species in black waters, of which only one is stenotopic to black water. However, the first well-recognised documentation of blackwater fish species of the north Selangor peat swamp forest (NSPSF) was done by Davies and Abdullah (1989). Numerous other surveys have also been conducted on the ichthyofauna of Peninsular Malaysia (Shiraishi et al., 1972; Lim et al., 1982; Mizuno & Furtado, 1982; Zakaria-Ismail, 1990; Ng & Lim, 1991; Ng et al., 1992, 1994; IPT-AWB, 1993; Lee & Ng, 1994; Zakaria et al., 1999; Lee, 2001; Beamish et al., 2003; Rezawaty, 2004; Shah et al., 2006; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013).

The objectives of this review are to provide a checklist of black water fish species in Malaysia, assess their economic importance, update the conservation status of the species and address the conservation problems of PSFs. An exhaustive search of published literature was conducted to

collate lists and determine the distribution of the peat swamp fishes from surveys in Malaysian PSF sites (Figure 1). Fish species provided in this review were recorded from the surveys between 1989 and 2015. Reference was made to Kottelat's catalogue of the fishes of southeast Asia (Kottelat, 2013) to verify and update taxonomic revisions of species.

FISH SPECIES RECORDED IN MALAYSIAN PEAT SWAMP FORESTS

Peat swamp forests have unique water quality and as a result, fish species that can survive and breed in the black water environment are limited. Researchers have reported many species of fish in PSFs that are not found in other habitats (Davies & Abdullah, 1989; Ng et al., 1994). A comprehensive list and a summary of the fish species of Malaysia PSFs are given in Table 1 and Table 2.

North Selangor Peat Swamp Forest

The NSPSF has been well studied relative to other PSFs in Malaysia. The most extensive surveys of the fish fauna of NSPSF were done by Davies and Abdullah (1989), IPT-AWB (1993), Ng et al., (1994), Beamish et al., (2003), Ahmad et al., (2013), Ismail et al., (2013) and Siow et al., (2013).

Davies and Abdullah (1989), in a survey of freshwater fish of NSPSF in 1989, recorded 42 fish species (actually 41 species, with the exception of *Osteochilus hasselti*, the synonym of *O. vittatus* recorded as another different species) belonging to

11 families (see Table 1). A lot of scientific names, from family to species level, have been changed over the past decade. The most recent comprehensive list of such changes is outlined in Kottelat's catalogue of the fishes of Southeast Asia (Kottelat, 2013).

An intensive survey in 1992 by IPT-AWB (1993) recorded 76 fish species belonging to 24 families (23 with exception of Belontiidae, now included in the family Osphronemidae). This is one of the most successful surveys in NSPSF in terms of diversity. Ng et al. (1994) recorded 47 species of fish living in the black waters of NSPSF, and 14 species of the fishes were stenotopic to acidic black waters. Six unique fish species relatively new to science were first recorded at NSPSF in 1989 (Ng et al., 1994).

Studies by Beamish et al. (2003) conducted from 1997 to 1998 recorded 35 fish species from NSPSF belonging to 14 families (actually 13 because Belontiidae and Luciocephalidae which are now included in Osphronemidae, were recorded separately), while Giam et al. (2012) recorded eight blackwater fish species belonging to the six families from NSPSF.

Recently, during the Selangor Scientific and Biodiversity Peatland Expedition of 2013, Ahmad et al. (2013), Ismail et al. (2013) and Siow et al. (2013) recorded 30 species from 13 families, 41 species from 13 families, and 24 species 10 families, respectively. The comprehensive list of fish species recorded from NSPSF is given in Table 1.
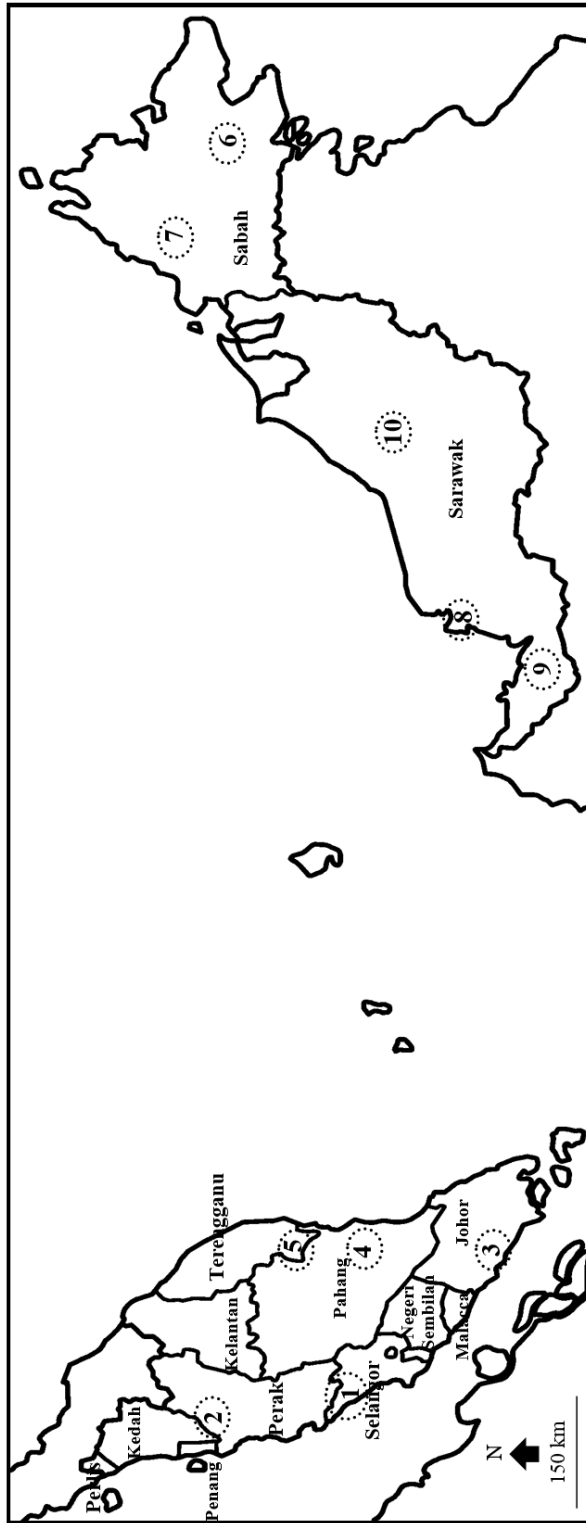
Key:

1- North Selangor peat swamp forest (NSPSF), Selangor

2- Paya Beriah peat swamp forest (PBPSF), north Perak

3- Black water sites, west Johor

4- Southeast Pahang peat swamp forest (SEPPSF)

5- Black water sites, part of Pahang and Terengganu

6- Segama River, Sabah

7- Maliau basin, Sabah

8- Rajang basin, Sarawak

9- Black water sites, Batang Kerang and Sadong, Sarawak

10- Black water sites, Nanga Merit area, Sarawak

Figure 1. Location of PSFs and black water sites surveyed

Table 1
Checklist of black water fish in Malaysia

Family	Species	IUCN Status	NSPSF	PBPSF	Johor PSFs	SEPPSF	EP PSFs	Sabah PSFs	Sarawak PSFs	References
Akysidae	*Acrochordonichthys ischnosoma* Bleeker, 1858	NE	x							Davies & Abdullah, 1989
	Acrochordonichthys rogosus (Bleeker, 1846)	NE	x							IPT-AWB, 1993
	Akysis cf. *hendricksoni* Alfred, 1986	LC	x							IPT-AWB, 1993
	Parakysis verrucosus Herre, 1940	NE	x							IPT-AWB, 1993; Ng et al., 1994
Anabantidae	*Anabas testudineus* (Bloch, 1792)	DD	x	x		x			x	Davies & Abdullah, 1989; IPT-AWB, 1993; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Khairul-Adha et al., 2009; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
Anguillidae	*Anguilla borneensis* Popta, 1924	VU						x		Martin-Smith & Tan, 1998
Aplocheilidae	*Aplocheilus panchax* (Hamilton, 1822)	LC		x						Zakaria et al., 1999; Rezawaty, 2004; Shah et al., 2006
Ariidae	*Cryptarius truncatus* (Valenciennes, 1840)	NE						x		Martin-Smith & Tan, 1998
	Batrachocephalus mino (Hamilton, 1822)	NE						x		Martin-Smith & Tan, 1998
	Cephalocassis borneensis (Bleeker, 1851)	NE						x		Martin-Smith & Tan, 1998
Engraulididae	*Setipinna melanochir* (Bleeker, 1849)	NE						x		Martin-Smith & Tan, 1998

cont'd Table 1

Family	Species	Status						References
Balitoridae	*Homaloptera* cf. *nebulosa* (Alfred, 1969)	NE	x	x				IPT-AWB, 1993; Ahmad et al., 2005
	Homaloptera ogilviei (Alred, 1967)	DD	x					IPT-AWB, 1993
	Homaloptera zollingeri (Bleeker, 153)	LC	x					IPT-AWB, 1993
	Neohomaloptera johorensis (Herre, 1944)	NE	x		x		x	Ng et al., 1994; Ng & Tan, 1999; Beamish et al., 2003; Giam et al., 2012; Ahmad et al., 2013
Barbuccidae	*Barbucca diabolica* Roberts, 1989	DD			x		x	Ahmad et al., 2005; Giam et al., 2012
Bagridae	*Bagrichthys hypselopterus* (Bleeker, 1852)	NE	x					Davies & Abdullah, 1989
	Bagrichthys macrocanthus (Bleeker, 1854)	NE	x					Siow et al., 2013
	Bagrichthys macropterus (Bleeker, 1854)	NE					x	Hassan et al., 2010
	Hemibagrus baramensis (Regan, 1906)	NE	x					Davies & Abdullah, 1989; Martin-Smith & Tan, 1998
	Hemibagrus capitulum (Popta, 1904)	NE			x			Ahmad et al., 2005
	Hemibagrus nemurus (Valenciennes, 1840)	LC	x	x			x	IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Khairul-Adha et al., 2009; Jimmy Shah et al., 2006; Ismail et al., 2010; Siow et al., 2013
	Hemibagrus planiceps (Valenciennes, 1840)	NE					x	Hassan et al., 2010
	Hemibagrus sabanus (Inger & Chin, 1959)	NE				x		Martin-Smith & Tan, 1998

cont'd Table 1

Family	Species	IUCN							References
	Hyalobagrus ornatus (Duncker, 1904)	NE			x	x			Ng & Kottelat, 1998; Ahmad et al., 2005; Giam et al., 2012
	Leiocassis leiacanthus (Weber & de Beaufort, 1912)	NE	x						Davies & Abdullah, 1989
	Leiocassis micropogon (Bleeker, 1852)	NE	x						Ng et al., 1994; Beamish et al., 2003
	Leiocassis poeciloptera (Valenciennes, 1840)	NE	x						Siow et al., 2013
	Leiocassis stenomus (Valenciennes, 1839)	NE	x						IPT-AWB, 1993
	Mystus bimaculatus (Volz, 1904)	NE	x	x					IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Giam et al., 2012; Ahmad et al., 2013; Ismail et al., 2013
	Mystus nigriceps (Valenciennes, 1840)	NE	x	x				x	Davies & Abdullah, 1989; IPT-AWB, 1993; Shah et al., 2006; Hassan et al., 2010; Ismail et al., 2013; Siow et al., 2013
	Pseudomystus robustus (Inger & Chin, 1959)	NE					x		Martin-Smith & Tan, 1998
Chacidae	*Chaca bankanensis* Bleeker, 1852	LC			x				Giam et al., 2012
Chaudhuriidae	*Bihunichthys monopteroides* (Kottelat & Lim, 1994)	NE	x						IPT-AWB, 1993; Ng et al., 1994
	Chendol keelini Kottelat & Lim, 1994	NE				x			Ahmad et al., 2005
	Nagaichthys filipes Kottelat & Lim, 1991	NE			x	x			Giam et al., 2012

cont'd Table 1

Family	Species	Status					References
Channidae	*Channa bankanensis* (Bleeker, 1853)	NE	x	x	x		IPT-AWB, 1993; Ng et al., 1994; Martin-Smith & Tan, 1998; Beamish et al., 2003; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013
	Channa gachua (Hamilton, 1822)	LC	x				Ng et al., 1994
	Channa lucius (Cuvier, 1831)	LC	x	x	x	x	Davies & Abdullah, 1989; Ng et al., 1994; Zakaria et al., 1999; Beamish et al., 2003; Ahmad et al., 2005; Parenti & Lim, 2005; Shah et al., 2006; Khairul-Adha et al., 2009; Ahmad et al., 2013; Ismail et al., 2013
	Channa marulioides (Bleeker, 1851)	LC	x	x			Ahmad et al., 2005
	Channa melosoma (Bleeker, 1851)	LC	x				IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003
	Channa micropeltes (Cuvier, 1831)	LC	x	x			Davies & Abdullah, 1989; Ahmad et al., 2005
	Channa striata (Bloch, 1797)	LC	x	x	x		Davies & Abdullah, 1989; IPT-AWB, 1993; Beamish et al., 2003; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Ahmad et al., 2013; Siow et al., 2013
	Channa sp.?	-		x			Shah et al., 2006
Cichlidae	**Oreochromis mossambicus* (Peters, 1852)	NT			x		Martin-Smith & Tan, 1998

cont'd Table 1

Family	Species	Status					References
Clariidae	Clarias batrachus (Linnaeus, 1758)	LC	x			x	IPT-AWB, 1993; Khairul-Adha et al., 2009; Siow et al., 2013
	Clarias leiacanthus Bleeker, 1851	NE	x		x	x	Davies & Abdullah, 1989; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Khairul-Adha et al., 2009; Ahmad et al., 2013
	Clarias macrocephalus Günther, 1864	NT	x		x	x	Rezawaty, 2004; Ahmad et al., 2005; Khairul-Adha et al., 2009; Siow et al., 2013
	Clarias meladerma (Bleeker, 1846)	LC	x				Ng et al., 1994; Ismail et al., 2013
	Clarias nieuhofii (Valenciennes, 1840)	LC	x		x	x	IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Khairul-Adha et al., 2009; Ismail et al., 2013
	Encheloclarias baculum Ng & Lim, 1993	NE	x			x	Ng & Tan, 2000; Giam et al., 2012
	Encheloclarias curtisoma Ng & Lim, 1993	CR	x		x		IPT-AWB, 1993; Ng et al., 1994; Giam et al., 2012
	Encheloclarias kelioides Ng & Lim, 1993	CR			x		Giam et al., 2012
	Encheloclarias prolatus Ng & Lim, 1993	VU				x	Giam et al., 2012
Cobitidae	Acanthopsoides molobrion (Seibert, 1991)	LC	x				IPT-AWB, 1993
	Acantopsis cf. dialuzona (van Hasseltt, 1823)	LC	x		x		IPT-AWB, 1993; Rezawaty, 2004
	Kottelatlimia katik (Kottelat & Lim, 1992)	NE		x		x	Giam et al., 2012

cont'd Table 1

Family	Species	Status					References
	Kottelatlimia pristes (Roberts, 1989)	NE	x				IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003
	Pangio kuhlii (Valenciennes, 1846)	NE	x				IPT-AWB, 1993
	Pangio muraeniformis (de Beaufort, 1933)	NE			x		Ahmad et al., 2005
	Pangio shelfordii (Popta, 1903)	NE			x		Ahmad et al., 2005
	Lepidocephalichthys tomaculum (Kottelat & Lim, 1992)	NE	x				Ng et al., 1994; Ahmad et al., 2013
Cyprinidae	*Boraras maculatus* (Duncker, 1904)	LC			x		Ahmad et al., 2005
	Barbodes banksi (Herre, 1940)	NE			x		Ahmad et al., 2005
	Barbodes sealei (Herre, 1933)	NE				x	Martin-Smith & Tan, 1998; Jimmy et al., 2010
	Barbonymus balleroides (Valenciennes, 1842)	NE				x	Martin-Smith & Tan, 1998
	Barbonymus gonionotus (Bleeker, 1949)	LC	x	x			Davies & Abdullah, 1989; Rezawaty, 2004; Siow et al., 2013
	Barbonymus schwanefeldii (Bleeker, 1854)	LC	x	x			Davies & Abdullah, 1989; IPT-AWB, 1993; Rezawaty, 2004; Ismail et al., 2013
	Brevibora cheeya (Liao & Tan, 2011)	NE	x				Ismail et al., 2013
	Crossocheilus cobitis (Bleeker, 1854)	NE				x	Martin-Smith & Tan, 1998
	Crossocheilus cf. *oblongus* (Kuhl & van Hasselt, 1823)	LC	x				IPT-AWB, 1993

cont'd Table 1

Species	IUCN status							References
Cyclocheilichthys apogon (Valenciennes, 1842)	LC	x	x	x				Davies & Abdullah, 1989; IPT-AWB, 1993; Zakaria et al., 1999; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Ismail et al., 2013
Cyclocheilichthys heteronema (Bleeker, 1853)	LC	x					x	IPT-AWB, 1993; Hassan et al., 2010
Cyclocheilichthys repasson (Bleeker, 1853)	LC	x				x		IPT-AWB, 1993; Martin-Smith & Tan, 1998
Desmopuntius hexazona (Weber & de Beaufort, 1912)	NE	x	x	x				IPT-AWB, 1993; Ng et al., 1994; Zakaria et al., 1999; Beamish et al., 2003; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Ahmad et al., 2013; Ismail et al., 2013
Desmopuntius johorensis (Duncker, 1904)	NE	x		x	x	x	x	IPT-AWB, 1993; Ng et al., 1994; Kottelat, 1996; Beamish et al., 2003; Ahmad et al., 2005; Parenti & Lim, 2005; Giam et al., 2012; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
Desmopuntius pentazona (Boulenger, 1894)	NE						x	Parenti & Lim, 2005
Hampala macrolepidota (Kuhl & van Hasselt, 1823)	LC	x	x					Davies & Abdullah, 1989; IPT-AWB, 1993; Ismail et al., 2013
Hampala sabana Inger & Chin, 1962	NE				x			Martin-Smith & Tan, 1998
Hypophthalmichthys molitrix (Valenciennes, 1844)	NE	x						Rezawaty, 2004

Pertanika J. Trop. Agric. Sci. 39 (1): 421 - 458 (2016)

431

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Species	Status							References
Kottelatia brittani (Axelrod, 1976)	NE		x					Zakaria et al., 1999; Rezawaty, 2004
Lobocheilos bo (Popta, 1904)	NE	x				x	x	Martin-Smith & Tan, 1998; Hassan et al., 2010
**Labiobarbus leptocheilus* (Valenciennes, 1842)	LC	x	x					Davies & Abdullah, 1989; Rezawaty, 2004; Shah et al., 2006
Labiobarbus sabanus (Inger & Chin, 1962)	NE					x		Martin-Smith & Tan, 1998
Luciosoma pellegrinii Popta, 1905	NE					x		Martin-Smith & Tan, 1998
Macrochirichthys macrochirus (Valenciennes, 1844)	NT	x						Davies & Abdullah, 1989
Mystacoleucus marginatus (Valenciennes, 1842)	LC	x						Davies & Abdullah, 1989; IPT-AWB, 1993
Nematabramis everetti Boulenger, 1894	NE					x		Martin-Smith & Tan, 1998; Sade & Biun, 2012
Osteochilus chini Karnasuta, 1993	NE					x		Martin-Smith & Tan, 1998
Osteochilus enneaporos (Bleeker, 1852)	LC	x						IPT-AWB, 1993
Osteochilus ingeri Karnasuta, 1993	NE					x		Martin-Smith & Tan, 1998
Osteochilus microcephalus (Valenciennes, 1842)	LC					x		Sade & Biun, 2012
Osteochilus spilurus (Bleeker, 1851)	LC	x		x	x		x	Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Giam et al., 2012
Osteochilus vittatus (Valenciennes, 1842)	LC	x		x				Davies & Abdullah, 1989; IPT-AWB, 1993; Zakaria et al., 1999; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006

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Species	Status	1	2	3	4	5	6	Reference
Osteochilus enneaporos (Bleeker, 1852)	LC	x						IPT-AWB, 1993
Oxygaster anomalura (van Hasselt, 1829)	LC	x	x				x	Davies & Abdullah, 1989; IPT-AWB, 1993; Shah et al., 2006; Khairul-Adha et al., 2009
Paedocypris micromegethes Kottelat, Britz, Tan & Witte, 2006	NE						x	Giam et al., 2012
Paedocypris sp. "North Selangor"	-	x						Giam et al., 2012
Paedocypris sp. "Pahang"	-				x			Giam et al., 2012
Parachela hypophthalmus (Bleeker, 1860)	LC	x		x				Ahmad et al., 2005
Parachela oxygastroides (Bleeker, 1852)	LC	x						Davies & Abdullah, 1989; IPT-AWB, 1993; Ismail et al., 2013
Puntioplites bulu (Bleeker, 1851)	DD	x						Davies & Abdullah, 1989; IPT-AWB, 1993; Martin-Smith & Tan, 1998
Puntius binotatus (Valenciennes, 1842)	LC	x	x					Davies & Abdullah, 1989; Rezawaty, 2004; Shah et al., 2006
Puntius fasciatus (Jerdon, 1849)	LC	x						Davies & Abdullah, 1989
Puntigrus partipentazona (Fowler, 1934)	LC			x				Ahmad et al., 2005
Rasbora argyrotaenia (Bleeker, 1849)	NE					x		Martin-Smith & Tan, 1998
Rasbora bankanensis (Bleeker, 1853)	NE	x		x				IPT-AWB, 1993; Ahmad et al., 2005
Rasbora borapetensis Smith, 1934	LC		x					Rezawaty, 2004

cont'd Table 1

Species	Status				References
Rasbora cf. *sumatrana* (Bleeker, 1852)	NE	x	x	x	Davies & Abdullah, 1989; IPT-AWB, 1993; Zakaria et al., 1999; Rezawaty, 2004; Shah et al., 2006; Sade & Biun, 2012; Siow et al., 2013
Rasbora dorsiocellata (Duncker, 1904)	NE	x	x		Ng et al., 1994; Ahmad et al., 2005
Rasbora maculata (Duncker, 1904)	NE	x			IPT-AWB, 1993; Ng et al., 1994
Rasbora gracilis (Kottelat, 1991)	NE	x	x		IPT-AWB, 1993; Beamish et al., 2003; Ahmad et al., 2005
Rasbora kalochroma (Bleeker, 1851)	NE	x	x		Davies & Abdullah, 1989; IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
Rasbora cephalotaenia (Bleeker, 1852)	NE	x	x	x	Davies & Abdullah, 1989; IPT-AWB, 1993; Ng et al., 1994; Zakaria et al., 1999; Beamish et al., 2003; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Ahmad et al., 2013; Ismail et al., 2013
Rasbora dusonensis (Bleeker, 1850)	NE	x			IPT-AWB, 1993; Ismail et al., 2013
Rasbora einthovenii (Bleeker, 1851)	NE	x	x		IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Ismail et al., 2013

cont'd Table 1

Family	Species	Status					References
	Rasbora elegans Volz, 1903	LC	x		x		Ahmad et al., 2005
	Rasbora paucisqualis Ahl, 1935	LC			x		Ahmad et al., 2005
	Rasbora tornieri Ahl, 1922	LC			x	x	Parenti & Lim, 2005
	Rasbora trilineata Steindachner, 1870	LC		x	x		Zakaria et al., 1999; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006
	Striuntius lineatus (Duncker, 1904)	NE	x	x			Zakaria et al., 1999; Rezawaty, 2004; Shah et al., 2006; Ismail et al., 2013
	Sundadanio margarition Conway, Kottelat & Tan, 2011	NE				x	Giam et al., 2012
	Thynnichthys thynnoides (Valenciennes, 1842)	LC	x				Davies & Abdullah, 1989
	Trigonopoma pauciperforatum (Weber & de Beaufort, 1916)	NE	x		x	x	IPT-AWB, 1993; Ng et al., 1994; Zakaria et al., 1999 ; Ahmad et al., 2005; Khairul Adha et al., 2009; Ahmad et al., 2013; Ismail et al., 2013
	Trigonopoma gracile (Kottelat, 1991)	NE	x				Ismail et al., 2013; Siow et al., 2013
	Trigonostigma heteromorpha (Ducker, 1904)	LC	x		x	x	IPT-AWB, 1993; Zakaria et al., 1999; Ahmad et al., 2005
Eleotrididae	*Oxyeleotris marmorata* (Bleeker, 1852)	NE	x		x		Davies & Abdullah, 1989; IPT-AWB, 1993; Rezawaty, 2004
Gobiidae	*Gobiid* sp.	-				x	Martin-Smith & Tan, 1998
	Pseudobiopsis oligactis (Bleeker, 1875)	NE	x				IPT-AWB, 1993

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Family	Species							References
Helostomatidae	*Helostoma temminkii* (Cuvier, 1829)	LC	x	x	x		x	IPT-AWB, 1993; Ng et al., 1994; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Khairul-Adha et al., 2009; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
Mastacembelidae	*Macrognathus aculeatus* (Bloch, 1787)	NE	x					IPT-AWB, 1993
	Macrognathus circumcinctus (Hora, 1924)	LC	x					IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2013; Ismail et al., 2013
	Mastacembelus armatus (La Cepède, 1800)	LC		x				Shah et al., 2006
Nandidae	*Nandus nebulosus* (Gray, 1835)	LC	x	x	x	x		IPT-AWB, 1993; Ng et al., 1994; Zakaria et al., 1999; Beamish et al., 2003; Ahmad et al., 2005; Ahmad et al., 2013
Nemacheilidae	*Nemacheilus selangoricus* (Duncker, 1904)	DD	x	x	x	x		Ahmad et al., 2005; Zakaria et al., 1999; Beamish et al., 2003; Ismail et al., 2013
Notopteridae	*Chitala chitala* (Hamilton, 1822)	NT	x					Davies & Abdullah, 1989
	Chitala lopis (Bleeker, 1851)	LC	x	x		x		IPT-AWB, 1993; Ahmad et al., 2005; Shah et al., 2006
	Notopterus notopterus (Pallas, 1976)	LC	x	x				Davies & Abdullah, 1989; Rezawaty, 2004; Shah et al., 2006; Ismail et al., 2013

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Family / Species	Status						References
Osphronemidae							
Belontia hasselti (Cuvier, 1831)	NE	x	x		x		Davies & Abdullah, 1989; IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Ahmad et al., 2013; Ismail et al., 2013
Betta akarensis Regan, 1910	NE					x	Parenti & Lim, 2005
Betta bellica (Sauvage, 1884)	LC	x			x		IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013
Betta brownorum Witte & Schmidt, 1992	NE					x	Giam et al., 2012
Betta hipposideros Ng & Kottelat, 1994	VU	x					Ng et al., 1994; Ahmad et al., 2013; Ismail et al., 2013
Betta ibanorum Tan & Ng, 2004	NE					x	Giam et al., 2012
Betta livida (Ng & Kottelat, 1992)	EN	x					IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Giam et al., 2012; Ahmad et al., 2013; Ismail et al., 2013
Betta imbellis (Ladiges, 1975)	LC	x					IPT-AWB, 1993
Betta persephone Schaller, 1986	CR			x			Giam et al., 2012
Betta pugnax (Cantor, 1849)	NE	x	x				Davies & Abdullah, 1989; Zakaria et al., 1999; Beamish et al., 2003; Ismail et al., 2013; Siow et al., 2013
Betta pulchra Tan & Tan, 1996	NE			x			Giam et al., 2012
Betta splendens Regan, 1910	VU		x				Rezawaty, 2004; Shah et al., 2006

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Species	Status							References
Betta taeniata Regan, 1910	NE		x					Rezawaty, 2004
Betta tomi Ng & Kottelat, 1994	VU			x				Giam et al., 2012
Betta tussyae Schaller, 1985	NE				x	x		Ahmad et al., 2005; Giam et al., 2012
Betta waseri (Krummenacher, 1987)	NE	x			x	x		IPT-AWB, 1993; Ahmad et al., 2005; Giam et al., 2012
Luciocephalus pulcher (Gray, 1830)	NE	x	x		x		x	Davies & Abdullah, 1989; IPT-AWB, 1993; Ng et al., 1994; Zakaria et al., 1999; Beamish et al., 2003; Parenti & Lim, 2005; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
Osphronemus goramy La Cepède, 1801	LC		x					Rezawaty, 2004
Parosphromenus alfredi Kottelat & Ng, 2005	NE			x				Giam et al., 2012
Parosphromenus allani Brown, 1987	NE						x	Giam et al., 2012
Parosphromenus filamentosus Vierke, 1981	NE		x					Zakaria et al., 1999
Parosphromenus harveyi (Brown, 1987)	EN	x						IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Giam et al., 2012; Ahmad et al., 2013; Ismail et al., 2013
Parosphromenus nagyi Schaller, 1985	NE				x	x		Ahmad et al., 2005; Giam et al., 2012
Parosphromenus tweediei Kottelat & Ng, 2005	NE			x				Giam et al., 2012

438

Pertanika J. Trop. Agric. Sci. 39 (4) 421 - 458 (2016)

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Family	Species	Status				References
	Sphaerichthys osphromenoides (Canestrini, 1860)	NE	x	x	x	IPT-AWB, 1993; Ng et al., 1994; Zakaria et al., 1999; Beamish et al., 2003; Rezawaty, 2004; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
	Trichopodus leerii (Bleeker, 1852)	NT	x	x		Davies & Abdullah, 1989; IPT-AWB, 1993; Ng et al., 1994; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013
	Trichopodus pectoralis (Regan, 1910)	LC	x	x	x	IPT-AWB, 1993; Ng et al., 1994; Rezawaty, 2004; Shah et al., 2006; Khairul-Adha et al., 2009
	Trichopodus trichopterus (Pallas, 1770)	LC	x	x	x	IPT-AWB, 1993; Ng et al., 1994; Martin-Smith & Tan, 1998; Beamish et al., 2003; Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Khairul-Adha et al., 2009; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
	Trichopsis vittata (Cuvier, 1831)	LC	x	x		IPT-AWB, 1993; Ng et al., 1994; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
Pangasiidae	*Pangasius lithostoma* Roberts, 1989	NE			x	Hassan et al., 2010
	Pseudolais micronemus (Bleeker, 1846)	DD	x	x		Martin-Smith & Tan, 1998; Ahmad et al., 2005

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Family	Species	IUCN					References
Pristolepididae	*Pristolepis grootii* (Bleeker 1852)	NE				x	IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ismail et al., 2013
	Pristolepis fasciata (Bleeker, 1851)	LC	x	x		x	Rezawaty, 2004; Ahmad et al., 2005; Shah et al., 2006; Siow et al., 2013
Siluridae	*Kryptopterus bircirrhis* (Valenciennes, 1839)	LC				x	Davies & Abdullah, 1989; IPT-AWB, 1993
	Kryptopterus limpok (Bleeker, 1852)	NE			x		Hassan et al., 2010
	Kryptopterus macrocephalus (Bleeker, 1858)	LC	x			x	Davies & Abdullah, 1989; IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013
	Kryptopterus sp.?	-				x	Siow et al., 2013
	Ompok bimaculatus (Bloch, 1794)	NT		x		x	Davies & Abdullah, 1989; Shah et al., 2006
	Ompok fumidus (Tan & Ng, 1996)	VU				x	Beamish et al., 2003; Siow et al., 2013
	Ompok leiacanthus (Bleeker, 1853)	DD				x	IPT-AWB, 1993; Ng et al., 1994
	Ompok sabanus Inger & Chin, 1959	NE			x		Martin-Smith & Tan, 1998
	Ompok sp.?	-		x			Zakaria et al., 1999; Shah et al., 2006
	Phalacronotus apogon (Bleeker, 1851)	LC	x			x	Ahmad et al., 2005
	Phalacronotus parvanalis (Inger & Chin, 1959)	NE			x		Martin-Smith & Tan, 1998

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Family	Species	Status					References
	Silurichthys hasseltii (Bleeker, 1858)	NE	x	x			IPT-AWB, 1993; Ng et al., 1994; Ahmad et al., 2005
	Silurichthys indragiriensis (Volz, 1904)	NE	x				Beamish et al., 2003; Giam et al., 2012; Ahmad et al., 2013; Ismail et al., 2013
	Silurichthys phaiosoma (Bleeker, 1851)	NE				x	Giam et al., 2012
	Wallago leeri Bleeker, 1851	NE	x	x			Ahmad et al., 2005; Siow et al., 2013
	Wallago maculatus Inger & Chin, 1859	NE			x		Martin-Smith & Tan, 1998
Symbranchidae	*Monopterus albus* (Zuiew, 1793)	LC	x	x	x		IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Ahmad et al., 2005; Shah et al., 2006; Siow et al., 2013
Syngnathidae	*Doryichthys deokhatoides* (Bleeker, 1854)	NE	x				IPT-AWB, 1993
	Doryichthys martensii (Peters, 168)	NE	x				IPT-AWB, 1993
Tetraodontidae	*Pao leirus* (Bleeker, 1850)	NE	x				Davies & Abdullah, 1989
Toxotidae	*Toxotes chatareus* (Hamilton, 1822)	NE			x		Martin-Smith & Tan, 1998
Zenarchopteridae	*Dermogenys pusilla* Kuhl & van Hasselt, 1823	NE	x	x			Shah et al., 2006
	Hemirhamphodon byssus Tan & Lim, 2013	NE		x			Hassan et al., 2010
	Hemirhamphodon kapuasensis Collett, 1991	NE		x			Hassan et al., 2010
	Hemirhamphodon kuekenthali Steindachner, 1901	NE		x			Hassan et al., 2010
	Hemirhamphodon phaiosoma (Bleeker, 1852)	NE		x			Hassan et al., 2010

cont'd Table 1

							References
Hemirhamphodon pogonognathus (Bleeker, 1853)	LC	x	x	x	x		IPT-AWB, 1993; Ng et al., 1994; Zakaria et al., 1999; Beamish et al., 2003; Rezawaty, 2004; Ahmad et al., 2005; Ahmad et al., 2013; Ismail et al., 2013

* Introduced species
**Confusing taxonomy
IUCN Status source: IUCN, (2015)
NE = Not Evaluated, DD = Data Deficient, LC = Least Concern, NT = Near Threatened, VU = Vulnerable, EN = Endangered, CR = Critically Endangered, EW = Extinct in the Wild, EX = Extinct
NSPSF = North Selangor peat swamp forest, PBPSF = Paya Beriah peat swamp forest, SEPPSF = Southeast Pahang peat swamp forest, EP = East Peninsular Malaysia (part of Pahang and Terengganu), PSFs = Peat Swamp Forests

442

Pertanika J. Trop. Agric. Sci. 39 (4) 421 - 458 (2016)

Table 2
Summary of black water fish species recorded in Malaysia

Peat swamp sites	Location	Region	No. of families	No. of species	Reference
NSPSF	Selangor	Peninsular Malaysia	23	114	Davies & Abdullah, 1989; IPT-AWB, 1993; Beamish et al., 2003; Ahmad et al., 2013; Ismail et al., 2013; Siow et al., 2013
PBPSF	Perak	Peninsular Malaysia	18	49	Zakaria et al., 1999; Rezawaty, 2004; Shah et al., 2006
Black water ditches and ponds of west Johor and Ambat	Johor	Peninsular Malaysia	7	13	Kottelat, 1996; Ng & Kottelat, 1998; Giam et al., 2012
SEPPSF	Pahang	Peninsular Malaysia	19	58	Ahmad et al., 2005
Endau drainage, black water sites including ponds and ditches	Pahang and Terengganu	Peninsular Malaysia	5	9	Kottelat, 1996; Ng & Tan, 1999; Giam et al., 2012
Segama River, Maliau Basin	Sabah	Malaysia Borneo	12	31	Martin-Smith & Tan, 1998; Sade & Biun, 2012
Rajang Basin, Sadong, Batang Kerang, Nanga Merit	Sarawak	Malaysia Borneo	13	40	Parenti & Lim, 2005; Khairul-Adha et al., 2009; Hassan et al., 2010; Giam et al., 2012; Tan & Lim, 2013

Paya Beriah Peat Swamp Forest, North Perak

The Paya Beriah peat swamp forest (PBPSF), which is located near Bukit Merah reservoir, Perak, has a total area of 5,500 hectares that are bordered by dykes, roads and railway tracks. Like most PSFs in Malaysia, it has been significantly impacted through conversion to residential, industrial and agricultural uses (Ismail & Ali, 2002). Nonetheless, the fish population and its biodiversity in PBPSF are not well documented, in comparison with NSPSF.

The most recognised and well-documented surveys in PBPSF were done by Zakaria et al. (1999), Rezawaty (2004) and Shah et al. (2006).

Zakaria et al. (1999) conducted a study of the swamp-riverine fish populations of two spatially isolated fresh water swamp ecosystems: Beriah Kiri River, which was mistakenly identified as Beriah Kanan River (Shah et al., 2006), located in northern Peninsular Malaysia, and the Ulu Sedili River in southern Peninsular Malaysia. The Beriah Kiri and Ulu Sedili river systems are separated by an extensive mountain

range and a north-south distance of about 900 km, creating an effective dispersal and/or migration barrier (Johnson, 1967a; Prentice & Parish, 1992; Krebs, 2009). Zakaria et al. (1999) reported a total of 24 fish species identified from their study, of which 20 species were from Beriah Kiri and 10 species from Ulu Sedili swamp-riverine area. Eight families were represented from the Beriah Kiri swamp-riverine area. However, only the fish species recorded from the Beriah Kiri swamp-riverine are outlined in this review.

A study from Rezawaty (2004) reported a total of 30 fish species from 12 families in a study carried out on the entire Sungai Beriah drainage system, while during an intensive survey of the PBPSF, specifically in Beriah Kanan, Beriah Kiri and Beriah rivers, Shah et al. (2006) recorded a total of 32 fish species belonging to 13 families (Table 1).

Fishes Recorded from the Peat Swamps of Johor

Information on the ichthyofauna of PSFs of the state of Johor is relatively patchy compared to the NSPSF. Kottelat (1996) and Ng and Kottelat (1998) recorded *Systomus lineatus* and *Hyalobagrus ornatus* respectively from PSF of Johor. Giam et al. (2012) recorded 13 fish species belonging to seven families from black water ditches, streams, pools, remnants of PSF and PSFs of West Johor and Ambat (Table 1).

Fishes Recorded from Southeast Pahang Peat Swamp Forest

Ahmad et al. (2005) recorded 58 fish species (with an additional unidentified species belonging to the family Mastacembelidae) from 17 families (19 following the recent taxonomic revisions detailed in Kottelat's catalogue) during a survey in Bebar River, a large river that flows out of the southeast Pahang peat swamp forest (SEPPSF, which is also known as Pekan peat swamp forest). This survey, which was conducted along Bebar River and Serai River (a tributary of Bebar River), brought the total fish species known in SEPPSF to 65 species (see Ahmad et al., 2005) (see Table 1).

Fishes Recorded from the Peat Swamps of East Peninsular Malaysia

Kottelat (1996) and Ng and Tan (1999) recorded *S. lineatus* and *Neohomaloptera johorensis* respectively from east Peninsular Malaysia. Similarly, Giam et al. (2012) recorded nine fish species from six families from the black waters of east Peninsular Malaysia comprising of part of Malaysia's Johor, Pahang and Terengganu (Table 1).

Fish Species Recorded in the Peat Swamp Forests of Sabah

Sabah, together with Sarawak, Kalimantan Barat, Kalimantan Timur and Brunei is situated in Borneo, the world's third largest island with some 743,107 km² of land area. As a result of "…difficulties of access to the interior part of tropical rainforest, a

lack of reliable dating of igneous rocks, poorly fossiliferous sedimentary rocks and an absence of a coherent stratigraphic scheme for many parts of the island" (Hall & Nichols, 2002), there is only fragmentary information on the geology and fish fauna of the island (Parenti & Lim, 2005). The fish species of Sabah are relatively less surveyed and documented than those of Peninsular Malaysia. Even when intensive surveys are carried out, they have not been widely published by the researchers.

Martin-Smith and Tan (1998) carried out an intensive collection of freshwater fishes over a period of two years from the catchment of the upper Segama River near Danum Valley Field Centre, headwater streams in the catchment of the Kuamut River and from the lower Segama River. They reported a total of 65 species from 20 families. A total of about 30 fish species were recorded in blackwater ditches, ponds and streams from the lower Segama River (Martin-Smith & Tan, 1998).

Also, Sade and Biun (2012) studied the ichthyofauna of Maliau Basin, a saucer-shaped depression enclosed by a mountainous rim in the remote part of Sabah, with an undisturbed flora and fauna. Although 15 fish species were recorded from the study, only three species were recorded from acidic tea-coloured blackwaters in the zone (Table 1).

Fish Species Recorded in the Peat Swamp Forests of Sarawak

Like Sabah, the ichthyofauna of the peat swamps of Sarawak has received little attention in comparison to that of Peninsular Malaysia. The ichthyofauna of Sarawak is generally neglected in comparison to the other political divisions of Borneo for which detailed checklists have been documented (Parenti & Lim, 2005).

Parenti and Lim (2005) carried out a study of the ichthyofauna of the Rajang Basin in Sarawak, Malaysia, Borneo, and presented a checklist of 164 fish species recorded from the headwaters all the way to the brackish waters, including Belaga and the Balui River, Kapit and the Baleh River, and Sibu area. A total of seven blackwater fish species were recorded from blackwater ditches and pools along Teku River and remnant of PSF behind the old Sibu airport.

A previous study from Khairul-Adha et al. (2009) recorded 36 species of fish belonging to 13 families from brownish and blackish water habitats of Batang Kerang in Balai Ringin, Sarawak, during wet and relatively dry seasons. Thirty-two species from 12 families were recorded in the brownish water, while only 12 species from seven families were recorded in the black water habitat. In a study by Hassan et al. (2010), seven out of 15 fish species recorded from 11 families in Nanga Merit area were from a water body with peat-like characteristics.

Recently, Tan and Lim (2013) recorded four species of fish of the genus *Hemirhamphodon* from blackwater ditches and ponds in Sarawak, Malaysia, Borneo, while Giam et al. (2012) recorded 13 fish species belonging to five families from the peat swamps of Rajang and Sadong in Sarawak (Table 1).

TAXONOMIC NOTES

Some of the taxonomic names of the fish species outlined in Table 1 and their placement within families are slightly or completely different from the originally recorded names from the surveys, as a result of the recent changes in the taxonomy of the fish species. A comprehensive list of taxonomic changes is given in Kottelat's catalogue (Kottelat, 2013). Davies and Abdullah (1989) recorded *O. vittatus*, along with its synonym *O. hasselti*, as two completely different species. The species *Belontia hasselti* now classified under the family Osphronemidae was placed in the Anabantidae family. Members of Osphronemidae family were placed in the family Belontia by IPT-AWB (1993) and Ng et al. (1994). In the same vein, *Luciocephalus pulcher* was classified under the family Luciocephalidae. Following the work of Britz (1994), and Kottelat and Whitten (1996), the earlier families of Osphronemidae, Belontiidae and Luciocephalidae were constituted as a single family, Osphronemidae. Recently, Beamish et al. (2003) classified *Nemacheilus selangoricus* under the family Nemacheiliidae in the family Bagridae, along with *N. johorensis*. In the same vein, Ahmad et al. (2005) classified *N. selangoricus* of the family Nemacheilidae and *Barbucca diabolica* of the family Barbuccidae under the family Balitoridae. Meanwhile, *Hemirhamphodon progonognathus* of the family Zenarchopteridae was placed in the family Hemiramphidae by IPT-AWB (1993), Ng et al. (1994), Beamish et al. (2003)

and Ahmad et al. (2005). The *Parakysis verucosus* of the family Akysidae was also classified under Parakysidae by IPT-AWB (1993) and Ng et al. (1994), while Siow et al. (2013) recorded *Pristolepis fasciata* of the family Pristolepididae as belonging to the family Nandidae. Also, a recorded fish species belonging to the genus *Kryptopterus* was not designated a species name and the reason was not stated appropriately. Considering there are about 17 distinct species in the genus *Krptopterus* (Kottelat, 2013), it is possible that the sample could be a newly discovered species.

The species recorded as *Puntius lineatus* is now named *Striuntius lineatus,* which is a "preferred" name or "authority", the latter being a synonym. Another species recorded as *Labiobarbus lineatus*, which is excluded from Kottelat's catalogue was regarded as a preferred name for the species *Dangila lineata* in Fish Base, Species 200 and ITIS Catalogue of Life: April 2013, and the NCBI Taxonomy (Torrres, 2000; EOL, 2015). *Dangila lineata* was also recorded as a synonym of *L. leptocheilus* in Kottelat's catalogue (Kottelat, 2013), suggesting that both names may actually be referring to the same species. However, Rainboth et al. (2012) considered that *L. lineatus* is a distinct species. There has been some confusion about the taxonomy of the species *L. leptocheilus* (*L. lineatus*). In the taxonomic outline provided in this review, the species is presented as *L. leptocheilus* in line with Kottelat's catalogue. Also, the fish earlier recorded as *Mystus micracanthus* is actually *M. nigriceps* (Roberts, 1993; Ng, 2002; Kottelat, 2013).

ECONOMIC IMPORTANCE OF PEAT SWAMP FISHES

Black water peat swamps are important catchment areas. The peat has great water retention ability and serves as reservoir of rain water, which is utilised in agriculture for irrigating the rice fields adjacent the NSPSF (Low & Balamurugan, 1989). The fish of NSPSF are harvested for consumption and also for the aquarium trade due to their ornamental value (Ng et al., 1994; Ismail et al., 2013) (Table 4). Most of the species harvested for consumption by the local people are sold at very low prices relative to their actual worth (Ng et al., 1994).

A breeding study has been carried out on some of the ornamental fish of peat black water such as pearl gourami (*Trichogaster leeri*), chocolate gourami (*Sphaerichthys osphromenoides*), clown rasbora (*Rasbora kalochroma*), six-banded barb (*P. johorensis*), bellicose Betta (*Betta bellica*), giant fighting fish (*B. waseri*) and pygmy rasbora (*R. maculata*), with the aim of conserving the species and improving their production (FFRC, 1995).

CONSERVATION STATUS OF PEAT SWAMP FISH SPECIES IN MALAYSIA

The conservation status of a species indicates if it still exists and how likely it is to become extinct in the near future (InfoNatura, 2007). The IUCN Red List of Threatened Species is the best known conservation status listing system in the world, which classifies species into nine categories based on the rate of decline of the population, population size, geographical range, and degree of population and distribution fragmentation (Mace et al., 2008; Biodic, 2013; IUCN, 2014). The categories are "Extinct (EX), Extinct in the Wild (EW), Critically Endangered (CR), Endangered (EN), Vulnerable (VU), Near Threatened (NT), Least Concern (LC), Data Deficient (DD) and Not Evaluated (NE)" (IUCN, 2001; InfoNatura, 2007; IUCN, 2012; IUCN, 2015; IUCN, 2014). A species is EX when there is no reasonable doubt that the last individual has died, as a result of failure to record an individual through exhaustive surveys in known and/or expected habitat, at appropriate times (diurnal, seasonal, annual), throughout its historic range. Extinct in the Wild is used to refer to species that are known only to survive in cultivation, in captivity or as a naturalised population(s) well outside the past range (InfoNatura, 2007; Biodic, 2013; IUCN, 2014).

