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

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

JTAS

Journal of Tropical Agricultural Science

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Contents

Foreword

Welcome to the **Second Issue 2015** of the Journal of Tropical Agricultural Science (JTAS)!

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

This issue contains **eleven articles**, out of which **one** is a review article and **ten** are regular research papers. The authors of these articles are from **Malaysia, Sri Lanka, China, Canada, Pakistan, Iran, Bangladesh, Philippines** and **India**.

The potential use of PCR and marker assisted selection (MAS) towards the assessment of genetic diversity of rice germplasm in breeding programmes is discussed in the review paper (*Wijerathna, Y. M. A. M., Perera, A. N. K., Hamama, I. B.* and *Hoang, L.*). The first research paper, researchers from Malaysian Rubber Board reports on the effects of combination ethephon and urea on productivity of *Jatropha curcas* L. Thai accession (*Shuib, N. H.* and *Sulaiman, Z.*). The second research paper discusses on the effective elements on technical knowledge of agricultural section for sustainable soil management (*Mohammad Sadegh Sabouri, Meysam Solouki* and *Marzieh Bordbar*). The next research paper from the University of Chittagong, Bangladesh presents an ethnomedicinal study of plants in Hathazari, Chittagong, Bangladesh (*Sajib, N. H.* and *Uddin, S. B.*).

In next research paper, a group of researchers from Universiti Putra Malaysia describes the relation between length-weight and relative condition factor of *Parapenaeopsis sculptilis* (Heller, 1862) from the coastal waters of Perak, Malaysia (*Amani A. A., Arshad, A., Yusoff, F. M.* and *Amin, S. M. N.*). Researchers from Philippines validates the dissemination of natural resource management technology for Irrigated Rice in the Philippines (*Corales, A. M., Sibayan, E. B.* and *Palis, F. G.*), and while in Malaysia, group of scientists reports a characterization and isolation of EgGST, a glutathione S-transferase protein transcript in oil palm (*Elaeis guineensis* Jacq.) (*Conie Toh, Parameswari Namasivayam, Ho Chai Ling* and *Sharifah Shahrul Rabiah Syed Alwee*).

Next research paper reports on the optimisation of solid liquid extraction of Orthosiphon stamineus leaves using response surface methodology technique (*Mohd Farhan, A. R., Pin, K. Y., Zamree, M. S., Luqman Chuah, A.* and *Nazira, M.*), followed by the reports from a group of Indian scientist on garlic: An effective functional food to combat the growing antimicrobial resistance (*Shivani Gupta, Suman Kapur, Padmavathi DV* and *Apoorva Verma*). The next research paper is on the comparison of protein extraction protocols for proteomic analysis of red algae, *Eucheuma cottonii* (*Lim, H. T.* and *Teo, S. S.*). The

last research paper discusses on the relationship between size of fish, temperature and parasitic intensity in snakehead fish species from Kepala Batas, Penang, peninsular Malaysia (*Rajiv Ravi* and *Zary Shariman Yahaya*).

I anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

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, the editorial assistance of the journal division staff is fully appreciated.

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

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Review Article

Application of PCR and MAS: Potential Use for Assessment of Genetic Diversity of Rice Germplasm in Breeding Programmes in Developing Countries

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ABSTRACT

Molecular characterisations of genotypes give precise information about the extent of genetic diversity, which assists in the development of an appropriate rice breeding programme. The latest approach in plant biotechnology and molecular breeding, which is the development of the polymerase chain reaction (PCR) for amplifying DNA, DNA sequencing and data analysis, is an effective technique that can be used for the screening, characterisation and evaluation of genetic diversity. Traits that serve as genetic markers are by definition polymorphic; the more polymorphic the trait, the greater its potential value to germplasm management. The issue of homology may seem insignificant for morphological markers, but the increasing use of molecular markers has heightened its importance. Application of molecular markers is still prohibitively expensive for most large-scale applications in rice breeding programmes, where performance parameters such as yield, quality, disease resistance and other desirable growth characteristics are upgraded. Therefore, marker assisted selection (MAS) methods are currently used for more targeted applications in order to keep up with the rising demand for rice consumption. Since conventional breeding methods will not be able to meet the satisfactory harvest, the application of biotechnological tools is one plausible option to tap into the significant yield potential of rice.

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INTRODUCTION

Rice is the most important staple food in Asia. More than 90% of the world's rice is grown and consumed in Asia, where 60% of the world's population live. Rice accounts for between 35-60% of the caloric intake of three billion Asians (Guyer *et al*., 1998). The two cultivated rice species is Oryza sativa, which is widely grown in Asia and other countries, and O. glaberrima, which is grown only in Africa. O. sativa evolved from perennial or annual type of O. rufipogon and diversified into two subspecies; indica and japonica (Oka, 1974; Chang, 1985). Ancient indica and japonica diversified at approximately 200,000~440,000 and 86,000~200,000 years ago, according to the nuclear genome and chloroplast DNA sequence, respectively (Ma & Bennetzen, 2004; Vitte *et al*., 2004). Over 150 million hectares of rice are planted annually, covering about 10% of the world's arable land. In 1999/2000, this amounted to some 600 million tonnes of rice seeds, equal to 386 million tonnes of milled rice. With the world's population estimated to increase from 6.2 billion in the year 2000 to about 8.2 billion in the year 2030, the global rice demand will rise to about 765 million tonnes, or 533 million tonnes of milled rice (FAO, 2002). For almost three decades since the Green Revolution, the rice yield growth rate was approximately 2.5% per year. During the 1990s, however, this decreased to only 1.1% (Riveros & Figures, 2000).

The aims of this review are to summarise the basic knowledge concerning the PCR based molecular markers to rice breeding and to explore the use of MAS in rice breeding programmes aimed at improving new varieties in this species. Various advantages and disadvantages, as well as uses of the molecular markers relative to other molecular marker types and importance of MAS for developing countries like Pakistan, Vietnam, Sri Lanka and Indonesia where rice is consumed as staple food are also discussed.

Rapid application of molecular markers has played an increasing role in rice breeding and genetics during last few decades. Of the different types of molecular markers, microsatellites have been utilized most extensively because they can be readily amplified by PCR and the large amount of allelic variation at each locus. Diversified molecular markers have been used to classify DNA polymorphism and are generally categorised as hybridization based markers and PCR based markers. DNA profiles are visualised by hybridising the restriction enzyme-digested DNA to a labelled probe, which is a DNA fragment of known origin or sequence. PCR-based markers engage in vitro amplification of particular DNA sequences or loci by specifically or arbitrarily chosen primers and a DNA polymerase enzyme which is thermostable. The amplified fragments are separated electrophoretically and banding patterns are visualised by using various methods viz. ethidium bromide or silver staining and autoradiography (Collard *et al*., 2005).

The application of molecular markers has risen as a productive and dominant access for augmenting traditional plant breeding techniques for the present day rice breeding programmes in China, Thailand and Japan. Series of molecular markers are now accessible viz. Restriction fragment length polymorphism (RFLP) which is based on Southern blot hybridization and Random amplified polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR)/ Microsatellites, Amplified Fragment Length Polymorphism (AFLP) and Cleaved amplified polymorphic sequence (CAPS) markers, which are based on PCR. The AFLP and CAPS markers comprise pre- and post-amplification restriction digestion. Single nucleotide polymorphism (SNP) is the latest marker system that utilises the vast DNA sequence resources available in different rice varieties. The most important characters for an appropriate molecular marker should be: a) polymorphism, b) co-dominant inheritance, c) random and frequent distribution throughout the whole genome, d) easy and cheap to detect, and e) reproducibility.

Polymorphic DNA markers are especially useful for divulging differences between individuals of the same or different species and also interpreted as co-dominant or dominant where markers can segregate between homozygotes and heterozygotes. In SSR, co-dominant markers illustrate the differences in the size of the alleles, while dominant markers display the presence or absence of an allele. In a single individual plant, there are only two alleles per locus for codominance and when referred to population codominance, there is a possibility to have more than two alleles for each locus (Collard *et al*., 2005). When considering the RAPD marker (dominant marker), there are only two maximum alleles both for the individual and population, which are referred to as the present and absence of loci.

PCR is a highly advantageous tool due to the simplicity of the procedure to set up and run and the minimal requirement of the amount of genomic DNA samples. This DNA can be isolated from the early stage of rice leaf tissue. PCR is a very time efficient procedure where millions of DNA copies can be made in a minimum of two hours. In particular, PCR allows the processing of many samples in a very short time with automated and robotic assistance where many small leaf tissue samples are collected from different individual plants. Finally, amplified products can be easily visualised on an agarose or acrylamide gel. These sample DNA can be PCR to determine the presence or absence of a particular marker of interest for interested trait. Only those tested positive would be kept for the next plant breeding stage. PCR based markers may either be allele specific (SSR, SNP) or allele unspecific (RAPD, AFLP). RAPD and AFLP are multi-loci markers that use one random primer (RAPD) or primer pairs (AFLP) and generate a number of marker loci in one PCR reaction. It can be used to screen and select plants for the required characteristics in any plant breeding programmes. Plants can be screened using one of the most suitable

PCR based methods for the presence of a particular gene of interest, and only those tested positive would be kept for the next plant breeding stage. At any given stage of the breeding programme, the testing can be repeated for screening purposes.

Plant breeders have been instrumental in the process of domestication and improvement of rice. After pollination, screening of the new genetic variant is the success of plant breeding. Today, rice plant breeders have the capabilities of altering the performances of some existing crop varieties by using targeted approaches of genomic research. To date, there is an urgent need to utilise new tools to assist in the screening of new genotype efficiently.