Out of the 198 black water fish species recorded from Malaysia, 106 (53.54%) species belonging to the category NE indicate that their conservation status has not been evaluated against the criteria. Seven (3.53%) species are DD as a result of inadequate information on their distribution and/or population status. Sixty-two species (31.31%) belonging to the LC category, indicate lowest risk, widespread and abundant species. Six species (3.03%) are NT, signifying likelihood to become endangered in the future. Six (3.03%) and two (1.01%) species are VU and EN, respectively, indicating a highly and very highly risk of extinction in the wild. Besides that, three species (1.52%) are CR and

facing an extremely high risk of extinction in the wild, while six species (3.03%) were not fully classified and so exempted from all the categories (Table 1).

A total of only 12 species (6.70%) are threatened, i.e. under CR, EN and VU category. The IUCN classification may not be a true expression of the conservation status of black water fishes, particularly in Malaysia as so many of the species (57.54%) remain unevaluated against the criteria for classification of threatened species. In data-poor situations, it is not uncommon for listing errors of species that otherwise should be classified as threatened (Gärdenfors, 2000; Keith et al., 2000; Gärdenfors et al., 2001; Keith et al., 2004). For instance, five of the ten black water fish species (*B. brownorum, Sundadanio margaration, B. ibanorum, Parosphromenus allani* and *H. ornatus*), listed as "most VU" by Giam et al., (2012) using the criteria of decline in population, geographical range and basin extinction, were classified under the NE in Red List category (Tables 1 and 3).

It is important to point out that the species recorded as 'rare' at a distinct time may be more abundant at other times. For example, the abundance of individual fish and species composition vary during the wet and dry seasons, which may be related to variations in migratory movements of fish species (Ng et al., 1994; Renato et al., 2000; Khairul-Adha et al., 2009). Some species migrate from downstream to upper reaches of a river during high water levels for breeding or food, then migrate back to the lower reaches after spawning as the water level reduces (Lowe-McConnell, 1975;

Welcomme, 1979). Therefore, conservation surveys should be exhaustive, in all known and/or expected habitats, at appropriate times covering seasons of high and low water levels over an extended period of time before a species is pronounced as rare or threatened.

Considering the extensive destruction of tropical PSFs worldwide and the decline in PSF biodiversity, more black water fish species will fall into the threatened (CR, EN and VU) category if evaluated against the criteria. However, it is important to note that the category of threat simply provides an assessment of the extinction risk under current circumstances, and is not necessarily sufficient to determine priorities for conservation action (Mace & Lande, 1991).

CONSERVATION OF PEAT SWAMP FORESTS

Peat swamp forests are an important component of the world's wetlands, providing a wide variety of goods and services in the form of carbon sequestration, flood mitigation and globally important biodiversity reservoirs (Parish, 2002; UNDP, 2006; Posa et al., 2011; Ismail et al., 2013), with many endemic species (Kottelat & Ng, 2005; Kottelat & Widjanarti, 2005; UNDP, 2006; Tan & Kottelat, 2009; Conway et al., 2011; Conway & Kottelat, 2011; Posa et al., 2011; Giam et al., 2012; Taskforce REDD, 2012; PIU-SERT, 2013; Muchlisin et al., 2015).

Malaysian PSFs make up about 75% of the country's total wetlands, with 80%

Table 3
Peat swamp fishes listed as Threatened in IUCN Red List

Family	Species	Status	Reference
Anguillidae	*Anguilla borneensis*	VU	Martin-Smith & Tan, 1998
Clariidae	*Encheloclarias curtisoma*	CR	IPT-AWB, 1993; Ng et al., 1994; Giam et al., 2012
	Encheloclarias keliodes	CR	Giam et al., 2012
	Encheloclarias prolatus	VU	Giam et al., 2012
Osphronemidae	*Ompok fumidus*	VU	Beamish et al., 2003; Siow et al., 2013
	Betta hipposideros	VU	Ng et al., 1994; Ahmad et al., 2013; Ismail et al., 2013
	Betta livida	EN	IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Giam et al., 2012; Ahmad et al., 2013; Ismail et al., 2013
	Betta splendens	VU	Rezawaty, 2004; Shah et al., 2006
	Betta tomi	VU	Giam et al., 2012
	Betta persephone	CR	Giam et al., 2012
	Parosphronemus harveyi	EN	IPT-AWB, 1993; Ng et al., 1994; Beamish et al., 2003; Giam et al., 2012; Ahmad et al., 2013; Ismail et al., 2013

found in east Malaysia (Sabah 8%; Sarawak 72%) and 20% in Peninsular Malaysia. Peat swamp forests in Malaysia have undergone severe degradation over the years. For example, the PSF cover of NSPSF was estimated to be 0.67 million hectares in 1981 but reduced drastically to 0.34 million hectares in the 1990s (UNDP, 2006) and there has been further destruction since then. As the nation becomes aware of the need to conserve PSFs, some percentage of PSFs have been protected within Permanent Forest Reserves and stateland forests (UNDP, 2006). However, this protection is inadequate. Agricultural conversion and fire have destroyed PSF within these protected areas and furthermore, unless the entire forests are protected, any drainage around the edges impacts the entire forest.

The main threats of PSFs in Malaysia are forestry (overexploitation), illegal logging, pollution (including oil, industrial, nutrient and sedimentation), waste disposal, airports, land reclamation, large scale land conversion for agriculture, industrialisation and settlement-urbanisation (Ng & Shamsudin, 2001; Chong et al., 2010). In addition, draining and clearing of the PSFs for agriculture and palm oil plantations have resulted in peat land fires (Parish, 2002; Page et al., 2011), which contribute to the annually recurring episodes of transboundary haze pollution in the Southeast Asian region (Lo & Parish, 2013).

Several studies on the diversity of fish in PSFs have revealed the existence of economically important fishes for consumption, aquarium trade and

Table 4
Commonly consumed and ornamental black water fish species in Malaysia

Consume	Ornamental
Anabas testudineus	*Betta bellica*
Belontia hasselti	*Betta livida*
Channa bankanensis	*Betta hipposideros*
Channa lucius	*Belontia hasselti*
Channa melosoma	*Channa gachua*
Clarias meladerma	*Helostoma temminkii*
Clarias nieuhofii	*Hemirhamphodon progonognathus*
Clarias leiacanthus	*Kryptopterus macrocephalus*
Helostoma temminkii	*Leiocassis micropogon*
Kryptopterus macrocephalus	*Luciocephalus pulcher*
Monopterus albus	*Macrognathus circumcinctus*
Mystus bimaculatus	*Mystus bimaculatus*
Macrognathus circumcinctus	*Osteochilus spilurus*
Hemibagrus nemurus	*Parosphromenus harveyi*
Pristolepis grootii	*Desmopuntius hexazona*
Trichopodus leerii	*Desmopuntius johorensis*
Trichopodus pectoralis	*Rasbora cephalotaenia*
Trichopodus trichopterus	*Rasbora dorsiocellata*
	Rasbora einthovenii
	Rasbora gracilis
	Rasbora kalochroma
	Rasbora maculata
	Trigonopoma pauciperforatum
	Silurichthys hasseltii
	Sphaerichthys osphromenoides
	Trichopodus leerii
	Trichopodus trichopterus

endangered species (IPT-AWB, 1993; Ng et al., 1994; Lee, 2001; Beamish et al., 2003), where the majority of fish are highly dependent on the rivers in the PSFs (Beamish et al., 2003; Yule, 2010). The degradation of PSFs is expected to affect the fish community structure in such a way that extinction of some already endangered species in the near future is probable. Gibson et al. (2011) reported that converted land-use types vary in their ability to support forest biodiversity, generally having a reduced capacity for biodiversity support than in their original unconverted form. Giam et al. (2012) predicted the number of fish species that would become extinct under different land-use conditions using the Matrix-Calibrated Species-Area Model (MCSAM) (Koh & Ghazoul, 2010) and Mote Carlo simulations to project PSF

basins extinctions. Under the scenario, the 10 most vulnerable species (*Encheloclarias prolatus, B. brownorum, S. goblinus, S. margarition, B. ibanorum, B. burdigala, E. tapeinopterus, Paedocypris progenetica, P. allani* and *H. ornatus*) were recognised and predicted to be extinct by 2050, if the present rate of PSFs conversion continues.

Meanwhile, Beamish et al. (2003) reported 22 species of fish from 43 sites in the NSPSF in 1998 as against 33 species from 27 sites during the preceding year. The PSF was largely forested during the 1997 survey, but the area was cleared and planted with oil palms in 1998. Thirteen species caught in 1997 were not caught in 1998. This supports the prediction by Giam et al. (2012) the extinction of fish species following PSF land conversion. Generally, riparian vegetation acts as a source of energy and matter (Kindler, 1998), and contributes matter to the PSF ecosystem through production of leaf litter (Tabacchi et al., 1998). Modifications in riparian vegetation through logging affect the structure and processes within the peat swamps. It leads to the alteration of the swamp characteristics, reduction of food resources (Tabacchi et al., 1998; Wright & Flecker, 2004) and subsequent loss of biodiversity (Bruenig & Droste, 1995).

Peat swamp forests also serve as an important global carbon storehouse (Parish, 2002; Chimer & Ewel, 2005; Jauhiainen et al., 2005; Rydin & Jeglum, 2006; UNDP, 2006). The PSFs of Malaysia and Indonesia alone store 67 gigatons of carbon in peat, which represents 75% of total tropical peat soil carbon storage (Page et al., 2011). Large scale conversion of this carbon sink, as is happening today, is expected to and will severely impact the earth's climate (Couwenberg et al., 2009; van der Werf et al., 2009; Miettinen et al., 2012) unless drastic measures are put in place to conserve PSFs of the world.

In the discussion of conservation of PSFs fish biodiversity, safe fishing techniques have always been ignored. Researchers, and fishermen alike, employ varying fishing techniques in PSFs and have always been more concerned with increase in catchability than safety and conservation of the fishes. One fishing technique, electro-fishing, that poses a threat to the health of fish has been employed in several surveys in Malaysia PSFs (Martin-Smith & Tan, 1998; Beamish et al., 2003; Shah et al., 2006; Siow et al., 2013). Electro-fishing establishes an electric field in the water (Lamarque, 1990) creating an epileptic response in fish resulting from electric shock to the central nervous system (Reynolds & Kolz, 1993; Sharber & Black, 1999). The reported health effects on fish include internal haemorrhaging and skin discolorations (Dalbey et al., 1996; Kocovsky et al., 1997; Muth & Ruppert, 1997; Thompson et al., 1997; Ainslie et al., 1998; Habera et al., 1999), spinal injuries (Kocovsky et al., 1997), lactacidosis and disturbance of the inter-renal stress response (Mitton & McDonald, 1994), retarded growth (Dalbey et al., 1996; Thompson et al., 1997; Ainslie et al., 1998; Hughes, 1998), and low gamete viability (Muth & Ruppert, 1997; Koupal et al., 1997). Although the

effects of electro-fishing on PSF biodiversity is minor compared to the enormous loss due to PSF drainage, clearance and fire, it is appropriate that only safe fishing techniques such as the use of scoop nets, cast nets, etc. should be employed in a unique habitat such as peat swamps.

CONCLUSION

It is apparent from the ongoing that one of the strongest justifications for conservation of peat swamps is the existing ichthyofauna. Therefore, maintaining fish populations of PSFs is very important as this will require maintaining the natural water tables of the swamps with seasonal flooding. Apart from the necessity to maintain appropriate aquatic habitats for the fish, another important reason is that when peat dries out due to drainage, it is extremely inflammable and thus, becomes highly vulnerable to destruction by fire (Langner et al., 2007; Langner & Siegert, 2009; Page et al., 2009; Posa et al., 2011).

A review of literature on PSF fish and also the IUCN Red List revealed the likelihood of many important fishes becoming extinct in the near future if the present PSFs degradation continues. Moreover, there is also an inevitable risk of increased global warming when the tons of carbon stored in peat lands is released through degradation. Consequently, a concerted effort for conserve regional PSFs is essential, and requires action plans involving all stakeholders (Azmi et al., 2009). The present measures aimed at protecting only some parts of the peat swamp forests in Malaysia are totally inadequate (and in any case, they are largely ignored).

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Pertanika J. Trop. Agric. Sci. 39 (1): 421 - 458 (2016)

453

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Review Article

Impact of Heat Stress on Immune Responses of Livestock: A Review

Sophia Inbaraj[1], Veerasamy Sejian[2]*, Madiajagan Bagath[2] and Raghavendra Bhatta[2]

[1]*ICAR-Central Island Agricultural Research Institute, Portblair, Andaman and Nicobar Islands, 744101, India*
[2]*ICAR-National Institute of Animal Nutrition and Physiology, Adugodi, Bangalore-560 030, India*

ABSTRACT

Climate change acts as a major threat to climate sensitive sectors such as agriculture and animal husbandry. This change in climate will be a greatest challenge to about 1.3 billion population who depends on animal husbandry as their livelihood. Heat stress is considered as one of the primary factors that imposes negative impacts on production and reproduction in farm animals. In addition, it also alters the immune functions of the animal and makes them susceptible to infectious diseases. Based on the duration exposure, heat stress either enhances or suppresses the immune functions in farm animals. The stress signal acts mainly through hypothalamo pituitary adrenal (HPA) axis to modulate the immune response. Generally, it is considered that heat stress acts to shift the adaptive immune function from cell mediated to humoral immunity and thus weakens the animal immune function. Another aspect of this climatic change is the threat of emerging and re-emerging pathogens and disease vectors for which livestock needs fine-tuned immune system to fight against naïve pathogens. Thus, the heat stress-immune system interactions need to be studied thoroughly in order to introduce various management and nutritional strategies to alleviate the ill-effects of heat stress in farm animals.

Keywords: Climate change, Heat stress, Immunity, Livestock, HPA axis, Pathogens

INTRODUCTION

Livestock are called as the living bank for farmers. They contribute about 53 percent of world agricultural GDP (World Bank,

2009), and also to the economy by means of milk, meat, hide, eggs, drought power, manure etc. Apart from that, livestock also provide employment to 1.3 billion world population (FAO, 2009). The current world population of 7.3 billion is expected to reach 8.5 billion by 2030, 9.7 billion in 2050 and 11.2 billion in 2100 (UN, 2015) and about significant proportion of the population in developing countries will migrate to towns leading to increased urbanisation of 56.9% by 2025 (UNFPA, 2008). Ultimately, urbanisation will increase the standard of living of people, while the demand for high quality protein will also increase (Steinfeld et al., 2006). Henceforth, the concept of animal husbandry currently changes with major focus on food animal production. Animal protein is a cheap source of high quality protein with essential vitamins and micronutrients. Thus, to ensure nutritional security to the current growing population, animal protein seems to be inevitable. In order to provide quality animal meat to the consumers' fork, the farm animals should be healthy in terms of physiology, temperament and immunity.

However, there are various stressors, both biotic and abiotic, which challenge the animal's wellbeing. Temperature, solar radiation, photoperiod, humidity, geographical location, nutrition and socio-economic signals are the major abiotic stressors Microbes such as bacteria, viruses, fungi and protozoa, as well as helminths and arthropod vectors, are the biotic stressors.

The term clinical disease is an outcome of the interaction of host factors, pathogen potential and environmental influence. Even though host-pathogen interactions are essential for clinical disease development, the environment also plays an equally important role in modifying the host and pathogen factors (Kelley, 2004). Due to global warming, abrupt climatic conditions like storms, droughts, floods and extreme hot and cold temperatures are prevailing around the globe. With the changing climatic scenario, the frequency and duration of exposure of livestock to abiotic and biotic stressors increases. Current incidences of global climatic change lead to the evolution of new concepts in host-microbe relationship. High temperatures, accompanied by high relative humidity, favour the survival and multiplication of animal disease vector like ticks, fleas, tabanid flies, etc.; thus, the risk of spread of vector borne diseases also increases (Wittman & Baylis, 2000). The intercontinental spread of blue tongue virus by *Culicoides* sps. is a typical example (Rao et al., 2012). Furthermore, the increasing trend of international trade also favours the introduction of new disease species, which the host immune system has never been exposed. Introduction of bovine spongiform encephalopathy (BSE) through bovine offals (Pattison, 1998) and introduction of porcine respiratory and reproductive syndrome (PRRS) in swine in India are few examples of these.

Abiotic stressors such as heat and nutritional stress have a major impact on livestock productivity (Sejian et al., 2011). In the changing climate scenario, other factors like solar radiation, photoperiod

and humidity also synergises with the above stressors. Temperature influences the animals' productive and reproductive traits in a major way. In particular, heat stress is one of the crucial factors affecting livestock productivity (Rivington et al., 2009). Furthermore, the concept of global warming is alarming the planet earth day by day. According to the latest report released by IPCC (2013), there is an average of about 1.53°F (0.85ºC) rise in global surface temperature from 1880-2012. Heat stress affects animal productive performances like milk yield, meat quality and reproductive performances like age at maturity, ovulation failure, embryo mortality, etc. (Shinde & Sejian, 2013). It also weakens the animal's immune system and makes them more prone to diseases. Although this has been observed by various researchers, the impact of heat stress on immune gene expression and process of heat stress mediating immune suppression at molecular level has not been dealt in detail in livestock. In order to withstand the existing and emerging pathogen challenges, the animal's immune system should be in a right tone. In order to mitigate immune suppression by means of various nutritional and hormonal interventions, an in depth understanding of the immunological pathways affected by heat stress and the mechanism by which it is affected is mandatory.

This review deals with the relationship between hypothalamo-pituitary-adrenal axis (HPA) and immune axis, the effects of heat stress on immunity, the mechanism by which heat stress affect immune response and

therefore, various nutritional interventions need to be taken to ameliorate heat stress mediated immune suppression.

STRESS AND IMMUNITY

Various researchers defined stress as per their observations. It was Seyle in 1946, who gave the first definition about the animal response to stress. He called it as the 'general adaptation syndrome'. Stress is also defined as the biological response elicited when an animal perceives a threat to its homeostasis (Moberg & Mench, 2000). The threat is the stressor. An animal during its lifespan experiences non-threatening stress situations too which did not affect its health normally. Stress at its minimal level is always beneficial. A minimal level of stress called as good stress is required by all living organisms to perform a task. Stress is called as bad or stress will become a distress when it alters the normal biological functions like production and reproductive performances of an animal. Stress becomes important or needs attention in an animal lifetime in the point at which its welfare is compromised i.e. its survival, production and reproduction are affected. Thus, stress needs to be an important matter of discussion in livestock production because in a stressed animal, the energy balance and the body reserves will be mobilised to counteract or alleviate the stressor. This, in turn, leads to decreased production and reproductive performances. Furthermore, stress mediated interactions with the immune system suppresses its normal functions and leads the animal to a disease-prone state (Moberg & Mench,

2000). Therefore, the type of stresses that the animals encounter and its impact need to be studied and documented thoroughly to enhance better animal production in the changing climatic scenario. In general, animals encounter various stressors such as thermal stress, production stress, transportation stress, nutritional stress, immune stress, stress due to crowding, drought and environmental conditions (Kelley, 1980). In the arid and semi-arid tropical conditions, animals have to walk considerably long distances which in turn leads to walking stress (Sejian et al., 2012; Maurya et al., 2012). Currently, the concept of multiple stresses is evolving in animal stress physiology. Studies in Malpura sheep of northern India revealed that multiple stresses had significant influence on productive and reproductive parameters (Sejian et al., 2011). In the event of climatic change, stress due to adverse climatic conditions and emerging microbes and vector – borne diseases are some of which the animals have to encounter (Epstein et al., 1998).

Whenever an animal encounters a stressor, the central nervous system first begins to respond by sending signals to any of the biological systems viz. behavioural, autonomic nervous system, neuroendocrine system and immune system in order to alleviate or compensate the threat. It is not mandatory that all the four biological systems should respond together whenever a stress occurs. The response of each animal to a particular stress condition varies depending upon its previous exposure to the stressors (Mason et al., 1991), genetic makeup (Marple et al., 1972), age (Blecha et al., 1983), season and physiological state. The neuroendocrine system responds mainly through the HPA by the release of glucocorticoids that are normally called as stress hormones (Webster & Glaser, 2008). They act to adapt the animal to cope up with the long-term stressors. The immune system responds to stress by enhancement or suppression of immune functions (Dhabhar, 2009). The immune system does not respond directly to stress but via neuroendocrine system. The stress related hormones act on the immune cell receptors to modulate the immune response.

IMMUNITY

The immune system functions can be broadly classified into: (i) innate immunity and (ii) adaptive immunity. Innate immunity is the germ line encoded, non-specific, preliminary line of defence against the invading pathogens. The first entry of the pathogens is prevented at body's entry site through antimicrobial components in mucosa, sweat, tears, saliva, etc. In spite of this, if the pathogens enter, the specialised immune receptors called Pathogen Recognition Receptors (PRR) will identify the conserved molecular signatures called Pathogen Associated Molecular Patterns (PAMPs) present in the pathogens (Janeway & Medzhitov, 2002) and recruit leucocytes, particularly neutrophils, chemical mediators called pro–inflammatory cytokines, followed by macrophages. They ultimately kill the pathogens through reactive oxygen/

nitrogen species production and engulf them up in a process called phagocytosis (Bassett et al., 2003). Their processed fragments are presented to another phase of the immune system for specific immune response called adaptive immune system. Based on the pro–inflammatory cytokines that are released, the adaptive immune response may be either antibody mediated or cell mediated. All these mechanisms will ultimately lead to clearing of pathogens from the host body.

THE LINK BETWEEN STRESS AND THE IMMUNE SYSTEM

Sympathetic innervation from brain connects the primary lymphoid organs and secondary lymphoid organs (Ader et al., 1995); thus, the catecholamine receptors in immune cells also contribute to immune cell activation by stress mechanisms. The end product of HPA axis activation results in glucocorticoid release, which has receptors in almost all organs including the immune cells (Ader et al., 2001).

THE STRESS AXIS

It includes neural and endocrine organs and its secretory products and hormones released in response to stress. The HPA axis and sympathetic-adrenal-medullary system (SAM) are the crucial components that receive stress signals and act to relieve the stress mechanism or adopt the animal to the stressful condition (Riedemann et al., 2010). Figure 1 depicts the interaction between various endocrine axes with the immune system during stress.

SYMPATHETIC-ADRENAL-MEDULLARY SYSTEM (SAM)

It is the sympathetic trunk of the autonomic nervous system (ANS) situated in the adrenal medulla. It acts by the release of epinephrine and nor epinephrine which are responsible for the characteristic flight or -fright mechanism. This helps the animals to overcome the stressor and it is the short term adaptation of the animal to acute stressor. These hormones act to enhance glycogenolysis causing increased glucose levels in circulation. The blood glucose will reach the stressed organ in order to meet the energy requirements and cope up with the stressor (Tort & Teles, 2011).

HYPOTHALAMO-PITUITARY ADRENAL AXIS

The core of stress response is believed to be based on the activation of HPA. The key components of this system include: (i) hypothalamus, and (ii) brain stem. The parvocellular neurons of CRH, arginine vasopressin (AVP) neurons of paraventricular nuclei of hypothalamus, CRH neurons of paragigantocellular and parabranchial nuclei of medulla and locus ceruleus innervate the system (Tort & Teles, 2011). They sense stressor and get activated to release CRH from paraventricular nucleus and arginine vasopressin from magnocellular neurons of hypothalamus. They act on chromaffin cells of anterior pituitary to release adrenocorticotropic hormone (ACTH). ACTH, in turn, acts on adrenal cortex to stimulate synthesis and release of steroids leading to cholesterol

uptake. Cholesterol further gets converted into cortisol and corticosterone. Cortisol is the major glucocorticoid (GC) secreted in response to stress in mammals. GCs have receptors called glucocorticoid receptors (GRs) in almost all body tissues (de Kloet & Derijk, 2004). Glucocorticoid receptor exists as a complex in the cytoplasm of most cells including the immune cells. Binding of the hormone – receptor complex to Glucocorticoid Responsive Elements (GRE), located in the promoter region of the target gene, regulates the expression of target genes either positively or negatively. Basal levels of GCs are always present in circulation to meet the day-to-day metabolic activities. During acute stress, there is stimulation of HPA axis, followed by GC surge. It acts on vital systems to maintain homeostasis. When the stressor is removed, negative feedback mechanism acts to impair GC secretion, thus, the cycle goes on. In contrast, chronic stress has long-term increase in glucocorticoids in circulation which causes deleterious effects on the productive parameters and immunity. During chronic stress, the number of GC receptors in hippocampus gets reduced. As a result, the negative feedback mechanism which regulates the HPA axis is affected (Webster & Cidlowski, 1994). Another reason may be that the chronic stress activates the adrenal gland resulting in adrenal hypertrophy and henceforth results in increased and prolonged glucocorticoid secretion (Miller & Tyrrell, 1995).

HEAT STRESS AS AN IMPORTANT FACTOR AFFECTING IMMUNITY IN LIVESTOCK

The process of disease involves three factors; namely, host, pathogen and environment. Environment modulates the host and pathogen interactions in such a way that the pathogen overcomes the immune barrier of the host and establishes itself and henceforth, the outcome is the disease condition. In calf neonates, the first 18 hours of post natal life is very crucial as it determines the immune status of the animal. During this period, the intestinal epithelium is permeable to colostral proteins, particularly immunoglobulins and thus passive transfer of immunity occurs from dam to offspring. Exposing dams and neonates to heat stress, however, has major impacts on the calf's immunity. Exposing heifers to high temperature during late pregnancy and early *postpartum* period not only reduces the concentrations of IgG, IgA, milk proteins and fatty acids in colostrums (Nardone et al., 1997) but also lowers the intestinal absorption of immunoglobulins (Stott et al., 1976). The low quality of Igs and reduced intestinal absorption in turn lead to calf mortality at high temperature (Martin et al., 1975).

HEAT STRESS AND DISEASE OCCURRENCE

Heat stress directly or indirectly favours disease occurrence in animal host. Directly, high temperature favours the survival of organisms outside the host for a long time. This can be seen in the case of spores of
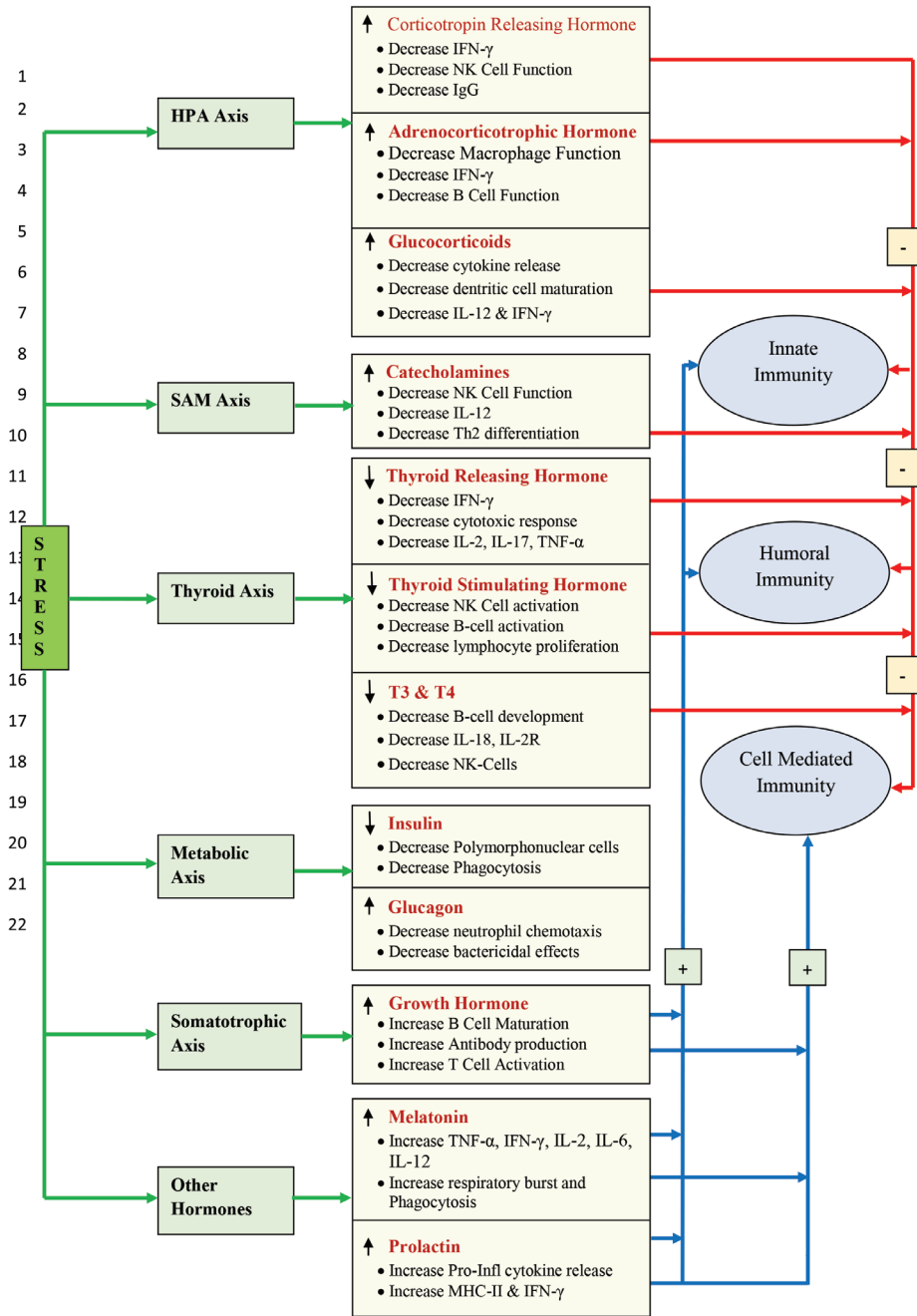
HPA-Hypothalamo-Pituitary-Adrenal; SAM-Sympathetic-Adrenal-Medullary; IFN-Interferon; IL- Interleukin; TNF-Tumor Necrosis Factor; IgG-Immunoglobulin G; T3- Tri-iodo-thyronine; T4-Thyroxine; MHC-Major Histocompatibility Complex; NK Cells- Natural Killer Cells; Th-T Helper Cell

Figure 1. The interaction between various endocrine axes with the immune system during stress

Bacillus anthracis and *Clostridium chauvoei* (Hall, 1988), which survive for a long period under high temperature and made available to infect the animals. Indirectly, chronic heat stress causes immune suppression in animals and makes them susceptible to diseases.

Moreover, global climatic change has created huge modifications on the macro and microclimate of both the host and the parasite and also altered certain trends of host-microbe interactions, namely: (i) shortened generation time of microbes which prefer high temperature leading to increased pathogen population and in turn increased risk of infection, and (ii) population of vectors like ticks, flies, midges, fleas normally requires high temperature, and hence they increase in number and their feeding frequency also increases. These lead to more chances for transmission of pathogen to animal host. The sub-tropical hot and humid conditions favour the tick population like *Boophilus microplus, Haemophysalis bispinosa and Hyalomma anatolicum* (Basu & Bandhopadyay, 2004). (iii) The extrinsic incubation period reduces due to high temperature has been seen in case of *Culicoides* (Wittman & Baylis, 2000). High ambient temperature combined with relative humidity predisposes dairy cows to clinical mastitis (Singh et al., 1996). The direct effect of heat compromises the udder immune system and also favours the housefly population (Sirohi & Michelowa, 2007).

HEAT STRESS AND VACCINATION RESPONSE

Culling of diseased livestock is not economically feasible in developing countries of south Asia and Africa. Thus, regular vaccination seems to be a better option in order to protect the livestock from dreadful diseases like foot-and-mouth disease (FMD), anthrax, haemorrhagic septicaemia (HS), PPR, etc. The effects of heat stress on the immune response to vaccines have been reviewed based on the available literature. Varied results have been obtained by various researchers under different circumstances.

High temperature combined with high relative humidity failed to elicit humoral immune response to canine distemper vaccine and also failed to protect against the virus challenge. /however, the same temperature conditions did not affect the immune response to hepatitis vaccine (Webstar, 1975). The effect of chronic heat stress on FMD vaccination in mice model revealed that that chronic heat stress had adverse effect on cell mediated immune response than humoral immune response. Th1 based cell mediated immune responses like IgGa production, T cell multiplication, IFN gamma expression and antigen specific cytotoxic T lymphocyte activities were affected severely (Hu et al., 2007). The experimental study conducted by the same team also proved that both humoral and cell mediated immune responses to H5N1 avian influenza vaccine were affected by chronic heat stress. A new concept of up regulation

of CD4$^+$ CD25$^+$ Foxp3$^+$ T reg cells, with increased TGF-β, IL-10, decreased Th1, Th2 cell responses, CD8$^+$ T cell proliferation and related cytokines was noticed in the study (Meng et al., 2013).

Various human studies revealed activation of latent viruses during stress conditions (McVoy & Adler, 1989). It was reported that glucocorticoids released during stress conditions activated the latent viruses by directly acting on the viral genome and also decreasing the immunological memory response (Glaser et al., 1995).

HEAT STRESS AND GUT HEALTH

The gastrointestinal tract health is crucial in farm animals like cattle, sheep, goat, pigs, etc., as optimum production depends upon efficient feed conversion. The integrity of gastrointestinal tract is essential in order to maintain the normal homeostasis of gut microbiota. During heat stress, however, the blood flow to internal organs like intestine gets compromised and peripheral blood flow increases to dissipate the internal body heat (Lambert et al., 2002). The decreased blood flow leads to ischemia and necrosis of intestinal epithelial cells (Hall et al., 1999), decreases tight junctions between enterocytes which favour paracellular entry of bacterial pathogens. Increase in intracellular permeability helps in translocation of bacteria (Pearce et al., 2013) and its antigenic components, particularly LPS, thus leading to endotoxemia (Pearce et al., 2013). The toxin in turn affects the normal liver metabolism, leading to

steatohepatitis and decreased productive performance.

EFFECT OF HEAT STRESS ON IMMUNE RESPONSES

Heat stress modulates various behavioural and physiological parameters in farm animals and in poultry species which have been discussed in detail by various reviewers (Lu, 1989; Kadzere et al., 2002; Marai et al., 2006; Yahav, 2009). Various authors recorded variable results indicating that heat stress as either an immune suppressing or immune enhancing factor in animal production. Stress affects both innate and adaptive immune response in animals.

HEAT STRESS AND INNATE IMMUNITY

Heat stress reduces the relative weights of lymphoid organs like spleen, thymus and cloacal bursa (Aengwanich, 2008). The mechanical barriers, namely mucosa and skin, act as the first line of defense in innate immune response. Experiments in poultry revealed that exposure to heat stress caused mild acute lymphocytic enteritis (Quinteiro-Filho et al., 2010). Chronic heat stress could affect the integrity of respiratory tract and reduce pulmonary alveolar macrophages in lungs of mice, thus making the animals susceptible to Highly Pathogenic Avian Influenza or H5N1(Jin et al., 2011).

NK cells are important components of the innate immune system present in systemic circulation and also in lymphoid organs like lymph nodes, spleen and bone marrow. They are involved in destruction

of tumour cells and infectious agents like bacteria, fungi and viruses. Studies in mice revealed that chronic heat stress in mice reduced the splenic NK cell cytotoxic functions. The inhibition may be due to increased glucocorticoid influence in the immune cell (Won & Lin, 1995).

The immune profile in PBMC of local Bama miniature pigs exposed to 21 days of heat stress revealed up regulation of the Toll Like Receptors (TLR) 2, 4 which are involved in identification of conserved molecules of microbes such as lipoprotein and lipopolysaccharides respectively (Ju et al., 2014). Heat stress also up regulated the TLR2, 4 genes in human monocytes (Zhou et al., 2005). Studies on the seasonal influences on TLR 1-10 mRNA expression in Black Bengal goats revealed that significant increases in TLR genes were noticed during summer season (Paul et al., 2015). The pro-inflammatory cytokines, namely IL-6, IFN-ß, which contributes to innate immune response were down regulated by heat stress (Jin et al., 2011).

HEAT STRESS AND MUCOSAL IMMUNITY

Heat stress reduces the blood flow to intestine, lowers the integrity of intestinal epithelium, causes villi desquamation and reduces the villi height and crypt depth (Yu et al., 2010; Yu et al., 2013). Furthermore, the innate immune components like mucosal barrier, toll like receptors, secretory Ig A, intestinal intraepithelial lymphocytes production (Deng et al., 2012), expression of cytokines responsible for humoral and cell mediated immune response were down regulated in intestine by heat stress. Reduction of intestinal immune function enabled bacterial translocation to mesenteric lymph node (Liu et al., 2012).

HEAT STRESS AND ADAPTIVE IMMUNITY

Adaptive immune response collectively represents the humoral and cell mediated immune response. Experimental results in farm animals and poultry revealed variable adaptive immune responses during heat stress. Table 1 represents the adaptive immune responses elicited by livestock during heat stress conditions.

MECHANISM OF HEAT STRESS IMPACTING LIVESTOCK IMMUNE SYSTEMS

Various stressors including heat stress induce the endocrine system to increase catecholamines and glucocorticoids. These hormones modulate the cytokine release and thereby regulate immune responses.

GLUCOCORTICOIDS MEDIATED LYMPHOLYSIS

Experiments in poultry revealed that decrease in relative weights of immune organs like spleen, cloacal bursa and thymus. This might be due to glucocorticoid induced lympholysis and redistribution of lymphocytes from systemic circulation to other organs (Jain, 1993).

MECHANISM OF INHIBITION OF INNATE IMMUNITY

Stress induced glucocorticoids act to inhibit the pro-inflammatory cytokines, namely TNF-α, IL-6, IL-8, which are required to initiate an innate immune response through inhibition of p38 MAPK pathway which helps in maintaining their stability (Abraham et al., 2006). GCs also enhance IL-10, an anti-inflammatory cytokine which is normally found at the end of immune response (Marchant et al., 1994). The cytotoxic function of NK cells are inhibited by catecholamines which act through adrenergic receptors to elevate cAMP levels that will in turn decrease the cytotoxic functions (Whalen & Bankhurst, 1990). The hyperthermia mediated suppression of NK cell activity is due to the inhibition of perforin and granzyme enzymes (Koga et al., 2005). GCs upregulates innate immune system via stimulation of Pattern Recognition Receptors TLR2, TLR4 (Galon et al., 2002).

MECHANISM OF INHIBITION OF ADAPTIVE IMMUNITY

Th1 cells are involved in cell mediated immune response whereas Th2 cells are responsible for humoral immune response. The modulation of cytokine gene expression in fact alters the immune function from Th1 to Th2 and vice versa. Th1 cells secrete IFN- γ, IL 2, TNF β which contributes to cellular immunity. Th2 cells secrete IL-4, IL-10 and IL-13, which contribute to humoral immunity. IL-12, in combination with IFN-gamma, converts uncommitted T helper (Th0) cells to Th1 cells, whereas cytokines IL-4 and IL-10 induce Th2 cell production. Both Th1 and Th2 cell mediated are inhibitory to each other (Elenkov & Chrousos, 1999). Glucocorticoids acts to inhibit the release of IL-12 and IFN γ which

Table 1

Effects of heat stress on adaptive immune responses of livestock

Animal	Immune Parameters studied	Effect of heat stress on the immune parameter	References
Poultry	Humoral immunity	Unaltered	Regnier et al., 1980
Calves	Mitogen stimulation index	Unaltered	Kelley et al., 1982a
Pigs	Mitogen stimulation index & antibody production	Unaltered	Bonnette et al., 1990
Poultry	Antibody response	Unaltered	Donker et al., 1990
Poultry	Antibody mediated primary immune response	Decreased	Thaxton et al., 1968; Subbarao & Glick, 1970
Poultry	Humoral immunity	Decreased	Mashaly et al., 2004
Cattle	Cell mediated immunity	Unaltered	Lacetra et al., 2002
Calves	Cell mediated immunity	Decreased	Kelley et al., 1982b
Cattle	B and T- cell mitogen response	Decreased	Elvinger et al., 1991

are the major cytokines involved in Th1 based cell mediated immunity (Elenkov et al., 1996). Furthermore, the expression of IL-12 receptors in NK cells and Th1 cells are down regulated by glucocorticoids (Wu et al., 1998), and thus the immune function get shifted from Th1 to Th2.

Dendritic cells are potent APCs and link the innate and adaptive immune response as they are involved in phagocytosis and antigen presentation. They also express more MHC Class II and co-stimulatory molecules like CD80, CD83 and CD86 on their cell surface. GCs act to inhibit expression of these molecules on DCs and thereby prevent its maturation (Girndt et al., 1998). In addition, catecholamines, which include adrenaline and noradrenaline, are also released during stress conditions to inhibit the production of IL-12, development of Th1 cells and Th2 differentiation (Elenkov et al., 1996). Treatment with adrenergic agonist revealed inhibition in IFN γ production by Th1 cells (Sanders et al., 1997).

MOLECULAR RESPONSES TO HEAT STRESS

Stress leads to activation of hypothalamic-pituitary adrenal axis and ultimately the release of glucocorticoids. Glucocorticoids, in normal pulsatile release, enhances the pro-inflammatory cytokine release. However, chronic rise in glucocorticoid levels is inhibitory to majority of immune cytokines. Glucocorticoid acts in various ways to inhibit cytokine release. Table 2 represents various genomic and non-genomic mechanisms by which GCs suppress cytokine release.

GENES ASSOCIATED WITH IMMUNE FUNCTIONS DURING HEAT STRESS

The stress related immune responses in poultry species revealed that acute stress is beneficial to the bird as it the immune system. On the other hand, chronic stress shifts the T helper cell response to T regulatory cell and also TGF-β, a regulatory cytokine production, thereby it suppresses immune response (Shini et al., 2010). Meng et al. (2013) who demonstrated that chronic heat could suppress both Th1 and Th2 lymphocyte based immune response for H5N1 avian influenza virus through upregulation of $CD4^+CD25^+Foxp3^+$ Treg cells, immune cytokine TGF-β gene expression. The heat stress signaling pathway was observed to be distinct from endotoxemic pathway in mice. It acts through HSFs such as HSP 70 and Stress Activated Protein Kinase pathways resulting in an early increase in the expression of *hsp*72, *c-fos* and *c-jun* genes. This in turn leads to the expression of a peculiar cytokine pattern consisting of increased IL-6 and IL-10 expression and decreased TNF-α, IL-1β expression. The pattern recognition receptor, namely *TLR*-4, is highly expressed whereas there was no significant difference in *TLR*-2 gene expression (Welc et al., 2013). The endotoxemic pathways differ from the earlier in which increased expression of *IL-1β, IL-6, TNF-α* genes are evident (Lang et al., 2003). Chronic heat stress could influence complement system, a component of the innate immune system in the intestine of rats (Lu et al., 2011). Furthermore, the

chemokine signalling pathways and HSP expression were reported to be down regulated during heat stress (Liu et al., 2014). This might be due to the inhibition of JAK-STAT signalling pathways, which play an important role in induction of immune cells to release cytokines and chemokines. The JAK-STAT proteins are thermolabile in nature and get decreased during heat stress (Nespital & Strous, 2012). Moreover, differential expression of adaptive immunity genes were noticed viz. up regulation of HSP-70 genes and down regulation of RT1-Bb genes, which encode for beta chain of MHC-class II. Both the genes are involved in antigen presentation to CD4^{+} T cells and ultimately the CD4+ T cell stimulation gets affected (Liu et al., 2012). Also, heat stress reduced the expression of MHC class II and co-stimulatory molecules like CD40, CD80 and CD86 by antigen presenting cells, thus delayed DC maturation (Jin et al., 2011). The cytokine genes for both Th1 (IL-2, IFN-γ) and Th2 (IL-4, IL-10) cell responses were down regulated during heat stress (Liu et al., 2012). Heat stress experiments in Bama miniature pigs revealed that IL-12,

the key cytokine gene to initiate cellular immune response was upregulated but IL-2 and IFN-γ, which are also involved in CMI process were down regulated, revealing differential expression of immune cytokine genes (Ju et al., 2014). Acute heat stress exposure in cattle led to up regulation of IL-17, a cytokine associated with innate immune response up to 48 hrs after heat stress. Moreover, the genes involved in humoral immune response (Cd83, HSPA1A & IL1A) showed increased expression during heat stress (Mehla et al., 2014). Table 3 describes the different immune response genes getting expressed during heat stress condition in animals.

SIGNIFICANCE OF OPTIMUM NUTRITION FOR IMMUNE FUNCTION UNDER HEAT STRESS

The various strategies need to be adopted for management of animals during heat stress include shelter management, evaporative cooling systems such as misting, fogging, pad cooling, sprinkling or dripping, modified nutrition strategy and genetic selection of animals for heat tolerant genes.

Table 2

Different mechanisms by which glucocorticoids suppresses cytokine function

General mechanisms involved	Cytokines affected	References
Suppression of cytokine gene transcription	IL-1, IL-2, IL-3, IL-8	Smoak & Cidlowski, 2007
Decrease in cytokine secretion	IL-1	
Destabilization of cytokine mRNAs	IL-1, TNF, GM-CSF	
Inactivation of the released cytokine by inducing decoy receptors	IL-1	

TABLE 3
Different immune regulatory genes expressed and their functions during heat stress

Species	Immune genes	Function	Up regulation/ Down regulation	References
Mice	IL-6	Pro-inflammatory cytokine	Up regulated	Welc et al., 2013
	IL-10	Anti-inflammatory, Immune regulatory cytokine		
	TLR-4	PRR for LPS		
	TNF-α	Acute inflammatory response	Down regulated	
	IL-1β	Pro-inflammatory cytokine		
Cattle	IL-17A	Innate immunity	Up regulated	Mehla et al., 2014
	HSPA1A	Induce antibody production & T cell activation		
	Cd83	Antigen presentation to B lymphocytes		
	IL1A	T cell dependant antibody production		
	CXCL5	Chemo attractant for neutrophils	Suppressed earlier; induced later	
	$F_C\gamma R1A$	Antigen presentation via MHC class I		
Poultry	IL-1β	Pro-inflammatory cytokine	Increased during acute stress; decreased during chronic stress	Shini et al., 2010
	IL-6	Pro-inflammatory cytokine		
	IL-18	Pro-inflammatory cytokine; involved in CMI		
	TGF β	Immunoregulatory cytokine	Increased during chronic stress	
	CCL16	Chemo attractant for lymphocytes		
Pigs	TLR2, TLR4	Innate immune response	Up regulated	Ju et al., 2014
	IL-12	Induction of Th1 based cell mediated immune response	Up regulated	
	IFN γ		Down regulated	
Rats	IL-2, IFN γ	Th1 based cell mediated immune response	Down regulated	
	IL-4, IL-10	Th2 based humoral immune response		

cont'd Table 3

Rats	HSPA1A, ZBTB16, CSF2, HPX, IL22RA2, PTDSR, LOC301133, BCL6, MAdCAM1, PPARA, CDK6, VEGFA		Up regulated	Liu et al., 2014
	TNFSF6, MS4A2, CCL5, SPN, CCR5, MM9, IR4, TLR2, CCL4, S100A9, RT2-Bb	Cytokine-cyokine interaction	Down regulated	
Rat	RGD1561028, HSP90AA1, HSP90AB1, HSPA11, and HSPA8		Up regulated	Lu et al., 2011
	RT1-Bb, CCL5, CCR5, CXCR3 and Fas	Cytokine-cytokine receptor interaction	Down regulated	
	IL22RA2		Up regulated	
	CSF2			
	TNFSF14			
	Cd55, F3	Complement & coagulation cascades	Down regulated	
	Fgb, Serpine1, and Serpine 2		Up regulated	
Mice	IL-10, TGF-b	Immunoregulatory cytokines	Up regulated	Meng et al., 2013
Mice	MHC-II, CD40, CD80, and CD86	Co-stimulatory molecules	Down regulated	Jin et al., 2011
	IL-6 and IFN-β	Pro-inflammatory cytokine		
	IL-10	Immunoregulatory cytokine	Up regulated	

Pertanika J. Trop. Agric. Sci. 39 (1): 459 - 482 (2016)

473

The nutrients which enhance immune response in heat stressed animals include vitamins, minerals, antioxidants, prebiotics, probiotics, synbiotics, etc.