Available rice genomic information on genes and gene functions is very important for plant improvement through biotechnological applications. The two major biotechnological applications improve rice genome, viz. (a) application of molecular markers to screen and select favourable genetic combinations, (b) application of genetic engineering (cisgenics/transgenics) to introduce interested foreign genes. These molecular markers help to screen the naturally occurring genetic variation within a species more efficiently. The association among the markers and phenotype needs to be established first and these markers can be used for indirect selection thereafter. MAS in a plant breeding context involves scoring indirectly for the presence or absence of a desired plant phenotype or phenotypic component based on the sequences or banding patterns of molecular markers located in or near the genes controlling the phenotype.

Breeders attempt to overcome limitations to rice yield by improving yield, resistance to pests and diseases, and adaptability to diverse growing conditions through breeding programmes and development of new rice varieties. MAS is an indirect selection process where a trait of interest is selected not based on the trait itself but on a marker linked to it (Ribaut & Hoisington, 1998).

MAS (also 'marker-assisted breeding' or 'marker-aided selection') is based on the concept that it is possible to infer the presence of a gene from the presence of a marker that is tightly linked to the gene. The use of genetic markers as a reliable tool for the plant breeder was recognized by Sax in 1923. However, its application was largely hindered by the lack of suitable markers and the non-availability of detailed genetic linkage maps. The rapid development of molecular techniques has opened up sources of genes to plant breeding that were not available previously through conventional breeding (Allen, 1994). With the help of molecular markers, plants can be screened at seedling stage to screen out multiple traits that would ultimately be epistatic with one another, and minimise linkage drag and rapidly recover the recurrent parent genotype; these are just a few attractions of MAS in plant breeding (Tanksley *et al*., 1989).

Genetic diversity estimation, high density genome maps construction, mapping and tagging of genes, map-based isolation

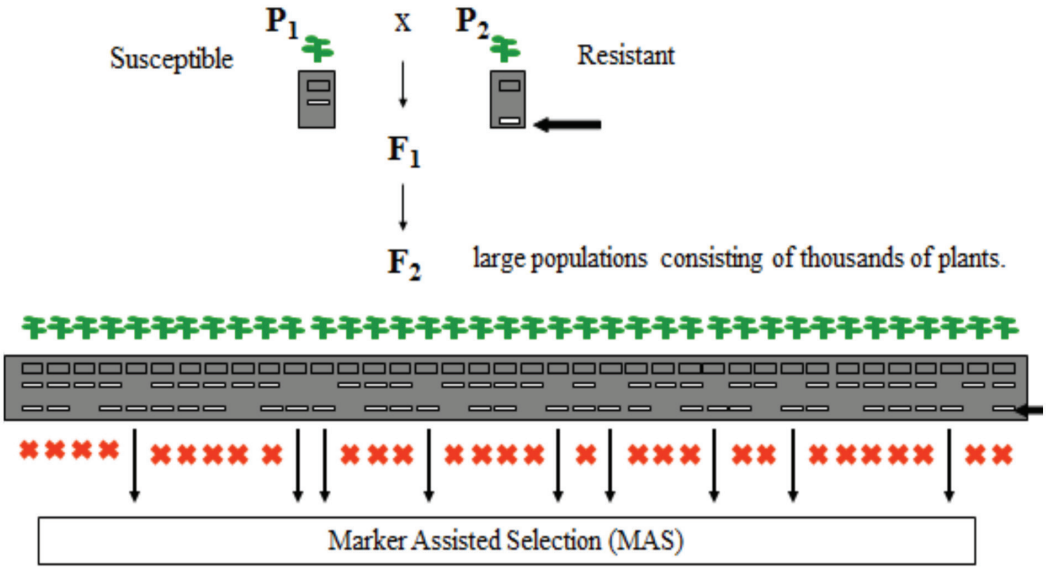
of genes and MAS are some aspects of molecular crop breeding. Genes that have been closely linked to molecular markers in rice are able to combine with conventional breeding approaches where MAS can be used to scan the existence or non-existence of these genes in breeding populations. MAS is done to quickly recover recurrent parent genome in backcross breeding and transfer the gene/s of interest from one genetic background to another using the closely linked markers.

Selecting plants in a segregating progeny that contains appropriate combinations of genes is a critical component of plant breeding (Ribaut & Betran, 1999; Weeden *et al*., 1994). Crop breeders work with huge populations, specifically ones that contain large number or hundreds or even thousands of crop plants (Ribaut & Betran, 1999; Witcombe & Virk, 2001). Thus, MAS has a great influence in plant breeding compared to conventional breeding methods that increase efficiency and effectiveness. Once markers that are closely linked to genes or quantitative trait loci (QTL) of interest have been identified prior to field evaluation of a large number of plants, breeders may use specific DNA marker alleles as a diagnostic tool to identify plants carrying the genes or QTLs (Michelmore, 1995; Ribaut *et al*., 1997). The presence or absence of a molecular marker is used as a substitute for or to assist in phenotypic selection in MAS (see Fig.1) (Collard *et al*., 2005).

In summary, there are four major schemes of MAS: a) Marker-assisted backcrossing (MAB), b) pyramiding, c) early generation selection, and d) combined approaches.

MAB has several advantages over conventional backcrossing that include effective selection of target loci, minimising linkage drag and accelerated recovery of recurrent parent. Gene pyramiding is widely used for combining multiple disease resistance genes for specific races of a pathogen. It is important to note that gene pyramiding is extremely difficult to be achieved using conventional methods which consider phenotyping a single plant for multiple forms of seedling resistance. Gene pyramiding is almost impossible but it is also important to develop 'durable' disease resistance against different races. In early generation of MAS, it conducted at F2 or F3 generations and plants with desirable genes/QTLs were selected and alleles could be 'fixed' in the homozygous state, while plants with undesirable gene combinations could be eliminated. This is an advantage for later stages of breeding programme because resources can be used to focus on fewer lines (Fig.1). In some cases, a combination of phenotypic screening and MAS approach may be useful to maximise genetic gain when some QTLs have been unidentified from QTL mapping and level of recombination between marker and QTL and to reduce population sizes for the traits where marker genotyping is cheaper or easier than phenotypic screening.

For example, marker-assisted backcross breeding can be used to integrate crucial genes with significant biological effects into a number of commonly grown rice varieties.



A susceptible (S) parent is crossed with a resistant (R) parent and the F1 plant is self-pollinated to produce a F2 population. In this diagram, a robust marker has been developed for a major QTL controlling disease resistance (indicated by the arrow). By using a marker to assist selection, plant breeders may substitute large field trials and eliminate many unwanted genotypes (indicated by crosses) and retain only those plants possessing the desirable genotypes (indicated by arrows). Note that 75% of the plants may be eliminated after one cycle of MAS. This is important because plant breeders typically use very large populations (e.g., 2000 F2 plants) derived from a single cross and may use populations derived from hundreds or even thousands of crosses in a single year.

Fig.1: MAS scheme for early generation selection in a typical breeding programme for disease resistance (Adapted from Ribaut & Betran, 1999)

The use of cost-effective, MAS strategies and finely mapped microsatellite markers should provide different opportunities for breeders to develop high-yield, selected trait rice cultivars.

Genetic diversity assessments of the traditional rice varieties, landraces are very essential component in germplasm characterization and conservation to identify potential parents for future breeding perspective. Morphological and seed traits have long been the means of studying taxonomy and variability among plant species. Microsatellites/ SSR are the most widely used DNA marker for many purposes such as diversity studies, genome mapping, varietal identification, etc. (Teixeira da Silva, 2005). Molecular markers are not stressed by environmental factors and growth practices as the morphological and biochemical markers (Ovesna *et al*., 2002). Application of these markers to investigate genotypic variations among different cultivars has previously been reported by some researchers (Singh *et al*., 2004; Joshi & Behera, 2006).

The knowledge of cereal genetics figures out of the structure and behaviour

of cereal genomics which are influenced rapidly with the advancement of molecular techniques. The present molecular genetics techniques and especially utilization of molecular markers help to scan the DNA sequence variation in and among the species and create new sources of genetic variation by introducing new favourable traits from landraces and related grass species. Markers linked to useful traits have enabled great advances for crop molecular breeding during recent years with the improvement of marker detection systems and in the techniques (Korzun *et al*., 2001).

Time consuming and environmental conditions are the main constraints of conventional cereal breeding. It usually takes between eight to twelve years for conventional rice breeding to take place, and even then, the release of an improved variety cannot be guaranteed within that time period.

Hence, molecular marker technology offers such a possibility by adopting a wide range of novel approaches to improving the selection strategies in cereal breeding (Korzun *et al*., 2001).

The genetic diversity of rice germplasm was assessed by the use of RFLP, RAPD and SSRs molecular markers. SSRs have been frequently used in genetic and breeding research because of the relatively high allelic polymorphism and easy genotyping by PCR. Moreover, approximately 20,000 SSR markers were mined from the genome sequence of japonica cv. Nipponbare and are publicly accessible (IRGSP, 2005). Mainly SSR markers have been extensively used in evaluating the genetic diversity of wild relatives, landraces, and cultivars of rice (Ram *et al*., 2007; Pusadee *et al*., 2009). However, each marker system has both advantages and disadvantages (Table 1).

Identification of markers linked to useful traits has been based on complete linkage maps and bulked segregant analysis. However, alternative methods such as construction of partial maps and the combination of pedigree and marker information have also been proven to be useful in identifying marker/trait associations. A revision of the current breeding methods by utilising molecular markers in breeding programmes is therefore crucial in this phase (Korzun *et al*., 2001).