VITAMINS

Vitamin A acts to maintain the normal mucosal and epithelial barrier, thereby helps to strengthen the mechanical barriers which are the components of innate immune response (Sordillo et al., 1997). Thus, it is required for maintaining udder health in milch animals. Moreover, supplementation of vitamin A augments the functions of anti-oxidant enzymes like glutathione peroxidase, superoxide dismutase, etc. (Jin et al., 2014). The supplementation of vitamin A also results in increased pro-inflammatory cytokines like IL-1, TNF-α , Ig M, Ig G and Ig A, soluble CD4, CD4/CD8 ratio, decreased CD8 (Jin et al., 2014) and aids in immunoglobulin transport proteins production (Tjoelker et al., 1990). Selenium, a trace element, plays an important role in immune function in combination with vitamin E. The anti-oxidant enzyme, glutathione peroxidase incorporates selenium within it, thus it is indirectly involved in protection of the body from oxidative stress. Moreover, selenium supplementation in diet decreased the incidence of clinical mastitis and somatic cell count (Smith et al., 1997). Selenium – vitamin E supplementation positively influences chemotaxis (Aziz & Klesius, 1986), phagocytic ability (Grasso et al., 1990) and oxidizing property of neutrophils (Aziz & Klesius, 1986).

TRACE MINERALS

Copper is essential for the development of immune organs and antibody production (Lukasewycz & Prohaska, 1990). Copper-deficiency was reported to reduce the phagocytic ability of neutrophils and macrophages (Babu & Failla, 1990a; Babu & Failla, 1990b), IL-2 production and thus lymphocyte proliferation (Bala & Failla, 1992). Keratin acts as a mechanical barrier in organs such as mammary gland and in appendages such as horn, hoof, etc. Zinc is involved in various stages of keratinization (Paulrud, 2005). Providing zinc supplementation in organic form during summer helps in maintaining udder health by strengthening keratin layer and preventing mastitis (Popovic, 2004). Meanwhile, iron helps in the development of immune organs like thymus, spleen, lymph node and cloacal bursa. Experiments with iron-glycine supplementation revealed iron particularly influences thymus development in animals (Feng et al., 2007) and birds (Sun et al., 2015). Chromium supplementation helps in the development of primary immune response with increased Ig M and Ig G concentration (Moonsie-Shageer & Mowat, 1993).

PROBIOTICS

Probiotics are mixture of live micro-organisms which are beneficial to animal's health when given in adequate amounts. These live organisms occupy the entire length of the gut to protect against pathogens entry and colonisation by a process called "competitive exclusion" or "bacterial

antagonism" or "bacterial interference" (Lloyd et al., 1977) and strenghthen the intestinal mechanical barrier. Furthermore, probiotics compete with pathogenic bacteria for nutrition and attachment site. They also produce antimicrobial peptides such as colicins and bacteriocins (Lee et al., 2008) and stimulate cytokine production (Marin et al., 1997).

PREBIOTICS

Prebiotics are said to be undigestible feed ingredients that beneficially affect the host by selectively stimulating the growth or activity of limited number of bacteria in colon (Gibson & Roberfroid, 1995). Feeding of fructose-oligosaccharide and mannon oligosaccharide increased antibody responses (White et al., 2002).

SYNBIOTICS

Synbiotics, a combination of probiotics and prebiotics, is supplemented in farm animals. The necessity for the combination is that prebiotics acts as nutritional substrate for probiotic organisms, thus helps in improving or maintaining the gut health. Synbiotics acts as a stimulant for phagocytosis and releases cytokines like TNF-alpha, IFN-gamma and antibody production (Kabir et al., 2004). Synbiotics are reported to increase the antibody response to vaccines (Hassanpour et al., 2013).

CONCLUSION

In the global climate change scenario, heat stress is the predominant stress affecting livestock production through emergence of different vector borne diseases. This review has thrown light that in addition to altering the productive and reproductive performance, heat stress can also alter the immune functions of animals to bring down their production. The stress hormones – cytokine interactions are responsible for altered immune functions during heat stress. In particular, the highly specific adaptive immune mechanisms are affected by heat stress. In more specific, heat stress deteriorates the cell mediated immune responses. The role of heat stress during critical events in an animal like passive transfer of maternal antibodies, vaccination and new pathogen challenge has been widely discussed. This reveals that heat stress needs to be considered as an important factor compromising animals' health. Furthermore, various managemental and nutritional interventions were discussed in detail during such instances to prevent the harmful impacts of heat stress on animal's welfare. This literature review can serve as a good reference material for researchers who aim at improving livestock production in the changing climate scenario by optimising the immune system functioning in livestock.

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478

Pertanika J. Trop. Agric. Sci. 39 (4) 459 - 482 (2016)

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Short Communication

A Note Comparing the Apparent Metabolisable Energy of Three Oil Sources and their Combination in Broiler Chickens

N. R. Abdulla[1,5], T. C. Loh[1,2]*, H. Akit[1], A. Q. Sazili[1,3] and H. L. Foo[4]

[1]*Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[2]*Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Halal Products Research Institute, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[4]*Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.*
[5]*Department of Animal Resource, University of Salah al- Din, Erbil, Kurdistan Region, Iraq*

ABSTRACT

Apparent metabolisable energy (AME) of palm oil (PO), soybean oil (SO), linseed oil (LO) and blend oil (BO) [PO, SO and LO in a ratio of 4:1:1] were evaluated. A total of 75, 21-day-old birds were a fed corn-soy basal diet and four test diets containing different oil sources (PO, SO, LO and BO), were developed by replacing 60 g/kg of the basal diet for eight days. Differences in the apparent metabolisable energy were found ($P<0.05$), with the higher values for broiler-fed BO. This study affirmed that BO increases AME of oil enriched with saturated fatty acid in poultry diets.

Keywords: Broiler chicken, blend oil, linseed oil, metabolisable energy, palm oil, soybean oil

INTRODUCTION

Broiler diets are supplemented with vegetable oils and animal fats to increase energy concentration and to improve productivity (Lopez-Bote et al., 1997). This is because the apparent metabolisable energy (AME) content in oil is thrice higher than that of other feedstuff (Mateos & Sell, 1981). Thus, oil is a vital component in compounding of high-energy broiler diets. As feeding cost accounts for about 65%

of total production cost, dietary oils may well be used as a way in which broiler chicken production might enhance growth performance and improve meat yield at a reasonably cheaper price (Corzo et al., 2005). Other advantages of using fats include increased palatability, reduced dustiness and improved feed texture (Baião & Lara, 2005; Ayed et al., 2015). Among all vegetable oils, soybean oil and oil palm are widely used in the feed industry. Abdulla et al. (2015, 2016a and 2016b) observed differences in dietary sources of palm oil, soybean oil and linseed oil and their effect on growth performance, gut morphology, composition of fatty acid, oxidative stability and cholesterol content of breast muscle in broiler chicken. Although previous findings have shown the influence of supplementing different kinds of oils on metabolism of fats, growth performance in broiler birds as well as the apparent metabolisable energy (AME), the information are still not consistent. For instance, Tancharoenrat et al. (2013) states that the use of soybean oil, palm oil and poultry fat had lower (P<0.05) apparent metabolisable energy than tallow while the differences were absent in poultry fat, soybean oil and palm oil. Kavouridou et al. (2008) found that the birds consuming linseed oil had a significantly higher percentage of apparent metabolisable energy matched with birds fed a diet containing palm oil but there was no significant difference from birds fed a diet supplemented with soybean and coconut oil. Although previous works have described the metabolisable energy (ME) of different

sources of oil that have been typically used in broiler diets, those data were recorded 25 to 50 years ago. Not only have oil sources changed since then (composition and quality indices), broilers have also gone through major heritable change. Consequently, consistent and current AME data on these sources of oil will allow for accurate formulation of the energy content in broiler feeds. Thus, the current study was aimed at assessing the AME in different sources of oils: Palm oil (PO), soybean oil (SO) and linseed oil (LO) when fed to broiler chicks. Also, the study aimed to examine the probable synergism of combination PO, SO and LO in a ratio of 4:1:1.

MATERIALS AND METHODS

Ethical Note

This study was conducted in accordance with the animal ethics guidelines of the Research Policy of University Putra Malaysia.

Birds, Husbandry and Experimental Procedure

One hundred one-day-old Cobb 550 broiler birds bought from a commercial farm were fed starter diets (22% crude protein) for 21 days. During the first week, their temperature was maintained at 35 ℃ and then reduced steadily to about 28 ℃ until the conclusion of the experiment. The birds were vaccinated against Newcastle disease (ND) and infectious bronchitis (IB) live vaccine (MyVac, Kuala Lumpur, Malaysia) through the intraocular route on day 7 and 14 of the raising period. The infectious bursal disease vaccine (IBD) (MyVac, Kuala

Lumpur, Malaysia) was administered on day 21 through intraocular route. The birds were given feed and water *ad-libitum*. On day 21, birds of similar body weight were picked at random and allocated to experimental units. Three birds per unit (cage) and five replicate units were randomly allocated to a basal diet and each of the four test diets: Palm oil (PO), soybean oil (SO), linseed oil (LO) and blend oil (BO). The blend oil was a combination of PO, SO and LO in a ratio of 4:1:1. The classic total excreta method was used to measure the AME test. The birds were fed in mash form for a period of eight days, with the first four days as an adaptation period. During the last four days, feed intake (FI) of each unit was recorded, and the chicks' faeces was collected on a daily basis, collected and weighed within a cage. Faeces collected was thoroughly mixed, and typical samples were obtained and freeze-dried (Model 0610, Cuddon Engineering, Blenheim, New Zealand). Dried fecal samples were crushed to pass through a 0.5 mm sieve and kept in airtight plastic containers at -4°C for further analyses. Dry matter (DM) and gross energy (GE) of the feeds and fecal samples were analysed.

Determination of Fatty Acid Composition of Oil Sources

The total fatty acids were extracted from the different oils following the protocol of Folch et al. (1957), modified by Ebrahimi et al. (2014) and described by Abdulla et al. (2015).

Apparent Metabolisable Energy Assay

The AME for PO, SO, LO and blend oil (BO) were determined following the procedures of Nalle et al. (2011). According to the procedure, the corn-soybean basal diet was compounded as shown in Table 1 and the test diets, each containing a different oil sample, were prepared by substituting 60 g/kg of the basal diet with a different oil.

The DM of samples was determined following the standard guidelines of AOAC (2007). Prior to oven drying, the weight of all the samples was taken and later placed in an oven dryer for a period of 24 hours at a temperature of 105 °C and the weights were recorded again after half an hour of cooling in a desiccator. To determine the GE an adiabatic bomb calorimeter (Gallenkamp Autobomb, UK) standardised with benzoic acid was used.

The AME was calculated for both the basal and test diets (different oils) applying the following formula:

AME of diet (kcal/kg)
= [(FI × GE diet) – (Excreta output × GE excreta)] ÷ FI

AME of oil (kcal/kg) =
[(AME of test diet) – (AME basal diet × 0.94)] ÷ 0.06

(Ravindran et al., 2014)

Pertanika J. Trop. Agric. Sci. 39 (1): 483 - 490 (2016)

485

Table 1

The Basal Diet Compositions (g/kg, as Fed Basis) Used in the AME Digestibility Assays

Ingredient (%)	AME assay[1]
Corn	65.40
Soybean meal	30.38
Mono di calcium phosphate 21%	1.35
Calcium carbonate	1.55
Salt	0.30
L-Lysine	0.30
DL-Methionine	0.20
Vitamin premix[2]	0.10
Choline chloride	0.10
Toxin binder[3]	0.15
Anti-oxidant[4]	0.02
Mineral premix[5]	0.15
Total, kg	100.00
Calculated Analysis	
ME (kcal/kg)	2887.00
Crude protein (%)	19.00
Fat (%)	2.66
ME/CP	151.95
Fibre (%)	3.76
Calcium (%)	0.92
Total Phosphorus (%)	0.64
Avail. P for Poultry (%)	0.36
L-Lysine (%)	1.21
DL-Methionine (%)	0.50

[1]Test diets were prepared by substituting 60 g/kg of the basal diet with palm oil, soybean oil and linseed oil. [2]Supplied per kg diet: Vitamin A 11,494 IU; vitamin D 1,725 IU; vitamin E 40 IU; vitamin K3 2.29 mg; cobalamin 0.05 mg, thiamine 1.43 mg, riboflavin 3.44 mg, folic acid 0.56 mg, biotin 0.05 mg, panthothenic acid 6.46 mg, niacin 40.17 mg, pyridoxine 2.29 mg. [3]Toxin binder contains natural hydrated sodium calcium aluminium silicates (HSCAS). [4]Antioxidant contains butylated hydroxyanisole (BHA). [5]Supplied per kg diet: Fe 120 mg, Mn 150 mg, Cu 15 mg, Zn 120 mg, I 1.5 mg, Se 0.3 mg, Co 0.4 mg.

STATISTICAL ANALYSIS

Data obtained were subjected to one-way ANOVA testing and analysed using the general linear model of SAS (SAS, 2007). The significant differences among the treatment means were compared using Duncan's multiple range tests while the Alpha level used for assessment of significance for all the analyses was set at 0.05.

RESULTS

Table 2 shows the composition of the fatty acids of the different oil sources. According to the results, the concentration of oleic (C18:1n-9) and palmitic acid (C16:0) in PO

486

Pertanika J. Trop. Agric. Sci. 39 (4) 483 - 490 (2016)

Table 2
The Composition of Fatty Acids (% of Total Identified Fatty Acids) of Different Oils[1]

Fatty acid	Palm oil	Soybean oil	Linseed oil	Blend oil	SEM[8]
C12:0	0.34[a]	0[c]	0[c]	0.23[b]	0.06
C14:0	1.00[a]	0.09[c]	0[d]	0.68[b]	0.16
C16:0	37.45[a]	10.80[c]	5.25[d]	27.64[b]	4.97
C16:1 n-7	0.21[a]	0.08[b]	0.05[b]	0.16[a]	0.03
C18:0	4.10[b]	4.40[a]	3.83[c]	4.11[b]	0.08
C18:1n-9	44.37[a]	25.38[c]	20.02[d]	37.14[b]	3.69
C18:2n-6	12.01[d]	52.45[a]	15.98[c]	19.41[b]	6.43
C18:3n-3	0.24[d]	6.44[c]	54.48[a]	10.31[b]	8.57
SFA[2]	42.66[a]	15.00[c]	9.00[d]	32.66[b]	5.18
USFA[3]	56.84[d]	84.35[b]	90.53[a]	67.02[c]	5.18
MUSFA[4]	44.57[a]	25.46[c]	20.06[d]	37.30[b]	3.72
PUFAn-3[5]	0.24[d]	6.44[c]	54.48[a]	10.31[b]	8.57
PUFAn-6[6]	12.01[d]	52.45[a]	15.98[c]	19.41[b]	6.43
n-6: n-3 ratio[7]	48.75[a]	8.14[b]	0.29[d]	1.88[c]	7.52
USFA: SFA	1.32[d]	5.51[b]	9.96[a]	2.05[c]	1.25
PUFA: SFA	0.28[d]	3.84[b]	7.75[a]	0.90[c]	1.08

[1]The data are expressed as the percentage of identified fatty acids. [2]Total saturated fatty acid= sum of C12:0+C14:0+ C16:0+C18:0. [3]Total unsaturated fatty acid= sum of C16:1n-7+ C18:1n-9+ C18:2n-6+C18:3n-3+C20:4n-6+C20:5n-3 +C22:5n-3+C22:6n-3. [4]Total monounsaturated fatty acid = sum of C16:1n-7+C18:1n-9. [5] polyunsaturated fatty acid n-3 = sum of C18:3n-3+C20:5n-3+C22:5 n-3+C22:6n-3. [6] polyunsaturated fatty acid n-6 = sum of C18:2n-6+C18:3n-6+C20:4n-6. [7] polyunsaturated fatty acid n-6: polyunsaturated fatty acid n-3= (C18:2n-6+C18:3n-6+C20:4n-6) ÷ (C18:3n-3 + C20:5n-3 + C22:5n-3 + C22:6n-3). [8]SEM: Standard error of means.

was significantly (P<0.05) higher than that in SO, LO and BO, while LO and SO were significantly higher in terms of α-linolenic acid and linoleic acid, respectively in comparison with PO. The ranking of n-6:n-3 ratios was in the order of PO>SO>BO>LO. The proportion of total saturated (SFA) and total mono-unsaturated fatty acids (MUSFA) of PO was higher (P<0.05), while total unsaturated fatty acid (USFA) content was lower (P<0.05) compared to those of SO, LO and BO.

The apparent metabolisable energy of different sources of oil in broiler chicken is summarised in Figure 1. The result showed that the BO had the highest (P<0.05) value

(9413 kcal/kg) of AME in comparison with PO (8277 kcal/kg), SO (8401 kcal/kg) and LO (8423 kcal/kg). On the other hand, there was no significant difference (P>0.05) between PO, SO and LO.

DISCUSSION

The values of ME in oils is mainly influenced by their ingestion, which is influenced by a myriad of factors such as the absence or presence of ester bonds (free fatty acids or tryglycerides), number of double bonds, stretch of the carbonic chain, the type and amount of the triglycerides augmented in the diets, free fatty acid compositions, detailed arrangement of SFA and USFA on
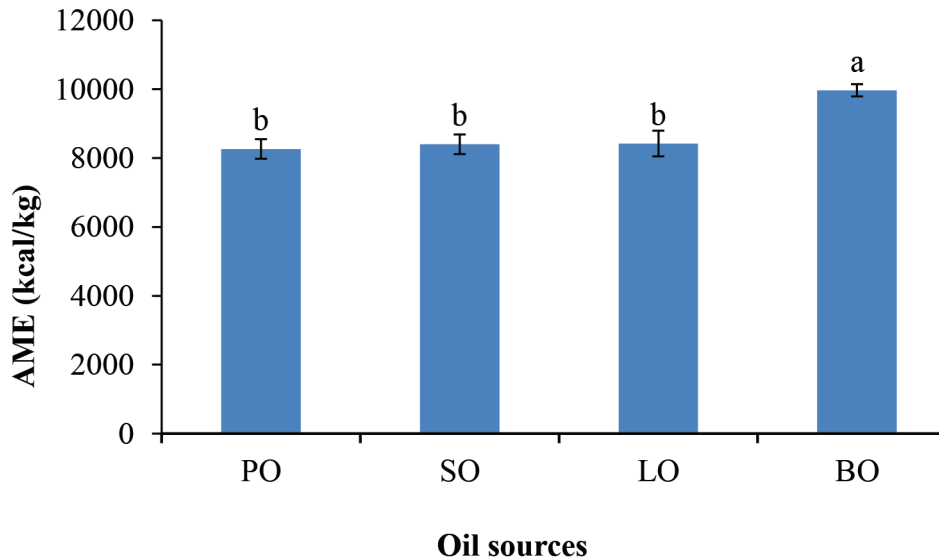
PO: Palm oil, SO: Soybean oil, LO: Linseed oil, BO: Blend of palm, soybean and linseed oil in a ratio of 4:1:1.
[a,b] indicate significant differences ($p<0.05$) between different oil sources.
Values are means ± 1 standard error.

Figure 1. Apparent metabolisable energy (AME) for various sources of oil in broiler chicken.

the backbone of glycerol, age, sex and the intestinal flora of birds (Garrett & Young, 1975; Ketels & De Groote, 1989; Leeson & Summers, 2001; Nascif et al., 2004). In the present study, although PO had a higher ratio of USFA to SFA compared to that of SO and LO, the AME in LO and SO was seen to be similar to that of PO. The similarity in the AME of the PO, SO and LO could be due to the low concentration of stearic acid (C18:0) in the oils. Stearic acid stops the activity of lipase (Van Kuiken & Behnke, 1994). Thus, similar concentration of acids such as stearic acid in oils may have similar activity on lipase, resulting in a similar AME. This statement agrees with the report of Tancharoenrat et al. (2013), who reported that the AME of PO was similar to that of SO. However, the present results

contradict the findings of Kavouridou et al. (2008), who reported that birds fed a diet supplemented with LO had significantly higher AME compared with birds fed a diet supplemented with PO but this was not significantly different from birds fed SO. The highest AME in BO could be due to the utilisation of SFA and this may be improved by the presence of USFA. These results are in agreement with the findings of Garrett and Young (1975). This interaction is triggered by the excellent blending abilities in USFA (Garrett & Young, 1975). This interaction affects the absorption of SFA. However, the utilisation of USFA was not influenced by changing the USFA:SFA ratio (Garrett & Young, 1975). Ketels and De Groote (1989) reported that oil was used at the bird's maximal capacity if the ratio exceeded

four. Diets containing saturated fat resulted in less feed gain ratio than those containing poly unsaturated fats (Zollitsch et al., 1997). Also, Sanz et al. (2000) posited that the rate of saturation in broiler chickens of dietary oils was affected by their accumulation of fats and metabolic use.

CONCLUSION

Based on the current results, it appears that the AME of PO, SO and LO in broiler chickens was similar despite differences in the fatty acid composition of the oils. In addition, the present evaluation showed that blend oil is an attractive way to increase the AME of oil that is rich with SFA for poultry by adding oil rich with USFA.

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Pertanika J. Trop. Agric. Sci. 39 (1): 483 - 490 (2016)

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Stability Analysis of Panicle and Grain Traits of Rainfed Upland Rice in Two Tropical Ecologies of Nigeria

Nassir, Adesola L.* and Alawode, Yetunde O.

Department of Crop Production, College of Agricultural Sciences, Olabisi Onabanjo University, Yewa Campus, Ayetoro, Nigeria

ABSTRACT

Stability of grain yield in upland rice due to the unpredictability of environmental indices is of important consideration in the development of cultivars adapted to fairly wide cultivation zone. A study was conducted with fifteen upland rice varieties in two locations in South-Western Nigeria to evaluate the contribution of panicle and grain characters to stable grain production. Data collection spanned five environments and was subjected to stability analyses. The effects of genotype, environment and their interaction were significant for all the panicle and grain characters. Broad sense heritability estimate (HB) was moderate for hundred grain weight (62.4) and grain length (58.9) but was generally low for other grain yield traits, particularly grain weight per panicle (11.6) and grain weight per plant (5.6), respectively. Stability variance identified different genotypes as stable for most of the characters. The crossover attribute of AMMI PC 1 however complimented the significant verdict returned by the stability variance though the former also specified the direction of instability. The Yield Stability index (YSi) harnessed the advantages of the two statistics to identify different genotypes as stable for different characters. Thus, there is a need to constitute a pool of genotypes for the evolution of superior synthetic but stable cultivars.

Keywords: AMMI, genotype x environment interaction, grain yield, *Oryza sativa*, yield-stability index.

INTRODUCTION

Genotype by environment (GE) interaction is an important issue in crop improvement efforts, especially considering its importance in the evolution of varieties with appreciably high and stable grain yield across seasons and specific target regions. Plant breeders are constantly guided by this to define

breeding strategies and direction (Ouk et al., 2007; Acuña et al., 2008; Nassir & Ariyo, 2011). For a genotype to be commercially successful, it must perform well across the range of environment likely to be encountered in a target region over the entire array of years in which the genotype could be in use. Beyond seasonal and location differences, however, cultivation conditions within season do transit from one condition to the other, as dictated by variability in moisture and other environmental indices. This, as affirmed by Acuña et al. (2008), makes the evaluation of genotypes with respect to dominant traits necessary in different environments to guide in efforts aimed at evolving varieties with reasonably stable yield. Stability implies that both yield and quality remain somewhat constant, and this draws from holding in steady state some aspects of morphology and physiology of the crop in question even when other cultivation factors change. This homoeostatic condition must necessarily derive from the stability of the characters that cumulatively determine grain yield.

Naturally, however, the presence of GE interaction makes it difficult to fully realise the potential of a genotype for a region in which weather varies from year to year. When the GE interaction is significant, the plant and environmental factors that play a major role in causing differential performance, and their significance in determining desirable breeding strategies, must be carefully considered (Kang & Martin 1987; Yan & Hunt, 2001). A number of approaches have been used overtime for various crops to evaluate interaction between genotype and environment and hence, stability. This included the computation of stability variance (Shukla, 1972; Kang & Pham, 1991), yield/stability biplots (Kempton, 1984), the Additive Main effect and Multiplicative method (AMMI) (McLaren & Chaudhary, 1994), the Genotype plus Genotype-by-Environment Method (GGE) (Yan et al., 2000, 2007; Acuña et al., 2008; Nassir & Ariyo, 2011; Acuña & Wade, 2012). The ultimate aim is to generate conclusions that would guide breeding direction to develop genotypes with good adaptation to fairly wide environments within seasons and across regions and cultivation conditions.

A lot of GE studies have placed emphasis on identification of megaenvironments. Rice production in the upland ecology however suffers from variation in cultivation conditions within and across seasons, thereby making evaluation of stability necessary, with some emphasis on within location environment factors. In most cases, genotypes that show most stable yield appear in the centre of the AMMI Biplot and thereby combine stability with average yield (Kempton, 1984; McLaren & Chaudhary, 1994; Yan et al., 2000; Gauch, 2006; Acuña et al., 2008). This average yield is often a compromise of many plant and environment factors and may not always meet the aspiration of farmers, hence the renewed emphasis on combining stability with high grain yield. The combination of recent techniques for analysing genotype-by-environment interaction with the Yield-

Stability index has been canvassed by Nassir and Ariyo (2011). This study consequently focused on the evaluation of stability of grain yield components of upland rice using the Yield-Stability method of Kang and Pham (1991), along with the AMMI model.

MATERIALS AND METHODS

Study Location

This study was conducted at the College of Agricultural Sciences, Olabisi Onabanjo University. The first four plantings were done at Ago-Iwoye, Nigeria (3.92^{o}N, 6.95^{o}E) tropical rainforest ecology from 2001 to 2004, either with the early or the late rains. Two plantings were done at Ayetoro, Nigeria (6.5^{o}N, 5°E); a location with derived savanna ecology in 2009 and 2010. The 2009 planting suffered from severe drought and was unable to produce panicles and hence not used in the analysis.

Varieties

Fifteen varieties of upland rice were obtained from the African Rice Centre (formerly West African Rice Development Association, WARDA) the substation of the International Institute of Tropical Agriculture (IITA) Ibadan and were used for the study. The varieties were: ITA 150 and OS 6 (which are frequently cultivated and established in the study region), ITA 257, ITA II7, ITA 315 and ITA 321 (which are improved release varieties), IGUAPE CATETO, LAC 23, IDSA 10 (which are cultivated in other upland ecologies in the west African sub-region), WAB 35-2-FX (hereinafter identified as WAB 35), WAB

56-60, WAB 33-25, WAB 96-1-1, WAB 99-1-1and WAB 375-B-5-H2-1 (WAB 375), which are breeding lines being developed for improved yield and especially tolerance to drought.

Plant Establishment

The upland rice varieties were sown in a nursery and later transplanted onto ploughed upland paddy as soon as rainfall became steady. There were fifteen plants per single row plot, arranged in a randomised complete block design with three replicates. The plants were separated by 30cm between and within rows. Similar planting procedure and agronomic practices were carried out for all the plantings. Only the five internal plants for each plot were used for data collection.

Data Collection

Data were collected on each plant included both panicle and reproductive characters, as described by Anon (1988) following the standard evaluation system for rice (SESR). In particular, the data were collected on panicle number, panicle length, primary branches on panicles, secondary branching, spikelets number per panicle, grain weight per panicle, grain weight per plant, 100-grain weight, grain length and width, and spikelets fertility.

Data Analysis

Results were analysed using the means recorded on the characters for each variety. Computer analysis of variance (ANOVA) was done using the SAS (2000) package. The analysis was based on the five-season

(year) environment data. Meanwhile, the Additive Main Effect and Multiplicative Interaction (AMMI) analysis was done to obtain Interaction Principal Components (IPC) using the GENSTAT package Version 12. The AMMI PC 1 particularly presents the non-crossover attribute of the data and quantifies the response of genotypes to the trial environments (Yan et al., 2000; Gauch, 2006; Yan et al., 2007). Broad sense heritability estimates H_B for characters were determined from the ANOVA results using the methods enumerated by Breese (1969).

Stability variance for grain weight per plant was calculated following the procedure established by Shukla (1972). In addition, the yield stability index (YSi) was calculated to determine the genotypes, which have a combination of high yield and stability using the procedure of Kang and Pham (1991).

RESULTS AND DISCUSSION

Analysis of Variance and Broad Sense Heritability

The mean squares (MS) and broad sense heritability (H_B) for the panicle characters of the rice genotypes over the five environments are presented in Table 1. All the characters showed significant genotype, environment and genotype by environment interaction. Heritability estimates were quite low with the least value of 1.9 for panicle number and the highest estimate of 18.0 for secondary branching.

Table 2 shows the mean squares and broad sense heritability (H_B) for grain characters. All the characters also recorded significant genotype, environment and genotype by environment interaction. Grain weight per panicle and grain weight per plant had low H_B of 11.6 and 5.6, respectively. The highest H_B estimates of 62.4, 58.9 and 40.0 were recorded by 100-grain weight (62.4), grain length (58.9) and spikelet fertility (40).

The significant mean squares for the genotype effect indicate genetic differences among the varieties for all the characters. Similarly, the significant environmental effect implies that the study seasons presented discriminatory conditions for a genotype by environment (G X E) study. Following other reports on rice (Nassir & Ariyo, 2005; Acuña et al., 2008; Shrestha et al., 2012), significant differences in environmental influence are to be expected when cultivating upland rice over seasons. The significant G X E interaction for all the characters clearly implies differential genotypic response to different environments such that character expression and hence genotypic performance cannot be expected to be stable over cultivation seasons. Hence, upland rice breeding effort must be conscious of this complex scope of interaction of environment with panicle and grain characters in plant improvement.

Low heritability estimate for a number of panicle and grain characters in upland rice has been similarly reported by Nassir and Ariyo (2006). Characters that show low heritability would require many cycles of hybridisation and selection in a fair range of environments for meaningful progress. However, gains from selection for hundred (100) grain weight and grain length is likely

to be faster, given the moderately high heritability estimate, and this may impact positively on larger grain weight per plant.

GE and Yield-Stability analysis for panicle and grain characters

The mean, stability variance (σ^2) and AMMI PC 1 and yield-stability statistic (YSi) for panicle characters of each genotype over the five environments are presented in Table 3. ITA 321 had the highest mean panicle number of 6.71 but was considered as unstable by the stability variance. However, it recorded a near zero, but negative AMMI PC 1 and was the only one selected by the YSi statistic among the genotypes

considered as unstable by the stability variance. Similarly, ITA 117 and ITA 315 had high mean panicle number, and returned stable by the stability variance. The two also had positive interaction with the environment and were consequently selected by YSi. ITA 257 recorded above average panicle number but was the most unstable according to σ^2 and had the largest negative interaction with the environment with the largest AMMI PC 1 score of -0.964.

The longest panicles were recorded by WAB 375 (27.22cm) and OS 6 (27.19cm). The two genotypes selected by the YSi were adjudged as unstable by the stability variance and had relatively large negative

Table 1

Mean squares and broad sense heritability (H$_B$) for the panicle character of upland rice varieties over five environments

Source of variation	Panicle number	Panicle length (cm)	Primary branches (No)	Secondary branching (s)	Spikelets per panicle (No)
Genotype (G)	4.59*	51.49**	28.32**	1.21**	5948**
Environment (E)	305.81**	580.75**	501.90**	9.24**	121737**
GE	6.56**	21.62**	6.79**	0.55**	2980**
Pooled Error	2.33	5.51	4.17	0.22	1064
H$_B$ (%)	1.9	13.5	11.5	18	6.9

* Significant at P< 0.05, ** Significant at P< 0.01

Table 2

Mean squares and broad sense heritability (H$_B$) for the grain characters of upland rice varieties over five environments

Source of Variation	Grain weight per panicle (g)	Grain weight per plant (g)	Hundred grain weight (g)	Grain length (mm)	Grain width (mm)	Spikelet fertility (s)
Genotype (G)	4.28**	146.44*	2.40**	1.09**	0.75**	5.94**
Environment (E)	39.38**	2914.58**	3.78**	0.88**	4.81**	14.27**
GE	2.60**	89.64**	0.36**	0.72**	0.16**	2.89**
Pooled Error	1.01	49.38	0.09	0.11	0.61	0.55
H$_B$	11.6	5.6	62.4	58.9	26.7	40.0

* Significant at P< 0.05, ** Significant at P< 0.01

AMMI PC 1. Indeed, most of the genotypes were deemed unstable by σ^2 and also had large AMMI PC 1 scores. WAB 33-25 had the highest YSi value on account of having above average panicle length, stable σ^2 and relatively lower PC 1 score.

WAB 99-1-1and WAB 375 had the highest mean primary and secondary branching scores and were returned unstable by the stability variance. WAB 375 however had positive interaction with improving environment in contrast to WAB 99-1-1. OS 6 had the highest YSi for primary branches. The genotype recorded above average mean primary branching and was considered stable by both the σ^2 and AMMI PC1. More varieties were considered as stable by σ^2 for the primary branching than secondary branching.

Table 4 presents the mean, stability variance, AMMI PC 1and the YSi values for grain and spikelets characters. The results indicated that only two genotypes (IDSA 10 and OS 6) were stable with respect to grain length. The two had positive interaction with improving environment. However, only OS 6 had above average mean grain length and was consequently selected by YSi. ITA 150, which recorded the mean longest grains and had a significant σ^2, showed positive interaction with the high environment with an AMMI PC 1 score of 0.42 and was also selected by YSi. For grain width, three of the genotypes (ITA 321, OS 6, and WAB-96-1-1) were declared as stable by σ^2 and also had small AMMI PC 1 scores. However, only the latter two, along with WAB 35-2-FX, IGUAPE CATETO and WAB 33-25, had mean grain width of up to 3mm, large YSi and were consequently selected.

WAB 99-1-1 had the highest mean spikelets number of 175.03 per panicle. It had the largest negative AMMI PC 1 score and was also highly unstable by the stability variance. WAB 375 also produced a high mean spikelets number of 168.2 per panicle, and was deemed as stable by σ^2. It also had the least AMMI PC 1 score and the best YSi. ITA 315, which had a non-significant σ^2 and WAB 35, which had positive interaction with improving environment, also had high YSi values and were therefore selected. In term of spikelets fertility, most of the genotypes were highly unstable by σ^2 verdict and also had relatively large AMMI PC 1 scores. ITA 315 had the lowest mean spikelets fertility scores, a non-significant σ^2, as well as a high and positive AMMI PC scores. It also had the best YSi and was consequently selected as a choice genotype for the character.

The stability variance, AMMI PC 1 and the YSi values for grain yield characters are shown in Table 5. Nine of the genotypes had mean 100-grain weight above 3.0g. Of these, however, only three (IDSA 10, ITA 150 and OS 6) had non-significant σ^2 and large YSi. WAB 33-25 recorded the largest mean panicle grain weight of 4.76g and the least non-significant σ^2, which consequently earned it the highest YSi. ITA 315 and WAB 99 also had high YSi but with lower mean panicle grain weight. WAB 35 and WAB 375 also had high mean panicle weight of 4.71g and 4.70g, respectively, and also with significant σ^2. Nine of the fifteen genotypes

Table 3

Means, stability variance (σ²) and AMMI PC 1 and yield-stability statistic (YSi) for the panicle characters of upland rice genotypes

Genotype	Panicle number				Panicle length (cm)				Primary branches				Secondary branching (s)			
	Mean	σ²	PC 1	YSi (s)	Mean	σ²	PC 1	YSi (s)	Mean	σ²	PC 1	YSi (s)	Mean	σ²	PC 1	YSi (s)
IDSA	5.53	3.01**	-0.84	-1	21.18	7.86**	-0.29	-7	9.69	1.87	-0.08	1	1.83	0.23**	-0.29	0
ITA 150	5.38	2.85**	-1.02	0	23.37	4.03*	0.68	4	9.33	1.34	-0.75	0	1.46	0.04	0.19	3
ITA 257	5.19	5.66**	-0.96	-5	24.27	4.15*	1.01	9+	10.33	2.70	-0.82	3	2.05	0.29**	0.68	6
WAB 35-2-FX	4.99	0.17	0.05	2	21.83	1.68	0.56	3	10.75	2.63	-1.03	0	1.85	0.13	-0.25	10+
WAB 56-60	5.37	0.62	-0.06	6+	23.09	8.84**	1.50	-2	11.51	3.85*	-1.28	9+	2.11	0.07	-0.26	15+
IGUAPE CATETO	5.53	3.93**	1.29	3	21.73	3.98*	0.74	-2	12.09	0.66	0.38	12+	1.33	0.01	0.07	0
LAC 23	5.87	4.28**	-0.80	5	24.13	8.48**	-0.57	4	11.24	1.92	0.86	5	1.40	0.02	0.07	1
0S 6	5.20	2.92**	1.07	-4	27.19	9.70**	-1.26	7+	12.73	0.91	0.05	14+	1.40	0.20*	0.35	-3
ITA 117	6.44	1.45	0.55	15+	23.33	4.63*	-0.85	5+	12.25	1.28	0.66	13+	1.53	0.12	0.33	4
ITA 315	6.05	0.37	0.11	14+	20.98	0.96	0.20	0	11.53	1.20	-0.56	10+	1.65	0.14	0.16	5
ITA 321	6.71	2.29**	-0.09	8+	22.55	2.24	-0.26	4	11.48	0.58	0.54	6	1.84	0.17*	-0.31	5
WAB 33-25	4.65	0.20	0.22	0	24.27	1.66	-0.15	14+	11.69	5.38**	1.30	3	1.97	0.27**	0.37	5
WAB 96-1-1	5.57	1.29	-0.63	12+	22.95	11.52**	-0.75	-3	10.17	0.80	0.32	2	1.95	0.21*	-0.27	8+
WAB 99-1-1	5.37	3.72**	0.88	-2	23.29	18.11**	1.32	-1	13.49	4.52**	-0.17	7	2.21	0.65**	-0.96	8+
WAB 375-B-5-H2-1	4.96	0.01	0.24	1	27.22	20.25**	-1.88	8+	14.37	4.34**	0.56	9+	1.93	0.22*	0.11	7+
Grand Mean(LSD) (P<0.05)	5.52 (2.05)				23.43 (3.14)				11.51 (2.74)				1.81 (0.64)			

*, ** significant at p<0.05 and 0.01, respectively;

+, selected based on one-third selection from total genotypes.

Table 4
Stability variance (σ²), AMMI PC 1 and yield-stability statistic (YSi) for the grain and spikelets characters of upland rice genotypes

Genotypes	Grain length (mm)				Grain width (mm)				Spikelets per panicle				Spikelets fertility (s)			
	Mean	σ²	PC 1	YSi	Mean	σ²	PC 1	Ysi	Mean	σ²	PC 1	Ysi	Mean	σ²	PC 1	Ysi
IDSA	8.86	0.00	0.10	5	2.85	0.27**	-0.83	5	113.25	254.40	1.68	1	3.25	0.37	-0.43	8[+]
ITA 150	9.32	0.27**	0.43	9[+]	2.71	0.08**	-0.39	-4	107.6	404.19	-3.15	0	3.21	0.87**	0.30	1
ITA 257	9.00	0.10*	-0.62	7[+]	2.76	0.03**	0.20	0	150.23	3073.92**	-5.87	3	3.55	0.62**	-0.60	-2
WAB 35-2-FX	9.00	0.14*	-0.25	6	3.15	0.01*	-0.08	13[+]	153.94	801.53*	3.30	10[+]	3.99	0.90**	-0.63	-5
WAB 56-60	9.25	0.63**	-0.55	7[+]	2.40	0.05**	0.10	-8	150.20	1331.65**	-3.17	2	2.83	0.35	0.22	10[+]
IGUAPE CATETO	8.84	0.15**	0.32	1	3.09	0.04**	-0.03	12[+]	125.47	771.14*	1.52	0	2.49	0.27	0.02	12[+]
LAC 23	8.91	0.15**	-0.24	3	2.50	0.19**	0.50	-3	119.58	1129.74**	4.07	-6	4.21	0.43*	0.04	-1
0S 6	9.11	0.05	0.23	13[+]	2.79	0.00	0.08	9[+]	125.14	944.45*	3.60	-1	3.26	1.21**	-0.37	0
ITA 117	9.31	0.17**	0.01	8[+]	2.62	0.05**	0.12	-5	128.91	235.97	1.88	5	3.43	1.42**	-0.90	-1
ITA 315	8.57	0.17**	0.01	-8	2.82	0.03**	0.24	3	151.48	216.79	-0.77	12[+]	1.93	0.44	0.47	13[+]
ITA 321	8.90	0.17**	0.23	-2	2.71	0.00	-0.09	5	136.89	782.09*	2.52	2	2.81	2.83**	1.35	3
WAB 33-25	9.14	0.33**	0.64	6	3.00	0.01*	0.17	11[+]	146.89	499.30	2.17	9[+]	2.37	1.14**	0.65	5[+]
WAB 96-1-1	8.66	1.00**	-0.98	-7	2.84	0.00	-0.01	12[+]	153.03	943.46**	-1.60	5	3.69	0.51**	0.24	-3
WAB 99-1-1	8.39	0.16**	0.38	-9	2.40	0.04**	-0.03	-9	175.03	2829.36**	-6.02	8[+]	3.77	1.01**	-0.78	-4
WAB 375-B-5-H2-1	8.80	0.07*	-0.26	2	2.80	0.01*	0.06	6	168.20	683.62	-0.16	15[+]	2.92	2.06**	0.42	1
Grand Mean(LSD) (P<0.05)	8.94 (0.12)				2.76 (0.16)				140.43 (43.8)				3.17(1.0)			

*, ** significant at p<0.05 and 0.01, respectively.
[+], selected based on one-third selection from total genotypes.

recorded above mean grain weight per plant with the highest grain weight per plant of 24.58g by WAB 96-1-1, followed by ITA 315 with 20.36g. The two genotypes were however deemed unstable by σ^2 although the latter was eventually selected by YSi. WAB 99-1-1, WAB 33 and ITA 117 had the next best grain weight of 19.24g, 18.59g and 17.26g respectively in that order per plant and were selected by YSi.

The significant stability variance of many of the genotypes for the panicle and grain characters underscores the necessity to shift focus away from yield alone in genotype – environment and stability analysis. It would appear that there is a necessity to attain a converging compromise involving the panicle characters in the development of high quality phenotypic expression, eventually in terms of yield and yield stability. This partly explains why the YSi, which attempts to adopt a compromise between higher and stable trait expression, selected different genotypes for many characters (Kang & Pham, 1991). The instability of ITA 257 for grain weight per plant, for instance, appeared to be the cumulative effect of the instability of most of the characters with the exception of primary branches and grain width. Similarly, WAB 96-1-1, which was the most unstable in terms of grain weight per plant, was also unstable for the characters except panicle number, primary branches and grain width. Conversely, ITA 357, which was unstable for grain weight per plant, was stable for most of the characters. It would seem that the instability of grain length, width and 100-grain weight were absorbed by the stable and above average performance of most of the other characters. Noteworthy, however is the advantage posed by high panicle number by ITA 321, the longest and most branched panicle by WAB 375 and the longest grains and largest 100-grain weight by ITA 150. These genotypes and others identified by superior character expression can for a pool of genotypes for the evolution of synthetic genotypes for overall increase grain yield.

Generally, the stability variance and the AMMI PC 1 appeared to be consistent to a reasonable extent in the value returned as a measure of stability of genotypes for different characters. However, AMMI PC 1 is only a fraction of the of the GE component of the AMMI 1 values and would not be expected to capture the entire interaction as much as the stability variance. While σ^2 does not give the direction and manner of instability, the non-crossover attribute AMMI PC 1 (Yan & Hunt, 2001; Samonte, 2005) helps to complement the decision on stability by specifying the manner of genotype reaction to improving (or declining) environment. The selection of different genotypes by the YSi for the characters attests to the need to concentrate traits for higher expression. For instance, WAB 33-25 can be improved for the panicle number without sacrificing its stability in respect of grain production. Similarly, ITA 315 would possibly have higher grain weight per plant through a carefully planned improvement in primary and secondary branching and spikelets number per panicle.

Table 5
Stability variance (σ²), AMMI PC 1and yield-stability statistic (YSi) for the grain yield characters of upland rice genotypes

Genotypes	100-grain weight (g)				Panicle grain weight (g)				Grain weight per plant (g)			
	Mean	σ²	IPC 1	YSi	Mean	σ²	IPC 1	YSi	Mean	σ²	IPC 1	YSi
IDSA	3.61	0.05	0.16	14[+]	4.12	0.49	0.29	8[+]	16.74	10.29	-0.26	4
ITA 150	3.68	0.03	0.10	16[+]	3.22	0.57	-0.64	1	11.31	10.03	0.22	0
ITA 257	3.26	0.08*	0.03	6[+]	4.23	0.86**	-0.71	1	15.86	65.24**	0.17	-5
WAB 35-2-FX	3.26	0.08*	0.08	11[+]	4.71	1.24**	-0.68	7[+]	17.40	-1.30	-0.17	10[+]
WAB 56-60	3.04	0.07*	-0.10	1	3.96	1.30**	-0.91	-4	16.96	6.98	0.42	5
IGUAPE CATETO	3.60	0.27**	-0.55	5	4.25	1.75**	0.12	2	17.32	24.82	-0.12	9
LAC 23	2.57	0.07	0.02	1	2.88	0.92**	0.72	-8	12.78	14.71	-0.52	1
OS 6	3.34	0.07	-0.23	11[+]	4.01	0.26	0.08	5	14.28	9.48	0.07	2
ITA 117	2.59	0.05	-0.29	2	3.69	1.25**	0.65	-6	17.56	5.59	0.40	11[+]
ITA 315	2.59	0.17**	0.15	-2	4.39	0.51	-0.45	13[+]	20.36	32.18*	-0.05	11[+]
ITA 321	2.80	0.06	-0.19	3	3.74	0.78*	0.46	-1	17.24	9.27	-0.02	7
WAB 33-25	3.36	0.43**	0.80	4	4.76	0.04	-0.12	16[+]	18.59	8.08	0.02	13[+]
WAB 96-1-1	3.11	0.21**	-0.59	-1	4.32	1.27**	0.82	3	24.58	200.45**	-0.11	8
WAB 99-1-1	2.56	0.10**	-0.10	-8	4.36	0.53	-0.07	12[+]	19.24	12.38	0.16	14[+]
WAB 375-B-5-H2-1	2.99	0.18**	0.49	-4	4.70	1.24**	0.43	6	18.28	39.99*	-0.19	8
Grand Mean(LSD) (P<0.05)	3.15 (1.31)				4.09 (1.35)				17.2 (9.44)			

*,** significant at p<0.05 and 0.01, respectively.
[+], selected based on one-third selection from total genotypes.