During domestication, some key agronomy traits such as grain shattering, grain dormancy and grain size were strongly selected, which led to greatly diminished genetic diversity in rice. The rice genome encountered a severe early domestication bottleneck; thus, landraces represented only small proportion of the genetic variation of wild rice (Kovach & McCouch, 2008). Moreover, modern breeding programmes continuously select desirable characters under highly controlled conditions to achieve an ideotype which exacerbates the reduction in gene pool of cultivars (McCouch, 2004).

In order to breed new varieties that can endure the effects of global climate changes, the gene pool of cultivars must be broadened by introducing wild species, landraces and exotic germplasm into the breeding programmes. Wild species that are resistant

TABLE 1
Advantages and disadvantages of most commonly used PCR based DNA markers

Markers type	Codominant or Dominant	Advantages	Disadvantages
RAPD	Dominant	More rapid No radioactive labelling Genomic abundance high Better genome coverage / Multiple loci from a single primer possible Does not need prior sequence information Perfect for automation Requires less DNA Inexpensive	No need of probe information Dominant markers Not reproducible Not well tested
SSR	Codominant	Easy to automate Genomic abundance high Highly reproducible High polymorphism Multiple alleles Moderately genome coverage No radioactive labelling Robust and reliable Highly reproducible Transferable between populations Require low amount of DNA Does not need high quality DNA	Not well examined Cannot suitable across species Sequence information needed Development of primer pairs is considerably expensive and time consuming Require prior sequence information Usually require polyacrylamide gel electrophoresis
AFLP	Dominant	High polymorphism Multiple loci detected Does not need prior sequence information Generate high levels of polymorphism High reproducibility Genomic abundance high Can be used across species Useful in preparing counting maps Works with smaller RFLP fragments	Very tricky due to changes in materials used Not reproducible Very good primers needed Complicated procedure Require large amount of DNA

to biotic and abiotic stresses are an important genetic resource (Khush, 1997). However, the incompatibility of the wild species with cultivars delimits the introgression of wild species' genes to cultivars (Brar & Khush, 1997). Landraces whose seeds are maintained by farmers still contain useful genes (Jackson, 1997; Pusadee *et al*., 2009). Many genes conferring resistance to abiotic and biotic stresses, viz. salinity, rice stripe virus, and rice blast, are preserved and used in modern breeding programmes (Shi *et al*., 2010).

There are many advantages of MAS over other screening techniques. A concise list of the most important features of MAS has been provided by Collard et al. (2005), Xu and Crouch (2008) and Koebner and Summers (2003).

a. It saves time especially during the substitution of complex field trials (experiments conducted at specific time period of year or at specific locations) with molecular tests.

b. It eliminates phenotypic evaluation associated with field trials due to environmental effects. These molecular markers are not products of translation; therefore, they are not affected by any environment factor.

c. It has the ability to screen extremely difficult, expensive or time consuming (score phenotypic score) traits (e.g., root morphology, resistance to quarantined pests / specific races / biotypes of diseases / insects, tolerance for certain abiotic stresses such as drought, salt, mineral deficiencies and toxicities).

d. Selection of genotypes at seedling stage. It is very efficient as organogenesis does not have to be completed; traits that are expressed later in the lifecycle of a plant (e.g., grain or fruit quality, male sterility, photoperiod sensitivity).

e. Gene pyramiding or simultaneous multiple genes combine effect.

f. Escapes transferring of undesirable genes ('linkage drag'; this is of particular relevance when the introgression of genes from wild species is involved). A common problem in a conventional breeding programme is when integrated genes are often found to be linked with other undesirable gene(s). This linkage drag can be avoided in MAS programme where flanking markers closely linked to the gene of interest are used to identify crossover break points. Therefore, unwanted recombinants within the target locus can be rouged out further to reduce the chances of selecting undesirable genotypes which leads to linkage drag. This strategy effectively and efficiently reduces population size and the time required to fix the genes of interest in the desired genetic background.

g. Low heritable trait selection.

h. Possibility to diagnose specific traits when phenotypic screening is not applicable (e.g., quarantine pathogens).

i. Ability to screen homozygous and heterozygous genetic makeup of many loci in a single generation by avoiding progeny evaluations (since molecular markers are codominant).

Rapid identification of individuals that contain complementary parts of a complex character by RFLP, SSR or SNP-tagged QTLs is allowed in the marker-aided selection. These individuals often oppose accurate phenotypic identification due to the complex gene interaction that may govern the trait of interest (Yamamoto *et al*., 2000). Therefore, molecular markers are better than morphological and biochemical markers and have great use in molecular breeding.

DISCUSSION

One crucial step for successful breeding programmes is the genetic diversity of the genotypes. The information of genetic diversity can be used to devise the best strategies for logical utilisation of genetic resources within and among closely related crop varieties. The analysis of genetic diversity within and among varieties is important for breeders because it can help to assess the variation in the germplasm and also predict potential genetic gains (Chakravarthi & Naravaneni, 2009).

Rice breeding has been upgraded by using the molecular markers system. In MAS, individuals carrying target genes are selected in a segregating population based on linked markers rather than on their phenotype. In MAS, the population can be screened at any growth stage and in various environmental conditions. Efficiency of a breeding programme can be increased by selecting markers which linked to target traits or QTLs (e.g., pest and diseases resistance, high yielding). The problem in relation to QTL is crucial. Identifying QTL/s for a particular phenotype is difficult due to polygenic characters.

Thus, molecular markers are powerful tools in basic and applied research for analysing genetic diversity within and among varieties. There are different molecular markers which are based on polymorphism of protein or DNA (Schnable *et al*., 2009). Molecular markers which can show differences between accessions at DNA level are more direct and reliable, making them ideal tools for germplasm conservation and management.

The success of MAS in a plant breeding programme depends on the following important factors:

i. co-segregation or tight linkage of markers (< 5 cM) with the desired trait,

ii. efficient, user-friendly, cost effective means to screen large populations for the molecular marker(s), and

iii. high reproducibility of the screening technique across laboratories.

Rice germplasm which are linked with very important agronomic traits can be improved and upgrade by using molecular markers system in efficiently and in an accelerated development program. However, considering the effort and expense of DNA marker analysis, it is important that the MAS program itself be as efficient as possible. The foundation of any plant-breeding

program is its germplasm collection, and it is important that it be well characterized so that the breeder can improve chances of success in developing lines for commercial release. With the impetus in recent years of using exotic cultivars and landraces to broaden the breeders' germplasm base (Eizenga et al., 2006) this characterization has become even more important. Data from genotyping the parental material make the MAS program more efficient by determining not only which current cross populations would benefit from marker analysis, but also which breeding lines to use as parents in future crosses and which cross combinations to make.

There are major possible reasons to fail MAS in rice breeding in Sri Lanka, Pakistan and Vietnam. One main reason is the lack of resources (scientists, skilled labors, chemicals, laboratory, and equipment and funds). Skilled scientists migrate to developed countries for better opportunities and the remaining scientists are located near big cities where fields are too far away for easy access. Most of these markers may not be cost-effective and poor integration between molecular genetics and conventional breeding has a big impact. Essential concepts of breeding may not be understood by molecular biologists and conventional breeders are not thorough enough with newly developed molecular techniques. Therefore many unforeseen issues arise at breeding stations regarding the transition from conventional breeding and molecular breeding aspects in developing countries like Sri Lanka. Mainly analyzing of generated data and result interpretation are crucial if a rice breeder doesn't thorough with molecular genetics and statistics. Therefore existing rice breeders are extremely interested in traditional technologies that could make this procedure more in-efficient.

Cost is a major obstacle and prohibitive in MAS. MAS are more expensive than conventional methods for most traits. Detailed study of cost-efficiency has rarely been studied or calculated. Cost is mainly determined by the interest trait or traits and method of phenotypic screening cost (greenhouse, field trials), cost of genotypic methods (equipment, consumables, and other laboratory facilities) and labour costs (field technicians, research fellows). Unfortunately in most cases, funding will largely determine the extent to which markers should use in breeding. The questions that should be answered would be which traits should get the highest priority for marker development and how to explore the advantage and importance of the molecular breeding over the conventional breeding by minimizing the costs and increase efficiency of MAS furthermore.

In conclusion, it is understood that MAS allows selection of rice plants at the juvenile stage at very early generation and pyramiding of different resistance genes. In addition, MAS provides opportunities to breeders to develop broad-spectrum of very valuable and interesting new rice varieties such as pest and disease resistance, high yielding with dominant characters (colour, taste, size, shape, etc.). PCR method, which

requires small amount of DNA, is becoming a very useful technique for screening large populations of segregating progenies for simply and complexly inherited traits, where unwanted genes can be eliminated or greatly minimized at the early stages of rice breeding. This would cut down the cost, time and labour of breeding in an efficient and effective way and eventually leads to a prosperous agricultural lifestyle in developing countries.

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Effects of Combination Ethephon and Urea on Productivity of *Jatropha curcas* L. Thai Accession

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ABSTRACT

Low number of fruit production becomes the main constraint to develop *Jatropha curcas* as one of the alternative biodiesel sources in Malaysia. Therefore, one study was carried out to evaluate the effects of ethephon by combining it with urea for induction of flowering in Jatropha, with the main objective of increasing the productivity of fruit. The experiment was carried out at Rubber Research Institute Experimental Station (RRIES) Sungai Buloh, Selangor, where the soil was identified as the Renggam series. The planting material used was *Jatropha curcus* from Thailand accession. There were a total of nine treatments which were arranged according to Randomized Complete Design (RCD). Results indicated that Treatment 2 (200ppm ethephon 2.5% + 2% urea) showed the best combination promote in balancing male-female ratio, and resulted in an improved yield of the plants expressed as an increase in fruit produce and ripe fruit. The efficacy of ethephon at a lower concentration for increased fruit yield, with the addition of urea, is of great deal as this will lead to decreased costs and increased survival of Jatropha plantation.

Keywords: Ethephon, *Jatropha curcas* L., Thailand accession, urea

INTRODUCTION

The price of crude oil was around USD95-100 due to limited production sources and increases in the demand for fuel, and the price could up above USD100 in certain time (www.oil-price.net/27 Jun 2013). In many countries including Malaysia, biofuel is now an alternative for renewable energy, after wind energy and solar energy. As a consequence, there is high a demand for biofuel materials. Palm, sunflower, rapeseed and Jatropha oils are the materials available as biofuel feedstock (wikipedia.org/wiki/Biodiesel/27 Jun 2013). Biofuel based on

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Jatropha requires further investment as supported by a broad body of information from various studies. As Jatropha is considered a new and developing crop to the plant community, there has been a great deal of research conducted on it. Based on its potential as an alternative source of biodiesel, the Malaysian government has planned to develop Jatropha as one of the commodity crops. The Ministry of Plantation Industries and Commodities through the Malaysian Rubber Board has been appointed as the leader in planning and executing a Jatropha project, in terms of its suitability and also the economic impacts.

Meanwhile, oil of Jatropha can be extracted from the fruit. However, poor number of fruit has been the major limiting factor for commercialisation of this crop, which has been highlighted in various publications (see Heller, 1996; Biswas *et al.,* 2006; ERIA, 2010,). Pollination of Jatropha is one of the limited factors on producing the fruit. It is due to the ratio of the male to female flowers, which is up to 27:1. In addition, Raju and Ezradanam (2002) reported that one of the most reasons for the poor yield is that Jatropha has few female flowers resulting from a very low female-to-male flower ratio, which, depending on the genotype, is about 1:29–1:13. Thus, pollination to produce fruit will be difficult due to limited female flowers. According to Bhattacharya *et al*. (2005), only 50% of flowers were reported to fruit due to the inconsistence inflorescence of flowers. One of the solutions is to involve a booster treatment or a growth regulator so as to improve the number of Jatropha fruit (Ghosh *et al*., 2010; Joshi *et al*., 2011; Xiurong *et al*., 2012). However, several researchers have focused on some specific chemicals as the growth regulator, where the high cost involved in the use of these chemical could become the limitation.

Ethephon was widely used in agriculture sector to accelerate pollination process and fruit maturity like application on pineapples (Dass *et al*., 1975). Unsaturated hydrocarbons like acetylene and ethylene were applied in inducing flowering in pineapple (Lewcock, 1937). However, ethephon (2-chloroethylphosphonic acid) was made available for commercial use. When applied to the plants, the chemical releases ethylene directly in the tissues, producing numerous physiological effects. Ethephon is stable at a pH below 4.0 and since the pH of cytoplasm of plant cells is higher than this, the ethephon is degraded to ethylene as it enters the plant tissues. Generally, urea is known as a supplier of nitrogen (N). Nitrogen is one of the important nutrients in the plant as a component to develop cell tissues and generate photosynthesis activity in plants.

Meanwhile, high concentrations of ethylene (300ppm to 500ppm) can cause dry and necrosis or red colouration in *Ptilotusnobilis* plants in *in vitro* experiment (Prameswara *et al*., 2009). The same researcher also suggested that high concentrations of ethylene may become toxic to plants. High concentrations of ethylene have been reported to lead to some effects of plant morpho-physiology of *Jatropha*

curcas, where the leaf discolouration resulted in the symptom of leave dry (Rosatikah *et al*., 2012). Therefore, using low concentration of ethylene can reduce the dryness but other added compounds need to be put in so as to maintain the efficiency of ethylene. According to Dass *et al*. (1975), some compounds such as urea and calcium carbonate are able to induce more flowering to pineapples. Therefore, a study was carried out to evaluate the efficacy of different concentrations of ethephon, specifically low concentration, combining it with urea on the productivity of Jatropha tree.

MATERIALS AND METHODS

Study Sites

The study was conducted at Field 15, Rubber Research Institute Experimental Station Sungai Buloh in Selangor, where the soil was identified as the Renggam series. During the experiment, the temperature was around 31-34^{o}C, while the average of humidity was 68%. The planting material used was *Jatropha curcus* from Thailand accession (Fig.1). The age of the Jatropha tree during the experiment was 5 years. Latex stimulant (Ethephon 2.5% and Ethephon 5%) was used as the main ingredient in the treatment. Urea used to be urea 46%. The treatments were stated as follows: T1: without spray (Control), T2: 200ppm ethephon 2.5% + 2% urea, T3: 200ppm ethephon 2.5% + 4% urea, T4: 400ppm ethephon 2.5% + 2% urea , T5: 400ppm ethephon 2.5% + 4% urea, T6: 200ppm ethephon 5% + 2% urea, T7: 200ppm ethephon 5% + 4% urea, T8: 400ppm ethephon 5% + 2% urea, T9: 400ppm ethephon 5% + 4% urea.

The treatments were applied as a spray in the month of March to May. The application of the treatments was done once a week. The experimental design was based on a randomized completed design (RCD).

Number of Flowers and Fruits

The number of flowers (male and female) (see Fig.2) was observed and recorded every week. Furthermore, the total fruit per tree was also recorded every week. The ripe fruit was harvested after the colour of fruit had changed from green to yellow (Fig.3). The harvesting period was stopped after one week from the last treatment sprayed. Chlorophyll content was measured using Konica Minolta SPAD Chlorophyll Meter. Meanwhile, the height of the trees was recorded to monitor their growth during the experiment.

Fig.1: Experimental trees of *Jatropha curcas*

Fig.2: The flowers structure of *Jatropha curcas*

Unmatured fruit Ripe fruit

Fig.3: A comparison between *Jatropha curcas*'s fruit

Statistical Analysis

The data obtained from the experiment were analyzed by using the Analysis of Variance (ANOVA). The standard error (SE) was evaluated at 5% level of significance. All the experiments were carried in three replicates for each treatment.

RESULTS AND DISCUSSION

Number of Fruit Produced

Total fruit in the whole trees for every treatment was determined every week. The results showed that the plants treated with the lowest concentration of ethephon, T2 (200ppm ethephon 2.5% + 2% urea) yielded the highest significance ($p<0.05$) in the average yield produced during the experiment (27 total fruits), followed by T3 (15 fruits), T1 (15 fruit), T4 (13 fruits), T9 (12 fruits), T7 (6 fruits), T5 (6 fruits), T6 (5 fruits) and T8 (4 fruits) (Table 1). Meanwhile, during week 0 to week 7, the highest fruit produced was shown for the T2 treatment group with a total of 63 fruits, followed by T3 (31 total fruit), T4 (26 total fruit), T1 (26 total fruit), T9 (22 total fruit), T7 (20 total fruit), T5 (16 total fruit), T6 (15 total fruit) and T8 (14 total fruit). However, from week 8 to week 9, the treatments mostly showed a decreasing trend in the total number of fruit, and this could be attributed to the harvesting period of the ripe fruit.

A considerably higher number of fruit yield was observed in the lowest concentration of 200ppm (i.e., for T2 and T3) added with 2-4% urea compared to the control after the application of the treatment. The high concentrations of ethephon at T5, T6, and T8 did not yield any encouraging results. This showed that the addition of urea had promoted absorption of ethephon and thus increased the availability of ethylene to the plant tissues (Yamada *et al*., 1965a, 1965b).

The variability in fruit production is generally affected by various factors. In this study, the weather conditions were implied to have influenced flowering. The weather could affect pollination, therefore, stigma receptivity, ovule fertility, ovule longevity and fruit set are directly affected as well (Burgos *et al*., 1993). In addition, genotype dependent factors related to floral biology influenced fruit set and productivity such as flower bud production and flowering time.

TABLE 1
The effects of plant growth regulators on the total fruit produce pattern of *Jatropha curcas*

Treatment	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Average fruit
T1 (Control)	Na	Na	1±0.67	1±0.58	4±2.08	5±2.08	33±11.67	26±7	37±8.76	37±4.26	15±4.75[a]
T2	Na	Na	1±2.91	13±2.60	20±4.80	52±14.70	52±14.70	63±4.30	52±3.28	11±4.91	27±7.74[b]
T3	Na	Na	Na	17±15.52	23±21.50	23±21.5	23±2.93	31±28.3	27±19.6	7±5.43	15±3.87[a]
T4	Na	Na	2±1.16	10±2.96	11±3.75	28±1.70	32±3.21	26±6.90	11±1.86	4±2.40	13±3.81[a]
T5	Na	Na	Na	3±2.39	9±3.21	9±3.21	9±3.21	16±6.24	4±1.73	18±6.24	6±1.65[a]
T6	Na	Na	Na	2±1.15	3±1.33	8±3.67	8±3.67	15±3.53	10±2.30	1±0.33	5±1.68[a]
T7	Na	Na	2±0.67	2±1.67	9±8.17	10±8.41	10±8.41	20±15.60	10±7.21	Na	6±2.13[a]
T8	Na	Na	1±0.57	3±1.53	3±0.88	3±1.45	4±1.53	14±6.93	12±5.85	Na	4±1.56[a]
T9	Na	Na	7±4.37	11±1.53	10±3.84	18±8.09	20±10.84	22±8.89	22±7.02	14±1.45	12±2.45[a]

Data are expressed as means ± S.E.
Na: Not available
Mean values with same superscript in column are not significantly different (p>0.05)

Number of Ripe Fruit

The results indicated that the plants treated with a lower concentration of mixture ethephon with urea could increase the yield of harvest fruit compared with the untreated plants. The highest results shown for T2 with the concentration of 200ppm ethephon 2.5%, added with 2% urea (Table 2). At the end of the harvesting period, T2 gave the significantly highest number of ripe fruit, with the mean of 44.33 ± 2.85, followed by T3 with 30.00 ± 29.01, T1 with 23.33 ± 8.99, T9 with 14.00 ± 11.59, T4 with 13.67 ± 3.76, T5 with 10.67 ± 3.84 ,T6 with 7.33 ± 4.33, and then T7 with 6.67 ± 5.70 and T8 with 6.00 ± 2.52. However, certain treatments gave some inconvenient results with reference to error bars (i.e., T3 and T9). It could be due to non-uniformed ripe fruit during the experiment. The results of this study are parallel with previous studies which suggested that the ripening of Jatropha fruits on the tree is not uniform (Heller, 1996; Biswas *et al.,* 2006). Therefore, in this study, the situation could affect the statistical analysis for ripe fruit, but in average, T2 showed a more pronounced effect on the ripe fruit of Jatropha.