CONCLUSION

Stability analysis in rice would continue to attract attention as different agro ecological zones present variable environmental conditions. The need to accumulate desirable genes into genotypes so as to have appreciable plastic response that would assure adequate character expression from intra- and inter-ecological variations is quite germane. The use of simultaneous selection for higher phenotypic expression and stability resulted in the selection of genotypes with significant stability variance. From the practical point of view, the YSi statistics is useful in identifying genotypes with high and stable expression for different characters. The complimentary role of AMMI PC 1 further shapes the decision on the genotypes that are compatible to season- or location-based improving or declining environmental conditions, particularly in upland rice cultivation.

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The Effect of Cultivation Techniques on the Growth Rate of Marine Microalgae Species from the Coastal Waters of Kudat, Sabah, Malaysia

Nurzafirah Mazlan* and Ridzwan Hashim

Department of Biomedical Science, Kulliyyah of Allied Health Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, 25200 Kuantan, Pahang, Malaysia

ABSTRACT

This study evaluates the growth of five species of marine microalgae; *Chaetoceros muelleri, Isochrysis galbana, Pavlova lutheri, Nitzschia acicularis* and *Navicula sp.,* isolated from coastal waters of Kudat in Sabah, Malaysia, using different cultivation techniques for mass outdoor culture. The microalgae were locally isolated and identified based on their morphology. The growth of the microalgae was compared between carboy and polythene bag culture. Results showed that cell count for all species was significantly higher in the polythene bag culture compared to carboy culture ($p < 0.05$). The time to harvest was also shorter (2-3 days) compared to carboy culture. In particular, *Chaetoceros muelleri* produced the highest cell count of 87 X 10^6 cells/ml and shortest time of 2 - 3 days to harvest. There were significant differences ($p < 0.05$) in cell count between indoor and outdoor mass culture, and these suggest the feasibility to culture the microalgae outdoor, reduce hatchery operation cost and save time.

Keywords: Carboy, microalgae, mass culture, polythene bag

INTRODUCTION

Microalgae are the primary producer of the food chain and have become the main source of food and feed in many aquaculture industries. The usage of microalgae as food for rearing larvae and juveniles has been extensively reported (Laing, 1991; Martinez-Fernandez & Souchgate, 2007; Galley et al., 2009). The larval and juvenile stages signify the importance of nutrition to ensure proper growth, survival rate and hence, successful hatchery operations. High mortalities during larviculture of marine animals such as fish, shellfish and echinoderms remain a

major bottleneck in aquaculture that limits production (Dhert et al., 2001). Many factors contribute to the high mortality cases such as diseases, parasites and food availability. Good diet requirement has been proven as the key factor in overcoming these problems (Rainuzzo et al., 1997).

Culturing of live algae is considered necessary as feed in the hatcheries. However, the process can be costly especially for the indoor culture (Cotteau & Sorgeloos, 1992). Feeding mixed algal diet consisting of two or more algae culture has been shown to produce optimal growth and development of larvae and juveniles compared to monoculture (Ivy & Giraspy, 2006). However, many of the microalgae have been proven difficult to culture in sizeable quantities necessary for large scale hatchery operations due to contamination, genetic unstability, light limitation and weather, which subsequently compromise its suitability for commercial applications. It is important to identify the specific diet requirements to determine which microalgae species to be cultured in the hatchery. Commercial hatcheries have consistently used reliable species such as *Isochrysis galbana, Pavlova leutheri* and *Chaeteceros calcitrans*. These species have been extensively studied for their biochemical composition, as well as nutritional value.

Microalgae can be used as live feed or processed into artificial feed. So far, no completely satisfactory alternative has been found as compared to the use of live microalgae in aquaculture. In spite of all effort to replace the live algae by artificial feeds, marine scientists are still dependent on the production and use of microalgae as live food for commercial aquaculture industries (Albentosa et al., 2002; Enes & Borges, 2003; Bonaldo et al., 2005; Espinosa & Allam, 2006; Chomaco et al., 2007).

Most algae cultivation can be described as either open or closed system. Closed system generally consumes more energy and requires a large capital investment but delivers a relatively high biomass yield. In contrast, open algae growth ponds have lower energy requirement but produce less biomass for the same area. Open ponds can also be affected by contamination and evaporation losses (Lardon et al., 2009; Jorquera et al., 2010; Stephenson et al., 2010). Various physiological and technological approaches have been proposed and investigated for maximising productivity in mass algal culture system. Numerous, more or less sophisticated systems have also been developed for culturing some 40 algal species to feed larvae and zooplankton organisms. Many studies have proven that a large scale production of monospecific algae would be feasible (Chaumont, 1993).

Cultivation technique is crucial in determining the growth of algae culture. While popular indoor algae culture utilises carboy as the culture recipient, polythene bag also shows promising results with better growth rates and shorter duration (Sipauba-Tavares et al., 2011). Thus, there

is a significant difference in the cultivation technique during indoor algae cultivation between carboy and polythene bag culture.

The aim of the present study was to isolate the microalgae from the coastal waters of Kudat in Sabah, Malaysia, and subsequently determine the effects of cultivation techniques on the growth of marine microalgae.

METHODS

Isolation and Identification of Microalgae

Water and rock samples were collected using transparent bottle from the coastal waters of Kudat, Sabah, Malaysia (6°49'12.1"N, 116°51'37.5"E) on May 13, 2012 and July 15, 2012, respectively. The samples were sieved using 50 μm mesh net to eliminate coarse particles and species such as sand and zooplankton.

The mixed algal samples were cultured in a 500 ml shake flasks filled with 400 ml of a standard f/2 medium (Guillard, 1975), with an addition of meta-silicate for diatoms species. After 10 days, the microalgae were identified using stereomicroscope and isolated from other algal species using capillary tube method (Blanco et al., 2008; Debenest et al., 2009). The isolated microalgae were identified morphologically based on size, form and colour. Identification results were verified by comparing with three books, namely, 'The Diatoms: Biology and Morphology of the Genera', 'Marine Algae: Biodiversity, Taxonomy, Environmental Assessment, and Biotechnology' and 'Algae' (Round et al., 1990; Graham et al.,

2008; Pereira et al., 2014). The species of *Chaetoceros muelleri, Isochrysis galbana, Pavlova lutheri, Nitszhia acicularis* and *Navicula sp.* were inoculated into fresh f/2 medium and used as inoculum for further studies. All the cultures were maintained at 25 ± 1 °C on a 12 h/12 h light/dark cycle with 1400 lux of light intensity.

Monoalgae Culture

The seawater used for culturing diatoms was filtered through sand filter and subsequently through 1 micron sediment filter cartridge and then treated with sodium hypochlorite for 12 h to kill microorganisms. The sodium hypochlorite in the seawater was neutralised with sodium thiosulphate before use. Non-axenic monocultures of the microalgae species were cultivated in f/2 medium (Guillard, 1975) with an addition of meta-silicate for diatom species in progressive volumes of 10 ml, 150 ml, 1 L. The size of inoculum was adjusted to 1.0×10^4 cells/ml for all the microalgae species.

Algal Culture in Polycarbonate Carboy and Polythene Bag

Monospecific diatom culture of 2 L was inoculated in either 20 L polycarbonate carboy or 20 L polythene bag filled with f/2 medium (Guillard, 1975), with an addition of meta-silicate for diatom species.

The culture from carboy of 20 L was inoculated in transparent fiberglass tank filled with 100 L f/2 medium and it was subsequently transferred into an outdoor tank filled with 1000 L f/2 medium.

The 20 L culture in the polythene bag, however, was combined with another 5 sets of similar polythene bag culture to make up 100 L culture and transferred directly into an outdoor tank filled with 1000 L medium. The differences of algal growth in both methods were recorded.

Continuous aeration was provided. Cell concentration was counted using a haemocytometer and expressed in cells/ml.

Statistical Analysis

Data were analysed using one-way analysis of variance (ANOVA), SPSS version 17 (SPSS Inc., Chicago, IL, USA). Significant differences were determined at 0.05 level of probability.

RESULTS

Isolation and Identification of Microalgae

There were 5 species of microalgae isolated from the mixed culture sample collected from the coastal area of the sea cucumber hatchery. The species were identified based on their morphological characteristics.

Diatoms have a characteristic that they are enclosed within a cell wall made of silica. *Chaetoceros muelleri* had frustules with long, thin spines. *Navicula sp.* was characterised by valves with bilateral symmetry and a well-developed raphe system. *Nitzschia acicularis* had extremely long and narrow valves, which were lightly silicified. *Pavlova lutheri* was characterised based on the spherical shape, with two flagellae and golden brown colour of the cell. Their sizes were about 3-5 µm. Isochrysis galbana had the same morphology as *Pavlova lutheri* but the size was bigger than *Pavlova lutheri*, i.e. about 5-6 um length and 2-4 µm wide.

Mass Culture of Microalgae

Isolated microalgae were cultured in progressive volume of 10 ml, 150 ml and 1 L culture. All the cultures were kept indoor at the temperature of 25°C. After 5 to 7 days of incubation period in 1 L shake flask, the samples were collected and counted using a haemocytometer. Table 1 shows the cell count for the 5 species of isolated microalgae. The cell for *Isochrysis galbana* showed the highest count of 48×10^6 cells/ml, while Navicula sp. showed the lowest count with an average of 10×10^6 cells/ml compared to the other four species of microalgae. Meanwhile, *Pavlova lutheri*

Table 1

Cell count for 5 species of microalgae measured after 5 to 7 days of incubation period in 1 L culture.

Species	Temperature (°C)	Cell Count (cells/ml)	Incubation period (day)	Volume (L)
Isochrysis galbana	25.0 ± 1.0	$48 \times 10^6 \pm 43 \times 10^5$	5 - 7	1
Pavlova lutheri	25.0 ± 1.0	$34 \times 10^6 \pm 33 \times 10^5$	5 - 7	1
Chaetoceros muelleri	25.0 ± 1.0	$33 \times 10^6 \pm 54 \times 10^5$	5 - 7	1
Navicula sp.	25.0 ± 1.0	$10 \times 10^6 \pm 12 \times 10^5$	5 - 7	1
Nitzschia acicularis	25.0 ± 1.0	$16 \times 10^6 \pm 12 \times 10^5$	5 - 7	1

and *Chaetoceros muelleri* had an average count of 34×10^6 and 33×10^6 cells/ml, respectively. Low cell count was also recorded on Nitzschia acicularis with 16×10^6 cell/ml.

Two litres of monoalgal cultures from the shake flask were used as the starter to inoculate 20 L carboy or polythene bag culture. The cultures were left to grow for 3 to 5 days indoor with temperature ranging from 24 – 26°C before they were used as starter culture for the mass outdoor culture. Table 2 and Table 3 show the cell count of the 5 species of microalgae in carboy or polythene bag culture respectively during the day of harvest.

Isochrysis galbana recorded the highest cell count in carboy culture with the average of 55×10^6 cells/ml, while *Nitzschia acicularis* recorded the lowest cell count, with the average of 14×10^6 cells/ml. *Isochrysis galbana* was significantly higher than other species ($p < 0.05$) in the carboy culture. However, in the polythene bag culture, *Chaetoceros muelleri* had the highest cell count of average 87×10^6 cells/ml [$F_{(4,10)} = 99.6$, p = 0.000)]) compared to other culture. Meanwhile, *Nitzschia acicularis* showed the lowest cell count in the polythene bag culture as well, with an average of 25×10^6 cells/ml. There were also increases in the cell count of *Nitzschia acicularis* and *Navicula sp.*, although not as much as *Chaetoceros muelleri* and *Isochrysis galbana*. The highest cell count for *Nitzschia acicularis* was in the polythene

Table 2
Cell count for 5 species of microalgae measured after 3 to 5 days of incubation period in 20 L carboy culture.

Species	Temperature (°C)	Cell Count (cells/ml)	Incubation period (day)	Volume (L)
Isochrysis galbana	25.0 ± 1.0	$55 \times 10^6 \pm 12 \times 10^5$	3 - 4	20
Pavlova lutheri	25.0 ± 1.0	$37 \times 10^6 \pm 22 \times 10^5$	3 - 4	20
Chaetoceros muelleri	25.0 ± 1.0	$42 \times 10^6 \pm 21 \times 10^5$	3 - 4	20
Navicula sp.	25.0 ± 1.0	$21 \times 10^6 \pm 37 \times 10^5$	3 - 5	20
Nitzschia acicularis	25.0 ± 1.0	$14 \times 10^6 \pm 16 \times 10^5$	3 - 5	20

Table 3
Cell count for 5 species of microalgae measured after 2 to 3 days of incubation period in 20 L polythene bag culture.

Species	Temperature (°C)	Cell Count (cells/ml)	Incubation period (day)	Volume (L)
Isochrysis galbana	25.0 ± 1.0	$80 \times 10^6 \pm 16 \times 10^5$	2 - 3*	20
Pavlova lutheri	25.0 ± 1.0	$47 \times 10^6 \pm 29 \times 10^5$	2 - 3*	20
Chaetoceros muelleri	25.0 ± 1.0	$87 \times 10^6 \pm 24 \times 10^5$	2 - 3*	20
Navicula sp.	25.0 ± 1.0	$33 \times 10^6 \pm 24 \times 10^5$	2 - 3*	20
Nitzschia acicularis	25.0 ± 1.0	$25 \times 10^6 \pm 21 \times 10^5$	2 - 3*	20

*All cultures show a significant difference at p <0.05 when compared to carboy culture

culture with average cell of 25 X 10^6 cells/ml while *Navicula sp*. recorded an average of 33 x 10^6 cells/ml. Meanwhile, *Pavlova lutheri* recorded an average of 37 X 10^6 cells/ml in the carboy culture and 47 X 10^6 cells/ml in polythene bag culture. All the cultures showed a significant ($p < 0.05$) increase in their cell count when cultured in polythene bag.

The time taken to harvest the cultures was also faster in polythene bag compared to the carboy. It took about 3 to 4 days to harvest *Isochrysis galbana, Pavlova lutheri* and *Chaetoceros muelleri* in carboy culture, whereby *Navicula sp*. and *Nitzschia acicularis* required longer period of 3 to 5 days to harvest. Meanwhile in the polythene bag culture, it took an average of 2 to 3 days for all the microalgal species to be harvested.

One hundred litres of culture from carboy or polythene bag was used as inoculum for outdoor mass culture with volume of 1 m^3 and temperature ranging from 30 to 32°C. Cell count was taken on the day of harvest. All microalgae culture showed a slightly lower cell count compared

to indoor culture, this may be due to changes in environment such as temperature and light. Table 4 and Table 5 present the cell count for the 5 species of microalgae using inoculum from carboy and polythene bag, respectively.

Nitzschia acicularis had the lowest cell count, while *Chaetoceros muelleri* grew well in outdoor mass culture. The *Chaetoceros muelleri* sample from outdoor mass culture tank using carboy culture as inoculum recorded an average cell count of 49 X 10^6 cells/ml, which was significantly higher [$F(4,10) = 117.7$, $p = 0.000$)] than the other microalgae culture and indoor culture as well. Following that, *Isochrysis galbana* showed a high cell count of an average of 47 X 10^6 cells/ml. *Navicula sp*. and *Nitzschia acicularis* recorded a low cell count with an average of 20 X 10^6 and 12 X 10^6 cells/ml, respectively. However, the amount was considered as feasible for an outdoor mass culture. There were no significant differences between the outdoor and indoor cultures, except for *Isochysis galbana* ($p < 0.05$).

Table 4

Mean temperature (°C) and cell count (cells/ml) for 5 species of microalgae measured at the harvest time of the outdoor algae culture using microalgae cultured in carboy as inoculum.

Species	Temperature °(C)	Cell Count (cells/ml)	Time taken to harvest (h)	Volume (m^3)
Isochrysis galbana	31.5 ± 1.5	47 X 10^6 ± 21 X 10^5	60 - 72	1
Pavlova lutheri	31.3 ± 1.3	34 X 10^6 ± 17 X 10^5	60 - 72	1
Chaetoceros muelleri	31.2 ± 0.6	49 X 10^6 ± 29 X 10^5	60 -72	1
Navicula sp.	31.7 ± 0.5	20 X 10^6 ± 24 X 10^5	60 - 72	1
Nitzschia acicularis	31.7 ± 0.5	12 X 10^6 ± 12 X 10^5	60 - 72	1

Table 5

Mean temperature (°C) and cell count (cells/ml) for 5 species of microalgae measured at the harvest time of the outdoor algae culture using microalgae cultured in polythene bag as inoculum.

Species	Temperature (°C)	Cell Count (cells/ml)	Time taken to harvest (h)	Volume (m^3)
Isochrysis galbana	31.3 ± 1.3	$73 \times 10^6 \pm 27 \times 10^5$*	60 - 72	1
Pavlova lutheri	31.7 ± 0.5	$40 \times 10^6 \pm 12 \times 10^5$	60 - 72	1
Chaetoceros muelleri	31.3 ± 1.3	$89 \times 10^6 \pm 42 \times 10^5$*	60 - 72	1
Navicula sp.	31.7 ± 0.5	$26 \times 10^6 \pm 22 \times 10^5$	60 - 72	1
Nitzschia acicularis	31.5 ± 1.5	$15 \times 10^6 \pm 12 \times 10^5$	60 - 72	1

*Cultures show significant difference ($p<0.05$) compared to culture using carboy as inoculum.

From the tank of outdoor mass culture using polythene bag culture as inoculum, the cell count for *Chaetoceros muelleri* was 89×10^6 cells/ml, which was significantly higher [$F(1,4) = 124.1$, $p = 0.000$)] than the sample from the tank of outdoor mass culture with carboy as inoculum and its indoor culture. All the cultures were generally higher in cell count compared to the cultures grown in the outdoor mass tank using carboy culture as inoculum. *Nitzschia acicularis* had the lowest cell count of an average of 15×10^6 cells/ml. There were significant differences ($p < 0.05$) in the cell growth for *Isochrysis galbana* [$F(1,4) = 108.6$, $p = 0.000$)] and *Chaetoceros muelleri* [$F(1,4) = 124.1$, $p = 0.000$)] between the indoor and outdoor cultures, while *Pavlova leutheri*, *Navicula sp.* and *Nitzschia acicularis* showed no significant difference between the indoor and outdoor cultures ($p > 0.05$).

The time taken to harvest the outdoor mass cultures was about 60 – 70 hours after inoculation, depending on the cell count and demand of the species cultured such as the sea cucumber larvae and juvenile.

DISCUSSION

The advantageous effects of dietary algae are their contributions to minerals, dietary fibre, carotenoids, chemical feeding attractants, vitamins and synergistic effects with vitamins and antioxidants. Microalgae are widely used in aquaculture due to their easy culture and fast growth. They have also been used as sources of essential fatty acids. There were 5 species of commonly used microalgae in the aquaculture industries isolated and identified from a sea cucumber hatchery in Kudat, Sabah, namely, *Isochrysis galbana, Pavlova lutheri, Chaetoceros muelleri, Navicula sp.* and *Nitzschia acicularis*.

Isochrysis galbana is generally considered the best food for bivalves larvae. *Isochrysis galbana* is small, naked, free-living motile flagellates, which are easily ingestible by small (larval) invertebrate (Martines-Fernandez et al., 2004). *Pavlova lutheri* has the same morphological features with *Isochrysis galbana*, but they differ in terms of size, whereby *Pavlova lutheri* cells are bigger than those of *Isochrysis galbana*.

Based on the results the monoalgal cultures were successfully cultured indoor and outdoor. The algal cultures were species specific. Each species carries specific characteristics and requirement for their growth (Cobelas et al., 1998). There were significant differences between the indoor and outdoor cultures for *Pavlova lutheri, Navicula sp.* and *Nitzschia acicularis* from the polythene bag cultures, as well as *Isochysis galbana* from carboy cultured. However, there were no differences in the rest of the species cultures. These results showed that the mass outdoor cultures for these species were feasible.

There were slight decreases in the cell number of a few species of microalgae in this study when they were being transferred outdoor. Numerous studies have shown that the biochemical composition and physiological status of microalgae cells are altered by different environmental factors (Alonso et al., 2000; Sanchez et al., 2000; Renaud et al*.,* 2002; Berges et al., 2002; Tzovenis et al., 2003). Optimum temperatures for diatoms range from 19°C to 27°C; thus, the high temperature outdoor may have killed the cells or delay the growth. However, there were also reports indicating that most algae can tolerate high temperature up to 36°C. This further explains why a few species of microalgae grew so well in outdoor culture. Furthermore, the presence of contaminants is unavoidable in outdoor culture. Culturing microalgae in outdoor environment can achieve higher yields, leading to a reduction of operating cost compared to the cultures cultivated indoors (Elias et al., 2005), however in this study,

slightly lower yields were observed in the outdoor culture compared to indoor culture.

All the microalgae cultures in the polythene bag have a higher cell count compared to the carboy culture. Lin et al. (2007) reported that light is the main factor that interferes with the growth of microalgae. Polythene bag has better light penetration compared to carboy. Furthermore, the surface area is wider in polythene bag compared to carboy which has round surface. All the five species of microalgae showed the same growth trend when the carboy and polythene bag were compared. The usage of polythene bag as culture recipient has proven to be efficient with good growth rates and shorter duration (Sipauba-Tavares et al., 2011). Furthermore, it incurs low cost and requires smaller space in the laboratory.

CONCLUSION

This study showed that the mass outdoor cultures of five species of microalgae isolated from Kudat in Sabah, Malaysia are feasible. Furthermore, there were significant differences between the indoor and outdoor cultures ($p < 0.05$). All the microalgae cultures in polythene bag showed significant differences ($p < 0.05$) compared to those of the carboy culture, suggesting that culturing using polythene bag is more efficient, resulting in a shorter harvest period. Future studies need to optimise the growth parameter of the mass outdoor culture further by studying other factors such as light, temperature, genetic stability and weather.

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Production and Characterisation of Cellulase from Solid State Fermentation of Rice Straw by *Trichoderma harzianum* SNRS3

Rahnama, N.[1,2#], Shah, U. K. M.[2,3,5*], Foo, H. L.[2,4], Rahman, N. A. A.[2,5] and A. B. Ariff[2,5]

[1]*Department of Biological Sciences, Urmia Branch, Islamic Azad University, Urmia, Iran*
[2]*Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4]*Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[5]*Bioprocessing and Biomanufacturing Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

Research on production and the use of cellulase and xylanase by commercial microbial strains is widely reported. However, research on production of cellulase and xylanase by local isolates of *Trichoderma harzianum* known as potential cellulase producers is still very limited. *T. harzianum* SNRS3 was used for cellulase and xylanase production from rice straw under solid state fermentation. Our study revealed that unlike *Trichoderma* sp. that is normally associated with low amounts of β-glucosidase, insufficient to perform an efficient hydrolysis, *T. harzianum* SNRS3 could be considered as a potential β-glucosidase producer, but not an efficient xylanase producer. As a result of storage of the crude cellulase at room temperature, β-glucosidase activity only decreased to above 80% of its original activity at the end of the 3rd week of storage. The crude cellulase produced by *T. harzianum* SNRS3 could be industrially applied as the enzyme is still highly active at 60°C and over a wide range of acidic pH.

INTRODUCTION

Plant biomass is composed primarily of cellulose, hemicelluloses, and lignin (Kuhad et al., 1997; Carpita et al., 2001). Lignocellulosic waste materials

obtained from energy crops, wood and agricultural residues represent the largest global renewable reservoir of potentially fermentable carbohydrates (Mtui & Nakamura, 2005; Talebnia et al., 2010). Therefore, Lignocellulosic wastes are regarded as attractive substrates for the production and recovery of a large number of value-added products such as enzymes (Mtui & Nakamura., 2005).

Having occupied 2% of the world's cultivated area; rice ranks the second most major crop worldwide. Rice is an extensive crop of Asia and Southeast Asia, which dominates tropical and sub-tropical belts (Devendra & Thomas, 2002; Leff et al., 2004). Rice straw is an abundant lignocellulosic waste in the world with several characteristics that make it an appropriate feed stock for biofuel production (Binod et al., 2010). Among the lignocellulosic crop residues, rice straw is the largest biomass feedstock in the world (Talebnia et al., 2010).

A variety of microorganisms such as bacteria and fungi are plant biomass decomposers in nature. This makes them interesting sources for enzyme discovery (Allgaier et al., 2010). Lignocellulosic enzymes are important commercial products of lignocellulosic wastes bioprocessing used in many industrial applications including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Howard et al., 2003; Rahnama et al., 2014). Currently, cellulases rank as the third largest volume of industrial enzyme with a wide range of applications in

industry. Cellulases are employed in cotton processing, paper recycling, as detergent enzymes, in juice extraction, and as animal feed additives apart from their immense use in the hydrolysis of lignocellulosic biomass as a prerequisite for biofuel production (Wilson, 2009; Singhania et al., 2010). The use of lignocellulosic biomass-derived sugar for biofuel production has been shown to be promising (Park et al., 2012). In fact, cellulase will become the largest volume industrial enzyme, in case fermentation-derived fuel such as bioethanol, and biobutanol could replace the current fossil-based transportation fuel (Wilson, 2009).

Solid state fermentation (SSF) is known as the fermentation whereby an insoluble substrate is fermented in the absence or almost absence of free moisture (Chahal, 1985). Submerged fermentation (SmF) and SSF are both the common techniques applied for production of cellulases. SSF is advantageous over SmF due to the production of the enzymes at a higher yield and higher pH or temperature stability (Holker & Lenz, 2005; Singhania et al., 2009; Saqib et al., 2010).

Cellulases are the most extensively studied multiple enzyme complexes. As hydrolytic enzymes, cellulases cleave the β-1,4- glucosidic bonds in the cellulose structure (Singhania et al., 2010). The major cellulase components include cellobiohydrolases (exo-1,4-β-D-glucanase, CBH), endoglucanases (endo-1,4-β-D-glucanase, EG) and β-glucosidase (Hong et al., 2001; Li et al., 2006; Sun et al., 2008; Singhania et al., 2010). Nonetheless,

β-glucosidase does not act upon cellulose chain directly. Instead, β-glucosidase is responsible for the cleavage of cellobiose into two glucose monomers and this characteristic of β-glucosidase is of great significance since accumulation of cellobiose causes a product inhibition on both cellobiohydrolase and endoglucanase. Therefore, in addition to glucose production, β- glucosidase reduces product inhibition by cellobiose, which in turn causes cellulolytic enzymes to function more efficiently (Workman & Day, 1982; Lymar et al., 1995; Joo et al., 2009). Hence, β-glucosidase is the rate-limiting factor in the enzymatic hydrolysis of cellulose (Lymar et al., 1995; Fadel, 2000; Elyas et al., 2010). Cellulases with low levels of β-glucosidase hydrolyse cellulose slowly, and in such cases, the addition of β-glucosidase enhances cellulose hydrolysis (Johnson et al., 1982; Lymar et al., 1995; Fadel, 2000; Elyas et al., 2010). Unfortunately, the majority of cellulolytic fungi, including hypercellulase-producing mutants of *Trichoderma reesei* show low production of β- glucosidase (Saha et al., 1994; Skory & Freer, 1995; Riou et al., 1998; Elyas et al., 2010).

Xylanases, the xylan degrading enzymes, are present everywhere and are diverse by nature (Collins et al., 2005). Xylan hydrolytic enzymes have been reported mainly from bacteria (Gilbert & Hazlewood, 1993), fungi (Jin et al., 2012) and yeast (Hrmova et al., 1984; Liu et al., 1998). Microbial xylanases are preferable since they are highly specific, reaction conditions are mild, and substrate loss and generation of side products are almost negligible (Kulkarni et al., 1999). As the xylan molecule contains several substituted groups and side chains synergistic action of multiple hydrolytic enzymes might be essential for the molecule to be completely hydrolysed (Dhiman et al., 2008). Xylanases have many biotechnological applications and are therefore categorised among the most important enzymes in industry. Xylanases have numerous applications in biotechnology, and are widely used in food, animal feed, paper, and pulp industries, as well as in bioconversion of hemicelluloses to value-added products (Dhiman et al., 2008; Chapla et al., 2010).

Since understanding the action of cellulolytic enzymes under different conditions is of great importance, many researchers have focused on the characterisation of cellulase in terms of various physio-chemical parameters including temperature and pH (Farinas et al., 2010).

This study provides a comparison between cellulase production from rice straw under SSF by the local *T. harzianum* SNRS3 and *A. niger* ATCC 6275 as a model fungus. Crude cellulase enzymes produced by *T. harzianum* SNRS3 were characterised in terms of physio-chemical parameters such as optimum temperature, and pH. Thermal stability and the effects of storage at room temperature (28ºC), 4ºC, −20ºC, and −40ºC on the enzyme activity have also been studied.

MATERIALS AND METHODS

Substrate Preparation and Pretreatment

Rice straw was obtained from a paddy field in Sekinchan, Selangor, Malaysia. The dried rice straw was ground to 2 mm lengths using an electric grinder (Model CW-1, Hsiang Tai, Taiwan), and kept in a cold room at 4°C prior to use.

Microorganism and Inoculum Preparation

A local isolate of *T. harzianum* SNRS3 (isolated from rice straw collected from a rice field in Sekinchan, Selangor, Malaysia) and *A. niger* ATCC 6275 (used as model fungus) were used as inoculum, respectively. The fungal spores were kept in 30% (v/v) glycerol at minus 20°C. Reactivation of the spores was performed by growing on Potato Dextrose Agar (PDA) for 7 to 9 days. Spore suspension was freshly prepared prior to fermentation experiment by washing the agar surface with sterilised distilled water. The spores were then quantified and adjusted to 1×10^6 spores mL^{-1} by using a haemocytometer (Rahnama et al., 2013).

Fermentation

Cellulase enzyme was produced by solid state fermentation. A series of 250 mL Erlenmeyer flasks with cotton stoppers were autoclaved and used for the production and collection of the enzymes. Three grams of untreated rice straw was placed in different flasks. Mandels medium (Mandels et al., 1974) was added to each flask containing the rice straw, and the moisture content was kept at 65% (w/v). Mandels medium (1 L) contained 1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.63 g urea, 0.3 g $CaCl_2$, 0.3 g $MgSO_4$.$7H_2O$, 1 mL Trace elements, 0.75 g peptone, and 2 mL Tween 80. The pH of the medium was adjusted to 5. The flasks were then incubated at 30°C prior to the extraction of crude enzyme. The extraction of crude enzyme mixture was carried out by adding 30 mL of 50 mM citrate buffer (pH 4.8) into each flask, followed by agitation for 30 min at 150 rpm and 30°C. The mixture was then centrifuged at 4°C and 1000 × g for 10 min. The supernatant was filtered and kept at 4°C prior to use (Rahnama et al., 2013).

Crude Cellulase Enzyme Characterisation

The crude cellulase enzyme produced from rice straw by *T. harzianum* SNRS3 was characterised in terms of temperature and pH optima, thermal stability and storage stability at various temperatures [room temperature (28°C, 4°C, −20°C, and −40°C)]. In order to study the optimum incubation temperature for the crude cellulase activity, the reaction mixtures were reacted in a temperature range of 40-90°C and incubated for 1 h for FPase and for 30 min for CMCase and β-glucosidase; the three major components of cellulases that act synergistically for the complete hydrolysis of cellulose. It was then followed by the assay under the standard assay conditions at pH4.8, as described in detail in the analytical procedure. CMCase activity was determined by estimating the reducing sugars produced from 1% (w/v) carboxymethylcellulose,

whereas FPase activity was determined by measuring the reducing sugars released from Whatman filter paper No.1. For β-glucosidase assay, the ρ-nitrophenol liberated from ρ-nitrophenyl-β-D- glucopyranoside (PNPG) was determined spectrophotometrically (Wood & Bhat, 1988).

The effect of pH on the activity of crude cellulase was determined in a pH range of 2.5-7.5 by using citric acid-disodium hydrogen phosphate (McIlvaine) buffer solutions, pH 2.6-7.6. The activity was measured under the standard assay conditions. In order to study the crude enzyme thermal stability, the crude enzyme was incubated at 50 and 60°C. The crude enzyme solution in the screw-capped glass tubes was withdrawn periodically at the time intervals of 30 min, 1 h, 2 h, 3 h, and 4 h. It was then cooled on ice and the residual enzyme activity was assayed under standard assay conditions. In order to investigate the crude enzyme stability over storage, the crude cellulase was aliquoted in four bottles. The bottles were each kept in different temperatures including room temperature (28°C), 4°C, −20°C, and –40°C. The enzyme activity was assayed under the standard assay conditions on a weekly basis for one month and a monthly basis up to two months.

Analytical Procedure

Crude cellulase activity was assayed according to the standard method (Wood & Bhat, 1988). Carboxymethylcellulase (CMCase) activity was determined by estimating the reducing sugars produced from 1% (w/v) carboxymethylcellulose, whereas Filter Paperase (FPase) activity was determined by measuring the reducing sugars released from Whatman filter paper No.1. The liberated reducing sugars were measured using the DNS method (Miller, 1959). One unit of CMCase or FPase activity was defined as the amount of enzyme that liberated 1 µmol reducing sugars/min under assay conditions and expressed as a unit of enzyme activity per gram fermented dry substrate (U/g).

For FPase, substrate blank contained 2.0 mL of 50 mM sodium citrate buffer (pH 4.8) in the presence of Whatman No.1 filter paper as the substrate. Conversely, the enzyme blank contained 1.8 mL of 50 mM sodium citrate buffer (pH 4.8), 0.2 mL of the crude enzyme mixture in the absence of Whatman filter paper, whilst the test was with the presence of filter paper. The reactions were carried out for 1 h at 40ºC.

As for CMCase assay, 0.2 mL of the crude enzyme was added to 1.8 mL of 1% (w/v) carboxymethylcellulose in 50 mM sodium citrate buffer (pH 4.8), and incubated at 40ºC for 30 min. The enzyme blank contained 0.2 mL of the enzyme and 1.8 ml 50 mM pH 4.8 sodium citrate buffer only [without 1% (w/v) carboxymethylcellulose], while for the substrate blank, 0.2 ml sodium citrate buffer (pH 4.8) was used instead of the crude enzyme.

For β-glucosidase assay, the reaction mixture, which consisted of 0.2 mL crude enzyme added to 2.0 mL of 0.5 mM ρ-nitrophenyl-β-D- glucopyranoside in 50 mM sodium citrate buffer (pH 4.8) was incubated at 40°C for 30 min. The reaction

was stopped by the addition of 2.0 mL of 1 M sodium carbonate (Na_2CO_3) immediately after the incubation time (Wood & Bhat, 1988). One unit of β-glucosidase was defined as the amount of enzyme that liberated 1 μmol ρ-nitrophenol/min under assay conditions and expressed as a unit of enzyme activity per gram fermented dry substrate (U/g). The substrate blank contained 0.2 mL of 50 mM sodium citrate buffer (pH 4.8) instead of the crude enzyme, while enzyme blank was prepared by adding 0.2 mL of crude enzyme into the sodium citrate buffer only (i.e., without ρ-nitrophenyl-β-D- glucopyranoside).

The xylanase activity was assayed by estimating the reducing sugars released from 1% (w/v) Birchwood xylan (Dong et al., 1992). The reaction was carried out by adding 0.2 mL of the crude enzyme to 1.8 mL of 1% (w/v) Birchwood xylan in 50 mM sodium citrate buffer pH 4.8 and incubated at 40℃ for 30 min. As for the substrate blank, 0.2 mL of 50 mM sodium citrate buffer (pH 4.8) was added to replace the crude enzyme, while for the enzyme blank, the sodium citrate buffer (pH 4.8) did not contain any xylan. One unit of xylanase activity was defined as the amount of enzyme that liberated 1 μmol reducing sugars/min under assay conditions and expressed as a unit of enzyme activity per gram fermented dry substrate (U/g). Reducing sugars released as a result of the reaction of the enzyme (FPase, CMCase, and xylanase) and the substrate was determined by using DNS method.

For the soluble protein concentration analysis, a modified method (Lowry et al., 1951) was used with bovine serum albumin as a standard.

The impacts of temperature, pH, and storage on crude cellulase enzyme activity were expressed as relative activity that is a percentage of the maximum activity. Thermal stability of the crude cellulase was expressed as the residual activity that is a percentage of the original activity.

Statistical Analysis

The data were analysed by using one-way analysis of variance (ANOVA). Meanwhile, t Tests (LSD) was used to compare the difference of means among treatment groups. Differences of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Cellulase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275 as a Model Fungus

Cellulase production from local *T. harzianum* SNRS3 was compared to that by *A. niger* ATCC 6275 as a model fungus. Untreated rice straw was used as the fermentation substrate for both fungi. Figure 1 presents the production of FPase by both fungi studied over a period of 10 days. FPase production by *A. niger* and *T. harzianum* was not significantly different ($p > 0.05$) during the first 5 days of fermentation. However, on day 6 of fermentation, FPase production by *A. niger* was significantly higher ($p < 0.05$) than that by *T. harzianum* and the maximum activity of FPase was obtained at 7.06 U/g substrate and 6.25 U/g substrate using *A. niger* ATCC 6275 and *T. harzianum* SNRS3, respectively.

CMCase and β-glucosidase production profiles were also studied over a period of 10 days. A comparison between the CMCase and β-glucosidase production from rice straw by *T. harzianum* SNRS3 and *A. niger* ATCC 6275 is presented in Figure 2. The CMCase production by *A. niger* was significantly higher ($p<0.05$) than that by *T. harzianum* between days 1 and 3 of fermentation. However, on day 6, the CMCase obtained (111.31 U/g substrate) was significantly higher ($p<0.05$) than the maximum CMCase production (86.35 U/g substrate) when *A. niger* was used. β-glucosidase production in *T. harzianum* was the highest on days 7 and 8 of fermentation ($p<0.05$), with a yield of 173.18-173.71 U/g. However, the highest production of β-glucosidase by *A. niger* ATCC 6275 only gave an activity of 17.41 U/g (Day 7). *Trichoderma* sp. is normally associated with insufficient production of β-glucosidase to perform efficient hydrolysis, whereas *Aspergillus* sp. has been reported as the most efficient producer of β-glucosidase (Wen et al.,

2005). According to the results of this study, however, *T. harzianum* SNRS3 proved to be a better β-glucosidase producer than *A. niger* ATCC 6275.

In comparison, a previous study on SSF of rice straw has reported the production of FPase (480.48 U/g substrate), CMCase (363.72 U/g substrate), and β-glucosidase (16.37 U/g substrate) by *A. terreus* ATTC 74135, and the production of 7.85 and 11.73 U/g substrate of FPase and CMCase respectively, by *A. niger* (Jahromi et al., 2011). On the other hand, using *A. niger* ATCC 6275 and palm cake under SSF, 23.8 U/g substrate CMCase was produced (Prasertsan et al., 1997).

The xylanase production profile was studied over a period of 10 days. A comparison between the xylanase production from rice straw using *T. harzianum* SNRS3 and *A. niger* ATCC 6275 is presented in Figure 3. As demonstrated in Figure 3, the xylanase production was increased significantly ($p<0.05$) when *A. niger* ATCC 6275 was used as the inoculum for enzyme
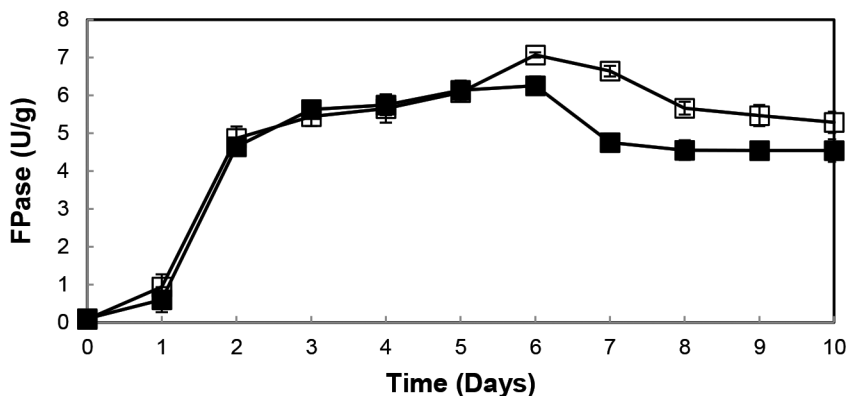

Figure 1. FPase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275
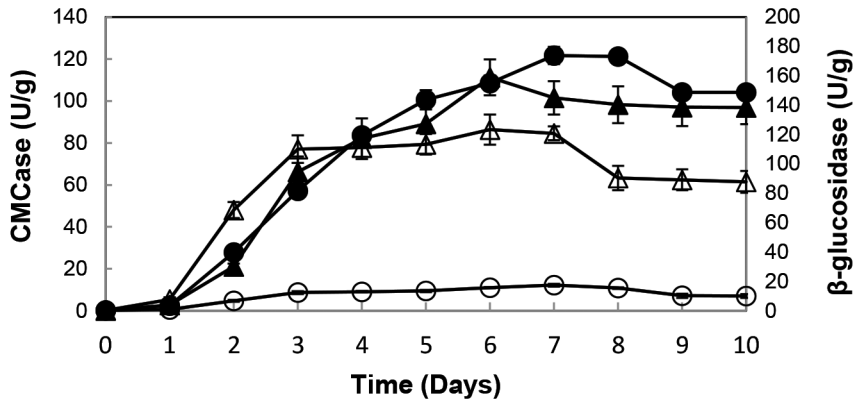
Figure 2. CMCase and β-glucosidase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Symbols represent: (▲) CMCase activity; (●) β-glucosidase activity. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275
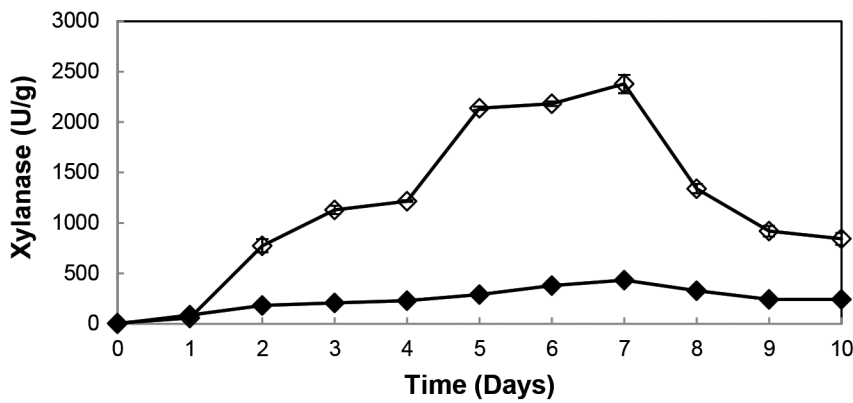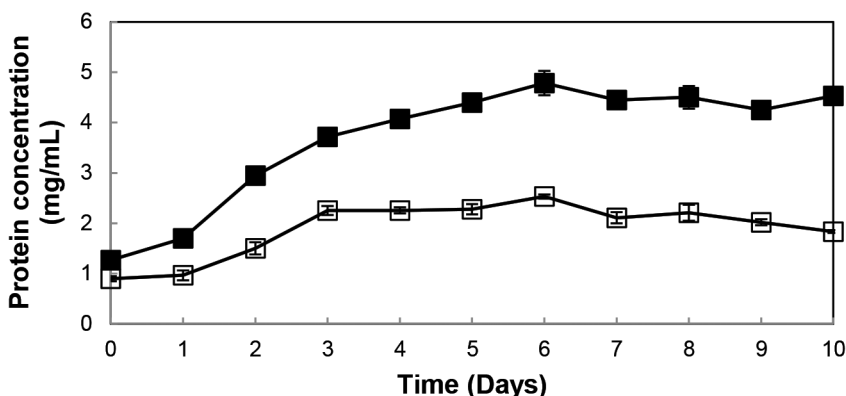

Figure 3. Xylanase from *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275

production. On day 7 of fermentation, xylanase was produced at the activity of 433.75 U/g substrate by *T. harzianum* SNRS3, as compared to 2378.64 U/g substrate obtained by *A. niger* ATCC 6275. Among the xylanolytic fungi, *Aspergillus* is known as an efficient and high xylanase producer (Fang et al., 2010). *A. terreus* ATCC 74135 was cultivated on untreated, ground rice straw under SSF and a very high

xylanase production of 6,166 U/g substrate was obtained. Table 1 shows cellulase and xylanase production by different fungi grown on various agricultural wastes under solid state fermentation.

The profiles of extracellular protein production by *T. harzianum* SNRS3 and *A. niger* ATCC 6275 grown on untreated rice straw studied over a period of 10 days were also monitored. Figure 4 indicates that

Figure 4. Extracellular protein profile of *T. harzianum* SNRS3 and *A. niger* ATCC 6275. Values are means of 3 replicates ± SD. Closed symbols represent: *T. harzianum* SNRS3; Open symbols represent: *A. niger* ATCC 6275

protein concentration increased significantly with time for *T. harzianum* (p<0.05), giving a higher protein concentration of 4.78 mg/mL on day 6 of fermentation compared to the maximum protein concentration of 2.43 mg/mL obtained on day 6 of fermentation for *A. niger*.

Characterisation of Crude Cellulase by *T. harzianum* SNRS3

Effect of Temperature on Crude Cellulase Activity

Figure 5 illustrates the effects of temperature on the activity of crude cellulase in a temperature range of 40-90°C. The temperature profile of the enzyme shows an optimal temperature of 50°C for FPase, CMCase, and β-glucosidase produced by *T. harzianum* SNRS3. The temperature profile of FPase illustrates an optimum temperature plateau ranging from 50-60°C. The same feature has been reported for FPase produced by *Penicillium notatum*

NCIM NO-923 (Das & Ghosh, 2009) and *Penicillium funiculosum* (Karboune et al., 2008) with an optimum temperature of 50°C and 60°C, respectively. It is worth noting that FPase produced from *T. harzianum* SNRS3 can remain almost up to 100% active at 60°C, and this characteristic could be considered as a major advantage.