TABLE 2
The effects of plant growth regulators on the ripening fruit pattern of *Jatropha curcas*

Treatment	Number of ripe fruit
T1 (Control)	23.33 ± 8.99^{a}
T2	44.33 ± 2.85^{c}
T3	30.00 ± 29.01^{a}
T4	13.67 ± 3.76^{a}
T5	10.67 ± 3.84^{ab}
T6	7.33 ± 4.33^{ab}
T7	6.67 ± 5.70^{ab}
T8	6.00 ± 2.52^{ab}
T9	14.00 ± 11.59^{a}

Data are expressed as means ± S.E.
Mean values with the same superscript in column are not significantly different (p>0.05)

Number of Flowers

Ethephon treatment can increase the number of inflorescence per plant and balance of both male and female flowers, and thus, increase the trend towards femaleness in comparison to the control. The trend gave encouraging results when treated with T2 (200ppm ethephon 2.5% added with 2% urea), but there was no significant difference (p>0.05) found among the treatment in the flower ratio during the experiment (Table 3). However, T2 had a more pronounced effect on increasing the trend towards femaleness in comparison to other treatment with the average ratio of male to female flowers shown to be 10.3±1.90 during the experiment. Although low concentrations of ethephon were used, the combination with 2% urea could increase absorption into the plant system, and thus, increase the number of inflorescence per plant.

TABLE 3
The effects of plant growth regulators on the flowering pattern of *Jatropha curcas*

Treatment	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	Week 9	Average ratio
T1 (Control)	6± 1.27	3± 1.76	20± 1.67	25±3.84	9± 1.86	20± 5.13	10± 2.50	12± 2.31	11±4.30	20± 2.52	13.6±2.30 [a]
T2	3± 0.61	2± 0.25	14± 0.67	5 ± 1.20	5± 1.20	10±2.19	13± 2.85	10± 2.52	11±4.49	14±0.88	10.3±1.90 [a]
T3	3± 1.45	3± 0	10± 1.45	16± 8.09	13±3.76	15± 1.45	19± 2.30	8± 2.03	14±2.31	14± 5.36	12.1±1.80 [a]
T4	4±0.64	3±0	16± 2.31	9± 1.20	4± 0.86	31± 5.56	32± 2.19	10± 1.86	12±0.88	30± 5.86	14.9±3.70 [a]
T5	3±0.75	3± 0.22	21± 1.16	19± 1.76	7± 1.76	17± 5.23	15± 4.30	24± 0.58	14±0.67	20± 4.33	14.1±2.38 [a]
T6	2±0.33	1±0.67	17± 1.53	24± 1.53	6± 2.52	21±7.55	16± 1.67	17± 5.51	22±7.42	20± 4.10	14.7±2.70 [a]
T7	3±0.58	2±0.74	19± 2.73	24± 4.0	9± 2.0	32±17.09	11± 7.30	24±10.40	8±4.33	18± 4.84	14.9±3.20 [a]
T8	3±0.28	2±0.29	12 ±1.0	27± 1.20	19±1.76	27± 0.88	21± 7.67	12± 6.39	21±3.18	12± 6.69	15.7±2.82 [a]
T9	2± 0.40	2±0.92	13± 1.86	12± 0.89	12 ± 4.0	28± 3.10	12± 1.45	12± 0.88	19±4.91	30± 3.38	14.3±2.90 [a]

Data are expressed as means ± S.E.
Mean values with same superscript in column are not significantly different (p>0.05)

Inconsistent flower ratio during the experiment could be due to weather conditions, where the number of flowers was affected by water level. Mwanamwenge *et al.* (1999) reported a significant abortion on faba bean flowers and small pods due to water deficit. Guitián (1993) mentioned that drop of flowers is a normal process in many species. It is due to improved fruit set (Jackson & Hamer, 1980). Abdelgadire *et al*. (2008) reported the fruit from cross-pollinated flowers in the crop was significantly larger and numerous than those produced by autogamous self-pollinated flowers.

Chlorophyll Content

The sprays of ethephon and urea on Jatropha plants positively affected the chlorophyll capacity. All the treatments gave increased chlorophyll capacity to the plants after the experiment (Table 4). The highest increment of chlorophyll content was from the plants treated with T8 (400ppm ethephon 5% + 2% urea), with 33.11 ± 0.90 SPAD units to 38.37 ± 1.90SPAD units, respectively. However, there was no significant difference (p>0.05) among the treatments in terms of the chlorophyll content during the experiment.

TABLE 4
The effects of plant growth regulators on the biochemical pattern (chlorophyll content) of *Jatropha curcas*

Treatment	Chlorophyll content	
	Before experiment	After experiment
T1 (Control)	32.40 ± 1.20[a]	34.80 ± 1.21 [a]
T2	33.30 ± 1.00 [a]	35.87 ± 2.02 [a]
T3	33.40 ± 1.20 [a]	35.70 ± 0.93 [a]
T4	32.20 ± 0.15 [a]	35.53 ± 0.19 [a]
T5	32.05 ± 1.20 [a]	35.53 ± 1.59 [a]
T6	34.10 ± 1.10 [a]	37.47 ± 0.98 [a]
T7	33.60 ± 1.50 [a]	37.40 ± 1.46 [a]
T8	33.11 ± 0.90 [a]	38.37 ± 1.90 [a]
T9	33.00 ± 1.20 [a]	36.70 ± 0.55 [a]

Data are expressed as means ± S.E.
Mean values with same superscript in column are not significantly different (p>0.05)

Height of Trees

Before the experiment was started, the trees were surveyed and selected to get the average same height of the trees. There was no significant difference (p>0.05) among the heights of the tree in all the treatments (Fig.4) at the start of the experiment. Meanwhile, Fig.5 shows that there is no difference between the heights, and the trend is similar in every treatment during the experiment. These show that the spray of combination ethephon and urea to Jatropha trees did not cause any side effect on the growth of the trees and this is in agreement to the findings of Joshi *et al*. (2011).

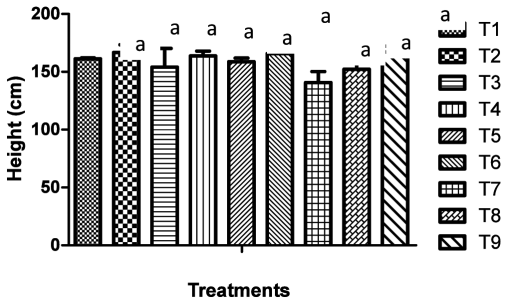

Fig.4: The height of the *Jatropha curcas* trees before the start of the experiment

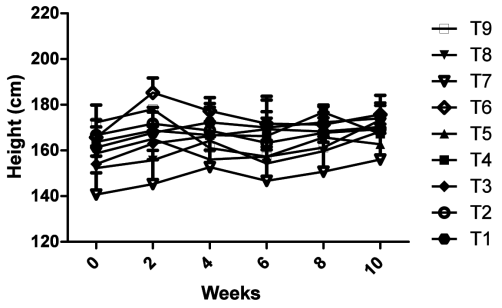

Fig.5: The effects of plant growth regulators on the growth pattern (height) of *Jatropha curcas*

It has been reported that high concentrations of ethylene (300ppm and 500ppm) caused dry and necrosis or red colouration in *Ptilotusnobilis* plants in *in vitro* experiment (Prameswara *et al*., 2009). In addition, Rosatikah *et al*. (2012) also reported that the application of high ethephon (300 to 600ppm) had induced leaf discolouration to Jatropha leaf resulting in the symptom of dry leaves. Thus, the study attempted to develop new mechanisms to use ethephon in low concentrations. Meanwhile, Dass *et al*. (1976) stated that the lowest effective concentration of ethephon which would induce flowering was previously believed to be around 100ppm. The efficacy of ethephon in increased yield at a low concentration can be improved by the addition of urea. It is probably due to the fact that urea promotes the absorption of ethephon and thus increases the availability of ethylene to the plant tissues (Yamada *et al*., 1965a, 1965b). In the present study, it was that ethephon added with urea could cause variable effects on different physiological properties of the Jatropha plants. The present results indicated that the concentration for pronounced effect when compared with the control, with the concentration of 200ppm (ethephon 2.5%) which was added with 2% urea.

The results presented in Table 1 and Table 2 show that the plants sprayed with low concentration of ethephon added with 2% urea had a significant increase in the fruit produced and ripe fruit. According to Joshi *et al*. (2011), this phenomenon could be attributed to the balanced water and also the concentration of ethephon use during vegetative phase, where stress condition was reduced during reproductive phase and there was an increase in the number of fertile female flowers. In addition, urea could promote increased photosynthesis activity to produce fruit.

The results shown in Table 3 indicate that the combination of ethephon and urea increased the number of inflorescence and balanced the flower ratio compared to the control, especially T2. The treatments with spray on the plants released ethylene and then increased the ethylene concentration at the meristem that might have induced the

development of floral organs. In addition, the efficacy of ethephon in inducing flowering by the addition of urea, even at a low concentration, was probably due to the fact that urea had promoted absorption of ethephon and thus increased the availability of ethylene to the plant tissues (Yamada *et al*., 1965a, 1965b). Nitrogen also gives response to the metabolic demands of developing reproductive and vegetative organs (Klein & Weinbaum, 1984; Fernández-Escobar *et al*., 2004). A lesser amount nitrogen will affect fruit set, yield and shoot growth (Freeman *et al*., 2005). In addition, nitrogen fertilization increased fruit set in rain-fed olives (Hartmann, 1958; Cimato *et al*., 1990). Meanwhile, Therios (2006) stated that nitrogen increased the proportion of hermaphrodite flowers and a concentration of less than 1% in the leaves led to the formation of staminate flowers and therefore decreased the potential level of fruit set.

Joshi *et al*. (2011) suggested that a suitable ethylene concentration might have increased the growth rate leading to a large apical meristem and greater number of inflorescence. The balance between the male and female flowers per plant in treating plants and the increase in the number of inflorescence in treating plants might be due to the synergistic effects of ethylene on the concentration of other hormones such as gibberellins and cytokinin within the cell (Joshi *et al*., 2011). The increase in femaleness after ethephon applications may be also related to effect of ethylene on auxin and gibberellins (GA) concentrations *in vivo* and their interaction (Joshi *et al*., 2011).

It is important to highlight that the chlorophyll content of Jatropha plants was increased after the application of ethephon and urea (Table 4). The effects of ethephon and urea on chlorophyll content might be due to the broadening of exterior mesophyll cells that provided more spaces for chloroplast arrangement (Joshi *et al*., 2011). Meanwhile, Fleischer (1934) reported that the rate of photosynthesis is proportional to the content of chlorophyll. The increase in the chlorophyll content for treating plants in the experiment could contribute to the increase in the number of inflorescence flowers and fruit.

CONCLUSION

In conclusion, the application of ethephon added with urea has been found to be beneficial for plant development and increasing the yield of the *Jatropha curcas* plants, although at low concentrations. It is suggested that 200ppm ethephon 2.5% added with 2% urea promotes balanced male-female ratio, and thus results in improved yield of the plants expressed as an increase in fruit production and fruit ripening.

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Effective Elements on Technical Knowledge of Agricultural Section for Sustainable Soil Management

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ABSTRACT

The main purpose of this study was to carry out a comparative analysis of effective elements of repercussion farmers of Garmsar province for sustainable soil management. The methodological approach in the research was a descriptive-correlation, where the survey type was applied. Using a proportional stratified sampling technique with Cockran formula, 184 farmers in Garmsar were selected. A total of 192 questionnaires were collected and analysed. The content and the face validity of the instrument was specified after several times of review and correction by the faculty members of Agricultural Extension and Education of Azad university of Garmsar, specialists in the Ministry of Jehad, graduate students of Agricultural extension and education, experts and local farmers in Garmsar. The reliability of the analysis was conducted through 30 questionnaires and Cronbach's Alpha values for the Varamin's farmers of the instrument were estimated as 91% using statistical SPSS software. Results show that participations in educational durations, educational level of beneficiaries, the quantity of media usage, type of their jobs, their knowledge of sustainable development, type of their drove, the amount of present extensional services to farmers were significant at 95% level, with the amount of technical knowledge of farmers about sustainable soil management. Meanwhile, the farmers' income, their family members, effectiveness of basic product, the amount of their experience in agriculture, their penchant to agricultural activities, social altitude, cultural trait, their attitude to soil sustainable development, the amount of convenience, farm areas were also found to be significant at 99% level, with the amount of technical knowledge of farmers about sustainable soil management. Multiple regressions indicated that 68% of the variance in the dependent variable could be explained by the effectiveness of basic product, economical situation, and social situation, personal elements, educational-extensional elements.

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INTRODUCTION

Soil is one of the most important resources of every country. Nowadays, soil erosion is considered as a serious threat to people's welfare and their lives. In some areas where people do not control the soil erosion, they will envisage soils with low productivity. Soil erosion has different unfavourable effects such as impoverish soil and canal tartar. As a result, soil safekeeping is an important topic.

The World Bank (2006) describes sustainable soil management as a retinue of the ability of meadow to product, preservation of commercial and productivity potential, conservation of canals to store water, some expediency to stoppage erosion, and also a way to decrease the damages of incorrect usage.

The Iranian Ministry of Forest and Natural Resources (2005) indicated that the current average level of erosion in the country is more than 35 tons per hectare, which is extremely high when compared with the corresponding values in Europe (0.9 t/h) and America (4 t/h); it is very calamitous. The result is deletion of desirable farms and of course social and economical crisis such as poverty and migration. The main reasons for this problem are violent decrease of pastures which is due to the lack of proper management, unfavourable technologies utilization to exploit farms and exploit farms regardless of soil and water sustainability.

The main reasons for farms destruction are some beneficiaries, which use the farms without technical knowledge. Linear development systems are designed based on wisdom, participation and ability of farmers; hence attention to beneficiaries' knowledge about development precinct can be an important principle which is unfortunately often ignored (Dialla, 1994). The main reason of such behaviour is the lack of relation between research science and the villagers information. In fact, it is an important point that agricultural development has an irrelevant meaning because many people ignore the needs, genius, experiences, knowledge and situation of the people in specific locations and change agents just to promote new and modern technologies regardless of their situation (Kazemi *et al*., 2007).

Latest studies have shown that when we consider indigenous knowledge and technology in order to soil conservation and it can have positive effects in farmers' participation in improvement, revision water and soil management methods (Kazemi & Shavali, 2003). Chizari and Ghadimi (2001) in a research on the comparison of multi-criteria evaluation methods on sustainable management of water resources of Khorasan's Qare-Qum watershed indicated that planners of water resource development prefer formulating plans in which a set of projects with the highest productivity of existing resources, as well as the most effective participation in meeting the conflicting goals of concerned groups and decision makers, were selected. The

results show that the evaluation methods based on UTA, depending on the type of problem, present a better approach for making decision in relation to prioritization compared to other evaluation methods.

Rezaei and Shafei (2007) carried out a study to determine the effects of technical knowledge of farmers on the production of grape in Ballo village in Urmia city with regard to the importance of reforms in traditional agricultural methods, usage of modem technologies and need of training farmers, modem techniques, the effect of farmers' technical knowledge on grape production. They found that the experience, training and use of cordon method had significant and positive effects on production. However, some variables like age, education and being farmer as a main job did not have any significant effects. These findings indicate that sharing of traditional knowledge is more than other variables in the production process and the importance of training farmers by modem technologies, and therefore, it is suggested.

Kalantari and Mirgohar (2003), in a research investigating the factors affecting the technical knowledge level and its application and their roles in irrigated wheat yield in Tehran and Isfahan province, indicated that the most important independent variables which describe the level of technology application are technical knowledge level, using knowledge sources, distance from agricultural service centres, attitude to extension programs and ownership of machinery. These factors constitute 46.9 percent of the technology application variation. It was also found that the level of technology application, times of urea fertilizer usage, technical knowledge level and using micro fertilizers described 34.4 percent of wheat yield variation. The t-test results indicated that there was no significant difference between technical knowledge level and technology application.

In Garmsar Township, irregular consumption of chemical muck and lack of sustainable management usage are the reasons for severe destructions of farms and low soil productivity. Jehad specialists reported that most of the farms do not have sufficient productivity and productions slump are seen frequently (Amininasab, 2008). Therefore, the main purpose of this study is the regression analysis of effective elements on technical knowledge of agricultural section about sustainable soil management in Garmsar township. The objectives of this study are to:

- determine the knowledge level of Garmsar agricultural beneficiaries for sustainable soil management. Priorities of Garmsar agricultural beneficiaries' attitudes about sustainable soil management.
- Priorities of Garmsar agricultural beneficiaries' viewpoint about cultural, social, economic, legal-infrastructural, educational-extension factors on sustainable soil management

MATERIALS AND METHODS

The methodological approach in the research was a descriptive correlation and applied

of the survey type. A questionnaire was developed from a review of literature to collect relevant data. The content and face validity of the instrument was specified after several times of review and corrections by the faculty members of Agricultural Extension and Education of Azad university of Garmsar, specialists in the Ministry of Jehad, graduate students of agricultural extension and education, as well as experts and local farmers in Garmsar. The reliability of analysis was conducted through 30 questionnaires and the Cronbach's Alpha values for the Varamin's farmers of the instrument were estimated at 91% using the statistical SPSS software. Using the proportional stratified sampling technique with Cockran formula, 184 farmers in Garmsar were selected out of a total of 8875 population. Finally, 192 questionnaires were collected and analysed.