Unlike FPase that exhibited almost a similar optimum temperature plateau between 50-60°C, CMCase showed a different trend and the relative activity dropped sharply between 50-60°C. Similar to CMCase produced by *T. harzianum* SNRS3, CMCase produced by *Penicillium notatum* NCIM NO-923 (Das & Ghosh, 2009) and *Streptomyces* transformant T3-1 (Jang & Chen, 2003) has been reported to be optimally active at 50°C. However, a lower optimal temperature of 40°C has been reported for endoglucanase produced by *Aspergillus niger* Z10 (Coral et al., 2002). The highest activities of CMCase from *Penicillium* sp. CR-316 have been reported

at 65°C (Picart et al., 2007). Whereas, CMCase from *T. aurantiacus* (Kalogeris et al., 2003a) and *Bacillus* sp. (Rastogi et al., 2010) have been reported to be optimally active at 75°C.

Similar to β-glucosidase from *T. harzianum* SNRS3, *Stachybotrys* sp β-glucosidase have also been reported to be optimally active at 50°C (Amouri & Gargouri, 2006). Interestingly, a closer look at β-glucosidase temperature profile revealed that at 60°C, β-glucosidase activity was still above 70% of its maximum activity. This is considered an advantage of the crude cellulase enzyme produced by local *T. harzianum* SNRS3. Its ability to retain high activity at 60°C, when other cellulases are inactivated, is an important characteristic for cellulases as industrial enzymes. Table 2 summarises the optimum temperature of the crude cellulase produced by *T. harzianum* SNRS3 and various other microorganisms.

Effects of pH on Crude Cellulase Activity

As illustrated in Figure 6, cellulolytic enzyme complex system produced by *T. harzianum* SNRS3 displayed cellobiohydrolase, endoglucanase, and β-glucosidase activities over a broad range of pH. This characteristic of the crude cellulase by *T. harzianum* SNRS3 is considered as an advantage for cellulases that are important industrial enzymes. However, the cellulose degrading enzymes are highly active in the acidic region (Table 2). Depending on the type of cellulase, the pH-activity profiles obtained were different. FPase retained above 50%

of its maximum activity in a broad pH range of 3.5-6.5, whereas CMCase retained almost above 70% of its maximum activity in the pH range of 3.5-7.0. However, β-glucosidase showed the highest activity at pH 5.0 and remained highly active in a narrow pH spectrum of 4.5-5.5.

As shown in Figure 6, the pH profile of the three components of the crude cellulase showed an increasing trend for the activity with the rise in pH value. In particular, the activity of FPase and CMCase increased sharply in the pH range between 2.5-4.0. For β-glucosidase, however, the activity increased drastically in the pH range between 3.5-4.50. Based on a relative activity of 100% for β-glucosidase at pH 5.0, the activity decreased to 12.2 %, and 8.1 % at pH3.5 and pH7, respectively.

Thermal Stability of Crude Cellulase

The results of thermostability of the crude cellulase produced by *T. harzianum* SNRS3 at 50 and 60°C are presented in Figure 7. As expected and can be observed, the higher the temperature, the higher the activity of the enzyme is likely to be lost. As shown in Figure 7, 30 min incubation of the crude enzyme at 50°C resulted in a reduction in the activity of FPase to around 70% of its original activity. However, incubation at 60°C for 30 min reduced the FPase activity to almost 40% of its initial activity. It is worth noting that increasing the incubation period up to 4 h did not further decrease the enzyme activity.

Unlike CMCase produced by *Penicillium notatum* NCIM NO-923 (Das & Ghosh, 2009) and *Penicillium citrinum* (Dutta et al., 2008) that proved to be more themostable at 50 and 60°C than FPase, CMCase produced by *T. harzianum* SNRS3 showed less thermal stability compared to FPase. The incubation of the crude cellulase at 50 and 60°C for 30 min caused the activity of CMCase to drop to almost 40%, and 30% of its original activity, respectively. At 50°C, however, and with the increase in the incubation time up to 4 h, no more reduction in the enzyme activity was detected. Consequently, in the incubation period range of 30 min to 4 h, a thermal stability plateau was observed. Meanwhile at 60°C, increasing the incubation time of the crude enzyme for up to 4 h steadily decreased the CMCase activity to less than 15% of its initial activity.

As depicted in Figure 7, β-glucosidase exhibited above 50% of its original activity after incubation at 50°C for 30 min and after that, any longer incubation of the enzyme up to 4 h did not have a significant effect on the residual activity of the enzyme. However, the enzyme was found to be less stable at 60°C and after 2-h incubation, and that almost all activities were lost probably due to the enzyme denaturation. Table 2 provides a comparison between thermostability of the crude cellulase produced by *T. harzianum* SNRS3 and that of cellulase by various microorganisms.

Effects of Storage on Crude Cellulase Activity

Results of the effects of storage on the activity of crude cellulase enzyme are depicted in Figures 8.A, 8.B, and 8.C.

Regardless of the storage temperature of the crude enzyme, the FPase activity was almost stable within the first week (Figure 8.A), and only at room temperature, a slight decrease occurred in the FPase activity from 100% up to 94%. Meanwhile, the activity of FPase for the crude samples kept at 4°C, − 20°C, and −40°C almost remained stable within the 2nd week, but the activity dropped to 77% at room temperature. After weeks 3 and 4 at 4°C, −20°C, and −40°C, the activity of FPase slightly decreased. However, at room temperature, the FPase activity of the crude enzyme decreased drastically at the end of week 4 and reached 27% of its original activity. Interestingly, the storage of crude enzyme at room temperature showed that FPase activity was still almost 60% of its original activity at the end of week 3. The FPase activity was still above 85% of its original activity after keeping the crude enzyme for 3 weeks at 4°C, −20°C, and −40°C.

As shown in Figure 8.B, CMCase proved to be more sensitive to storage temperature compared to FPase. At room temperature, the CMCase activity dropped sharply from 71% after week 1 to merely 25% and 7% at the end of weeks 3 and 4, respectively. However, CMCase retained 60% of its original activity at the end of the 2nd week of storage at room temperature. The effect of storage of the crude enzyme at

Table 1

Cellulase and Xylanase Production by Different Fungi Grown on Various Agricultural Wastes under Solid State Fermentation

| Enzyme source	Carbon source	Enzyme activities (U/g dry substrate)				References
		FPase	CMCase	β-glucosidase	Xylanase	
Aspergillus niger ATCC 6275	Palm cake	_	23.8	_	282.9	(Prasertsan et al., 1997)
Aspergillus niger KK2	Rice straw	19.5	129	100	5070	(Kang et al., 2004)
Aspergillus terreus M11	Corn stover	243	581	128	_	(Gao et al., 2008)
Aspergillus terreus MTCC 8661	Palm oil fiber	_	_	_	115,269	(Suvarna Lakshmi et al., 2009)
Aspergillus terreus ATCC74135	Rice straw	480.48	363.72	16.37	6,166.01	(Jahromi et al., 2011)
Aspergillus ustus	Wheat bran	3.78	11.84	60.00	615.26	(Shamala &Sreekantiah, 1986)
Aspergillus ustus	Rice straw	5.82	12.58	15.82	740	(Shamala & Sreekantiah, 1986)
Myceliophthora sp. IMI 387099	Corn cob	0.31	11.38	5. 49	411.6	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Bagasse	0.70	6.62	2.01	620.1	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Wheat bran	0.74	26.6	3.83	128.9	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Wheat straw	1.37	30.8	6.78	656.6	(Badhan et al., 2007)
Myceliophthora sp. IMI 387099	Rice straw	2.44	32.9	7.48	900.2	(Badhan et al., 2007)
Thermoascus aurantiacus	Wheat straw	4.3	956	46.1	2973	(Kalogeris et al., 1999)
Trichoderma aurantiacus	Wheat straw	_	1572	101.6	_	(Kalogeris et al., 2003a)
Trichoderma aurantiacus	Wheat straw	5.5	1709	79	4490	(Kalogeris et al., 2003b)
Trichoderma koningi F244	Wheat bran	94	287.3	184	_	(Li et al., 2004)
Trichoderma longibrachiatum	Wheat bran and wheat straw	_	_	_	592.7	(Azin et al., 2007)
Trichoderma reesei MCG77	Rice bran	2.314	_	_	_	(Latifian et al., 2007)
Trichoderma reesei ZU-02	Corncob	158	_	_	_	(Xia & Cen, 1999)
Aspergillus niger ATCC 6275	Rice straw	7.06	86.35	17.41	2378.64	Present study
Trichoderma harzianum SNRS3	Rice straw	6.25	111.31	173.71	433.75	Present study
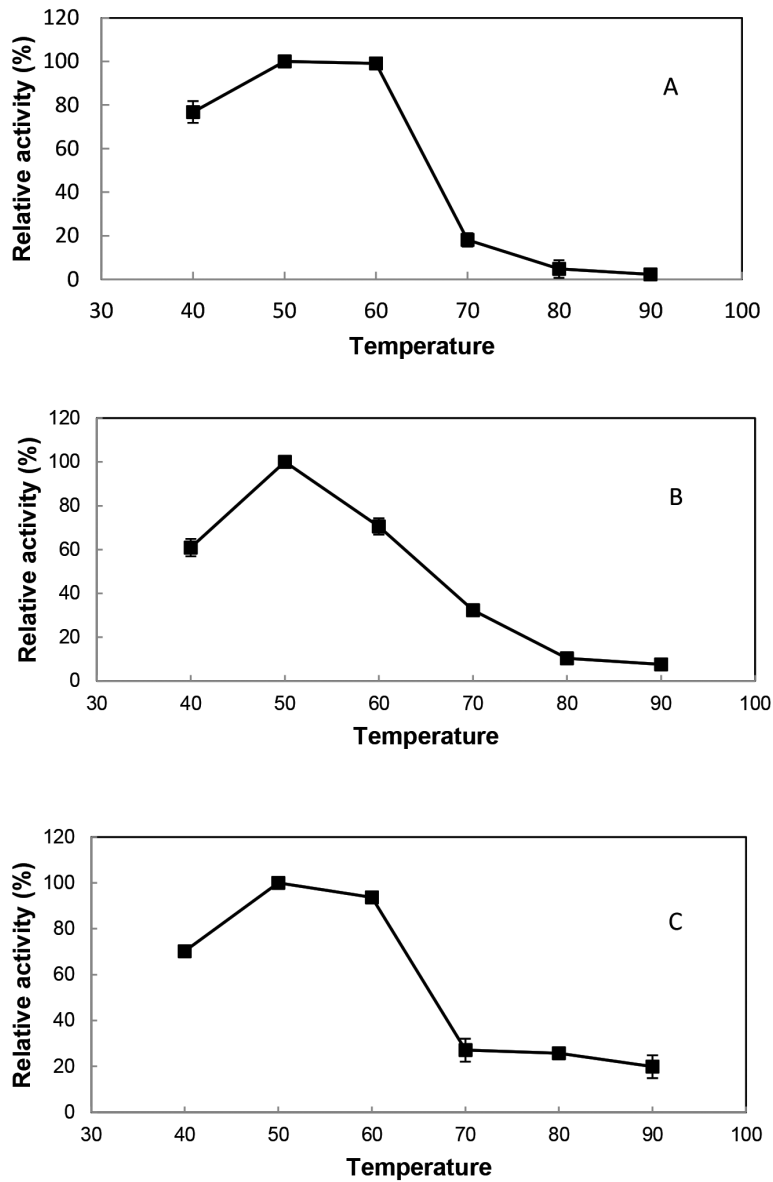
Figure 5. The effects of temperature on the activity of crude cellulase by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ±SD. A: FPase, B: CMCase, C: β-glucosidase
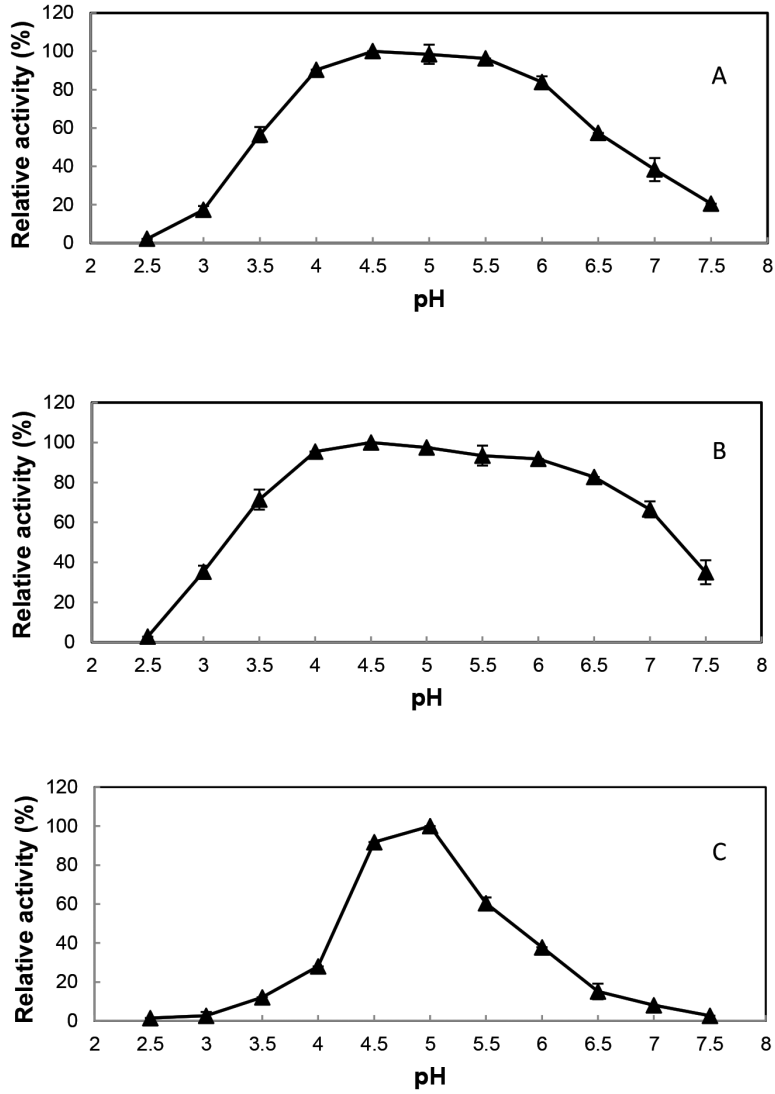
Figure 6. The effects of pH on the activity of crude cellulase by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ± SD. A: FPase B: CMCase C: β-glucosidase
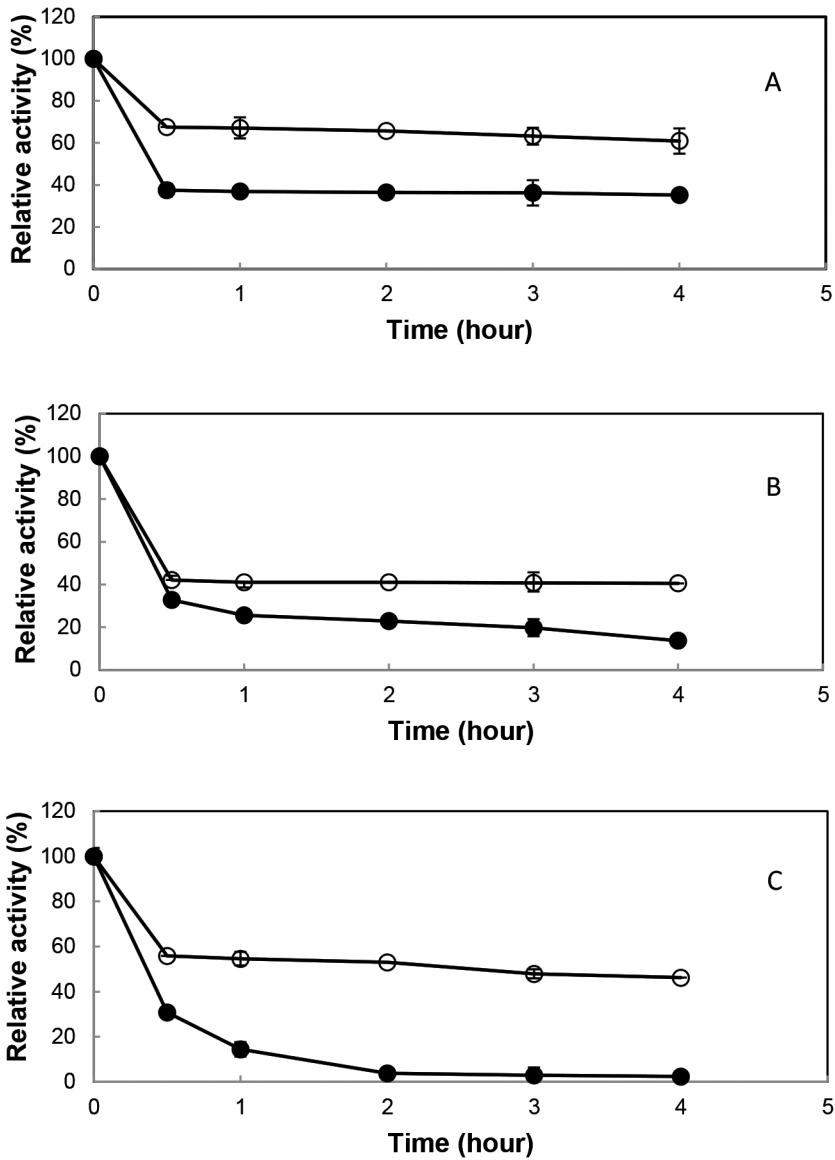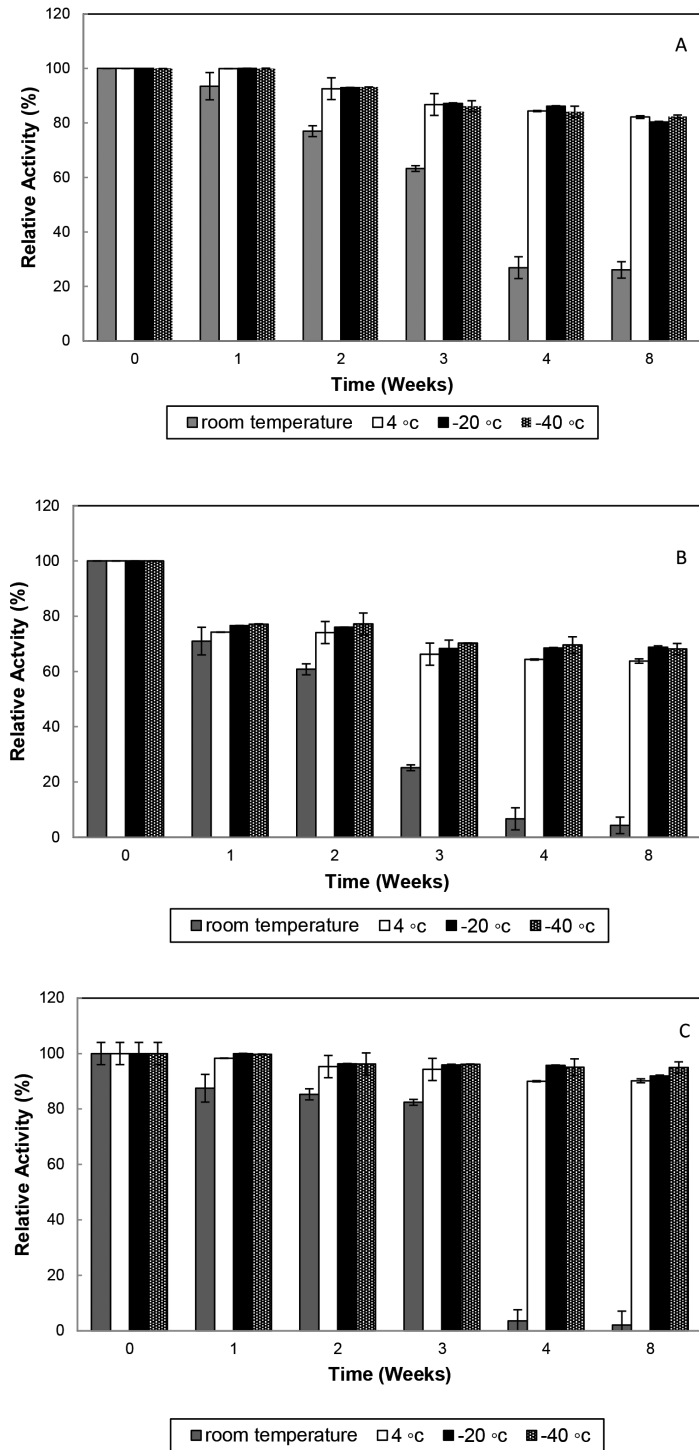
Figure 7. Thermostability of FPase (A), CMCase (B), and β-glucosidase (C) by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ± SD. Closed symbols represent: Residual activity at 60°C; Open symbols represent: Residual activity at 50°C

Figure 8. The effects of storage on the activity of crude cellulase by *T. harzianum* SNRS3 from rice straw in SSF. Values are means of 3 replicates ± SD. A: FPase B: CMCase C: β-glucosidase

Table 2
Optimum Temperature, pH, and Thermostability of Cellulase Produced by Various Microorganisms

Microorganism	Enzyme	Temperature optimum (°C)	pH optimum	Thermostability	References
Penicillium citrinum	FPase	-	6.5	less thermostable than CMCase	(Dutta et al., 2008)
Penicillium funiculosum	FPase	60	4.0-5.0	thermostable at up to 55°C	(Karboune et al., 2008)
Penicillium notatum NCIM NO-923	FPase	50	4.0	stable at 40°C; at 50 and 60°C FPase less thermostable	(Das & Ghosh, 2009)
Alternaria alternate	CMCase	55-60	5.0-6.0	-	(Macris, 1984)
Aspergillus fumigatus	CMCase	64.3	-	-	(Saqib et al., 2010)
Aspergillus niger Z10	CMCase	40	4.5 and 7.5	above 40% of activity at 90°C after 15 min	(Coral et al., 2002)
Aspergillus terreus M11	CMCase	70	2.0	highly stable; retained 65% of activity after 6 h	(Gao et al., 2008)
Bacillus sp.	CMCase	75	5.0	Stable at 50°C after 1 day; 97% activity remained at 60°C after 1 day	(Rastogi et al., 2010)
Ceriporiopsis subvermispora	CMCase	60	3.5-5.0	rapid loss of activity at 40 and 50°C	(Heidorne et al., 2006)
Geobacillus sp.	CMCase	70	5.0	100% active at 50 and 60°C after 1 day; 7% loss of activity at 60°C after 2 days	(Rastogi et al., 2010)
Penicillium sp. CR-316	CMCase	65	4.5	stable at 60°C after 3 h; lost 75% of activity at 65°C after 1 h	(Picart et al., 2007)
Penicillium citrinum	CMCase	-	5.5 and 8	stable at 50-70°C after incubation for 2 h	)Dutta et al., 2008)
Penicillium funiculosum	CMCase	60	4.0	thermostable at up to 55°C	(Karboune et al., 2008)
Penicillium notatum NCIM NO-923	CMCase	50	4.0	stable at 40°C	(Das & Ghosh, 2009)
Streptomyces drozdowiczii	CMCase	50-60	5.0	stable at 50°C after 1 h; 40% of activity remained after 2 h; 20% of activity remained after 8 h	(Grigorevski de Lima et al., 2005)
Streptomyces transformant T3-1	CMCase	50	7.0-8.0	stable at 50°C after 5 days; 98% of activity remained at 50°C after 7 days; half-life 15 h at 60°C ; half-life 5 h at 70°C	(Jang & Chen, 2003)

Pertanika J. Trop. Agric. Sci. 39 (1): 515 - 539 (2016)

531

cont'd Table 2

Organism	Enzyme	Temp	pH	Stability	Reference
T. aurantiacus	CMCase	75	3.5 and 4	half-life 42 min at 80°C; half-life 1 day at 70°C	(Kalogeris et al., 2003a)
Alternaria alternata	β-glucosidase	70-75	4.5-5.0	Half-life 3.5 days at 60°C, 1.8 h at 65°C, and 10 min at 70°C	(Macris, 1984)
Aspergillus niger	β-glucosidase	55	4.5	-	(Watanabe et al., 1992)
Aspergillus pullulans	β-glucosidase	75	-	-	(Saha et al., 1994)
Aspergillus terreus M11	β-glucosidase	70	3.0	highly thermo stable; retained 53% of original activity after 6 h	(Gao et al., 2008)
Aureobasidium sp.	β-glucosidase	80	4.0	stable at 80°C for 15 min	(Hayashi et al.,1993)
Aureobasidium pullulans	β-glucosidase	80	4-4.5	retained 98% of activity after 1 h incubation at 75°C	(Leite et al., 2007)
Ceriporiopsis subvermispora	β-glucosidase	60	3.5-5.0	rapid loss of activity at 40 and 50°C	(Heidorne et al., 2006)
Penicillium decumbens	β-glucosidase	65-70	4.5-5.0	96% of activity remained at 50°C after12 h; 50% of activity remained at 70°C after 4 h	(Chen et al., 2010)
Penicillium funiculosum	β-glucosidase	60	4.5	stable at 25-40	(Karboune et al., 2008)
Stachybotrys sp.	β-glucosidase	50	5.0	-	(Amouri & Gargouri, 2006)
Thermoascus aurantiacus	β-glucosidase	70	4.5	retained 98% of activity after 1 h at 70°C	(Leite et al., 2007)
T. aurantiacus	β-glucosidase	80	4.5	half-life 18 min at 80°C; half-life 2.5 days at 70°C	(Kalogeris et al., 2003a)
Aspergillus niger	Crude cellulase	35-60	4.0-5.5	-	(Farinas et al., 2010)

cont'd Table 2

Aspergillus niger ATCC-16404	Crude cellulase	54-57	5.0-5.5	-	(Machado de Castro et al., 2010)
Trichoderma harzianum IOC-4038	Crude cellulase	47- 54	4.9-5.2	rapid thermal denaturation at 60°C corresponding to half-life < 1 h	(Machado de Castro et al., 2010)
Trichoderma reesei RutC30	Crude cellulase	52-57	5.1-5.3	-	(Machado de Castro et al., 2010)
T. harzianum SNRS3	FPase	50	4.5-5.5	At 50°C after 30 min and up to 4 h, 70% of activity remained, at 60°C after 30 min and up to 4 h, 40% of activity remained	Present study
	CMCase	50	4.0-5.0	At 50°C after 30 min up to 4 h, 40% of activity remained; at 60°C after 30 min 30% of activity remained and after 4 h, < than 15% of activity remained	
	β-glucosidase	50	5.0	At 50°C after 30 min up to 4 h, > 50% of activity remained; inactivated after 2h at 60°C	

4°C, −20°C, and −40°C was almost similar. After week 1, the activity first decreased to almost above 75% for the three samples kept at 4°C, −20°C, and −40°C. It is worth noting that storage at 4°C for 1 month caused the CMCase activity to drop to almost 65% of its initial activity.

Similarly, the β-glucosidase activity was also affected by storage temperature and duration (Figure 8C). Storing the crude enzyme at room temperature caused the activity to drop sharply from 82% at the end of week 3 to 4% at the end of week 4. However, the β-glucosidase activity was only slightly affected when the enzyme was kept at 4°C, −20°C, and −40°C for one month. Like FPase, β- glucosidase was also proven to be almost stable for the first three weeks of storage at room temperature. The β- glucosidase activity only decreased to above 80% of its original activity at the end of the 3rd week of storage at room temperature.

It is worth noting that after keeping the crude cellulase for 2 months at 4ºC, −20ºC and −40ºC, CMCase retained almost above 60% of its original activity. However, FPase and β- glucosidase remained active, i.e. above 80% and 90% of their original activity, respectively.

CONCLUSION

Unlike *Trichoderma* sp. that are normally associated with the production of low amount of β-glucosidase for an efficient hydrolysis, *T. harzianum* SNRS3 was shown to be a potential β-glucosidase producer. Meanwhile, *T. harzianum* SNRS3 produced β-glucosidase at a much higher activity (173.71 U/g substrate) compared to 17.41 U/g substrate β-glucosidase by *A. niger* ATCC 6275, belonging to a genus reported as the most efficient producer of β-glucosidase. However, *T. harzianum* SNRS3 was not an efficient xylanase producer (433.75 U/g substrate) compared to *A. niger* ATCC 6275 (2378.64 U/g substrate). FPase showed an optimum temperature plateau in the temperature range of 50 to 60ºC, indicating that this enzyme can remain active almost up to 100% at 60ºC. At 60ºC, the β-glucosidase activity was still above 70% of its maximum activity. This is a significant characteristic of cellulases with a wide range of industrial applications. Cellulose degrading enzymes were highly active in the acidic region and could be mostly applied over a wide range of acidic pH.

ACKNOWLEDGEMENTS

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Pertanika J. Trop. Agric. Sci. 39 (1): 515 - 539 (2016)

537

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Effect of Higher Density Planting on Irrigation Water Use and Yield of Sugarcane

D. Khodadadi Dehkordi

Department of Water Engineering and Sciences, Ahvaz Branch, Islamic Azad University, Ahvaz, Iran

ABSTRACT

Sugarcane is one of the important crops that needs plentiful water in tropical and semi-tropical regions. This crop is widely grown in Khuzestan province of Iran. In this study, triple-row planting of sugarcane was evaluated and compared to double-row planting as currently being practiced in the region. Results showed that in spite of the higher density planting in triple-row planting, there was no significant difference between triple-row planting and double-row planting in terms of leaf sheath moisture, leaf nitrogen, ridge EC, qualitative situations of cultivar, plant height and stem diameter. However, the number of shoots and stems per m^2 in the triple-row planting were found to be higher than in the double-row planting and the difference between them was significant at 5% level. In addition, water application efficiency, water use efficiency (WUE), mean yield of sugarcane and sugar content in triple-row planting were higher than double-row planting.

Keywords: Sugarcane, triple-row planting, double-row planting

INTRODUCTION

Sugarcane is one of the most important crops in Southwest Iran. Sugarcane is a C_4, a high biomass crop which requires large amount of water for maximum production. About 60 percent of the world's sugar content is supplied from sugarcane production and the rest is produced from sugar beet. The favourable temperature for growing is between 30-34°C and the favourable relative humidity is between 60-80%. These conditions are available in Khuzestan province that is located in southwest Iran. The current production of sugarcane in Iran is about 7 million t year^{-1} and the average of sugarcane yield is about 70 t ha^{-1} and cultivated area is about 100000 ha (Anon, 2011). Van der Merwe et al. (2003) reported that the water use efficiency of sugarcane

in alternate furrow irrigation method was better than other irrigation methods with sugar yield of 14.5 t ha⁻¹ obtained from that method. Meanwhile, Sheini-Dashtgol (2007) reported that alternate furrow irrigation method consumed less water than current irrigation methods. Noori et al. (2008) reported that by decreasing furrow depth, water application efficiency of sugarcane increased. Bull and Bull (2000) reported that a method for increasing sugarcane production was higher density planting, in the form of double-row planting. Seeruttun and Ismael (2003) and Ismael et al. (2007) showed that sugarcane production increased with double-row planting. Songsri et al. (2009) reported that water application efficiency of sugarcane can be increased by having an extended root system in drought stress conditions. Smith et al. (2005) reported that sugarcane cultivars that have extended root systems, are more resistant to drought stress. There is a contradiction regarding the effect of row spacing and seeding densities on the quality parameters such as Brix, sucrose contents, juice extraction and commercial cane sugar, etc. (Sharar et al., 2000; Asokan et al., 2005; El-Geddawy et al., 2005; Pawar et al., 2005). Nevertheless, most of the studies agreed that sugarcane quality was not affected by row spacing and seeding densities (Asokan et al., 2005; El-Geddawy et al., 2005). In contrast, Pawar et al. (2005) reported that wider row spacing improved the sucrose contents and commercial cane sugar percentage. Similarly, according to Sharar et al. (2000), higher seeding density (100000 setts ha⁻¹)

improved sucrose content and commercial cane sugar compared to seeding density of 75000 setts ha⁻¹. Patel et al. (2005) reported that higher seeding densities increased the commercial cane sugar. The current cultivation of sugarcane in Iran is in the form of double-row planting by hilling up activities. Thus, this research was conducted to investigate the effects of planting density on variation of water application efficiency, water use efficiency and crop yield. The purposes of this study were to introduce the triple-row planting of sugarcane and compare its advantages with those of the double-row planting in Iran.

MATERIALS AND METHODS

Geographical Location and Weather Characteristics

This study was conducted in farms located in the tropical climate of northwest Shush County in the Khuzestan province of Iran. Two different farms (labelled as A-01 farm and B-01 farm, respectively) were selected for the purpose of this experiment. Triple-row planting was done in A-01 farm and double-row planting was done in B-01 farm. The irrigation water of the farms is from Karkheh River. This research was carried out from October 2014 to October 2015. Some weather parameters and Karkheh River EC are shown in Table 1.

Soil Characteristics and Sugarcane Variety

Composite samples of 5 random points were taken from 0-30, 30-60, 60-100, 100-150 and 150-200 cm depths of the cultivated

Table 1
Some weather parameters and Karkheh River EC

Month (2014-2015)	Mean of min temperature (^{o}C)	Mean of max temperature (^{o}C)	Mean of temperature (^{o}C)	Mean of min humidity (%)	Mean of max humidity (%)	Sum of rainfall (mm)	Sum of sunshine hours	EC of Karkheh River (dS m^{-1})
October	20.0	39.1	29.8	17.7	58.3	0	287.60	1.69
November	14.4	28.1	20.3	37.1	75.7	25.8	178.60	1.37
December	5.2	20.9	13.1	35.5	81.2	0	260.36	1.40
January	6.4	20.9	13.4	43.6	83.5	12.5	178.60	1.39
February	6.1	19.9	13.1	40.4	78.2	28.4	174.27	1.46
March	9.0	23.1	16.0	30.3	70.1	29	210.10	1.78
April	15.3	31.1	23.2	33.1	80.0	5.5	215.07	1.67
May	21.7	39.8	29.9	28.7	65.0	2.5	246.78	1.72
June	24.8	45.2	35.0	19.0	49.0	0	300.38	2.18
July	26.6	47.0	36.7	20.6	52.6	0	356.47	2.57
August	27.4	48.6	38.0	21.5	54.8	0	352.20	2.68
September	23.3	44.4	33.7	24.3	68.1	0	332.60	2.51
October	19.2	39.4	29.1	33.6	76.3	0	278.45	2.49

Table 2
Some physical and chemical properties of the soil before planting test

Depth (cm)	Texture	EC (dS m^{-1})	pH	Hco$_3^-$ (meq l^{-1})	CL$^-$ (meq l^{-1})	Mg^{2+} (meq l^{-1})	Ca^{2+} (meq l^{-1})	OM (%)
0-30	Silt Loam	2.00	8.27	0.10	1.4	13.6	13.5	0.51
30-60	Silt Loam	2.68	8.11	0.05	3.2	22.5	16.1	0.41
60-100	Silt Loam	2.74	8.10	0.06	7.5	21.1	17.3	0.25
100-150	Silt Loam	2.86	8.00	0.00	8.1	16.4	21.2	0.31
150-200	Silt Loam	3.03	8.04	0.05	7.6	17.1	21.2	0.31

land in the farms. The results are presented in Table 2. The sugarcane variety used in this study was CP69-1062. It is a middle-mature variety tolerant to drought stress and suitable for cultivation in subtropical regions. This variety is sensitive to cold weather with acceptable yield and sugar content.

Farming operations

A special furrow maker was used to create three small furrows for the sugarcane cuttings (see Figure 1). Space between the rows in this step was 50 cm, whereas the depth of small furrows was 14 cm; the width of ridge in the triple-row planting was 130 cm, the width of ridge in the double-row planting was 90 cm and the length of the furrows was 50 m (see Figures 3 and 4). After the furrower activities, cuttings of sugarcane were put in small furrows (Figure 2). Then, covering activities were done by using special covering apparatus.

Meanwhile, irrigation was done based on soil moisture measurement at FC, PWP and readily available moisture by sampling of root zone soil. Fertilisers were given according to the current fertilizer schedules of the sugarcane farms. A-01 farm (triple-row planting) and B-01 farm (double-row planting) were cultivated simultaneously and all the cultivation activities were equal for both of farms. Moreover, the measurements of ridge soil EC, leaf nitrogen and leaf sheath moisture content were simultaneously done for both the farms. The number of cuttings in the triple-row planting was about 23000 per ha and this was about 15000 per ha in the double-row planting.

Besides, in A-01 farm (triple-row planting), three furrows with three replications were evaluated, and generally, nine furrows including three groups of triplex furrows were therefore evaluated. These conditions were repeated exactly for B-01 farm (double-row planting). The comparison between two cultivations was according to the average data of nine furrows in every farm. The means comparison was done according to Duncan's multiple range test (P < 0.05).

RESULTS AND DISCUSSION

Evaluation of Leaf Sheath Moisture and Leaf Nitrogen

Leaf sheath moisture content for both farms was found to tbe equal and there was no significant difference between them (Table 3). In spite of the higher density planting in the triple-row planting, the leaf sheath moisture content was equal to that of the double-row planting.

Table 3
Percent of leaf sheath moisture of Sugarcane in triple-row planting and double-row planting

| Date | Leaf sheath moisture | |
	Double-row planting	Triple-row planting
2015/27/03	81.01	80.21
2015/10/04	80.31	80.34
2015/24/04	81.02	79.85
2015/06/05	79.75	79.52
2015/20/05	80.42	79.01
2015/11/06	78.15	78.46
2015/24/06	78.84	78.03

Figure 1. Triple-row furrower

Figure 2. The sugarcane cuttings in triple-row planting

Figure 3. The width of ridge in triple-row planting

Figure 4. The width of ridge in double-row planting

Leaf nitrogen in both farms was found to be equal and there was no significant difference between them (Table 4). In spite of the higher density planting in triple-row planting, the leaf nitrogen was nearly equivalent to that of the double-row planting. Thus, it was not necessary to apply more nitrogen fertiliser for the triple-row planting (higher density planting) compared with the double-row planting.

Table 4
Percent of leaf nitrogen in triple-row planting and double-row planting

| Date | Leaf nitrogen (%) | |
	Double-row planting	Triple-row planting
2015/27/03	2.05	2.19
2015/10/04	2.22	2.00
2015/24/04	2.03	1.94
2015/06/05	2.02	1.75
2015/20/05	2.08	1.78
2015/11/06	1.61	1.56
2015/24/06	1.52	1.50

Evaluation of the Ridge Soil EC

The soil EC of the ridge for both the farms was nearly equal and there was no significant difference between them. The soil EC of the ridge for the double-row planting was 4.1 dS m^{-1} and this was 4.03 dS m^{-1} for the triple-row planting. Thus, despite the wider ridge which allows more salt to enter in the triple-row planting compared to that of the double-row planting, the soil EC of the ridge for the triple-row planting was nearly equal with that of the double-row planting. This might be due to the less height of ridge in the triple-row planting (14 cm) in comparison with the height of ridge in the double-row planting (25 cm). In other words, the lesser height of ridge in the triple-row planting caused better entrance of water in the ridge and reduced ridge soil EC.

Evaluation of Qualitative Parameters of CP69-1062 Cultivar

As shown in Table 5, there was no significant difference between the qualitative parameters of CP69-1062 cultivar in both farms (Table 5). Therefore, sugarcane can be cultivated in the farm with higher density although its quality parameters can be stable.

Evaluation of Plant Height and Stem Diameter

The height of plant for both farms was observed to be nearly equal and there was no significant difference between them, whereas the tallest plant was relevant to the double-row planting (313 cm) and the shortest was relevant to the triple-row planting (303.8 cm) numerically. Hence, in the triple-row planting with higher density planting, the height of plant was not significantly shorter than that of the double-row planting (see Figure 5).

Similarly, the diameter of sugarcane stem for both the farms was nearly equal, and there was no significant difference between them. The largest diameter (2.28 cm) was associated with the double-row planting and the smallest diameter (2.07 cm) was noted for the triple-row planting numerically. Thus, in higher density planting, the stem diameter was not significantly smaller than that of the double-row planting.

Table 5
Qualitative parameters of CP69-1062 cultivar in triple-row planting and double-row planting

Farm	Pol (%)	Brix (%)	Purity (%)	Purity Coefficient	Quality Ratio	Refined Sugar (%)
Triple-row planting	16.50	18.96	90.25	130.45	7.66	10.36
Double-row planting	16.55	18.68	90.26	130.46	7.64	10.39

Pol: The juice sucrose percent or Pol percent is the actual cane sugar present in the juice.
Brix: Juice Brix refers to the total solids content present in the juice expressed in percentage.
Purity coefficient: It refers to the percentage of sucrose present in the total solids content in the juice.
Purity Percentage = (Pol Brix^{-1}) 100

Number of Shoots and Stems

The difference between the number of shoots per m^2 in the triple-row planting in comparison with double-row planting was significant at 5% level. The number of shoots in the triple-row planting was 385.83, while the number of shoots in the double-row planting was 334.15. Hence, the number of shoots in the triple-row planting was higher than the number of shoots in the double-row planting because of higher number of cuttings.

Meanwhile, the difference in the number of stems per m^2 in the triple-row planting in comparison with the double-row planting was significant at 5% level. The number of stems per m^2 in the triple-row planting was 265.42, while the number of stems per m^2 in the double-row planting was 208.25. Hence, the number of stems per m^2 in the triple-row planting was higher than the number of stems per m^2 in double-row planting because of bigger number of cuttings and also the higher number of shoots in the unit area.

Moreover, only strong stems were included in the counts. Therefore, the number of stems was less than the number of shoots.

Evaluation of Water Application Efficiency and Water use Efficiency (WUE)

The mean of water application efficiency in the triple-row planting was about 49.6%, which was higher than the water application efficiency of 45% in the double-row planting. The lower down-slope of A-01 farm (triple-row planting) in comparison with B-01 farm (double-row planting) had probably caused more water application efficiency in the triple-row planting. Figure 6 shows the variation of water application efficiency in the period of 4 months.

Water use efficiency in the triple-row planting was more than WUE in double-row planting (Table 6). This is because less water was consumed and more sugarcane and sugar yield were produced in the triple-row planting compared to that of the double-row planting.

Table 6
Water use efficiency (WUE) in triple-row planting and double-row planting

Farm	The volume of consumed water (m³ ha⁻¹)	The mean of produced Sugarcane (t ha⁻¹)	The mean of produced cane sugar (t ha⁻¹)	WUE of produced Sugarcane (kg m⁻³)	WUE of produced cane sugar (kg m⁻³)
Triple- row planting	26623.53	138.45	14.05	5.20	0.528
Double-row planting	29673.46	119	12.55	4.01	0.423
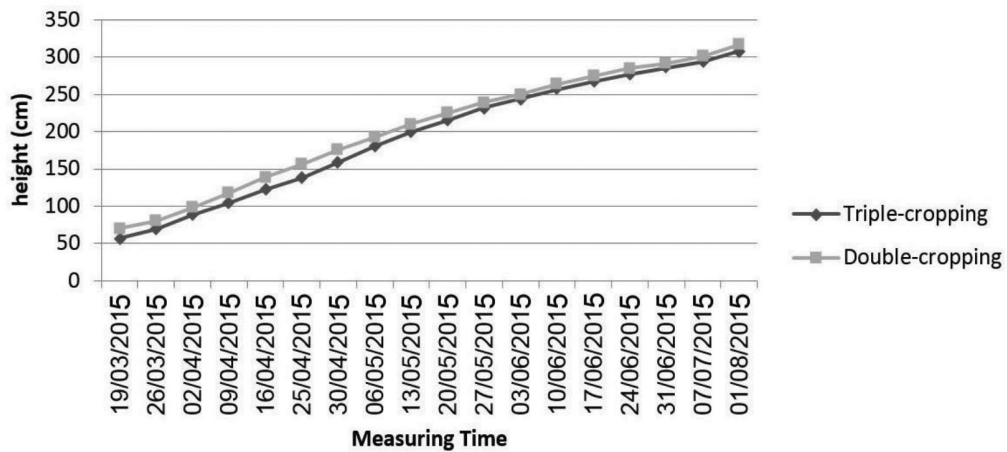
Figure 5. The height of sugarcane in both farms
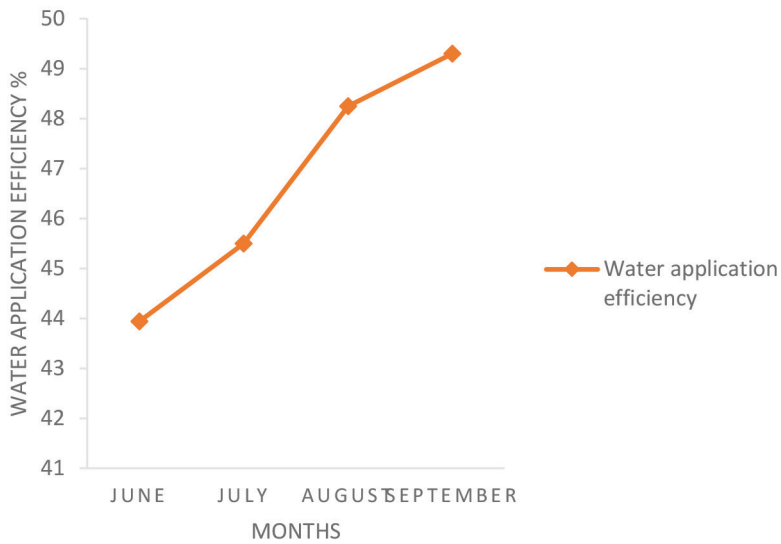
Figure 6. Water application efficiency in triple-row planting

EVALUATION OF THE MEAN YIELD OF SUGARCANE AND SUGAR CONTENT

The mean yields of sugarcane and sugar content in triple-row planting were 138.45 t ha^{-1} and 14.05 t ha^{-1}, respectively. These were numerically higher than the mean yields of sugarcane and sugar content in the double-row planting of 119 t ha^{-1} and 12.55 t ha^{-1}, respectively, although the difference between them was not significant. The reason was that the higher number of shoots due to the increase in the number of cuttings in the triple-row planting compared to that of the double-row planting. It is important to explain that in the end of growing season of sugarcane, all the nine furrows in every farm were harvested and the mean weight of stems was considered as the sugarcane yield. Then, the harvested stems were sent to laboratory to determine the qualitative and quantitative characteristics of sugarcane. After extracting stems by using press apparatus, the qualitative characteristics of sugarcane were identified by Saccharimeter and Polarimeter, followed by determining the other characteristics.