RESULTS AND DISCUSSION

Table 1 shows that the maximum frequency (61 persons) covers the 41-50 age range. In addition, 99.78% (191 persons) are males and on 1 (0.5%) female. Educational level analysis of farmers shows that the maximum frequency includes reading and writing only and 35% of them have more than a diploma or a degree (Table 2). Table 4 shows three (3) maximum frequencies (23.4%) of the farmers participated in educational durations as very low and 55% of the farmers do not participate in these durations. In fact, most of the beneficiaries do not participate in the educational durations.

TABLE 1
Frequency distribution of respondents' age

Age	Frequency	Percentage	Cumulative Percentage
Until 20 years	3	1/6	1/6
20-30 years	30	15/6	17/2
31-40 years	55	28/6	45/8
41-50 years	61	31/8	77/6
51-60 years	27	14/1	91/7
More than 60 years	15	7/8	99/5
No response	1	0/5	100
Total	192	100	---

TABLE 2
Frequency distribution of respondents' educational measure

Educational measure	Frequency	Percentage
Illiteracy	28	14/6
Reading & writing	49	25/5
Elementary	11	5/8
Guidance	20	10/4
High school	15	7/8
Diploma	30	15/6
Associate degree	22	11/5
Bachelor of science	12	6/3
No response	5	2/6
Total	192	100

TABLE 3
Frequency distribution of respondents' participation in educational durations

Participate in educational durations	frequency	percentage
Zero	43	22/4
Very low	45	23/4
Low	19	9/9
Moderate	20	10/4
High	41	21/4
Very high	22	11/5
Total	192	100

This section discusses the agricultural knowledge of farmers to evaluate their knowledge about sustainable soil management. The information presented in Table 4 indicate that their knowledge about sustainable soil management is partly low (2/57).

Meanwhile, priority of farmers' incompetence in knowledge shows that the top priorities are optimization of products nutrition, distinguishing of chemical shortages symptom, the amount of soils acidity and salty, soil porosity, tillage alternation and planting systems, the amount of organic materials of soil and water consumption.

In order to study the farmers' attitude, 22 statements were chosen to be scored using the Lickert scale. The results were found to be quite varying, ranging from complete disagreement to complete agreement. The farmers stated that they must undertake the responsibility of soil sustainability protection and that the chemical muck has a destruction effect on soils and the most important factor on soil sustainability is preservation of leftover herbaceous, cultivate various plants and used animal wafer in the farms. The last priorities of the farmers in this section include the only attendance to profit, accomplishing educational activities in order to sustainable soil management and encourageing other farmers to do sustainable soil management.

Correlation matrix was done to study the role of different variables on farmers' knowledge about soil management (Table 7). Some variables such as participation in educational durations, level of education, the level of media usage, type of beneficiaries' job, the level of farmers' knowledge on sustainable development, drove number and the level of extension services presentation to beneficiaries were significant with 95%

TABLE 4
Frequency distribution of respondents' knowledge about sustainable soil management

Respondents' knowledge about sustainable soil management	Very Low		Low		Moderate		High		Very High		PRIORITY
	Freq	Perc	Freq	perc	freq	perc	freq	perc	freq	perc	
Optimization of products nutrition	28	14.6	112	58.3	32	16.7	12	6.25	8	4.2	1
Distinguish of chemical shortages symptom	30	15.6	99	51.6	43	22.4	14	7.3	6	3.1	2
The amount of soils acidity and salty	19	9.9	95	49.5	51	26.6	16	8.3	11	5.7	3
Soil porosity	8	4.2	54	28.1	95	49.5	19	9.9	16	8.3	4
tillage alternation and planting systems	8	4.2	44	22.9	87	45.3	42	21.9	11	5.7	5
The amount of organic materials of soil	5	2.6	29	15.1	85	44.3	59	30.7	14	7.3	6
Water consumption	4	2.1	25	13	77	40.1	60	31.3	24	12.5	7

TABLE 5
Priority of farmers' attitude in relation to sustainable soil management

Farmers' attitude in related to sustainable soil management	Mean	Standard Deviation	Priority
Undertake the responsibility of soil sustainability protection with farmers			1
Chemical muck has a destruction effects on soils			2
The most important factor on soil sustainability is Preservation of leftover herbaceous			3
cultivate various plants			4
Used **Animal wafer** in the farms is better than chemical muck			5
People Wrong activities is one of the reasons of soil destruction			6
Soil is the natural resources that produced in long time			7
Sustainable soil management don't do because of farmers low financial			8
Sustainable management and perfect use of water make soil sustainability			9
Without good soil the farming is impossible			10
Soil reinforcement is a special activity and need high knowledge and experience			11
Soil test has a basic role in soil management and Improvement			12
Planting some plants such as alfalfa is the reason of soil productivity			13
I emphasize on others guidance and instructions in related to soil sustainability protection			14
I'm enthusiastic to maintain the farms			15
Soil erosion is a serious problem that unsuitable soil management is the reason of this problem			16
It's a duty to respect next generation to Have proper soil			17
Farmers have to been obligate to use sustainable soil techniques			18
Farmers know everything about sustainability soil management and they don't need to any education			19
Profit maximization is more important than soil sustainability			20
Agricultural specialists must educated farmers to know what's sustainability soil management			21
I encourage the other farmers to use sustainable soil management			22

TABLE 6
The correlation relationship between the variables for respondents' viewpoint

First variable	r	P
Participate in educational durations	*0.212	0.04
Level of education	*0.186	0.017
The level of media usage	*0.147	0.028
Level of beneficiaries' income	**0.241	0.004
The number of family members	- **0.384	0.002
Distances until Agricultural service center	*-0.241	0.009
Efficiency of main production	**0.536	0.000
Number of beneficiaries' contacts with change agents	0.097	0.095
Number of beneficiaries' contacts with state institutions	0.101	0.075
Type of beneficiaries' job	*0.125	0.047
Type of beneficiaries' job	**0.361	0.001
Level of interest to agricultural activities	**0.220	0.004
Social altitude of beneficiaries	**0.282	0.003
Cultural trait of beneficiaries	**0.257	0.001
The level of farmers knowledge about sustainable development	*0.199	0.012
drove number	*-0.143	0.042
Attitude of beneficiaries to sustainable soil development	**0.312	0.002
The level of extension services presentation to beneficiaries	*0.112	0.042
The amount of loans	**0.124	0.034
The watery farms area	**0.236	0.007

confidence and level of beneficiaries' income. Meanwhile, the number of family members, efficiency of main production, level of experience in farming, level of interest to agricultural activities, social altitude of beneficiaries, cultural trait of beneficiaries, attitude of beneficiaries to sustainable soil development, the amount of loans and the watery farms area were found to have significant relationships with 99% confidence and technical knowledge of beneficiaries about sustainable soil management.

TABLE 7
The role of independent variables in sustainable soil management

Regression model	R	R^2	Adj R^2	S.E
Stepwise	o.792	0.737	0.684	0.241

Regression model was also significant (sig=0.000). The regression analysis showed the variables that were statistically significant. The results indicated that 68% (R^2_{adj}=0.68) of the variance in the technical knowledge of beneficiaries about soil management could be explained by these variables.

TABLE 8
The result of multiple regressions (stepwise method) to determine the role of independent variables in technical knowledge of beneficiaries about sustainable soil management

Independent variables	B	Beta	T	sig
Constant	3.097	---	3.376	0.000
Efficiency of main production	0.674	0.638	8.364	0.000
Economical situation	0.468	0.389	5.975	0.000
Social situation	0.339	0.307	4.579	0.002
Individual factors	0.309	0.293	3.098	0.003
Extension factors	0.218	0.177	2.429	0.014

The regression equation can be written using the information presented in Table 11, as follows:

Y= Efficiency of production (0.638) + economical situation (0.389) + social situation (0.307) + individual factors (0.293) + extension factors (0.177)

- There is significant and positive relation between educational level of farmers and their knowledge on sustainable soil management, whereas more than 52% of the farmers have educational level that is lower than guidance school, leading to their low knowledge of sustainable soil management. This finding agrees with the finding by several researchers including Touri (2009), Dinpanah (2004), Karbasi (2001), Jahani (2001), Bagdi (2005), and Najouki (2003).
- There is a significant and negative relation between the number of family members and their knowledge about sustainable soil management. The higher the number of the family members, there is more pressure on the farms to have further income. Some researchers like Shahroodi and Chizari (2005), Touri (2009), Bagdi (2005) have reported the same result.
- There is a significant and positive relationship between efficiency of main production and their knowledge about sustainable soil management. The latter is evident because with increasing farmers' knowledge, they will use the references to the optimum and consciously. Some researchers like Dinpanah (2004), Hayati *et al*. (2000), Shahroodi and Chizari (2005), and Touri (2009) have also reported the same finding.
- There is a significant and negative relationship between distance of farms to service centre and the farmers' knowledge about sustainable soil management. The same result has also been highlighted by some other researchers like Dinpanah (2004), Shahroodi and Chizari (2005), Hayati *et al*. (2000), whereas no relationship between the two was reported in Touri's (2009) research.

- There is a significant and positive relationship between farmers' experience in agricultural activities and their knowledge about sustainable soil management. The same result was also reported by some other researchers like Dinpanah (2004), Shahroodi and Chizari (2005), Nooripur and Shahvali (2006), Tabraeei (2005), and Najooki *et al*. (2008).
- There is no relationship found between the number of beneficiaries' contacts with change agents and the number of beneficiaries' contacts with state institutions with sustainable soil management. However, some researchers like Dinpanah (2004) and Touri (2009) reported a positive relationship for the two variables.
- There is a significant and positive relationship between farmer's altitude and their knowledge about sustainable soil management. This result was not expected because social altitude is a synthetic variable that is relevant to people's knowledge and information related to their job and life. Some studies have also reported the same finding (see Dinpanah, 2004; Tabraei, 2005).
- There is a significant and positive relation between the amount of loans and farmers' knowledge about sustainable soil management. This confirms the work of Dinpanah (2004) and Bagdi (2005).