CONCLUSION

The research work of the current study showed that in spite of higher density planting, there was no significant difference between the triple-row planting and double-row planting on the ridge in terms of leaf sheath moisture, leaf nitrogen, ridge soil EC, quality parameters of the sugarcane cultivar, plant height and stem diameter. However, the difference in the number of shoots and number of stems per m^2 in the triple-row planting than the double-row planting was significant at 5% level. The numbers of shoots and stems per m^2 in the triple-row planting were higher because of the increased numbers of cuttings and in the unit area that were higher than the numbers of shoots and stems per m^2 in the double-row planting. Besides, water application efficiency, water use efficiency (WUE) and the mean yield of sugarcane and sugar in the triple-row planting were also higher than those of the double-row planting, although the difference between them was not significant. Actually, we can use yield data from the triple-row planting to increase the mean yield of sugarcane and sugar in spite of higher density planting in comparison to the double-row planting and also increase water application efficiency and water use efficiency of sugarcane and cultivate more lands in arid and semi-arid regions with lower water storage requirement.

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PERTANIKA JOURNALS

Effects of Feeding Different Levels of Low Crude Protein Diets with Different Levels of Amino Acids Supplementation on Layer Hen Performance

M. Tenesa[1], T. C. Loh[1,4], H. L. Foo[2,3]*, A. A. Samsudin[1], R. Mohamad[2] and A. R. Raha[2]

[1]Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Institute of Biological Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4]Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

The use of synthetic amino acids (methionine, lysine, threonine) makes the formulation of diets with minimum levels of crude protein possible. The objective of this study was to determine the effects of low crude protein diet with amino acids supplementation on the performance, egg production, small intestine villus height and faecal LAB and ENT count in laying hens. A total of 144 16-week old layer hens of Hisex brown were assigned to four dietary treatments: 17.5% CP (control); (ii) 17.5% CP; (iii) 17% CP; and (iv) 16.5% CP supplemented with amino acids. Treatment group supplemented 17% CP was significantly higher ($P<0.05$) in egg production than other groups. Nonetheless, there were no significant differences ($P>0.05$) in egg quality. Results showed significant differences ($P<0.05$) in both villi height and crypt depth in duodenum, jejunum and ileum. The treatment supplemented with 17% CP showed a significant reduction ($P<0.05$) in faecal pH compared to the other groups. There was a significant different ($P<0.05$) in LAB and ENT count and LAB/ENT ratio among the dietary treatments. In conclusion, 17% CP treatment supplemented with amino acids demonstrated the best effects in improving the hens' egg production, small intestine villus height and even promoting beneficial effects of faecal microflora.

Keywords: Methionine, lysine, egg production, egg quality, microflora, morphology

INTRODUCTION

Inclusion of synthetic amino acid in poultry rations is becoming very common in poultry industry as chickens can only utilise approximately 40% of the dietary protein (NRC, 1994; Saki et al., 2012) and it has been proven that decreasing crude protein level in the diet has many advantages. However, commercial amino acid must be supplemented to meet the requirements of limiting amino acid as the dietary protein is decreased (Mousavi et al., 2013). In corn soybean meal diets for laying hens, methionine, lysine and threonine are generally the first and next limiting amino (Gheisar et al., 2011). Numerous studies on low crude protein diet with amino acid supplementation have been conducted on various classes of poultry essential amino acids such as methionine, lysine, threonine and tryptophan that have become economically available in recent years due to technological advances (Yakout, 2010). Recent reports indicated that promising results in terms of maintaining birds' performance and maximising profitability can be obtained by the use of low protein diets with supplementation of amino acid for laying hens (Zeweil et al., 2011; Kashani et al., 2014).

According to Rao et al. (2013), low protein diet with supplementation of synthetic amino acid is able to minimise nitrogen excretion, production cost, intestinal disorder and amino acid excess. Reducing dietary crude protein increased the efficiency of utilisation of dietary crude protein and improved poultry tolerance to high ambient temperature (Zeweil, 2011). Laudadio et al. (2012a) also stated that the dietary protein level reduction in the broilers' rations under hot environmental conditions could be advantageous compared to the conventional feeding programmes.

Thus, it has become more evident that synthetic amino acid inclusion in diets allows nutritionists to further decrease crude protein while more effectively meeting the birds' amino acid requirements for maintenance and tissue accretion (Gheisar et al., 2011). Several investigators have reported that the requirements for certain essential amino acids for laying hens increase with the increase in dietary level of protein (Adeyemo et al., 2012).

Commercial amino acids must be used in order to meet the requirements of limiting amino acids due to the dilution of amino acids as the dietary protein is reduced. Inadequate knowledge about the essential amino acid requirements of laying hens, essential amino acid content of feed ingredients, digestibility and bioavailability of amino acids in feed ingredients and proper ratio between essential amino acids in low protein diets may have been the reasons for the inferior performance of hens fed a low protein, amino acid-supplemented diet (Mousavi et al., 2013). A few studies have been done with layer hen fed with 17.5% CP as control and 16.5 % CP for lower level in early laying phase. The current trial was carried out to determine how much dietary crude protein could be reduced, while supplementing diets with constant amount of synthetic amino acids such as

DL-methionine, L-lysine and L-threonine on production performance, egg quality, villus height and crypt depth and faecal pH, LAB and ENT count of commercial laying hens during production phase, i.e. from 20 to 32 weeks of age.

MATERIALS AND METHODS

In present experimental study, 16 week-old birds were kept in Ladang 2 Poultry Unit, Universiti Putra Malaysia, Serdang. A total of 144 Hisex Brown birds were randomly divided into four treatment groups with 6 replications of 36 birds per treatment. The feeding period lasted for 17 weeks, starting from the time when the birds were 16 weeks of age and ending when they were 32 weeks of age, with the addition of four weeks for acclimatisation purpose. Four dietary treatments consisted of: (T1) 17.5% CP (without supplementation of lysine and threonine; (T2) 17.5% CP diet; (T3) 17% CP and (T4) 16.5% CP diets supplemented with methionine, lysine and threonine. Diets were formulated to meet or exceed the minimum amino acid standards, as recommended by Hisex Brown Management Guide (2008), and water was provided *ad-libitum*. The compositions of the basal diet are shown in Table 1.

The individual feed intake was recorded weekly, while feed conversion ratio and live body weight gain were calculated at the end of the study. Eggs from individual cages were collected daily and weighed. The hen/day egg production and feed conversion ratio were calculated as the rate of production per hen per day and feed intake/egg mass. Live body weight gain (LBWG) was calculated as the difference between the initial and final body weights. At the end of the experiment, twelve birds of each treatment were randomly selected and slaughtered for digesta and small intestine samples for further analysis.

A total of 30 eggs per treatment were randomly collected and used to determine the egg quality. These measurements involved Haugh unit, eggshell thickness, eggshell weight, yolk percentage and albumen percentage. The Haugh unit was calculated using the egg analyser ORKA EggAnalyzer®. Eggshell thickness was measured in micrometer (μm) using a digimatic micrometre on the large end, equatorial region and small end. Meanwhile, albumen percentage was calculated as albumen weight over egg weight meanwhile yolk percentage was calculated as yolk weight over egg weight.

Different sections of small intestine were obtained for the morphometric analysis, as described by Choe et al. (2012). Segments of about 5cm long were removed from the small intestines (duodenum, jejunum and ileum) at the following locations: (i) from gizzard outlet to the end of pancreatic loop, (ii) segment between pancreatic loop and Meckel's diverticulum, and (iii) segment between Meckel's diverticulum and ileo-caecal junction was removed. The intestinal segments were flushed with 10% neutral buffered formalin solution and then used for the morphometric analysis. Then, the segments were fixed in 10% neutral buffered formalin solution overnight.

Table 1
Ingredients and composition of the experimental diets

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Crude Protein (%)	17.5	17.5	17.0	16.5
Corn	46.30	46.34	47.00	47.24
Crude Palm Oil	3.45	3.45	3.44	3.52
Wheat Pollard	11.80	11.97	12.45	12.77
Soy Bean Meal	22.86	22.67	21.75	21.64
Fish Meal	4.30	4.34	4.00	3.24
Dicalcium Phosphate	0.80	0.79	0.85	0.99
Calcium Carbonate	8.00	8.00	8.00	8.00
Vitamin Premix[2]	0.55	0.55	0.55	0.55
Mineral Premix[3]	0.55	0.55	0.55	0.55
Salt	0.06	0.06	0.06	0.08
Antioxidant	0.55	0.55	0.55	0.55
Toxin Binder	0.55	0.55	0.55	0.55
L-Lysine	0.00	0.01	0.04	0.07
DL-Methionine	0.15	0.16	0.16	0.17
L-Threonine	0.00	0.01	0.03	0.05
Calculated Analysis [4] (%)				
Crude Protein	17.5	17.5	17.0	16.5
Energy kcal/Kg	2811	2811	2811	2811
Fat	5.65	5.67	5.66	5.61
Fiber	3.61	3.68	3.68	3.68
Calcium	3.62	3.63	3.61	3.66
Available Phosphorus	0.43	0.43	0.43	0.43
Arginine	1.10	1.09	1.05	1.02
Lysine	0.97	0.98	0.97	0.98
Methionine + Cysteine	0.73	0.74	0.73	0.73
Methionine	0.46	0.47	0.46	0.47
Threonine	0.64	0.65	0.65	0.65
Tryptophan	0.20	0.20	0.20	0.19

[1] T1: 17.5% CP without essential amino acid supplementation (negative control);
T2: 17.5% CP with amino acid supplementation (positive control);
T3: 17% CP with amino acid supplementation;
T4: 16.5% CP with amino acid supplementation.
[2] Provided per kg diet: Fe 100mg; Mn 110mg; Cu 20mg; Zn 100mg; 12mg; Se 0.2mg; Co 0.6mg.
[3] Provided per kg diet: Vitamin A 6,667 IU; Vitamin D 1,000 IU; Vitamin E 23 IU; Vitamin K3 1.33mg; Cobalamin 0.03mg; Thiamin 0.83mg; Riboflavin 2mg; Folic Acid 0.33mg; Biotin 0.03mg; Pantothenic Acid 3.75mg; Niacin 23.3mg; Pyridoxine 1.33mg.
[4] The diets were formulated using Feedlive International Software (Thailand).

Intestinal samples were then excised, dehydrated in a tissue processing machine (Leica Microsystems K. K., Tokyo, Japan) and embedded in paraffin wax. Sections of 4 mm were cut from each of the sample, fixed on slides, stained with haematoxylin and eosin, mounted and examined under light microscopes. The distance from the tip of the villus to the villus crypt junction represents villus height, while crypt depth was defined as the depth of the invagination between adjacent villi. The villi height and crypt depth were measured using an image analyser.

Fresh faecal droppings were collected aseptically once a week using a sterile plastic bag from every treatment group and stored in the chiller prior to the laboratory analysis. The faecal lactic acid bacteria (LAB) and *Enterobacteriaceae* (ENT) population were determined using the method described by Foo et al. (2003) and Loh et al. (2010). The faecal samples were diluted in sterile peptone water to 10% (w/v) and left at room temperature for an hour prior to tenfold serial dilutions (v/v). Enumerations of LAB were carried out on Lactobacillus agar DE Man, ROGOSA and SHARPE (MRS-agar, Merck, KgaA, Darmstadt, Germany). The plates were incubated anaerobically at 30ºC for 48 hours. ENT were spread plated and enumerated on Eosin-methylene-blue lactose sucrose agar plates (EMB-agar, Merck, KgaA, Darmstadt, Germany) and incubated aerobically for 24 hours at 37ºC. The number of colony forming units (CFU) was expressed as the base 10 logarithm of CFU (log10 CFU) per gram. All the

samples were repeated in triplicates and the LAB/ENT ratio was also calculated. One gram of fresh faecal sample was mixed homogeneously with 9ml of deionised distilled water in a sterile tube. The pH value was measured using a Mettler-Toledo pH meter with a glass electrode (Mettler-Toledo, England). The faecal pH values were determined as stated by Choe et al. (2012).

All the collected data were subjected to SAS statistical analysis. The means and their standard errors were computed using One Way Analysis of Variance (ANOVA). Duncan's Multiple Range Test System was used to compare the significant difference between the treatments at P<0.05. The results were expressed as the mean ± standard error of mean.

RESULTS

The growth performance for the layer hen fed low crude protein diet supplemented with commercial amino acid is shown in Table 2. In particular, the LBWG and egg weight of hens had no significant differences (P>0.05) when fed with different levels of low crude protein diets. Treatment 3 (17% CP) had significantly higher (P<0.05) egg production and egg mass than other groups. As for the feed intake, there were significant differences (P<0.05) among the treatment groups. T4 had the lowest feed intake and T1 had the highest feed intake. FCR was significantly different (P<0.05) among the treatments, with T3 having the lowest FCR at 1.79.

The egg quality of layer hen fed with low crude protein diet supplemented with amino acids is shown in Table 3. There were no significant differences (P>0.05) in Haugh units, yolk and albumen weight percentage, eggshell weight and thickness among the treatment groups.

The intestinal villus height and crypt depth for birds fed with low crude protein diet with amino acid supplementation are presented in Table 4. The results showed significant differences (P<0.05) in both villi height and crypt depth in duodenum, jejunum and ileum. Duodenal, jejunum and ileum villi height in T1 and T3 were significantly higher (P<0.05) than T2 and T4. The duodenal, jejenum and ileum crypt depth showed similar results as the villi

Table 2

Production performance of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
HDEP[2] (%)	82.77 ± 0.04^b	75.76 ± 0.05^c	87.79 ± 0.01^a	82.77 ± 0.04^b
Egg weight (g)	55.15 ± 0.64	54.99 ± 0.65	55.44 ± 0.59	55.50 ± 0.56
Egg mass (g/hen/day)	45.65 ± 0.02^c	41.66 ± 0.03^d	48.68 ± 0.01^a	45.94 ± 0.02^b
Feed intake (g/hen/day)	87.67 ± 0.02^a	85.55 ± 0.05^c	87.04 ± 0.04^b	82.94 ± 0.04^d
LBWG[3] (kg)	0.33 ± 0.03	0.29 ± 0.02	0.28 ± 0.03	0.28 ± 0.02
FCR[4]	1.92 ± 0.01^b	2.06 ± 0.01^a	1.79 ± 0.01^d	1.81 ± 0.01^c

abcd Means in the same row not sharing a common superscript are significantly different (P<0.05).
[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation
[2]HDEP: Hen day egg production
[3]LBWG: Live body weight gain
[4]FCR: Feed conversion ratio

Table 3

Egg quality of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Haugh units	50.31 ± 0.86	50.66 ± 0.78	50.60 ± 0.73	50.47 ± 0.92
Yolk weight (%)	25.53 ± 0.55	25.60 ± 0.09	26.68 ± 0.59	27.30 ± 0.92
Albumen weight (%)	65.43 ± 0.92	65.76 ± 0.28	64.01 ± 0.93	63.86 ± 0.68
Eggshell weight (g)	5.26 ± 0.03	5.10 ± 0.01	5.28 ± 0.15	4.87 ± 0.09
Eggshell thickness (mm)	0.59 ± 0.01	0.63 ± 0.01	0.59 ± 0.03	0.58 ± 0.02

[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation

height, whereby the values of T1 and T3 were significantly higher (P<0.05) than T2 and T4. For VH/CD ratio, however, there were no significant differences (P>0.05) in duodenum, jejunum and ileum between all the groups.

The faecal pH, LAB and ENT counts are presented in Table 5. The lowest (P<0.05) faecal pH was observed in T3 and T4 compared to the rest of the treatment groups. However, the treatment supplemented with 17% CP showed the lowest reduction (P<0.05) in faecal pH compared to the other groups. There was a significant difference (P<0.05) in the LAB and ENT count and LAB/ENT ratio between the dietary treatments. T3 showed significantly higher (P<0.05) LAB counts compared with other

treatment groups. T3 also had the lowest (P<0.05) ENT counts. As for the LAB/ENT ratio, T3 had the highest ratio among all the treatment groups.

DISCUSSION

The egg weight and live body weight gain (LBWG) were not significantly different among the treatment groups. This finding suggests that low protein is well fortified with essential amino acids and has adequate level of total nitrogen that can maintain egg weight. Zou and Wu (2005) reported that the no response of egg weight to protein might be due to over consumption of feed of hens fed with low protein diet. In addition, a study conducted by Khajali et al. (2007) revealed that egg weight was not significantly

Table 4

Villus height and crypt depth of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Villi height, μm				
Duodenum	668.59 ± 44.15[a]	149.99 ± 7.81[b]	726.29 ± 91.59[a]	182.55 ± 16.25[b]
Jejunum	496.17 ± 31.80[a]	107.24 ± 7.92[b]	433.71 ± 52.87[a]	127.86 ± 14.19[b]
Ileum	361.00 ± 21.88[a]	81.01 ± 3.00[b]	317.73 ± 36.44[a]	111.78 ± 14.60[b]
Crypt depth, μm				
Duodenum	123.24 ± 11.17[a]	23.86 ± 2.31[b]	138.07 ± 15.27[a]	31.61 ± 4.30[b]
Jejunum	105.32 ± 5.91[a]	29.93 ± 1.37[b]	110.69 ± 11.03[a]	29.70 ± 6.22[b]
Ileum	90.51 ± 6.23[a]	21.70 ± 1.64[b]	74.18 ± 6.70[a]	24.12 ±3.59[b]
Villi height to crypt depth ratio				
Duodenum	2.87 ± 0.74	2.98 ± 0.64	3.75 ±1.31	3.38 ± 1.04
Jejunum	2.43 ± 0.57	2.31 ± 0.47	2.22 ± 0.70	2.70 ± 0.83
Ileum	2.20 ± 0.53	2.47 ± 0.52	2.27 ± 0.71	2.99 ± 1.05

[ab] Means in the same row not sharing a common superscript are significantly different (P<0.05).
[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation

Pertanika J. Trop. Agric. Sci. 39 (1): 551 - 563 (2016)

557

Table 5

Faecal pH and faecal LAB and ENT count of layer hen at different levels of crude protein diets with amino acid supplementation

Parameter	Dietary Treatment[1]			
	T1	T2	T3	T4
Faecal pH	6.83 ± 0.09^a	6.68 ± 0.05^a	6.37 ± 0.06^b	6.46 ± 0.04^b
LAB counts (log CFU/g)	5.71 ± 0.09^c	5.91 ± 0.07^b	6.31 ± 0.04^a	6.03 ± 0.01^b
ENT counts (log CFU/g)	5.82 ± 0.02^a	5.75 ± 0.01^b	5.61 ± 0.02^c	5.47 ± 0.03^d
LAB/ENT ratio	0.98 ± 0.01^c	1.03 ± 0.01^b	1.12 ± 0.01^a	1.10 ± 0.01^a

[abcd] Means in the same row not sharing a common superscript are significantly different (P<0.05).
[1]T1: 17.5% CP without supplemented essential amino acid (negative control)
T2:17.5% CP with amino acid supplementation (positive control)
T3: 17% CP with amino acid supplementation
T4: 16.5% CP with amino acid supplementation

affected by feeding reduced crude protein diet. This result is in agreement with a study conducted by Yakout (2010), which observed no difference in egg weight and body weight gain when birds were fed with low crude protein diets supplemented with amino acid. Meanwhile, no significant changes were observed in the initial, final and change in body weight of hens at 21 to 33 weeks of age fed different dietary treatments, indicating that different dietary treatments with higher levels of protein and methionine are not sufficient to make alteration in the body weights (Kumar et al., 2012). In the current study, higher hen day egg production was observed in the T3 (17% CP) treatment group compared to other dietary groups. This could be due to the basic amino acid and dietary protein fulfilment in the dietary treatment. This was supported by a previous research that indicated better performance was observed in animals fed with the low dietary crude protein supplemented with amino acid

(Banarjee et al., 2013). Results of this experiment are also in disagreement with those reported by Liu et al. (2004) and Wu et al. (2005), who claimed that reducing dietary protein would reduce egg production.

In both these diets supplemented with amino acids in this study, there were increases in the feed intake when birds were fed with 17% CP compared with those fed with 17.5% CP diet supplemented with amino acid. Our results are in agreement with Kidd et al. (2001), who reported that 20% CP diet with amino acids supplementation fed to broilers had significant increase in feed intake than those fed a diet with 23% CP. Feed intake was increased when reduced protein diets were fed to chickens. This happened as the result of an amino acid "appetite" that occurred when feeding amino acids marginal diets. In terms of FCR, 17% CP had significantly lower value compared to hens fed with higher level of dietary protein treatments. According to Novak et al. (2006), feed efficiency of

birds improved as dietary protein decreased. Variation in FCR could also be attributed to the differences in egg mass as the resultant effects of feed intake (Adeyemo et al., 2012). In a study conducted by Kumar et al. (2012), FCR was similar in hens fed medium (16%) or high (18%) protein diets during 20 to 43 weeks of age.

In our study, egg qualities are not affected by reducing dietary crude protein. This was supported by a previous study (Adeyemo et al., 2012), which revealed egg qualities were not affected by reducing protein level in poultry ration. There were no differences observed in Haugh units in the early phase (20-43 weeks of age) of the production cycle when feeding low protein diets (Novak et al., 2006). There was no effect of lysine on the variables Haugh unit, yolk and albumen percentage, as reported by Torki et al. (2014), Figueiredo et al. (2012) and Jardim et al. (2008, 2010). Eggshell percentage and thickness were not influenced by the dietary treatments, which could be explained by the fact that eggshell synthesis requires only a small amount of protein (Praes et al., 2014).

The dietary nutrient utilisation efficiency is affected by gastrointestinal tract development and can be assessed through measurements of villi height and crypt depth (Swatson et al., 2002). Meanwhile, Tufarelli et al. (2010) found that the changes in gut morphology influenced the nutrient utilisation and performance of rabbits. The intestinal villi and cells in chickens are affected by dietary components (Incharoen & Yamauchi, 2009; Incharoen et al., 2009).

Small intestine is the predominant site for protein digestion. Dietary protein not only plays a major role in poultry growth and reproductive performance, but also on the gastrointestinal tract features (Laudadio et al., 2012). Maximum digestion and absorption are believed to occur as the villus height to crypt depth ratio increases in weaned pigs. The increased villus height to crypt depth ratio produced an intestinal structure more oriented to absorption (Xu et al., 2012). Villi are important structures in the small intestine that involves mainly in nutrient absorption. Therefore, an increased villus height would increase the surface area for nutrient absorption (Choe et al., 2012). Lengthening of villi may increase total luminal villus absorptive area and subsequently result in satisfactory digestive enzyme action and higher transport of nutrients at the villus surface. However, deeper crypt indicates faster tissue turnover and high demand for the renewal of the villus, which suggests that the host's intestinal response mechanism is trying to compensate for normal sloughing or atrophy of villi due to inflammation from pathogens and their toxins (Incharoen et al., 2010). The villus height to crypt depth ratio is a very useful measure to estimate the absorption capacity of the small intestine. Higher villus height to crypt depth ratio is a positive aspect as it results in a decreased turnover of the intestinal mucosa and leads to lower maintenance requirement, which ultimately leads to a higher animal growth rate (Tufarelli et al., 2010; Laudadio et al., 2012).

Pertanika J. Trop. Agric. Sci. 39 (1): 551 - 563 (2016)

559

The faecal pH, LAB count, ENT count and LAB to ENT ratios were significantly different among the treatment groups. The decrease in the faecal pH with low CP regimens provides a more favourable pH environment for digestive activity, allowing for greater digestion and absorption of nutrients, particularly in the small intestine. The acidic environment favours growth of LAB. Results for the ENT count showed a clear alteration of faecal microflora in low level of CP. It has been proven that increased LAB count could decrease ENT count (Loh et al., 2007; Choe et al., 2012). According to Loh et al. (2007), LAB has antagonistic effect on harmful bacteria either *in vitro* or *in vivo* studies. These beneficial bacteria have been shown to be able to reduce the faecal ENT count. Intestinal LAB is able to produce antimicrobial compounds such as bacteriocin and organic acids, which are bacteriostatic against pathogenic bacteria. The undissociated organic acid decreases the pH when it passes through. The LAB competes against enteropathogens for nutrient, binding and receptor sites. They have strong inhibitory effects in preventing the adherence, establishment and replication of pathogenic bacteria. The LAB is important component for a balanced microflora in the gastrointestinal tract. In this study, 17% CP has beneficial effects on laying hens, and this is most likely due to the optimal intestinal environment as confirmed by the faecal pH (Laudadio et al., 2012).

CONCLUSION

The findings of this study indicate that reducing dietary protein level supplemented with essential amino acids in layer ration could be advantageous compared to conventional feeding programmes. Furthermore, the diet containing 17% CP with addition of synthetic amino acids offers an advantage on improving the hen/day egg production and shows positive effects such as increased small intestine villus height and promoted beneficial effects of faecal microflora. These findings indicate that the response of layers to lowered levels of dietary crude protein can be used in feeding layer hens.

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562

Pertanika J. Trop. Agric. Sci. 39 (4) 551 - 563 (2016)

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Characterisation of Plant Growth-Promoting Bacteria from Kacip Fatimah (*Labisia pumila*) under Natural Tropical Forest

Yasmi, A.[1], Radziah, O.[1,2]*, Hawa, ZE. J.[3], Zainal Abidin, M. A.[4] and Panhwar, Q. A.[2,5]

[1]*Department of Land Management, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Department of Crop Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4]*Department of Plant Protection, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[5]*Soil and Environmental Sciences Division, Nuclear Institute of Agriculture Tandojam, Sindh, Pakistan*

ABSTRACT

Medicinal plants play a major role in many cultures, not only as medicines, but also as trade commodities to fulfil the demands of distant markets. A study was conducted to characterise growth-promoting bacteria from two varieties of Kacip Fatimah *(Labisia pumila)* found at two different locations, Sungai Tekala, Semenyih, Selangor for *Labisia pumila* var. *lanceolata* and Bukit Slim Permanent Forest Reserve, Perak for *Labisia pumila* var. *alata.* Soil and plant samples were taken for the physico-chemical analyses and characterisation of the indigenous plant growth-promoting bacteria (PGPB). Both Labisia varieties were found to thrive in areas with quite similar soil chemical and physical properties in natural forest environments except for altitude, where *Labisia pumila* var. *alata* was found in higher elevation compared to *Labisia pumila* var. *lanceolata.* The soil in both places was found to be slightly acidic and low in nutrient content. Total bacterial population found on var. *alata* was higher than in var. *lanceolata* and the highest population was found in the root endosphere (8.68×10^7 cfu g^{-1} soil). Morphologically-isolated bacteria were circular in shape, with flat/raised elevation, entire margin, moist texture and smooth and glistening surface but varied in colour and size. Most of the bacterial strains showed several plant-growth promoting traits like plant-growth hormones (indole acetic acid (IAA)), N_2 fixation and P solubilisation activities and beneficial enzymes. Two of the bacterial isolates showing most of the beneficial properties were identified as *Exiguobacterium* sp. and *Stenotrophomonas* sp. These PGPB have the potential to enhance the growth of Kacip Fatimah.

INTRODUCTION

Microorganisms play an important role in agriculture by supplying nutrients to plants and reducing the demand of chemical fertilisers (Cakmakci et al., 2006). Soil microbes affect soil fertility through their influence on organic matter turnover, mineral immobilisation and dissolution and soil aggregation (Davis & Abbott, 2006). Several bacteria have ability to fix atmospheric N_2 and are known as diazotrophs (Ladha & Reddy, 2000; Osivand et al., 2009). Poorly soluble P in soil can be solubilised by microorganisms and converted to soluble forms by the process of acidification, chelation and exchange reactions (Chung et al., 2005). Soil microorganisms such as the phosphate-solubilising bacteria (PSB) and arbuscular mycorrhizal fungi have the ability to mobilise P in soil and reduce inputs of chemical fertilisers (Arpana & Bagyaraj, 2007). Species of *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia* are well-known as plant growth-promoting bacteria (PGPB).

Higher populations of microorganisms occur in the rhizosphere than non-rhizosphere soil and they are metabolically more dynamic than those from other sources (Vazquez et al., 2000). Microorganisms are found everywhere in soils with various diversities in their functionalities. The population of these microorganisms is affected by soil physical and chemical properties, organic matter and P content, including cultural practices (Kim et al., 1998).

Several plant growth-promoting bacteria (PGPB) have been known to enhance growth of numerous plant species including some medicinal plants. Mixed bacterial inoculations were reported to have a higher success in improving plant growth as they provide more balanced nutrition. They significantly improved the adsorption of nitrogen, phosphorus and other mineral nutrients (Bashan & de-Bashan, 2005).

Kacip Fatimah *(Labisia pumila)* is one of the most popular traditional medicinal herbs found in the natural forests of Malaysia, Indonesia, Thailand, Indochina, Philippines and New Guinea (Wiart & Wong, 2002; Ong, 2004). Kacip Fatimah belongs to the family Myrsinaceae, and is a small wild sub-herbaceous plant with creeping stems growing naturally on the forest floor of 20-1500 m altitude. Malaysia is listed as the twelfth most bio-diverse nation in the world (Foster, 2009) with a diverse flora and fauna that are important for the phyto-pharmaceutical, phytocosmetic and nutraceutical industries. Herbal industry is one of the important industries in the development of health food. Currently, there is growing interest in medicinal plants as sources of new pharmaceutical products in Malaysia. Malaysia stands to gain from strong growth of natural herbal products. The growth rate of the herbal industry in Malaysia is estimated at 15% per annum and the industry is projected to have an

estimated market of RM15 billion and RM29 billion, respectively, for the years 2015 and 2020 (ECERDC, 2011).

Kacip Fatimah is a popular herb among local women (Ezumi et al., 2007); it is frequently consumed by women of reproductive age. It has been widely used by many generations for various ailments, especially those associated with childbirth and the feminine reproductive system. It has essential astringent, antibacterial and antifungal properties due to the presence of rich natural phytoestrogens. Other bioactive phytochemical contents are flavonoid (rutin, quercetin, kaempferol, myricetin), phenols (gallic acid, pyrogallol, caffeic acid), saponin, iron, benzoquinoid derivatives, alkenylresorcinols, triperpenoid and other antioxidative compounds consisting of β-carotene, vitamin C and anthocyanin (Foster, 2009; Norhaiza et al., 2009; Karimi et al., 2011). There is an increased demand for Kacip Fatimah but the plant source in natural forests are becoming scarce due to extensive harvesting. There are a few efforts to propagate the plant using seeds, leaves or root cuttings, but the growth rate in unnatural habitats is very slow and not profitable. In general, this plant thrives best in shady areas and non-waterlogged humus-rich soil.

Previous studies showed that PGPB can stimulate plant growth directly or indirectly in various ways through manipulation of their surrounding environment to support growth or through secretion of growth-promoting substances that enhance plant growth. Limited studies have been conducted on the beneficial effects of PGPB on the growth of Kacip Fatimah. The use of these beneficial bacteria may enhance sustainable production and reduce dependency on chemical fertilisers. The literature records many beneficial effects of PGPB in various crops but few studies have been conducted on Kacip Fatimah. Hence, the present study was carried out to isolate PGPB from the roots and shoots of Kacip Fatimah (*Labisia pumila*) obtained from natural forests and to characterise their plant-growth promotion traits.

MATERIALS AND METHODS

Plant and Soil Sampling

Kacip Fatimah plants were sampled from two different locations i.e. Sungai Tekala, Semenyih, Selangor for *Labisia pumila* var. *lanceolata* and Bukit Slim Permanent Forest Reserve, Perak for *Labisia pumila* var. *alata*. A total of 20 plants from each location were sampled together with the adhered soil for bacterial isolation. Soil samples at a depth of 15 cm were taken using an auger for nutrient analysis.

Locations and Climatic Conditions

The location and climatic conditions of the natural forest areas where Kacip Fatimah (*Labisia pumila*) were found were not significantly different. *Labisia pumila* var. *lanceolata* was found at elevation 66-116 m above sea level while *Labisia pumila* var. *alata* was found at the higher elevation of 266-356 m (Table 1). The light intensity and relative humidity of the area for var. *alata* was slightly higher than that for the location for var. *lanceolata* but the soil and

leaf temperatures were higher in area of var. *lanceolata* than that of var. *alata*.

Determination of Soil Chemical Properties

The pH of soil at ratio of 1:25 (soil:water) was determined using Beckman Digital pH meter. Nitrogen concentration in soil was determined using Kjeldahl method (Bremner, 1996). The available P was determined by the method of Bray and Kurtz (1945), while exchangeable K and cation exchange capacity (CEC) were determined using the modified leaching method of Chapman (1965).

Determination of Moisture in Soil

Ten grams of fresh soil were placed in a crucible and dried in an oven at 105 °C until a constant weight was obtained. The soil moisture content was expressed as follows:

$$\text{Soil moisture \%} = \frac{\text{Weight of water}}{\text{Dry weight of soil}} \times 100$$

Enumeration and Isolation of PGPB from Soil and Plant

Bacteria were isolated from the non-rhizosphere (bulk soil), rhizosphere and endosphere (root, stem and leaf) of Kacip Fatimah plants using serial dilution and plate count method (Johnson & Curl, 1972). Colony morphology of bacteria on a nutrient agar plate (NA) was observed and characterised according to shape, elevation, margin, texture, colour, surface and size within seven days of incubation.

Determination of Gram Reaction (Gram Staining and KOH Test)

The Gram reaction was determined using potassium hydroxide (KOH) test (Halebian et al., 1981) and the Gram-staining modified method of Hucker (1921). In a KOH test, Gram-negative bacteria turn viscous/mucoid, while there is no change in Gram-positive bacteria.

Table 1

Chemical Properties of Soil Surrounding Kacip Fatimah Plant at 15cm Depth

Soil Characteristics	Location[a]	
	Sungai Tekala (*L.pumila* var.*lanceolata*)	Permanent Reserve Forest Bukit Slim (*L.pumila* var. *alata*)
pH	4.00 ± 0.03	4.42 ± 0.43
Total nitrogen (%)	1.03 ± 0.04	0.99 ± 0.7
Extractable phosphorus (mg kg^{-1})	5.23 ± 0.5	2.21 ± 0.18
Exchangeable potassium (mg kg^{-1})	20.68 ± 2.13	21.26 ± 1.49
Cation exchange capacity (cmol$_c$ kg^{-1})	18.09 ± 1.42	10.27 ± 0.48
Soil moisture (%)	37.51 ± 5.13	35.08 ± 1.36

[a]Average of five samples ± standard error of the mean

Screening for Phosphate Solubilising Activity

The ability of bacterial isolates to solubilise phosphate was determined using the National Botanical Research Institute's phosphate growth medium (NBRIP) modified method (Nautiyal, 1999). Four drops of 20 µl of bacteria from a nutrient broth was placed on a plate containing the NBRIP medium. The plate was incubated at a temperature of 28 ℃±2 ℃ for 24 hours. Positive growth was observed through the visible halo zone surrounding the bacterial colony.

Screening for Potential Nitrogen Fixation Ability

The ability of the isolated bacteria to fix nitrogen was qualitatively determined using an N-free solid malate medium as used in the modified method of Dobereiner and Pedrosa (1987). Nitrogen fixing is indicated by a change in agar colour from green to blue.

Screening for Hydrolytic Enzyme Reaction

The ability of the isolates to produce enzymes *viz*. cellulase and pectinase was determined using a carboxy methyl cellulose (CMC) and citrus pectin media, respectively. Two grams of substrate (CMC/citrus pectin) were added into 1 l of nutrient agar (NA). Bacterial cultures were dropped on a media plate containing CMC and citrus pectin substrates. The plates were incubated for 24 hours at a temperature of 28 ℃±2℃ before staining with 0.1 % Congo red for 30 minutes. The plates were then washed with 1 m of sodium chloride (NaCl). A positive reaction was indicated by a visible halo zone around the bacterial colonies.

Screening for Starch Hydrolysis

Starch hydrolysis of bacterial isolates was carried out using the method of Seeley and Dain (1960). The bacterial isolates were incubated in a 250-ml Erlenmeyer flask containing 100 ml of a nutrient broth (NB) for 24 hours before inoculating a starch agar plate consisting of 5 g L^{-1} of starch, 14g L^{-1} of nutrient agar (NA) and a teaspoon of agar powder. After incubation for three to five days at a temperature of 28℃ ± 2℃, the plate was flooded with a dilute iodine solution (Gram's iodine solution). The iodine was allowed to remain in the medium for 30 seconds and then decanted. Hydrolysis activity was indicated by a clear zone around the growth.

Screening for Catalase Production

Catalase production of bacterial isolates was determined according to the modified method of Duke and Jarvis (1972). The bacterial isolates were incubated in 250-ml Erlenmeyer flasks containing a 100-ml nutrient broth (NB) for 24 hours before streaking on nutrient agar (NA) plates. The plates were incubated for 24-48 hours at a temperature of 28 ℃±2℃. Three or four drops of 3% hydrogen peroxide were added over the entire surface of the bacterial culture. Catalase production was determined by the presence of bubbling or foaming on the culture.

Determination of Indole-Acetic Acid (IAA) Production

IAA production by bacterial isolates was determined using the colorimetric method, modified from Gordon and Weber (1951). The bacterial isolates were incubated in 250-ml Erlenmeyer flasks containing a 100-ml nutrient broth (NB) with addition of 0.2% of L-tryptophan. The bacterial cultures were grown on a shaker for 24 hours at a temperature of 28 ^{o}C±2 ^{o}C. Two mm of bacterial cultures were placed in an Eppendorf tube and centrifuged for 7 minutes at 7000 rpm. One ml of the supernatant from each isolate was mixed with 2 ml of Salkowsky's reagent. The colour absorbance was determined using a spectrophotometer at 535 ηm after 20-25 minutes.

Strain Identification Using Polymerase Chain Reaction (PCR)

Bacterial identification was determined using 16S rRNA gene partial sequence analysis. DNA for bacterial isolates was extracted using the Genomic DNA Mini Kit (blood cultured cell) Bacteria Protocol (GENEAID). The cell harvesting/pre-lysis for the isolates was conducted following the Gram-negative bacteria procedure. Cultured bacterial cells was transferred to a 1.5-ml microcentrifuge tube and centrifuged for 1 minute at 10 000 rpm. Then, 200µl of GT Buffer was added and the cell pellet was suspended using a vortex. For the lysis step, 200µl of GB Buffer was added to the sample and vortexed for 5 seconds. The sample was incubated at 70 ℃ until the sample lysate was clear. For DNA binding, 200µl of absolute ethanol was added to the sample lysate and immediately vortexed for 10 seconds. All the mixture was transferred to the GD column and placed in a 2-ml collection tube and centrifuged at 12 000 rpm for 2 minutes. The 2-ml collection tube containing the flow-through was discarded and the GD column was placed in a new 2-ml collection tube. For DNA washing, 400µl of WI buffer was added to the GD column and centrifuged at 12 000 rpm for 30 seconds. The flow-through was discarded and the GD column was placed back in the 2-ml collection tube before 600 µl of wash buffer (ethanol added) was added to the GD column. The sample was centrifuged at 12 000 rpm for 30 seconds. The flow-through was discarded and the GD column was replaced in the 2-ml collection tube before being centrifuged again at 12 000 rpm for 3 minutes to dry the column matrix. For DNA elution, the dried GD column was transferred to a clean 1.5-ml microcentrifuge tube before 100 µl of pre-heated elution buffer was added to the centre of the column matrix. The mixture was allowed to stand for 3 minutes until the elution buffer was absorbed by the matrix before being centrifuged at 12 000 rpm for 30 seconds to elude the purified DNA. For sample mix preparations, Dream TAq Green PCR master mix-fermentas was used. The PCR amplification was carried out using the MJ Mini Personal Thermal Cycler, Bio-Rad. The universal primer-F used was 27f, AGAGTTTGATCMTGGCTCAG while the primer-R was 1492r,

GGTTACCTTGTTACGACTT. The DNA was visualised using gel electrophoresis under UV light in a UV transluminater. The PCR products were purified using a GeneJet PCR Purification Kit and the purified DNA was stored at -20 ºC before being sent to 1stBase DNA Sequencing Division for sequencing. The 16s rDNA gene sequence was compared with a nucleotide database of the National Centre for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) at http://blast.ncbi.nih.gov/Blast.cgi.

Determination of Growth pH for Bacterial Strains

The bacterial strains were tested to determine the suitable pH condition for growth. The strains were cultured in 100-ml Erlenmeyer flasks containing a sterilised nutrient broth adjusted to pH 3, pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 for 24 hours at 28±2 ºC on an orbital shaker. A ten-fold serial dilution was made and total bacterial population was determined using the drop-plate technique and was calculated as follows:

$$cfu = \frac{(Mean\ plate\ count)\ (Dilution\ factor)}{0.1\ ml}$$

Determination of Optimum Growth of Bacterial Strains

The bacterial strains were tested to determine optimum cell growth. The strains were cultured in 250-ml Erlenmeyer flasks containing a 100-ml sterilised nutrient broth with adjusted pH 3, pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 accordingly on the orbital shaker. Two ml of the nutrient broth was taken after 12 hours, 24 hours and 36 hours of culturing and put into Eppendorf tubes. The Eppendorf tubes were centrifuged at 7000 rpm for 7 minutes. The supernatant was taken out, leaving only the bacterial cells. The cells were then washed with 0.85% of phosphate buffered saline (PBS). The final solution was read using a spectrophotometer at 600 nm for an optical density reading.

RESULTS

Chemical Properties

Different species of *Labisia pumila* were sampled from two different locations. Table 2 shows that the two species could grow in slightly acidic soil at pH 4.00±0.03 (var. *lanceolata*) and 4.42±0.43 (var. *alata*). For the soil nutrient content, total soil nitrogen in surrounding soil was not significantly different for both species, which was 1.03±0.04 % for the area with var. *lanceolata* sampled from Sungai Tekala and 0.99±0.7 % for var. *alata* sampled from the Permanent Reserve Forest Bukit Slim. Extractable phosphorus of var. *lanceolata* soil was higher (5.23±0.5 mg kg⁻¹) than in var. *alata* soil but exchangeable potassium was higher in var. *alata*. (21.26 mg kg⁻¹). Cation exchange capacity in var. *lanceolata* soil was also greater at 18.09±1.42 cmol$_c$ kg⁻¹ compared to 10.27±0.48 cmol$_c$ kg⁻¹ for var. *alata*.

Table 2
Location and Climatic Conditions of Kacip Fatimah (Labisia pumila) in a Natural Forest

Species	Location and climatic conditions						Relative humidity (%)
	N (^{0})	E (^{0})	Elevation (m)	Light intensity (μmol m^{-2}S$^{-1)}$)	Temperature (^{o}C)	Leaf temperature (^{o}C)	
L.pumila var. *lanceolata* (Sungai Tekala)	3.0598-3.0605	101.8718-101.8722	66-116	10.2 ± 1.56	29.58 ± 0.25	27.44 ± 0.09	80.6 ± 0.75
L.pumila var. *alata* (Permanent Reserve Forest Bukit Slim)	3.9658-3.9671	101.4223-101.4226	266-356	11.4 ± 1.72	27.66 ± 0.11	25.62 ± 0.18	82.62 ± 0.49

[a]Average of five samples ± standard error of the mean

Total Bacterial Population

Total bacterial population of var. *alata* was higher than var. *lanceolata* in most plant parts except in phyllosphere (Table 3). The bacterial populations were highest in the root endosphere (8.68×10^7) for both species and the lowest was in the non-rhizosphere of var. *lanceolata* and in phyllosphere of var. *alata*.

Plant Growth-Promoting Traits of Bacterial Isolates

There were 20 bacterial strains isolated from the soil and Kacip Fatimah plants obtained from Sungai Tekala, Selangor for var. *lanceolata* (Table 4). Six of them were isolated from the stem, five from the root, seven from the rhizosphere and two from the soil. Results of the Gram reaction showed that 16 isolates were Gram-positive and four were Gram-negative. All bacteria were able to grow in the N-free solid malate medium except for one of the isolates, which was KLR 06. Out of 20 isolates, only five strains were able to solubilise calcium phosphate and none of the strains were able to synthesise either cellulase or pectinase. However, 15 of the strains isolated were able to synthesise catalase enzyme while half of the strains were able to hydrolyse starch (Figure 1).

There were 17 bacterial strains isolated from which six were isolated from the rhizosphere, six from the roots, four from the soil and one from leaf of Kacip Fatimah (*L. pumila* var. *alata*). Results of the Gram reaction showed that nine isolates were Gram-positive and eight were Gram-negative. All the bacteria were able to grow in the N-free solid malate medium except two of the isolates (KAH03 and KAR18). Nine of the isolates were able to solubilise calcium phosphate while the rest showed a negative result. For hydrolytic enzyme reaction, six of the isolates were positive for

Table 3
Total Bacterial Populations from Non-Rhizosphere, Rhizosphere and Endosphere of Kacip Fatimah

Soil/plant parts	Total bacterial population (cfug^{-1} soil plant^{-1} dw)[a]	
	Sungai Tekala (*L. pumila* var. *lanceolata*)	Permanent Reserve Forest Bukit Slim (*L. pumila* var. *alata*)
Non-rhizosphere	1.04×10^{4d}	4.28×10^{5c}
Rhizosphere	2.53×10^{6b}	3.28×10^{7a}
Root endosphere	3.14×10^{6b}	8.68×10^{7a}
Stem endosphere	2.94×10^{4d}	1.86×10^{5c}
Phyllosphere	1.13×10^{6b}	1.57×10^{5c}

[a]Average of 19 samples
Means within the same column followed by the same letters are not significantly different at $P>0.05$.

Table 4
Plant Growth Promoting Trait of Bacterial Isolates in Kacip Fatimah (L. pumila var. lanceolata)

Bacterial strain	Gram reaction	N$_2$-fixation ability (N- free solid malate medium)	Phosphate solubilising activity	Hydrolytic enzyme reaction		Starch hydrolysis	Catalase production
				CMC	Citrus pectin		
KLS01	+	+	-	-	-	+	+
KLS02	+	+	-	-	-	+	+
KLS03	-	+	+	-	-	-	+
KLR05	+	+	-	-	-	-	-
KLR06	+	-	-	-	-	+	+
KLR07	+	+	-	-	-	+	-
KLS09	+	+	-	-	-	+	+
KLR10	-	+	+	-	-	-	+
KLR11	+	+	-	-	-	+	-
KLH12	-	+	+	-	-	-	+
KLH13	-	+	+	-	-	-	+
KLS14	+	+	-	-	-	-	-
KLH19	+	+	-	-	-	-	-
KLT22	+	+	-	-	-	-	+
KLT23	+	+	-	-	-	+	+
KLH24	+	+	-	-	-	+	+
KLH25	+	+	+	-	-	-	+
KLH27	+	+	-	-	-	+	+
KLH29	+	+	-	-	-	-	+
KLS35	+	+	-	-	-	+	+

both the carboxymethyl cellulase (CMC) and citrus pectin test but one isolate (KAR18) showed positive for carboxymethyl cellulase (CMC) and negative for citrus pectin. Nine isolates produced catalase and 11 isolates could hydrolyze starch (Table 5 and Figure 1).

Indole-Acetic Acid (IAA) Production

All the strains isolated from var. *lanceoalata* were able to produce IAA. The highest production was 5.77 µg/ml from KL S14, while the lowest was 1.45 µg/ml from KL T24 strains isolated from *L. pumila* var. *lanceolata* (Figure 2). However, the bacterial strains isolated from the *L. pumila* var. *alata* were also able to produce IAA,

except for one strain (KAT14). The highest production was 5.84 µg/ml from KAH19, while the lowest was 1.14 µg/ml from KAR12 isolated from *L. pumila* var. *alata* (Figure 3).

Bacterial Identification Using Polymerase Chain Reaction (PCR)

Two of the isolates (KAR12 and KAR23) that possessed the most beneficial traits were taken for identification, while similar characteristics possessed by the isolates were not chosen for isolation, such as KAT 13. Both are from *L. pumila* var. *alata* sampled from the Permanent Forest Reserve Bukit Slim, Perak. BLAST output of isolate KAR12 after the 16s rRNA gene partial

Table 5

Plant Growth Promoting Trait of Bacterial Isolates in Kacip Fatimah (L. pumila var. alata)

Bacterial strain	Gram reaction	N_2-fixation ability (N- free solid malate medium)	Phosphate solubilising activity	Hydrolitic enzyme reaction		Starch hydrolisis	Catalase production
				CMC	Citrus pectin		
KAL01	+	+	+	-	-	+	-
KAH02	+	+	-	-	-	+	+
KAH03	+	-	+	+	+	+	-
KAH05	-	+	+	-	-	-	-
KAH06	+	+	-	-	-	+	+
KAR08	-	+	+	-	-	-	-
KAR09	-	+	-	-	-	-	+
KAR12	+	+	+	+	+	+	+
KAT13	+	+	+	+	+	+	+
KAT14	+	+	-	-	-	+	-
KAT15	-	+	-	+	+	-	+
KAT16	+	+	+	+	+	-	-
KAR17	-	+	-	-	-	+	+
KAR18	+	-	-	+	-	+	-
KAH19	-	+	+	-	-	+	+
KAR20	-	+	-	-	-	-	+
KAR23	-	+	+	+	+	+	-

sequence PCR procedure showed that the strain belonged to *Exiguobacterium* sp. NIU-K2 (Table 6), while isolate KAR23 belonged to *Stenotrophomonas* sp. Y06 (Table 6) with 100% and 99% similarity, respectively.

Growth of Bacterial Strains at Different pH

Growth of both bacterial strains was significantly affected by culture pH. The strains were able to grow at a pH range of 5-9 and could not grow at pH 3 (Figure 4). After 24 hours, growth of *Exiguobacterium* sp.

Table 6
BLAST Output of Isolates KAR12 and KAR23 (Sequences Producing Significant Alignment)

Isolates	Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KAR12	HQ385945.1	*Exiguobacterium* sp. NIU-K216S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	HQ385944.1	*Exiguobacterium* sp. NIU-K416S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	HM047519.1	*Exiguobacterium acetylicum* strain KNUC604 16S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	JF227852.1	Uncultured bacterium clone ncd2602b09c1 16S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
	JF71564.1	Uncultured bacterium clone ncd1967d06c1 16S ribosomal RNA gene, partial sequence	939	1848	100%	0.0	100%
KAR23	JF220295.1	Uncultured bacterium clone ncd2628f03c1 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	JF163965.1	Uncultured bacterium clone ncd1904d07c1 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161884.1	*Stenotrophomonas* sp. Y06(2010) 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161883.1	*Stenotrophomonas* sp. Y05(2010) 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161882.1	*Stenotrophomonas* sp. Y04(2010) 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
	HM161866.1	Uncultured bacterium clone hdb_SIPB653 16S ribosomal RNA gene, partial sequence	1064	2107	99%	0.0	99%
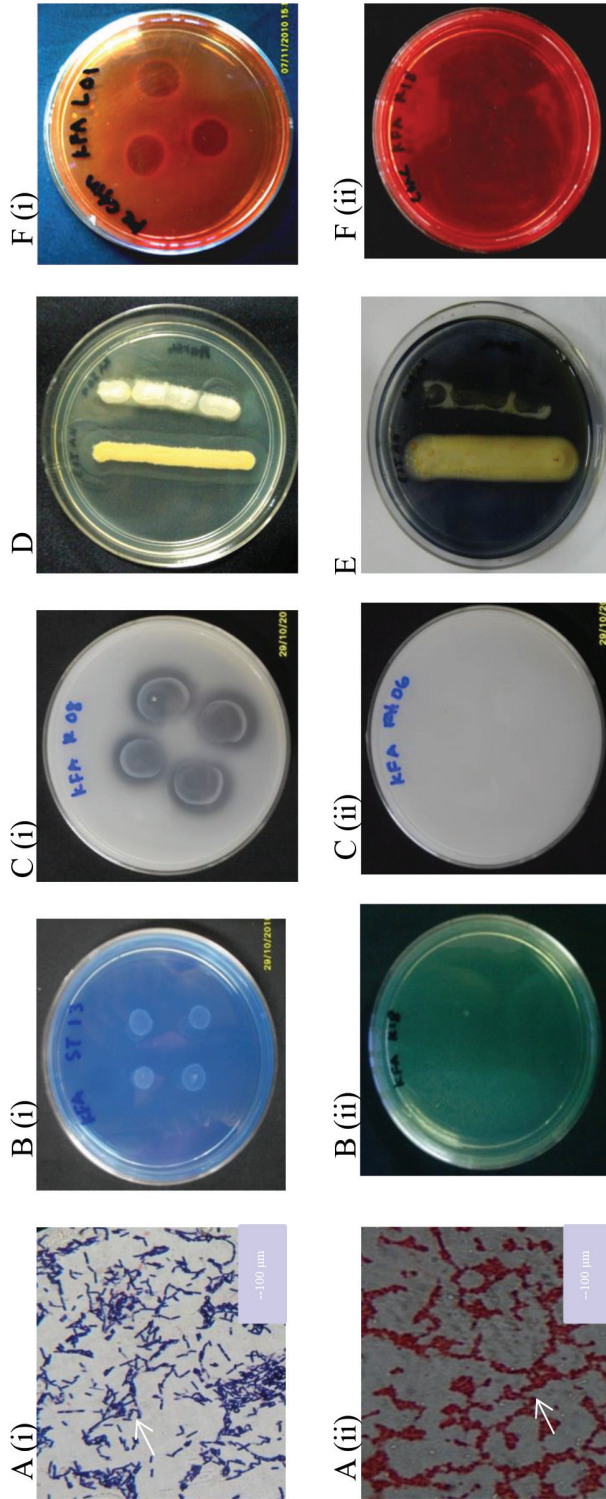
Figure 1. Plant-growth promoting trait of bacterial isolates in agar plates. A (i) Positive result, and A (ii) Negative result of Gram staining. B (i) Positive result and B (ii) Negative result of N₂-fixation ability on N-free solid malate medium. C (i) Positive result and C (ii) Negative result of phosphate solubilisation on NBRIP media plate. D, Negative result. D (left) and positive result (right) of catalase production on nutrient agar (NA) plate. E, positive result (left) and negative result (right) of starch hydrolysis on starch agar media plate. F (i) positive result and F (ii) negative result of hydrolytic enzyme on citrus pectin media (CMC) plate.
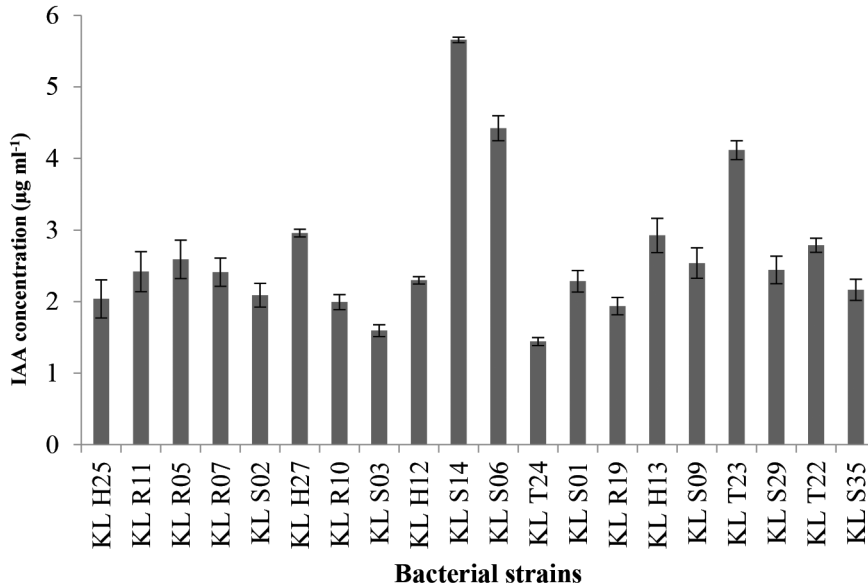
Figure 2. Indole-acetic acid (IAA) production by bacterial strains isolated from *L. pumila* var. *lanceolata.* Bars indicate standard error, n=3.
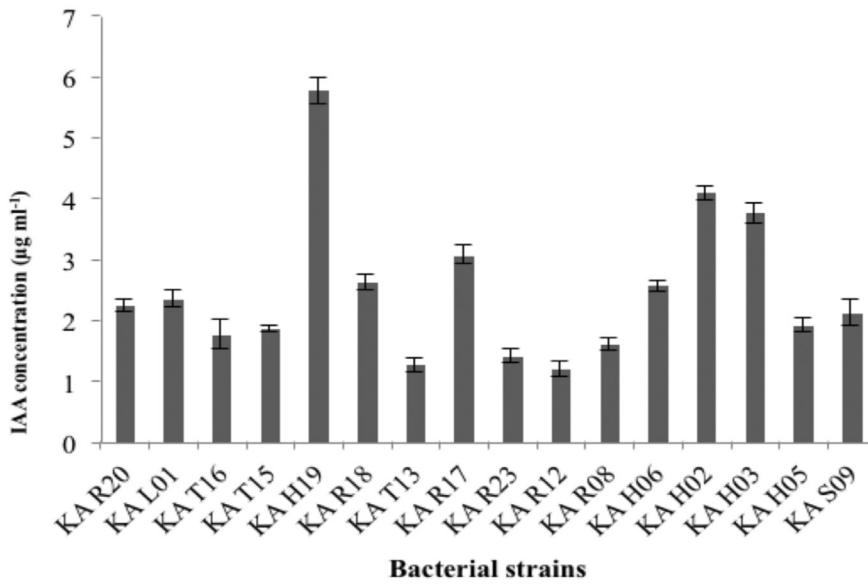
Figure 3. Indole-acetic acid (IAA) production by bacterial strains isolated from *L. pumila* var. *alata.* Bars indicate standard error, n=3.
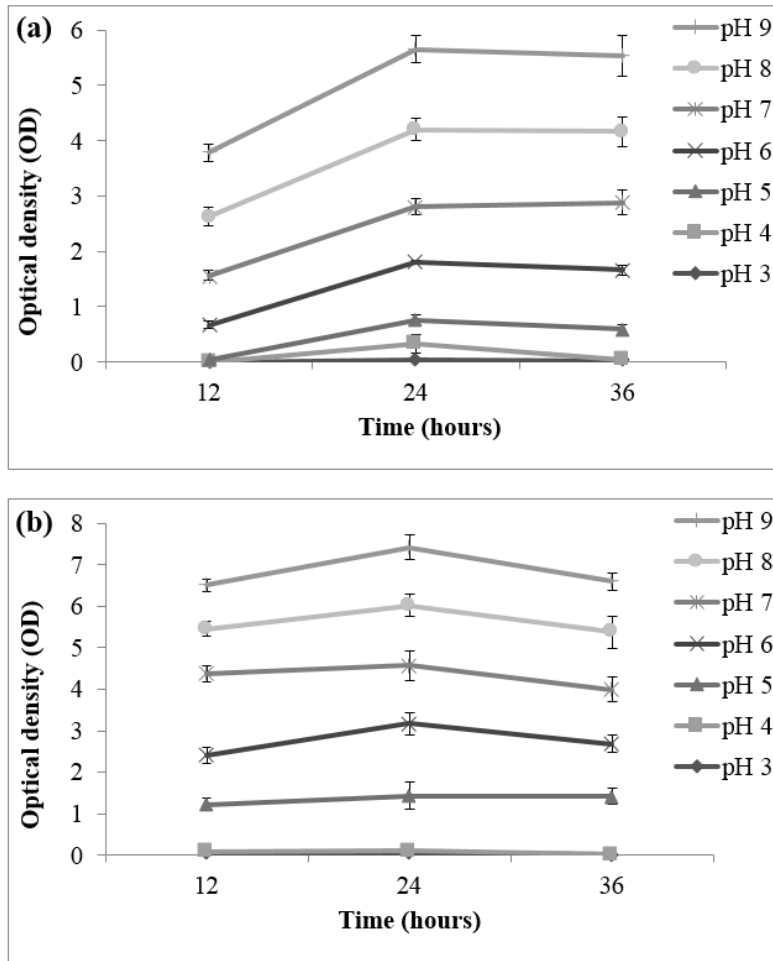
Figure 4. Cell growth of *Exiguobacterium* sp. (a) and *Stenotrophomonas* sp. (b) at different pH of culture solution. Bars indicate standard error, n=3.

was optimum at pH 8 (Figure 4a). However, the growth of *Stenotrophomonas* sp. was optimum at pH 6 and the highest growth was observed after 24 hours culturing (Figure 4b).

DISCUSSION

The bacterial populations of Kacip Fatimah differed between plant varieties and the location from which they were collected. The bacterial population in most of the plant parts except for phyllosphere was higher in var. *alata* than in var. *lanceolate*, whereas the population in phyllosphere for var. *lanceolata* was higher than for var. *alata* perhaps because the leaf surface of var. *lanceolata* was greater than that of var. *alata.* Variety *lanceolata* lives on a higher surrounding where the temperature is 29.58 $^{\circ}$C±0.25 $^{\circ}$C) and leaf temperature is 27.44

$^{\circ}$C±0.09 $^{\circ}$C as compared to 25.62 $^{\circ}$C±0.18 $^{\circ}$C of var. *alata*. Higher leaf temperature may increase leaf dehydration, reduce water availability and restrict phyllosphere bacterial populations as the bacterial populations within leaves are strongly correlated with water availability. Leaf water availability may have direct impact on bacterial growth as it can restrict bacterial growth (Freeman & Beattie, 2009). Most of the bacterial strains were Gram-positive and showed the ability to grow under fixed-nitrogen limiting conditions (N-free solid medium), indicating that there was strong possibility that the strains could fix nitrogen. Nitrogen fixation is a biological reaction where atmospheric N_2 is converted into NH_3; this is carried out by nitrogenase enzyme complex, which is activated under nitrogen-limiting conditions (Ladha & Reddy, 2000; Naher et al., 2011). The bacteria isolated from Kacip Fatimah could be said to be diazotrophs, which have the ability to fix nitrogen from the atmosphere and supply it to plants.

Phosphate-solubilising bacteria (PSB) produce organic acids to solubilise inorganic insoluble phosphorus. The starch hydrolysis test indicated the ability of bacterial strains to produce certain exoenzymes, including α-amylase, amylomaltase and α-glucosidase (Hii et al., 2012). The exoenzymes are able to degrade starch molecules into subunits that can be utilised as a source of carbon and energy for growth (Mutalib et al., 2012).

The catalase test was carried out to determine the ability of bacterial cultures to degrade hydrogen peroxide by producing the enzyme, catalase. Catalase-positive organisms use catalase to deactivate peroxide radicals, thus allowing them to survive unharmed within the host (Rao et al., 2003).

Bacterial strains can directly influence shoot development by secreting IAA as a plant-growth promoting hormone. The IAA is one of the most physiologically active auxins and several microorganisms including PGPB have the ability to produce IAA through the L-tryptophan metabolism. The IAA-producing bacteria use this phytohormone to interact with plants as part of the colonisation strategy, including phytostimulation and basal plant defense mechanisms (Spaepen et al., 2007). Several studies showed that IAA-producing bacteria promote plant growth and yield (Tsavkelova et al., 2007; Giongo et al., 2007). However, the growth of both bacterial strains was significantly affected by the pH culture. Generally ,strains were able to grow at the pH range of 5-9 and could not grow at the low pH level of pH 3. Similar findings were reported by Small et al. (1994).

The bacterial strain that possessed the most beneficial traits tested was *Exiguobacterium* sp., which has been characterised as a Gram-positive, facultative anaerobe that can grow profusely under aerobic conditions (Tatiana et al., 2014). Several researchers found that Exiguobacterium strains possess unique properties for application in biotechnology, bioremediation, industry and agriculture (Lee et al., 2009; Margesin & Feller, 2010; Kumar et al., 2010). This genus can also

survive in a wide range of temperatures from -12 to 55 ℃ and environmental conditions and have been isolated from different habitats (Vishnivetskaya & Kathariou, 2005; Vishnivetskaya et al., 2007). Hence, these two strains were able to survive well in a wide range of temperatures in the Permanent Reserve Forest Bukit Slim. Earlier studies also showed that *Exiguobacterium* sp. can influence growth and nutrient uptake of seedlings, improve yield and enhance the content of secondary metabolites of a salt-tolerant plant and wheat (Selvakumar et al., 2010; Bharti et al., 2013).

The other bacterial strain identified was *Stenotrophomonas* sp., which is a Gram-negative, aerobic bacterial strain that can be found in a wide range of environments particularly in close association with soil and plants (Kim et al., 2010; Ramos et al., 2011). *Stenotrophomonas* sp. has been isolated from various plants such as coffee (Nunes & de Melo, 2006), dune grass (Dalton et al., 2004) and medicinal herb (Lata et al., 2006). This strain can survive in a broad range of temperatures from 4-35 ℃, has potential to be used for bioremediation, phytoremediation, biocontrol and also as a plant-growth promoter (Liu et al., 2007; Ryan et al., 2009).

CONCLUSION

Two varieties of Kacip Fatimah were found to thrive in areas with quite similar soil chemical and physical properties in natural forest environments except for the altitude where var. *alata* was found in higher elevation compared to var. *lanceolata.* The study also showed that there is a diverse growth of microorganisms in Kacip Fatimah plants. Most of the bacterial strains isolated from *Labisia pumila* showed plant growth-promoting traits and several possessed multiple traits. Two of the bacterial isolates showing most of the beneficial properties were identified as *Exiguobacterium* sp. and *Stenotrophomonas* sp. These PGPB have the potential to enhance growth of Kacip Fatimah for future plant propagation.

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Impact of Daily Supplement of Probiotic on the Production Performance of Akar Putra Chickens

Hasan, S. A. Jawad[1,4]*, Lokman, I. H.[1], Saad, A. Naji[2], Zuki, A. B. Z.[1] and Kassim, A. B.[3]

[1]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[2]Department of Animal Resources, Faculty of Agriculture, University of Al-qadisiya, Iraq.
[3]Department of Animal Sciences, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia
[4]Department of Animal Production, Faculty of Agriculture, Baghdad University, Iraq

ABSTRACT

This study was conducted to investigate the effects of prepared probiotic (PP) on the live body weight, weight gain, feed intake and feed conversion ratio in Malaysian chicken (Akar Putra). A total of 72 day-old Akar Putra chicks were reared for 12 weeks and randomly assigned to three dietary treatments (24 chicken/treatment), with 3 replications for each (8 chicken/replicate). The treatments consisted of a control group (T1), and the supplemented diet with probiotic in the second treatment was prepared at the rate 1:1 (1 kg of commercial broiler feed + 1 g PP). While the rate was 1:2 (1 kg of commercial broiler feed + 2 g PP) in the third treatment. Supplementing probiotic in both rates revealed significant improvement in terms of males' and females' growth rates, final live body weight, weight gain and feed conversion ratio. Based on the research findings, the best results were obtained when chickens received 1 g PP in males and 2 g in females.

Keywords: Akar Putra chicken, probiotic, production performance

INTRODUCTION

Akar Putra is a Malaysian chicken species that is characterised by a slow growth rate compared to modern broiler chicken (Jawad et al., 2015). Since 2006, antibiotics has been banned for use as feed additives by the European Union because the continued use results in common problems such as

development of drug resistant bacteria, imbalance of normal microflora and drug residues in animal products (Chen et al., 2009). This necessitates the need for other alternatives like probiotics. Probiotics have become important as replacement feed additives (Steiner, 2006). A probiotics is a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. Probiotics have been classified as Generally Recognised as Safe (GRAS) by Food and Drug Administration (FDA). The concept of their use relates to maintaining the equilibrium of intestinal microflora by the addition of beneficial microorganisms (Goldin, 1998). Many studies have reported

the benefits of probiotic utilisation on productive indices (Cavazzoni et al., 1998; Jin et al., 1998). Dhama et al. (2012) and Al-Gharawi (2012) also reported that use of probiotics, including *Lactobacillus sp.*, improved the growth performance, feed efficiency, immunity parameters and disease resistance. The major probiotic strains include *Lactobacillus*, *Saccharomyces*, *Streptococcus* and *Aspergillus* (Tannock et al., 2001). At present, *Bacillus*, *Lactobacillus* and *Saccharomyces* are the major strains commonly used in broilers (Zhang et al., 2005; Chen et al., 2009).

The mixture of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium* and *Saccharomyces cerevisia* was used as

Table 1
Composition of basal diet

Items	Basal Diet	
	1 to 22 d	23 to 84 d
Corn	44.9	53.10
Wheat	18.0	15
Soybean meal (45%)	33	27
Mineral and vitamin premix	1	1
Oil	2	3
Limestone	0.8	0.6
Dicalcium phosphate	0.3	0.3
Total	100 %	100 %
Calculated analysis		
Crude protein (%)	21.92	19.70
Metabolism energy (kilo calorie per kg. Diet)	2990	3100
Calcium (%)	0.93	0.85
Phosphorus (%)	0.48	0.45
Methionine (%)	0.55	0.50
Lysine (%)	1.35	1.25
Methionine + Cysteine (%)	0.85	0.91
Folic acid	1.1	1.2

Calculated analysis according to NRC (1984).

probiotic for the first time by Lokman et al. (2015) when one and two gram of the prepared probiotic was fermented with the daily feed of Akar Putra chicken. The authors reported that noticeable enhancement in the production parameters was obtained especially for 2g of the probiotic. Moreover, a few studies have investigated the production effectiveness of adding a mixture of probiotics in the chicken diet. Thus, the present study was undertaken to evaluate the effects of daily supplementing probiotic including *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium* and *Saccharomyces cerevisia*, with feed (without fermentation) on the production performance of Malaysian chicken (Akar Putra).

MATERIALS AND METHODS

Preparation of Probiotic

Feed shown in Table 1 was offered *ad libitum* the same diets (1–13 days: starter; 14 day-slaughter: finisher) with continuous provision of water.

Prepared probiotic (PP) was made at Universiti Putra Malaysia (UPM). Each one g of PP contained at least 10^9 CFU (Colony Forming Unit) of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium* and at least 10^8 CFU of *Saccharomyces cerevisia*.

Chicken Husbandry and Experimental Design

The experiment was carried out at the poultry farm of the Faculty of Veterinary Medicine in Universiti Putra Malaysia (UPM), Malaysia, for the period of three months (15th December 2014 to 15th March, 2015). A total of 72 one-day old Akar Putra chicks were randomly assigned as (CRD) chicks in the three experimental groups were fed, as follows:

T1: Control group fed on dry feed (without probiotic supplementation).

T2: Fed on supplemented diet prepared at the rate 1:1 (1 kg of commercial broiler feed+ 1 g PP).

T3: Fed on supplemented diet prepared at the rate 1:2 (1 kg of commercial broiler feed+ 2 g PP).

Each treatment group was replicated three times with 8 chicks per replicate. The birds were housed in the battery cages with eight birds (4 males and 4 females) per pen (5"x 4"x1.5"). Since the chicks were reared in the open house, stable temperature, humidity and constant light schedule were therefore provided, along with *ad libitum* access of water and feed throughout the experiment. It is important to note that no vaccination was used during the whole experiment period.

Sampling Procedure and Analytic Methods

Body weight, weekly weight gain, feed intake and feed conversion ratio for the males and females were recorded separately from week 1 until week 12. Growth rate was calculated at the marketing age based on the formula proposed by Brody (1945). In the same regard, the variation ratio of the production performance parameters was calculated based on the formula, which was mentioned by Jawad et al. (2015).

Statistical Analysis

Data generated from the present experiment were subjected to statistical analysis using the General Linear Model (GLM) procedure of SAS (2001) statistical software package. When significant differences were noted, means were compared by using Duncan's multiple range tests (1955).

RESULTS AND DISCUSSION

The effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on the mean weekly body weight (g) of males and females Akar Putra chicks reared to 12 weeks of age are presented in Tables 2 and 3. Supplementing 1 and 2 g of PP caused improvement (P<0.01, P<0.05) in the final body weight of males and females chicken. The best results were

observed in T2 for males (1503.3g) and T3 for females (1274.7g). These findings are the opposite to the results described by Ahmad (2004), and Yousefi and Karkoodi (2007). These authors reported that the production parameters were not affected by the dietary probiotic and yeast supplementation. Alternatively, the results consistently showed that natural feed additives such as probiotic are very important materials that can improve, growth rate, daily weight gain, feed efficiency utilisation and productive performance (Wysong, 2003).

Total feed intake in males did not significantly differ between the groups receiving probiotic and the control group (Table 4), corroborating some previous results reported for feed intake at 21 days (Sato et al., 2002) and at 42 days of age (Mohan et al., 1996). Nevertheless, total

Table 2

Effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on mean weekly body weight (g) and growth rate gauge of males Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	62±2.887	62.333±2.728	63.667±2.963
2	104±2.887[b]	128±2.082[a]	126.333±2.333[a]
3	150±4.041[b]	217±3.215[a]	210.333±3.48[a]
4	277±6.928[b]	303.333±4.807[a]	306±5.292[a]
5	345±11.547[b]	443±9.074[a]	443.333±10.138[a]
6	499±14.434[b]	582.667±12.468[a]	609±13.577[a]
7	610±9.815[c]	737.333±9.244[b]	772.667±8.686[a]
8	869±11.547[b]	890±10.693[b]	988±9.866[a]
9	1037.667±15.103	1033±16.197	1059±15.373
10	1165±19.053	1157.333±18.478	1219±18.193
11	1290±20.207	1323.333±18.782	1324±19.348
12	1390±20.785[b]	1503.333±19.359[a]	1484±19.925[a]
Growth Rate	190.455±0.178[b]	191.837±0.242[a]	191.132±0.251[ab]

• Mean values with common superscript in row differ significantly (P < 0.01).
• Mean values at weeks 4 and 12 differ significantly (P<0.05).
• The values of growth rate differ significantly (P<0.05).

Table 3

Effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on mean weekly body weight (g) and growth rate gauge of females Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	61.667±3.756	63.733±3.813	65±4.041
2	104.2±3.062[b]	129.667±3.48[a]	128±3.786[a]
3	178.3±4.304[b]	218.333±4.333[a]	211.667±4.631[a]
4	276.667±6.642[b]	305.333±6.36[a]	307±6.083[a]
5	344.667±11.26[b]	444.667±10.414[a]	444.333±10.975[a]
6	468.333±13.86	517.667±14.146	507±14.434
7	516.667±9.528[b]	640±9.815[a]	626±8.963[a]
8	624.267±11.779[c]	810±11.547[a]	741.667±11.26[b]
9	714.667±17.61[b]	856±17.898[a]	863±17.039[a]
10	815.333±18.478[b]	968.333±17.629[a]	978±19.053[a]
11	876.667±19.919[b]	1075±20.207[a]	1124±19.348[a]
12	937.333±20.21[c]	1201±20.785[b]	1274.667±20.497[a]
Growth Rate	186.155±0.522[b]	189.405±0.481[a]	189.328±0.52[a]

•   Mean values with common superscript in row differ significantly ($P < 0.01$).
•   Mean values at week 4 differ significantly ($P<0.05$).

Table 4

Effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on weekly feed consumption (g) of males Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	44±4.041	44.333±3.48	43.667±3.756
2	82±2.887[b]	110.167±3.032[a]	105.667±2.603[a]
3	126±6.928[b]	174.333±6.36[a]	140±6.083[b]
4	196±5.196[c]	219±4.359[b]	254.333±4.631[a]
5	270±6.928[a]	254.333±4.807[ab]	242.667±5.044[b]
6	269±9.815[b]	218.333±9.244[c]	348±8.963[a]
7	407±11.547[b]	406±10.693[b]	504.333±10.975[a]
8	410±13.279[c]	279±12.423[b]	534.333±12.706[a]
9	500±12.124[b]	627.667±10.99[a]	352.333±11.552[c]
10	440±14.434[b]	386±13.577[c]	585.667±14.146[a]
11	534±16.166[a]	425.333±15.592[b]	375±15.308[b]
12	507±15.588[a]	410.333±15.015[b]	484±14.731[a]
Total	3785±118.934	3554.833±109.406	3970±110.387

•   Mean values with common superscript in row differ significantly ($P < 0.01$).
•   Mean values at weeks 5 and 12 differ significantly ($P<0.05$).

Table 5

Effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on weekly feed consumption (g) of females Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	44.1±4.128	44.667±3.756	44±4.041
2	82.3±3.15[b]	109.667±2.603[a]	106±2.887[a]
3	125.333±6.36[b]	174.667±6.642[a]	141±6.928[b]
4	195.667±4.91[c]	220±5.196[b]	253.667±4.096[a]
5	230.667±6.642	255.667±5.812	245±6.928
6	276.333±9.244[a]	244±8.963[a]	183±9.815[b]
7	248.333±10.975[c]	453.667±10.414[a]	304.667±9.597[b]
8	289.667±12.991[b]	433.667±11.319[a]	202±12.423[c]
9	266.667±11.837[b]	90.333±10.713[c]	426.333±11.552[a]
10	357.667±14.146[b]	501.333±13.017[a]	301±13.577[c]
11	260±15.308[b]	373±14.468[a]	374.667±15.026[a]
12	307.333±14.17[b]	400.333±13.346[a]	320.333±12.548[b]
Total	2684.067±113[b]	3301±106.209[a]	2901.667±109.266[b]

- Mean values with common superscript in row differ significantly ($P < 0.01$).
- The total feed consumption value differ significantly ($P < 0.05$).

feed intake was slightly higher when 1 g probiotics were administered in females (Table 5), thus corroborating the previous finding by Lokman et al. (2015).

Tables 6 and 7 show that superiority in the weight gain for birds receiving probiotics than the control group starting from the starter phase (1-21 days). These findings are in contrast to the results reported in previous trials by Fethiere and Miles (1987), Maiorka et al. (2001) and Sato et al. (2002). Moreover, the distinction continued from the growing period until the marketing age.

Noticeable ($p<0.01$) enhancement in the total feed conversion ratio was observed in supplementing 1 g PP with diet in males and 2 g PP with diet in females. This improvement in feed conversion ratio was the principal reason to improve the weight

gain indexes since almost the treatments had similar feed intake. These findings are similar to the results described by Jin et al. (1998), Besnard et al. (2000), and Ayanwale et al. (2006). The authors reported worse feed conversion in the control group when compared to groups of broilers, and turkeys fed probiotics based on *Lactobacillus* sp and *Saccharomyces cerevisiae* in the diets, respectively.

Birds fed probiotics had lower feed intake ($p<0.01$) associated to improve the feed conversion in almost the evaluated periods ($p<0.01$), which were decisive to result in the high weight gain ($p<0.01$) seen in these birds. Although the significant differences in performance were observed between these groups in the finishing phase (36-84 days), the increase ($p<0.05$) in the

Table 6
Effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on weekly weight gain of males Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	28±1.732	31±1.732	30±1.732
2	42±0[c]	65.667±0.667[b]	62.667±0.667[a]
3	46±1.155[c]	89±1.155[b]	84±1.155[a]
4	127±2.887[a]	86.333±1.667[c]	95.667±1.856[b]
5	68±4.619[b]	139.667±4.333[a]	137.333±4.91[a]
6	154±2.887[b]	139.667±3.48[c]	165.667±3.48[a]
7	111±4.619[b]	154.667±3.283[a]	163.667±4.91[a]
8	259±1.732[a]	152.667±1.453[c]	215.333±1.202[b]
9	168.667±3.844[a]	143±5.508[b]	71±5.508[c]
10	127.333±4.372[b]	124.333±2.404[b]	160±3.055[a]
11	125±1.155[b]	166±0.577[a]	105±1.155[c]
12	100±0.577[c]	180±0.577[a]	160±0.577[b]
Total	1356±19.63[b]	1472±18.475[a]	1450.333±18.765[a]

- Mean values with common superscript in row differ significantly ($P < 0.01$).
- The total weight gain value differ significantly ($P<0.05$).

Table 7
EFFECTS of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on weekly weight gain of females Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	28±1.732	31±1.732	30±1.732
2	42.533±0.742[c]	65.933±0.581[a]	63±0.577[b]
3	74.1±1.242[c]	88.667±0.882[a]	83.667±0.882[b]
4	98.367±2.36[a]	87±2.082[b]	95.333±1.667[a]
5	68±4.619[b]	139.333±4.055[a]	137.333±4.91[a]
6	123.667±2.603[a]	73±3.786[b]	62.667±3.48[b]
7	48.333±4.333[b]	122.333±4.333[a]	119±5.508[a]
8	107.6±2.272[c]	170±1.732[a]	115.667±2.333[b]
9	90.4±5.839[b]	46±6.351[c]	121.333±5.783[a]
10	100.667±0.882[b]	112.333±0.882[a]	115±2.082[a]
11	61.333±1.453[c]	106.667±2.728[b]	146±0.577[a]
12	60.667±0.333[c]	126±0.577[b]	150.667±1.202[a]
Total	903.667±18.187[c]	1168.267±18.707[b]	1239.667±18.187[a]

- Mean values with common superscript in row differ significantly ($P < 0.01$).
- Mean values at week 4 differ significantly ($P<0.05$).

Table 8
Effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on weekly feed conversion ratio (g .feed/ g .gain) of males Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	1.566±0.048	1.426±0.035	1.451±0.043
2	1.952±0.069[a]	1.679±0.061[b]	1.687±0.058[b]
3	2.735±0.082[a]	1.957±0.046[b]	1.665±0.05[c]
4	1.543±0.006[c]	2.537±0.02[b]	2.659±0.015[a]
5	3.994±0.17[a]	1.822±0.024[b]	1.769±0.029[b]
6	1.746±0.031[b]	1.562±0.029[c]	2.1±0.013[a]
7	3.688±0.258[a]	2.63±0.123[b]	3.091±0.16[ab]
8	1.582±0.041[c]	1.826±0.064[b]	2.481±0.046[a]
9	2.965±0.031[c]	4.396±0.094[b]	4.998±0.234[a]
10	3.456±0.046[b]	3.103±0.056[c]	3.66±0.033[a]
11	4.27±0.09[a]	2.562±0.09[c]	3.569±0.107[b]
12	5.069±0.127[a]	2.279±0.076[c]	3.024±0.081[b]
Total	2.79±0.047[a]	2.414±0.044[b]	2.736±0.041[a]

- Mean values with common superscript in row differ significantly (P < 0.01).
- Mean values at weeks 2 and 7 differ significantly (P<0.05).

Table 9
Effects of diet supplementation with probiotic at the rate of (1 and 2g PP: 1 Kg food) on weekly feed conversion ratio (g .feed/ g .gain) of females Akar Putra chicks reared to 12 weeks.

Week	Treatments		
	T1	T2	T3
1	1.569±0.051	1.436±0.042	1.461±0.051
2	1.938±0.105	1.664±0.05	1.683±0.055
3	1.689±0.058[b]	1.969±0.056[a]	1.684±0.066[b]
4	1.989±0.005[c]	2.529±0.014[b]	2.661±0.016[a]
5	3.41±0.135[a]	1.836±0.012[b]	1.785±0.013[b]
6	2.233±0.028[c]	3.348±0.055[a]	2.921±0.016[b]
7	5.263±0.7[a]	3.724±0.217[b]	2.579±0.201[b]
8	2.689±0.065[a]	2.55±0.041[a]	1.744±0.073[b]
9	2.958±0.062[b]	1.974±0.041[c]	3.521±0.074[a]
10	3.551±0.11[b]	4.464±0.131[a]	2.615±0.074[c]
11	4.232±0.152[a]	3.495±0.063[b]	2.566±0.098[c]
12	5.064±0.21[a]	3.176±0.092[b]	2.125±0.068[c]
Total	2.968±0.066[a]	2.824±0.046[a]	2.339±0.054[b]

- Mean values with common superscript in row differ significantly (P < 0.01).
- Mean values at weeks 3 and 7 differ significantly (P<0.05).

growing rate was enough to positively influence the performance of birds fed probiotics in the total period of rearing (1-84 days). Similar results were obtained when fermented feed with probiotic in a dry form was used as a daily diet of Akar Putra chickens (Lokman et al., 2015). The results of that experiment revealed remarkably significant ($P<0.01$) enhancement for supplementing treatments than the control group in all of the males' and females' body weight, weight gain, feed intake and feed conversion ratio measurements. Furthermore, the best results were obtained in the chickens fed on dry feed mixture with 1g of probiotic. Moreover, such results corroborate the findings of Santoso et al. (1995), Yeo and Kim (1997), and Cavazzoni et al. (1998), but are nevertheless opposite to those reported by Buenrostro and Kratzer (1983).

With regard to the growth rate criteria (see Tables 2 and 3), the males and females of T2 and T3 treatments outperformed the control group in the growth rate criteria values. The males' growth rate variations ratio of T2 and T3 than the males in control group was 1.315% and 0.486% respectively. Meanwhile, the variation ratios of the growth rate in females were 1.898% for T2 and 1.335% for T3. Genetic and non-genetic factors are controlling growth trait in animals (Selvaggi et al., 2015). Growth in the domestic chicken is commonly measured by body weight and body conformation, which are the most important parameters. The techniques included in the control for the growth in chickens are too complex to be explained only under univariate analysis because all related traits are biologically correlated due to the pleiotropic effect of genes and linkage of loci (Udeh & Ogbu, 2011). Consequently, and based on the view point of animals genetic and improvements, the principal components such as growth rate and live body weight are simultaneously considered as a group of attributes, which may be used for selection purpose (Pinto et al., 2006).

Based on the results of this research, it can be concluded that supplementing 1 and 2g of prepared probiotic caused dependent improvement of the production performance in Akar Putra chickens. Furthermore, the best results were obtained when chickens received 1 g PP in males and 2 g in females. The prominent influence of the probiotic was shown in the live body weight, as well as the growth rate traits.

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595

TROPICAL AGRICULTURAL SCIENCE

The Effects of Fermented Feed on Broiler Production and Intestinal Morphology

Saad A. Naji[1], I. F. B. Al-Zamili[2], Hasan, S. A. Jawad[3, 4]* and J. K. M. Al-Gharawi[2]

[1]*Department of Animal Resource, Faculty of Agriculture, Al-Qadisia University, Iraq*
[2]*Department of Animal Resource, Faculty of Agriculture, Al-Mthanna University, Iraq*
[3]*Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[4]*Department of Animal Production, Faculty of Agriculture, Baghdad University, Iraq*

ABSTRACT

The present experiment was conducted to evaluate the effects of wet feed and fermented feed on the intestinal morphology and histology of broiler chicks. A total of 360 one day old Ross 308 broiler chicks were randomly assigned (CRD) into six treatment groups. Chicks were fed: (T1) Control group of dry feed; (T2) Fed on wet feed (1:1, feed: water); (T3) 25% fermented feed + 75% dry feed; (T4) 50% fermented feed + 50% dry feed; (T5) 75% fermented feed + 25% dry feed and (T6) 100%, fermented feed throughout the experimental period. Each treatment group was replicated three times using 20 chicks per replicate. The chicks were raised at a temperature and in humidity controlled room with a 24-h. constant light and had ad. libitum access to water and feed throughout the experimental period which lasted for six weeks. The results showed that all diets containing fermented feed, especially 100%, had dependent effects on the evaluated production characteristics. The feeds had significantly ($P < 0.05$) increased the relative weight and length of the small intestine. Likewise, it raised the villi height, crypt depth and the percentage of the villi height to crypt depth in duodenum, jejunum and ileum increased. In conclusion, the results of the current experiment indicated that fermented feed with probiotic would be beneficial economically since the broiler feed conversion ratio had been improved (2.4% in T2; 4.1% in T3; 5.3% in T4; 5.3% in T5 and 7.7% in T6) as a consequence to enhance their intestinal morphology.

Keywords: Fermented feed, broiler, intestinal morphology, histology

INTRODUCTION

The main concept of fermenting feed with probiotic is to improve the quality of feed. That application was practiced by Lokman et al. (2015) when one and two grams of prepared probiotic was fermented with the daily feed of local Malaysian chicken (Akar Putra). The authors reported that noticeable enhancement in the production parameters especially when 2 grams of probiotic was used. Fermentation process could improve the nutritive value of the soybean (Mathivanan et al., 2006), copra meals (Hatta & Sundu, 2009) and tofu waste (Rasud, 2009). Fermented feed influenced the bacterial ecology of gastrointestinal tract and reduced the level of *Enterobacteriaceae* in the different parts of gastrointestinal tract in pigs (Winser et al., 2001) and broiler chicks (Heres et al., 2003). *Lactobacilli* and yeast in the kefir, which supplemented in drinking water, significantly increased the population of *Lactobailli* spp. and total aerobic bacteria, thus decreasing the population of *Enterobactciaceae* and coliform in the goose intestine (Yaman et al., 2006). Primarily fermented feed caused a reduction of pathogenic bacteria, including *Salmonella* and *Campylobacter* in the digestive tract, most particularly in the crop and gizzard. Since the crop often ruptures during slaughter, the decrease level of pathogens in this area, in particular, makes contamination of meat less likely (Donkor et al., 2006).

Antibiotic has been used as feed additive to improve growth performance and control disease in animals. However, the continued use of antibiotics has resulted in common problems such as the development of drug-resistant bacteria, imbalance of normal microflora and drug residues in animal products (Chen et al., 2009). Since 2006, antibiotics have been banned for use as feed additives in the European Union. Probiotics has therefore become important as the replacement of feed additives (Steiner, 2006). It is a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. After feeding of probiotics, improvements in growth performance, feed efficiency, immunity parameters and disease resistance have been reported (Al-Gharawi, 2012). The major probiotic strains include *Lactobacillus*, *Saccharomyces*, *Streptococcus* and *Aspergillus* (Tannock et al., 2001). At present, *Bacillus*, *Lactobacillus* and *Saccharomyces* are the major strains applied in broilers (Zhang et al., 2005; Chen et al., 2009).

Since very few investigations on the fermentation of broiler feed with probiotic have been done, therefore, the present study was undertaken to evaluate the effects of fermented feed and fermented feed with probiotic on the broiler production performance, as well as the intestinal morphology and histology of broiler chicken.

MATERIALS AND METHODS

Preparation of the Fermented Feed

A commercial broiler starter and finisher diet (Table 1) was purchased from local markets. The chicks were fed the starter diet during

the first three weeks, followed by the finisher diet until 6 weeks.

Whole starter and finisher feed were fermented with and without the addition of probiotic. The feed was moistened with water (1 liter water: 1 kg feed), placed in a plastic tray and inoculated with prepared probiotic (PP) at the rate 10 grams of PP for each one kilogram of feed. Then, the plastic trays were kept and incubated in a small room for 48 h. at 37±2ºC.

The PP was purchased from laboratory of poultry technology at the Agriculture Faculty, University of Baghdad. According to the manufacture information label, each gram of PP contained at least 10^9 cfu of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium* and at least 10^8 cfu of *Saccharomyces cervisia*. Fermented feed was characterised by high lactic acid concentration (up to 260 mmol/ kg feed) and moderate amounts of acetic acid (20-30 mmol/ kg feed), high number of lactic acid bacteria (Log 9-10 cfu/ g. feed) and pH of approximately 4.5-5.0, as described by Cutlure et al. (2005).

Broiler Husbandry and Experimental Design

The experiment was carried out at the poultry research farm in the Faculty of Agriculture, University of Al-Mothanna. A total of 360 one-day-old Ross308 broiler chicks were randomly assigned (CRD) into six experimental groups, which were daily fed, as follows:

T1: Control group fed on dry feed.
T2: Fed on wet feed (1:1, feed: water).

T3: 25% fermented feed + 75% dry feed.
T4: 50% fermented feed + 50% dry feed.
T5: 75% fermented feed + 25% dry feed.
T6: 100% fermented feed.

Each treatment group was replicated three times with 20 chicks per replicate. The chicks were reared in battery cages (1.5 × 1.0 m) with four tiers. The chicks were raised in a temperature and humidity controlled room with a 24-h. constant light and had *ad. libitum* access to water and feed throughout the experiment.

Sampling Procedure and Analytic Methods

Production parameters, which included final body weight and weekly feed conversion ratio, were recorded. At the end of the feeding trial, six birds per treatment were selected at random and slaughtered for sampling. The intestinal parts' weight and length and relative parts; weight and length were calculated according to live body weight.

The intestinal parts were separated carefully; duodenal loop, jejunum the middle sections of the intestine between the duodenum and mackles diverticulum, and ileum, which are located between mackles diverticulum to the ileocecal junction. The cecum was also separated from the site of ileocecal junction and their relative weight and length were calculated. Samples of the intestinal parts were taken for histological study. The samples were fixed at 10%

Table 1
Composition of basal diet.

Items	Basal Diet	
	1 to 22 d	23 to 35 d
Corn	44.9	53.10
Wheat	18.0	15
Soybean meal (45%)	33	27
Mineral and vitamin premix	1	1
Oil	2	3
Limestone	0.8	0.6
Dicalcium phosphate	0.3	0.3
Total	100 %	100 %
Calculated analysis		
Crude protein (%)	21.92	19.70
Metabolism energy (kilo calorie per kg. Diet)	2990	3100
Calcium (%)	0.93	0.85
Phosphorus (%)	0.48	0.45
Methionine (%)	0.55	0.50
Lysine (%)	1.35	1.25
Methionine + Cysteine (%)	0.85	0.91
Folic acid	1.1	1.2

Produced by Ghadeer Babylon, calculated analysis according to NRC (1994).

buffered formalin and embedded in paraffin. Three micron sections were microtome cut and stained with haematoxylin and eosin. Slides were measured by light microscopy to measure crypt depth, villi height and villi height/ crypt depth. Measurements of villus height and crypt depth were taken only from the sections where the plane of section ran vertically from the top of villus to the base of an adjacent crypt. Values presented are means from the seven samples of villi measured from the tip to the crypt mouth and seven associated crypts measured from the crypt mouth to the base (Xu et al., 2003).

Statistical Analysis

Data generated from the present experiment were subjected to statistical analysis using the GLM procedure of SAS (SAS, 2001) statistical software package. When significant differences were noted, means were compared using Duncan's multiple range tests (Duncan, 1955).

RESULTS

There was noticeable significant interaction for using fermented feeds with probiotic compared to the control group. The results revealed that using 25%, 50%, 75% and 100% fermented feed with prepared probiotic (PP) in the daily feed had dependent effects on the evaluated characteristics. Chicken in

T6 achieved higher (P < 0.01) body weight (2092.87 g), followed by T5 (1971.44 g), T4 (1901.99 g), T3 (1862.97 g), T2 (1799.88 g) and the control (1700.30 g). Meanwhile, chicken received fermented feed with PP exhibited lower (P < 0.05) feed conversion ratio than the control group at the end of the experiment (Table 2).

Morphological measurements of the duodenum, jejunum, ileum and cecum are shown in Table 3 and Table 4. Birds fed on fermented feed had higher (P ≤ 0.05) relative weight and relative length of duodenum, jejunum, ileum and cecum when compared

with birds fed on the control dry feed. As the percentages of the fermented feed in the diet increased, these parameters were significantly (P ≤ 0.05) increased.

Dietary treatment had a significant (P ≤ 0.05) effect on villus height, crypt depth, and villus height to the crypt depth ratio in the duodenum (Table 5), jejunum (Table 6) and ileum (Table 7). Birds fed on the fermented feed had higher (P ≤ 0.05) villi height and crypt depth in the duodenum, jejunum, and ileum than birds fed control dry feed. These birds also had a higher (P ≤ 0.05) villi high to the crypt depth ratio in all these three parts.

TABLE 2
The effect of 25%, 50%, 75% and 100% fermented feed with probiotic on the feed conversion ratio FCR (g food/ g weight gain) of broiler.

Treatments	Week					Mean FCR
	1	2	3	4	5	
T1	1.57± 0.01[a]	1.60± 0.03[a]	1.66± 0.03[a]	1.73± 0.02[a]	1.87± 0.03[a]	1.69± 0.04[a]
T2	1.54±0.02[ab]	1.57± 0.02[ab]	1.63± 0.03[ab]	1.70± 0.04[ab]	1.80± 0.02[b]	1.65± 0.02[b]
T3	1.52± 0.03[ab]	1.55± 0..01[b]	1.61± 0.01[b]	1.67± 0.01[bc]	1.75± 0.03[c]	1.62± 0.03[b]
T4	1.51± 0.01[b]	1.54± 0.03[b]	1.60± 0.03[bc]	1.65± 0.03[c]	1.72± 0.02[c]	1.60± 0.02[b]
T5	1.51± 0.02[b]	1.54± 0.02[b]	1.59± 0.01[bc]	1.64± 0..01[cd]	1.70± 0.01[c]	1.60± 0.03[b]
T6	1.49± 0.02[b]	1.52± 0.03[b]	1.56± 0.02[c]	1.60± 0.02[d]	1.65± 0.03[d]	1.56± 0.01[c]
Significant level	*	*	*	*	*	*

T1: Control group fed on dry feed. **T2:** fed on wetting feed (1:1, feed: water). **T3:** 25% fermented feed + 75% dry feed. **T4:** 50% fermented feed + 50% dry feed. **T5:** 75% fermented feed + 25% dry feed. **T6:** 100% fermented feed throughout the experimental period. Mean values with different superscripts ([a,d]) in columns differ significantly (P < 0.05).

Table 3

The effect of 25%, 50%, 75% and 100% fermented feed with probiotic on the relative weight of the small intestine and cecum (%) of broiler.

Treatments	The relative weight				
	The small intestine	Duodenum	jejunum	Ileum	Cecum
T1	3.7 ± 0.031^d	0.5 ± 0.007^d	1.46 ± 0.021^d	1.7 ± 0.018^d	0.45 ± 0.004^c
T2	4.1 ± 0.026^c	0.69 ± 0.005^c	1.5 ± 0.012^c	1.84 ± 0.022^c	0.50 ± 0.005^c
T3	4.80 ± 0.022^b	0.84 ± 0.008^b	1.8 ± 0.021^b	2.0 ± 0.020^b	0.57 ± 0.006^b
T4	4.90 ± 0.020^b	0.8 ± 0.007^b	1.8 ± 0.023^b	2.13 ± 0.018^b	0.6 ± 0.005^b
T5	4.96 ± 0.015^a	0.8 ± 0.016^b	1.9 ± 0.010^b	2.16 ± 0.022^b	0.6 ± 0.007^b
T6	5.7 ± 0.008^a	1.14 ± 0.007^a	2.17 ± 0.009^a	2.4 ± 0.011^a	0.72 ± 0.009^a
Significant level	*	*	*	*	*

T1: Control group fed on dry feed. **T2:** fed on wetting feed (1:1, feed: water). **T3:** 25% fermented feed + 75% dry feed. **T4:** 50% fermented feed + 50% dry feed. **T5:** 75% fermented feed + 25% dry feed. **T6:** 100% fermented feed throughout the experimental period. Mean values with different superscripts ([a,d]) in columns differ significantly (P ≤ 0.05).

Table 4

The effect of 25%, 50%, 75% and 100% fermented feed with probiotic on the relative length of the small intestine and cecum (cm/ 100 g body weight) of broiler.

Treatments	The relative weight				
	The small intestine	Duodenum	jejunum	Ileum	Cecum
T1	9.97 ± 0.14^c	1.66 ± 0.19^d	3.96 ± 0.40^c	4.35 ± 0.42^c	1.01 ± 0.1^c
T2	10.1 ± 0.18^c	1.70 ± 0.21^c	4.02 ± 0.43^c	4.41 ± 0.47^c	1.04 ± 0.11^c
T3	10.8 ± 0.16^b	1.86 ± 0.19^b	4.18 ± 0.47^b	4.78 ± 0.51^b	1.14 ± 0.13^b
T4	10.9 ± 0.21^b	1.89 ± 0.17^b	4.22 ± 0.37^b	4.80 ± 0.43^b	1.15 ± 0.11^b
T5	10.9 ± 0.16^b	1.91 ± 0.2^b	4.24 ± 0.5^b	4.83 ± 0.48^b	1.21 ± 0.17^b
T6	11.3 ± 0.21^a	2.07 ± 0.18^a	4.42 ± 0.22^a	4.96 ± 0.32^a	1.34 ± 0.14^a
Significant level	*	*	*	*	*

T1: Control group fed on dry feed. **T2:** fed on wetting feed (1:1, feed: water). **T3:** 25% fermented feed + 75% dry feed. **T4:** 50% fermented feed + 50% dry feed. **T5:** 75% fermented feed + 25% dry feed. **T6:** 100% fermented feed throughout the experimental period. Mean values with different superscripts ([a,d]) in columns differ significantly (P ≤ 0.05).

Table 5
The effect of 25%, 50%, 75% and 100% fermented feed with probiotic on villus height, crypt depth (μm) and the ratio of villus height to crypt depth in the duodenal of broiler.

Treatments	Villus height (μm)	Crypt depth (μm)	The ratio of villus height to crypt depth
T1	117.42 ±1.15[c]	15.35 ± 0.18[c]	7.65 ± 0.05[c]
T2	122.33 ± 2.06[c]	15.59 ± 0.21[c]	7.85 ± 0.05[c]
T3	141.16 ± 1.23[b]	17.52 ± 0.19[b]	8.06 ± 0.04[b]
T4	146.21 ± 2.33[b]	17.72 ±0.18[b]	8.25 ± 0.08[b]
T5	150.05 ± 1.17[b]	17.97 ± 0.2[b]	8.35 ± 0.07[b]
T6	166.36 ± 1.42[a]	18.82 ± 0.18[ab]	8.84 ± 0.05[a]
Significant level	*	*	*

T1: Control group fed on dry feed. **T2:** fed on wetting feed (1:1, feed: water). **T3:** 25% fermented feed + 75% dry feed. **T4:** 50% fermented feed + 50% dry feed. **T5:** 75% fermented feed + 25% dry feed. **T6:** 100% fermented feed throughout the experimental period. Mean values with different superscripts ([a,c]) in columns differ significantly (P ≤ 0.05).

Table 6
The effect of 25%, 50%, 75% and 100% fermented feed with probiotic on villus height, crypt depth (μm) and the ratio of villus height to crypt depth in the jejunum of broiler.

Treatments	Villus height (μm)	Crypt depth (μm)	The ratio of villus height to crypt depth
T1	101.12 ±1.07[c]	14.32 ± 0.15[c]	7.06 ± 0.09[c]
T2	103.67 ± 1.12[c]	14.44 ± 0.12[c]	7.18 ± 0.07[c]
T3	113.22 ± 1.61[b]	15.18 ± 0.13[b]	7.46 ± 0.06[b]
T4	115. 17 ± 1.22[b]	15.25 ± 0.21[b]	7.55 ± 0.05[b]
T5	117.20 ± 1.16[b]	15.37 ± 0.2[b]	7.62± 0.06[b]
T6	132. 18 ± 1.36[a]	16.41 ± 0.19[a]	8.05 ± 0.08[a]
Significant level	*	*	*

T1: Control group fed on dry feed. **T2:** fed on wetting feed (1:1, feed: water). **T3:** 25% fermented feed + 75% dry feed. **T4:** 50% fermented feed + 50% dry feed. **T5:** 75% fermented feed + 25% dry feed. **T6:** 100% fermented feed throughout the experimental period. Mean values with different superscripts ([a,c]) in columns differ significantly (P ≤ 0.05).

Table 7

The effect of 25%, 50%, 75% and 100% fermented feed with probiotic on villus height, crypt depth (μm) and the ratio of villus height to crypt depth in the ileum of broiler.

Treatments	Villus height (μm)	Crypt depth (μm)	The ratio of villus height to crypt depth
T1	41.14 ±0.53[c]	8.62 ±0.79[c]	4.77 ± 0.05[c]
T2	41.77 ± 0.6[c]	8.70 ± 0.68[c]	4.80 ± 0.04[c]
T3	50.18 ± 0.54[b]	9.81 ± 0.72[b]	5.12 ± 0.06b[c]
T4	51.25 ± 0.65[b]	9.87 ± 0.71[b]	5.19 ± 0.07[b]
T5	53.09 ±0.49[b]	10.11 ± 0.81[b]	5.25 ± 0.06ab
T6	61.55 ± 0.56[a]	11.04 ± 0.64[a]	5.58 ± 0.05[a]
Significant level	*	*	*

T1: Control group fed on dry feed. **T2:** fed on wetting feed (1:1, feed: water). **T3:** 25% fermented feed + 75% dry feed. **T4:** 50% fermented feed + 50% dry feed. **T5:** 75% fermented feed + 25% dry feed. **T6:** 100% fermented feed throughout the experimental period. Mean values with different superscripts ([a,c]) in columns differ significantly (P ≤ 0.05).

DISCUSSION

Dependent effects on production parameters were achieved by using 25%, 50%, 75% and 100% fermented feed with prepared probiotic (PP) in the daily broiler feed. The positive effects of probiotics to the Akar Putra chicken also used *Lactobacillus acidophilus*, *Bacillus subtilis*, *Bifidobacterium* and *Saccharomyces cervisia* with dry fermented feed (Lokman et al., 2015) and wet fermented feed (Jawad et al., 2016). This result could be strongly supported when the worst feed conversion ratio was reported for the control group compared to the groups of layer and turkeys fed probiotics based on *Lactobacillus* sp. and *Saccharomyces cerevisiae* in the diet (Besnard et al., 2000; Ayanwale et al., 2006; Lokman et al., 2015). Adversely, Mutus et al. (2006) did not find any influence of probiotic to feed conversion ratio. Furthermore, Ahmad (2004), and Yousefi and Karkoodi (2007) reported that broiler feed intake and feed conversion ratio

was not affected by the dietary probiotic and yeast supplementation. Some researchers reported that when chicks were housed in a clean environment, probiotic did not affect performance (Anderson et al., 1999).

In the current study, the morphology and histology parameters of the digestive tract parts were improved in the broiler chicks feeding on fermented feed than the broilers feeding on control diet. These results are similar to those reported by Chaing et al. (2010) and Xu et al. (2012).

The villus height to crypt depth is a very useful measure to estimate the absorption capacity of the small intestine. Maximum digestion and absorption are believed to occur as villus height to the crypt depth ratio increased (Chaing et al., 2010). Changes in the intestinal morphology such as reduced villus height and deeper crypt may also indicate the presence of toxins (Xu et al., 2003). In the present study, increased villus height and increased villus height

to the crypt depth ratio were observed in the broilers feeding on fermented feed compared with unfermented feed. The increased villi height and villus height to the crypt depth ratio might be associated with the increased number of beneficial bacteria like *Lactobcilli*, *Bifidobacterium*, *Bacillus subtilis* and *Sacchromyces cervisia* (Xu et al., 2003; Naji & Al-Mosawi, 2014). Fermented feed was characterised by a high number of lactic acid bacteria (Log 9-10 cfu/ g feed) and pH of approximately 4.5-5.0, as described by Cutlure et al. (2005). The increased villus height and villus height to the crypt depth ratio produced an intestinal structure more oriented to digestion, with improved absorptive and hydrolysis potential, as well as fewer nutrients requirement for intestinal maintenance. Thus, the intestinal structures of duodenum, jejunum and ileum are more favourable for the bird and may help to explain the improvements in weight gain and feed conversion (Feng et al., 2007a). The good results obtained from the solid state fermentation feed might be attributed to the higher production of secondary microbial metabolites during fermentation. These metabolites include organic acid (lactic acid) produced by Lactobacilli, enzymes (amylase and protease) and antimicrobial substances like iturin and surfactin produced by *Bacillus subtilis* bacteria during solid fermentation (Feng et al., 2007b).

Conclusively, the results of this study indicated that using fermented feeds with probiotic caused significant improvement in the intestinal structure of the broilers, including villus height and villus height to the crypt depth ratio, as well as higher body weight and improvement in the feed conversion ratio.

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607

Characteristics and Potential Usage of Dissolved Silica in Rice Cultivation in Sumani Watershed, Sumatra, Indonesia

Hiroaki Somura[1]*, Darmawan[2], Kuniaki Sato[1], Makoto Ueno[1], Husnain[3], Aflizar[4] and Tsugiyuki Masunaga[1]

[1]*Shimane University, Faculty of Life and Environmental Science, 1060 Nishikawatsu, Matsue, Shimane 6908504, Japan*
[2]*Andalas University, Faculty of Agriculture, Padang 25163, West Sumatra, Indonesia*
[3]*Indonesian Soil Research Institute, Bogor 16114, West Java, Indonesia*
[4]*State Polytechnic Payakumbuh for Agriculture, Payakumbuh 26271, West Sumatra, Indonesia*

ABSTRACT

Research on watershed silica dynamics in Indonesia has been sparse as most of the focus on water environment has centred on suspended sediments, nitrogen and phosphorous. Thus, Si concentrations in rivers and their seasonal and spatial variations are not well understood. Silicon helps rice plants to overcome abiotic and biotic stresses by preventing lodging and increasing resistance against pests and diseases. Rice is one of the more important crops in the country, and information on Si concentrations in rivers is useful because river water is a primary irrigation source. In this study, we conducted a preliminary research on temporal and spatial variations in dissolved Si (DSi) concentrations at watershed scale to help achieve an efficient use of Si resources through irrigation water management. The Sumani Watershed, located approximately 50 km east of Padang City in West Sumatra, Indonesia, was selected as the target area. Lake Dibawah lies on the upstream end of the watershed, and water is discharged from the watershed into Lake Singkarak. The results verified that Lake Dibawah had a dam effect of naturally reducing DSi concentrations in water. In addition, the average DSi concentration from the samples obtained from rivers, small channels, and ditches from October 2013 to December 2014 did not show strong seasonal patterns at each site but revealed clear spatial differences among sub-watersheds linked to the groundwater from Mt. Talang. The watershed has a high capability of supplying DSi to paddy fields via irrigation water.

INTRODUCTION

Rice is a staple crop for over half of the world's population and it is grown on nearly every continent (Seyfferth et al., 2013). Rice production has been increasing since the mid-1960s, with the implementation of new rice cultivation systems known as green revolution technology (Pingali, 2012). By 2013, rice crops covered an area of about 165 million ha for an annual production of 741 million tons (FAO, 2013).

Rice is a typical silicon accumulator because of the high capability of its roots to take up Si from soil (Mitani & Ma, 2005). Silicon is an important beneficial nutrient for healthy and competitive growth of all cereals including rice (Brunings et al., 2009). Beneficial nutrients are equally important as macronutrients like nitrogen and phosphorus (Ahmad et al., 2013), and balancing these nutrients in rice cultivation can enhance the quality and yield of the crop (Ma, 2004). Thus, many studies have investigated the functions and mechanisms of Si in terms of plant pathology, plant physiology, as well as soil science and plant nutrition (Idris, 1975; Hso-Freng & Yann-Shee, 1978; Richmond & Sussman, 2003; Ma et al., 2006; Kraska & Breitenbeck, 2010; Dufey et al., 2014; Makabe-Sakaki et al., 2014).

Several studies have indicated that continuous rice cultivation affects the silica content of soil. Darmawan et al. (2006) reported that the average content of available Si decreased from 707 ± 269 to 575 ± 260 kg ha^{-1} in the 0–20-cm soil layer and from $3,121\pm1,668$ to $2,755\pm1,576$ kg ha^{-1} in the 0–100-cm soil layer between 1970 and 2003, especially in the upper paddy fields. Additionally, Husnain et al. (2010) found that long-term fertilisation imbalances in the intensive rice-farming system led to surpluses of nutrients such as N and P and deficits of K and Si. This occurred because between 230 and 470 kg ha^{-1} of Si was removed during rice harvest, while only 75–120, 20–25, and 23–257 kg ha^{-1} of N, P, and K, respectively, were removed (Yoshida et al., 1981; Dobermann et al., 1996a,b; Casman et al., 1997). A lack of Si available to plants may have adverse effects on rice yield by decreasing resistance to lodging, diseases and pests (Winslow, 1992).

In Japan, silicate fertiliser containing several types of slag is applied to paddy fields to improve Si uptake in rice plants, although the application rate per unit area of paddy field is being gradually decreased to reduce production costs (Ma & Takahashi, 2002). In South and Southeast Asia, where most of the world's rice is grown, the straw and husks are typically removed from the field and used for various purposes, including animal fodder, fuel for stoves or burning (Savant et al., 1997). Since most Si taken up by rice is found within the straw and husk, the removal of rice straw accelerates soil desilication with no return of Si via biocycling (Seyfferth et al., 2013).

However, the supply of Si from irrigation water may slow the rate of Si depletion (Drmawan et al., 2006). Imaizumi

and Yoshida (1958) reported that irrigation water supplied approximately 30% of the Si taken up by rice in a paddy field in Japan. Silicon is the second most abundant element in the Earth's crust (Conley, 2002). Silicon in rivers, reservoirs, and lakes mainly originates from rock weathering, a process enhanced by high temperatures, moisture and active vegetation (Cochran & Berner, 1996; Conley, 2002; Humborg et al., 2006; Billen and Garnier, 2007), while direct input of Si through urban or industrial wastewater is minor (Sferratore et al., 2006; Garnier et al., 2006). Thus, the management of irrigation water in terms of Si and macronutrient supply and water quantity may help farmers to stabilise rice quality and production.

Indonesia is the third largest producer of rice in the world (FAO, 2013), and because of the importance of macronutrients for rice growth, Si in irrigation water has received limited attention. Research on DSi dynamics in the watersheds of Sumatra Island has not yet been conducted. In this study, therefore, we investigated temporal and spatial variations in DSi concentrations at the watershed scale to improve the efficiency of Si resource use for rice cultivation through irrigation water management.

STUDY AREA

Sumani Watershed is located approximately 50 km east of Padang City in West Sumatra, Indonesia (Figure 1) and covers about 586 km². Lake Dibawah lies on the upstream end of the watershed, and water flows from the watershed into Lake Singkarak. The average annual precipitation is 2,201 mm (Farida et al., 2005), and the watershed lies between 338 and 2,739 m.a.s.l. The watershed is situated in a humid tropical zone (Aflizar et al., 2010a), with average annual temperatures ranging from 19 to 30°C, and varying along an altitudinal gradient. The average annual humidity ranges from 78.1 to 89.4% (Aflizar et al., 2010b).

The various land uses of the watershed are summarised according to the GIS data of the Ministry of Forestry in Indonesia (2010) in Table 1. This range of land uses includes primary and secondary forests, tree crop gardens (mixed gardens, coconuts and tea gardens), vegetable gardens, *sawah*, bushes (shrubs, grasses, and alang-alang or *Imperata cylindrica*) and settlements (Aflizar et al., 2010b). A *sawah* is a levelled and bounded rice field with an inlet and outlet for irrigation and drainage (Wakatsuki et al., 2009). In a mixed garden, perennial crops, primarily trees such as coconut, clove, coffee, teak, mahogany, avocado, melinjo (*Gnetum gnemon*), rubber and cinnamon are planted in combination with annual crops (Karyono, 1990). Chilies (*Capsicum annum* L.), onions (*Allium cepa* L.), soybeans (*Glycine max* L.), corn (*Zea mays* L.) and sweet potatoes (*Ipomea batatas* L.) are the major crops in vegetable gardens (Aflizar et al., 2010b).
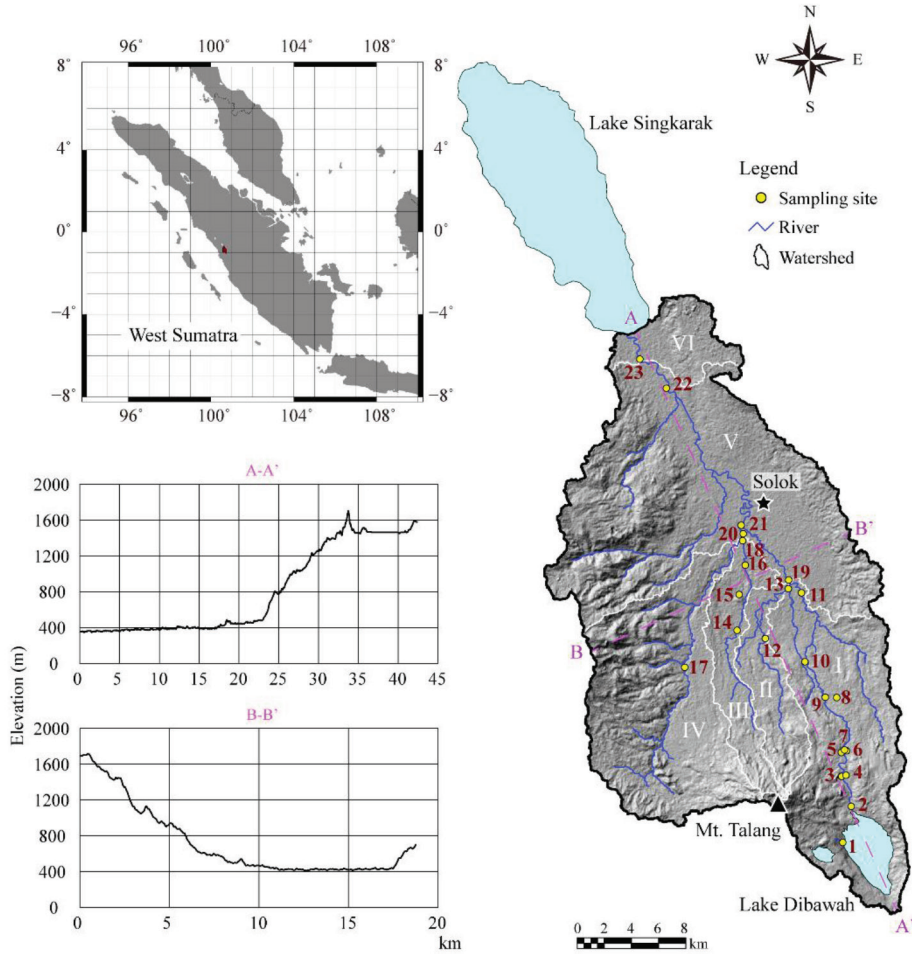
Figure 1. Location of the Sumani Watershed and sampling sites

Table 1
Landuse characteristics of Sumani Watershed

	Forests	Tree crop gardens	Vegetable gardens	Paddy fields/ sawah	Settlements	Water
Sub-watershed I	9%	1%	37%	40%	3%	10%
Sub-watershed II	27%	15%	4%	51%	3%	0%
Sub-watershed III	24%	10%	2%	48%	16%	0%
Sub-watershed IV	57%	15%	12%	14%	2%	0%
Sub-watershed V	16%	18%	36%	26%	5%	0%
Sub-watershed VI	0%	82%	0%	14%	4%	0%

Source: Ministry of Forestry, Republic of Indonesia (2010)

METHODOLOGY

Field sampling was conducted once per month between October 2013 and December 2014 (with the exception of March 2014). Water samples from rivers, irrigation channels and ditches, and a paddy field were collected from 23 sites. Due to the lack of detailed information on river locations and low road accessibility in the area, sampling points were increased with knowledge gained throughout the field investigation in this study. Thus, sampling occurred at only 11 sites in October 2013, 16 sites between November 2013 and January 2014, and 22 sites in June and July 2014. All the sites were sampled in the remaining months, except for March 2014 when road and weather conditions were not suitable for any fieldwork. In total, 287 water samples were collected. Of these, 263 were collected from rivers, channels and ditches. Additional samples were obtained from a paddy field (site no. 8) and spring water (site no. 15). Surface waters of paddy fields were also sampled at five sites downstream, eight sites midstream, and five sites upstream in the watershed in November 2013.

The water samples were filtered through a 0.45-µm membrane filter (Advantec Dismic-25CS, Japan). The concentration of DSi was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Shimadzu ICPE-9000).

All the statistical analyses were performed with EZR (Saitama Medical Centre, Jichi Medical University, Saitama, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS AND DISCUSSION

Roles of Lake Dibawah in DSi concentration

Lake Dibawah, which is located on the upstream end of the watershed, is primarily precipitation- and spring-fed and it provides the main water source for streams. A portion of the lake's water is allocated for irrigation via small channels at the outlet of the lake. Of the small ditches along the lake, one from Mt. Talang was selected as a regular sampling site because of its accessibility (site no. 1). Figure 2 compares the DSi concentration at this site with that at the outlet of the lake (site no. 2). The average DSi concentrations were 35.8 mg L^{-1} (n = 14) at site no. 1 and 6.21 mg L^{-1} (n = 14) at site no. 2. The standard deviations were 1.75 and 0.41 mg L^{-1} at site nos. 1 and 2, respectively. The concentrations at both sites were relatively stable during the research period from month to month. A comparison of the two sites indicated that water with high DSi concentrations flowed into the lake, while water with low concentrations was discharged from the lake ($p < 0.001$).

It has been previously reported that the construction of large dams leads to decreased input of Si into coastal zones because of increased water residence times and net losses of Si to the sediments within reservoirs (Conley et al., 1993; Garnier et al., 1999; Friedl et al., 2004; Koch et al., 2004; Ahearn et al., 2005; Humborg
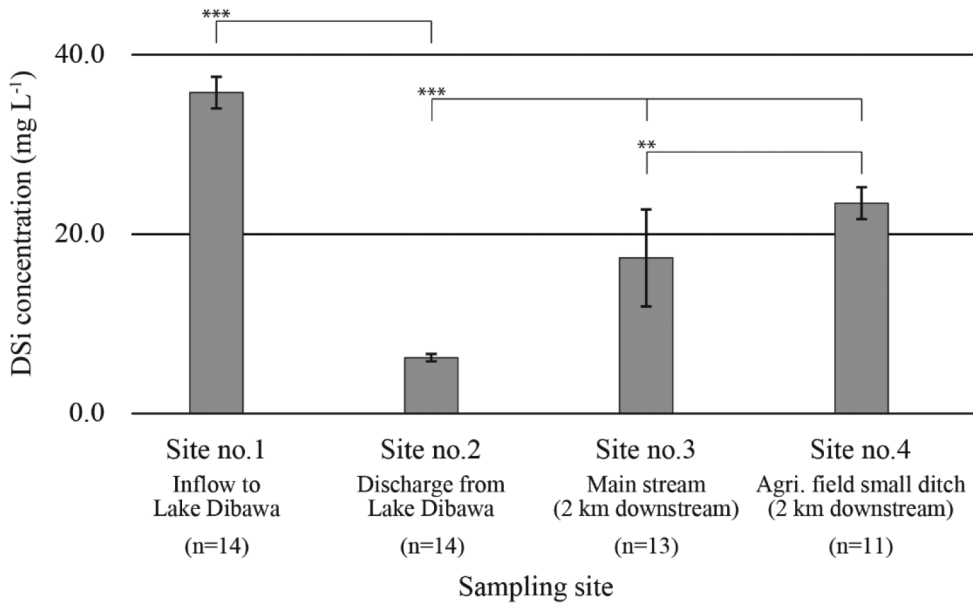
Figure 2. Average DSi concentrations and standard deviations. Statistical analysis was performed using Welch t-Test against site nos. 1 and 2. In addition, Kruskal-Wallis Test with the Bonferroni adjustment was applied against site nos. 2, 3 and 4. **$p < 0.01$, ***$p < 0.001$.

et al., 2006). Development, such as dam construction or concrete canal installation within a watershed, was found to result in a decrease in Si concentrations in irrigation water (Ma and Takahashi, 2002). Thus, the lake naturally becomes akin to a dam reservoir, and dilution through precipitation, consumption of Si through diatom growth, and subsequent settling of diatom frustules in the lake might occur.

These natural functions may have adverse effects on rice, sugarcane and other tall plants because of reduction of Si levels in irrigation water. However, the concentrations of DSi 2 km downstream of the lake outlet (site no. 3) were higher than at site no. 2 ($p < 0.001$). These differences varied from 1.37 to 19.4 mg L^{-1} during the research period and exceeded 10 mg L^{-1} in all, except for four months (November 2013 and May, November, and December 2014). In addition, at site no. 4, which is also located about 2 km downstream of the lake outlet and close to sampling site no. 3, containing a small ditch flowing through an upland field, the DSi concentrations averaged 23.4 mg L^{-1}, with a maximum of 26.1 mg L^{-1} and a minimum of 20.5 mg L^{-1}. These relatively high concentrations exhibited little variation, with a standard deviation of 1.78 mg L^{-1}. These results pointed to a possible Si source for river water between site nos. 2 and 3. As the DSi concentrations were consistently higher at site no. 4 than site no. 3 ($p < 0.01$), agricultural fields were also likely to contribute Si to the water.

Variations in the DSi concentrations

The average DSi concentration for the whole watershed, obtained from river, channel and ditch samples was 18.0 mg L^{-1} (n = 263). The concentrations varied over 4.2–38.3 mg L^{-1} during the research period, with a standard deviation of 7.1 mg L^{-1}. Husnain et al. (2010) reported that the DSi concentration of irrigation water in the Citarum Watershed of Java, Indonesia, was 13.64±3.13 mg L^{-1} (n = 15) from September 2006 to November 2007, with monthly sampling. Thus, the average DSi concentrations of the Sumani and Citarum Watersheds exhibited similar tendencies.

Figure 3 provides a histogram and standard statistical values of DSi concentrations. For this analysis, the samples at site nos. 1, 8, and 15 were excluded. This is because water at site no. 1 flows into the lake, where high DSi concentrations appeared to be diluted. Water is then discharged from the lake as river water and irrigation water. Thus, the concentrations at sampling site no. 1 were not influenced by downstream water quality in sub-watershed I. Additionally, the samples at site nos. 8 and 15 were not collected from a river or small ditch, but rather from the surface water on a paddy field and spring water, respectively. In order to compare DSi characteristics among sub-watersheds, four independent sub-watersheds (sub-watersheds I through IV) were selected. In this comparison, sub-watershed V was excluded because its water was influenced to an unknown degree by water from the other four sub-watersheds. For this discussion, the individual sub-watersheds were divided into two groups based on the DSi concentration levels. The group with lower concentrations included sub-watersheds I and IV, and that with higher concentrations included sub-watersheds II and III. Sub-watershed I had an average concentration of 13.7 mg L^{-1} and a standard deviation of 5.00 mg L^{-1}, with measurements frequently falling between 10 and 15 mg L^{-1}. The average concentration in sub-watershed IV was 17.0 mg L^{-1}, and the standard deviation was 4.62 mg L^{-1}. Measurements were concentrated between 15 and 20 mg L^{-1}. On the other hand, in sub-watersheds II and III, the average DSi concentrations were 26.2 and 24.0 mg L^{-1}, respectively. Measurements between 25 and 30 mg L^{-1} were most frequent in sub-watershed II, and those from 20 to 25 mg L^{-1} were most frequent in sub-watershed III.

The DSi concentrations in sub-watershed V were determined by inflow quantity and quality from sub-watersheds I through IV. In this study, as river discharge was not observed, the impact ratios of each sub-watershed against the concentration in sub-watershed V were unknown. In sub-watershed V, the average DSi concentration was 17.6 mg L^{-1}, and the standard deviation was 2.0 mg L^{-1}. Measurements tended to fall between 15 and 20 mg L^{-1}. The concentrations in sub-watershed V were similar with those in sub-watershed IV, but fluctuations were smaller in sub-watershed V than in sub-watershed IV.

In order to investigate possible sources of differences in the DSi concentrations among the sub-watersheds, we examined groundwater. For the preliminary analysis,
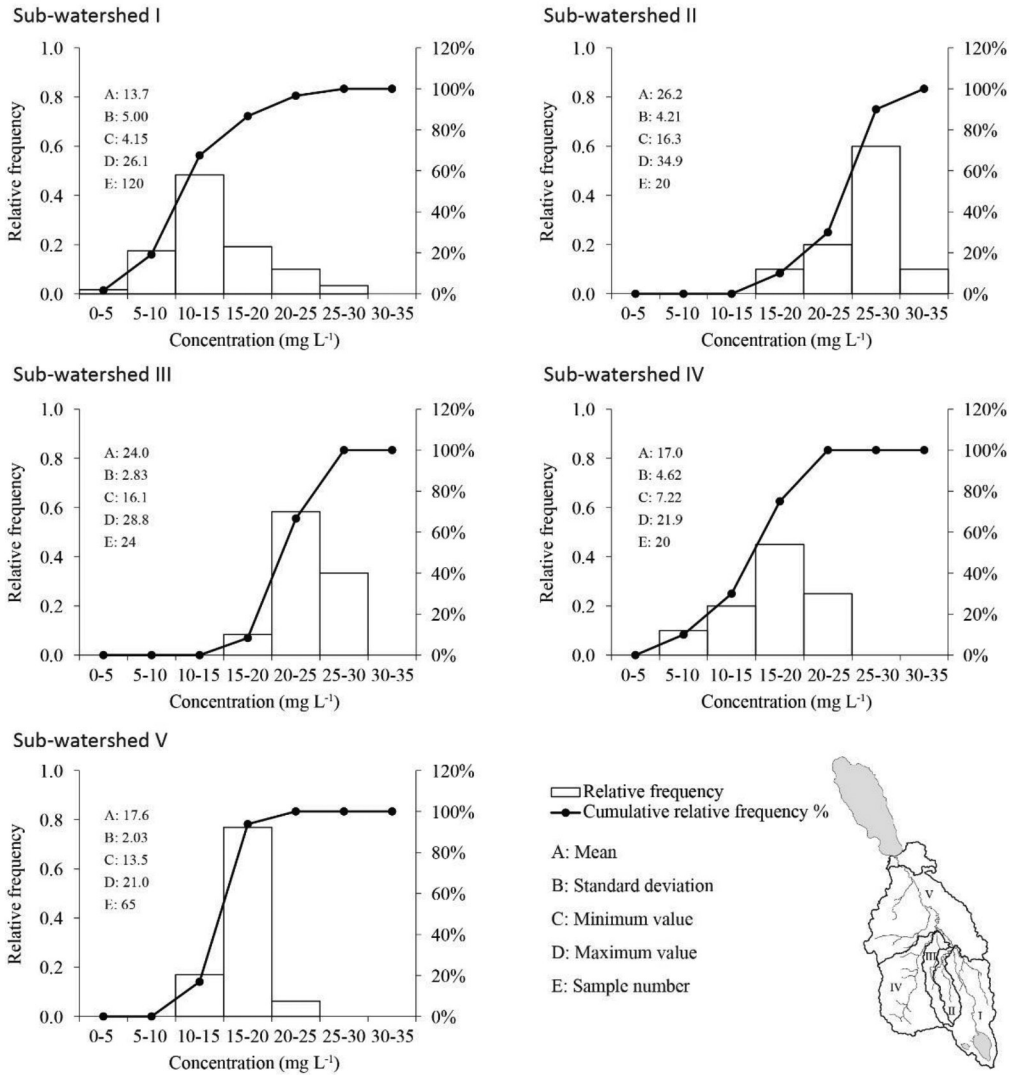
Figure 3. Histogram and statistical values of DSi concentrations among sub-watersheds, excluded site nos. 1 (Inflow water to Lake Dibawa), 8 (Surface water of a paddy field), and 15 (Spring water). Kruskal-Wallis Test with the Bonferroni adjustment was applied against sub-watersheds I to IV. $p < 0.001$ in sub-watershed I vs. sub-watersheds II and III, and in sub-watershed IV vs. sub-watersheds II and III. No statistically significant difference was detected between sub-watersheds II and III.
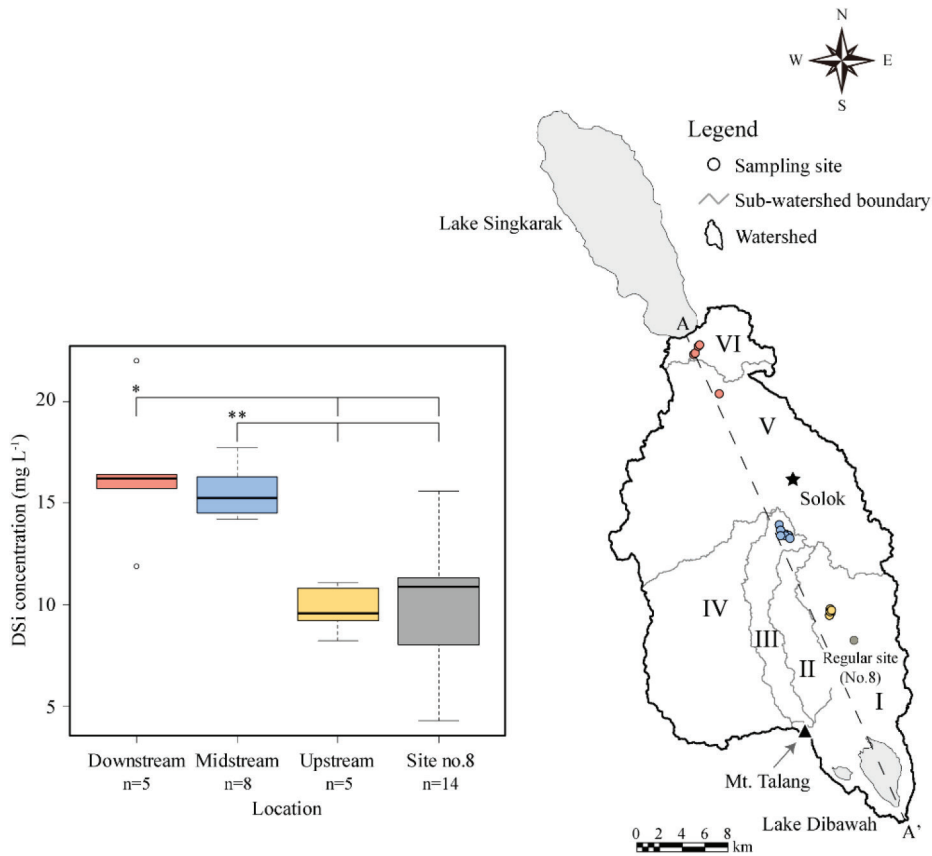
Figure 4. DSi concentrations in the surface water of paddy fields. Box=25th and 75th percentiles; bar=minimum and maximum values. Kruskal-Wallis Test with the Bonferroni adjustment, *p* < 0.05, **p* < 0.01.

DSi concentrations in spring water upstream of sub-watershed II were analysed on-site in February 2014 using the pack test (DPM-SiO₂). The average concentration was 72.1 mg L^{-1} (n = 2), indicating that spring water might be a source of Si in the area. Thus, to examine variations in DSi concentration in spring water (groundwater), the highly accessible spring water of site no. 15 located in sub-watershed III was sampled. The averaged DSi concentration of this site was 62.1 mg L^{-1} (n = 10), with a standard deviation of 2.8 mg L^{-1}. This finding indicated that water with high concentrations of DSi was steadily discharged as spring water with little variation.

Based on the consistently high DSi concentrations at site nos. 1 and 15, it was concluded that water from Mt. Talang provided a source of Si through groundwater to surface water within the watershed.

Spatial Distribution of DSi concentrations in the Surface Water of Paddy Fields

The DSi concentrations in the surface water of upstream through downstream paddy fields in the watershed were studied in November 2013 (see Figure 4). The water samples were collected along the longitudinal (A-A') line from Lake Dibawah (upstream) to Lake Singkarak (downstream). As variations in the growth stages of rice on each paddy field might affect DSi concentrations of surface water, the water samples were collected under conditions similar to those before and after transplanting of rice seedlings and during the growing stages as much as possible. The upstream sampling sites were located in sub-watershed I, midstream sites in sub-watershed III and downstream sites in sub-watersheds V and VI. The average DSi concentrations were 9.8, 15.5, and 16.4 mg L^{-1} for upstream, midstream and downstream sites, respectively. Most of the DSi concentrations between 5 and 10 mg L^{-1} occurred in upstream paddy fields, while those between 15 and 20 mg L^{-1} occurred more frequently downstream. For the midstream fields, values were concentrated between 10 and 20 mg L^{-1}. Along the A-A' line, there were clear differences in these intervals between upstream and downstream sites ($p < 0.05$). Our findings indicated that water with relatively low DSi concentrations from Lake Dibawah provided irrigation water via small channels for upstream areas. Downstream, river water mixed with water with high DSi concentrations from sub-watersheds II and III, and was allocated to the paddy fields.

Moreover, the DSi concentrations of surface water at a regular sampling site (no. 8) exhibited a trend that is similar to that of the upstream sampling from November 2013. The concentration at this site was relatively low, with an average of 10.1 mg L^{-1}. The DSi levels seemed to be lower during the rainy season and higher during the dry season, especially in June and July. Although the cause of this phenomenon is not quite clear, the dilution of surface water by precipitation in the rainy season and the long retention time of water in the soil during the dry season might provide an explanation.

Si Supply from Irrigation Water

Irrigation water may provide an important source of Si for rice. Thus, the amount of Si delivered to a paddy field via irrigation was estimated. Ma and Takahashi (2002) reported that paddy soil was irrigated with an average of 1,400 mm (14,000 m^3 ha^{-1}) of water during the single growth period of rice. Average Si concentrations from 380 rivers in Japan ranged from 4.0 to 19.5 mg L^{-1}, with an overall average of 10.1 mg L^{-1}. From these results, Si supply from irrigation water in Japan was estimated to be 141.4 kg ha^{-1}, or about 30% of the crop requirement. In this study, the amount of irrigation water was also assumed to be 1,400 mm because of the lack of information on irrigation water levels in the area. DSi load to the paddy fields was then estimated from this irrigation

water value for an average of 238±81.2 kg ha⁻¹ per rice cultivation period (Table 2), accounting for 53.6±18.3% of the Si taken up by rice.

On average, 141.4 kg ha⁻¹ of DSi is supplied by irrigation water in Japan, and 30±15 kg ha⁻¹ of DSi is supplied by irrigation in France (Desplanques et al., 2006). Thus, the Sumani Watershed has a higher capacity for supplying Si to paddy fields via irrigation. DSi loads varied among the sub-watersheds. The highest DSi load occurred in sub-watershed II at 366.8±58.8 kg ha⁻¹. The spring water at site no. 15 had a DSi load of 869.4±39.2 kg ha⁻¹, constituting approximately 195.8±8.8% of the crop requirement. Thus, Si input via irrigation could increase with the efficient introduction of spring water into paddy fields. Numerous beneficial effects of Si have been reported; these include prevention of lodging (falling over), increased photosynthetic activity, increased insect (e.g., brown planthopper) and disease (e.g., rice blast) resistance,

reduced mineral toxicity, improvement of nutrient imbalance, enhanced drought and frost tolerance, and improved grain yield (Deren et al., 1994; Kim et al., 2002; Ma, 2004). Therefore, aggressively introducing Si via irrigation water may enhance the stability of rice production and quality in the region.

CONCLUSION

The dynamics of DSi concentrations in Sumani Watershed were investigated for the first time between October 2013 and December 2014. From this research, it was found that Lake Dibawah had a dam effect of naturally reducing DSi concentrations in the water, based on a comparison between inflow and outflow concentrations. Further, it was revealed that the average DSi concentration of samples obtained from rivers, small channels and ditches was 18.0 mg L⁻¹, with the levels varying from 4.2 to 38.3 mg L⁻¹ during the research period. Among the sub-watersheds, there

Table 2

DSi loads supplied by irrigation and spring water in Sumani Watershed. The load was assumed as 1,400 mm irrigation water per cultivation period.

	DSi load (kg ha⁻¹)	DSi concentration (mg L⁻¹)	Standard deviation (mg L⁻¹)	Sample number
Whole watershed	238.0±81.2	17.0 [a]	5.8	249
Sub-watershed I	191.8±70.0	13.7 [b]	5.0	120
Sub-watershed II	366.8±58.8	26.2	4.2	20
Sub-watershed III	336.0±39.2	24.0 [c]	2.8	24
Sub-watershed IV	238.0±64.4	17.0	4.6	20
Sub-watershed V	246.4±28.0	17.6	2.0	65
Spring water (no. 15)	869.4±39.2	62.1	2.8	10

[a] verage value of DSi concentrations in the watershed, excluding site nos. 1, 8, and 15.
[b] average value of DSi concentrations in sub-watershed I, excluding site nos. 1 and 8.
[c] Average value of DSi concentrations in sub-watershed III, excluding site no. 15.

were significant differences in the DSi concentrations, with higher concentrations in sub-watersheds II and III than other sub-watersheds. Based on an analysis of spring water, the differences in DSi concentrations among sub-watersheds may be closely related to the groundwater from Mt. Talang. In addition, it was understood that spatial variation in DSi concentration existed upstream to downstream in the surface water of paddy fields. Then, by using the measured DSi concentrations and a literature review, it was determined that more than 50% of Si taken up by rice can be provided from irrigation water in the area based on the average DSi concentration value. Moreover, from the highest average DSi concentration $(26.2\pm4.2$ mg L^{-1}) from sub-watershed II, up to 69-96% of Si taken up by rice may be supplied from the irrigation water.

Although the total Si absorbed by rice in a growing season is easily determined, no study has yet investigated which Si source (soil or irrigation water) is the most critical for the absorption of Si during a rice cultivation period. Additionally, the relationship between the spatial distribution of Si concentrations and rice quality in this watershed is not yet understood. Thus, such research should be conducted as the next step to help produce high-quality rice through the efficient allocation of irrigation water.

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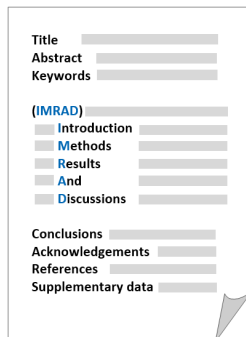
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Contents