CONCLUSION

In order to carry out the regression analysis, the most important factor in the educational level of farmers in relation to sustainable soil management in Garmsar is the efficiency of the main production. As a result, suitable orientation is needed to introduce some techniques that could improve and enhance the productions. An example of this technique is enhancement of farmers' technical knowledge about sustainable soil management (nutrition management of productions, shortage signs, etc.).

Farmers' knowledge about sustainable soil management is still low. Hence, improving the capacity building of farmers to educational classes and educational durations attend to adult education principles and different level of knowledge, attitudes and skills.

The descriptive statistics shows that farmers' participation in extension programmes is still low. Therefore, educational centres must find ways to increase farmers' willingness to partake in educational classes and provide schedules and duration of programmes that are suitable and relevant to farmers' needs.

As indicated previously, farmers' educational level has a significant role in sustainable soil management and it is necessary to farmers' literacy to regulate and execute comprehensive programmes.

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Ethnomedicinal Study of Plants in Hathazari, Chittagong, Bangladesh

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ABSTRACT

An ethnomedicinal survey of the rural community, mainly Chakma from Hathazari, Bangladesh, was conducted from May 2010 to January 2013. The methods used for ethnomedicinal data collection were field interviews, plant interview and group interview techniques. Local (Bangla) names, habit, parts used, mode of preparation and medicinal uses of plants were recorded by interviewing the locals of different age groups (mostly between 25 to 75 years) and also herbal practitioners (Kabiraj). A total of 75 plant species consisting of 67 genera categorised under 44 families were documented for the treatment of 35 ailments. Among the total documented species were herbs (41%), shrubs (19%), trees (28%) and climbers (12%). The most encountered medicinal plant families are Asteraceae, Lamiaceae, Rutaceae, Solanaceae, Liliaceae, Malvaceae, Combretaceae and Amaranthaceae. Analysis of the parts used showed that leaves are mostly used in majority of medicinal plants, followed by roots, fruit, stems, bark, rhizome, flowers, bulbs, seeds, thorns and latex. The most common preparations include juice, paste and extract. The present investigation was the first attempt undertaken in Hathazari to document the traditional uses of plants for the treatment of different ailments. This study also recorded new ethnomedicinal information. It can be concluded that data collected in the present study will be useful for any future ethno-pharmacological research for the discovery of new drugs.

Keywords: Medicinal plants, Ethnobotany, Hathazari, Bangladesh.

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INTRODUCTION

Plants have been used as medicinal plants for human welfare in healing ailments as drugs and natural therapies since long ago in Bangladesh. Medicinal plants play a

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significant role in primary health care service of rural people (Roy *et al.,* 2008; Mohiuddin *et al.,* 2012). They are considered one of the crucial components as far as the contribution of biodiversity to society is concerned. Indigenous knowledge of herbal medicine for the cure of several types of diseases exists among different rural communities of Bangladesh (Rahman *et al.,* 2007). Bangladesh, a country of a very fertile land, has a rich flora of medicinal plants. A total of 4939 angiosperm plant species are scattered throughout the forests, jungles, hills, plains, crop fields, road-sides, gardens, marshy lands and watery places of Bangladesh, out of which 750 species are used in traditional medicine (Pasha & Uddin, 2013; Uddin, 2010). Tropical forests contain more than half of the world's estimated 500,000 plant species and less than 1% of these plants have been researched for medicinal activity (Conte, 1996). Tropical rainforest of Suriname contains 400 medicinal plants (Andel & Ruysschaert, 2011). Traditional practitioners of India use about 6000 plants as traditional medicine (http://crdd.osdd.net/indipedia/index.php/Medicinal_plants_of_India). More than 10% of the species from the national flora of Pakistan and Nepal are used for traditional medicine, with 2000 and 701 species, respectively (Chomchalow & Henle, 1995; DPR, 2007). Meanwhile, Sri-Lanka uses 40% plants of their native plants as medicinal (Chomchalow & Henle, 1995). Almost 50% of the medicines are derived from plants and 25% of the prescription drugs have their genesis of tropical plants (Haq, 2004). Malaysia has about 15,000 species of flowering plants, of which about 10% are said to be medicinal (Faridah Hanum *et al.,* 2001). The World Health Organization (WHO) listed 21,000 medicinal plants that were used in different parts of the world. WHO reported that 80% of the world's population still depend on traditional medicine for their primary health care needs (Islam, 2006).

The rural community of Hathazari gathered the knowledge about plant used for the treatment from their environment through observations, intuition, experimentation and enriched the knowledge through selection and rejection, and passed it from generation to generation through various channels. Hathazari is one of the well-known upazila in the Chittagong district of Bangladesh, located at 22°24′ - 22°38′ N and 91°41′ - 91°54′ E, with an area of 246.32sq km, bounded by Fatik chhari upazila in the north, Panchlaish and Chandgaon thanas in the south, Raozan upazila in the east, and Sitakunda upazila in the west (Sajahan, 2012). The total population of Hathazari is about 0.4 million, out of which, 402 families are Chakma. According to Patwari (2012), the main occupations of the people are agriculture (14.65%), agricultural labourer (7.73%), wage labourer (2.6%), commerce and industry (17.88%), transport (4.68%), service (27.16%) and others (19.4%).

A number of ethno-medicinal studies in Bangladesh have been carried by Rahman *et al.* (2007), Yusuf *et al.* (2006, 2007), Roy *et al.* (2008), Faruque and Uddin (2011), Mohiuddin *et al.* (2012), Uddin *et al.* (2006, 2011, 2012), as well as Sajib and

Uddin (2013), who are from the Chakma community of hill tract districts, Rangamati district, Tripura Community of Hazarikhil, Bandarban district, Phulbari of Dinajpur district, Subarnachar of Noakhali, Lawachara and Sandwip Island of Chittagong district. However, no specific work has been done on the medicinal plants for the rural community of Hathazari. The present study intends to document the information on the plants used for medicinal purposes at Hathazari.

MATERIALS AND METHODS

Documentation of the uses of medicinal plants as ethnomedicinal data sheet was done through field interview, plant interview and group interview techniques (Alexiades, 1996; Alcorn, 1984; Boom, 1978) carried out between May 2010 and January 2013 in the study area. Ten field trips were made in different flowering seasons and 60 interviews were conducted among 20 local informants. Field interview is also referred to as bagging interview (Alcorn, 1984); it consists of walking in one or more vegetation zones with an informant, collecting and taking notes on plants and their uses. Meanwhile, plant interview refers to the plants that have been collected and brought back and presented to the informants for their information (Alcorn, 1984; Boom, 1978). In addition, audio recordings were also done using a digital voice recorder. The authenticity of the information on each plant was confirmed through repeated interviews. Ethnomedicinal information was also obtained through informal interviews following semi-structured and structured techniques. The interviews were conducted among the locals of different age groups, mostly between 25 to 75 years, including herbal practitioners (Kabiraj). The interviews focused on basic questions concerning the informant's knowledge of the uses of local plants. A typical question would be: which local plants do you know and/or use? How many people in your area use the plant as medicine? Depending on the response, more specific questions concerning the uses of plants were gradually formulated. In order to help assure that the information is as unbiased as possible, efforts were made to avoid the presence of other people during the interviews. Participant observation was used to enrich the information gathered. All the information regarding plant species, habit, family, local names and mode of application was documented. Voucher specimens were preserved, examined and identified in the laboratory of Chittagong University Herbarium (CTGUH). In some cases, standard literatures such as Heinig (1925), Ghani (1998), Siddiqui *et al.* (2007), Yusuf *et al.* (2009), Ahmed *et al.* (2008, 2009), Pasha and Uddin (2013) were also referred to for identification of species. On the other hand, world wide website, Catalogue of Life (2012) and the Plant List (2010) were also taken into consideration for the identification and recent nomenclature of all the specimens.

RESULTS AND DISCUSSION

In the present ethno-medico-botanical survey, a total of 75 species under 67 genera of 44 families which are used for

Fig.1: Map of the study area

the treatment of 35 types of ailments were documented. For each species, botanical name, family, local name (Bangla name), habit, and mode of application are identified and presented in Table 1. The survey indicated that the common medicinal plant families in the study area are Asteraceae, Lamiaceae, Rutaceae, Solanaceae, Liliaceae, Malvaceae, Combretaceae, Amaranthaceae, Acanthaceae, Anacardiaceae, Apiaceae, Apocynaceae, Euphorbiaceae, Menispermaceae, Mimosaceae, Moraceae, Piperaceae, Verbenaceae and Zingiberaceae. This finding of common medicinal plant families in the study is in agreement with that of Ghani (1998) and Yusuf *et al.* (2009). Among the recorded species, herbs (41%) were found to be dominating over trees (28%), shrubs (19%) and climbers (12%). Meanwhile, analysis of the plant's parts used showed that leaves are the mostly used plant parts (43%), followed

by roots (14%), fruit (11%), stems (9%), bark (6%), rhizomes (5%), flowers (4%), bulbs (4%), seeds (2%), thorns (1%) and latex (1%). It is important to highlight that such a wide harvesting of leaves and seeds, compared to roots which are important for survival of plants, has a less negative influence on the survival and continuity of useful medicinal plants and hence does not affect sustainable utilisation of the plants (Yirga, 2010). Cutting off roots, bulbs and rhizomes is considered a destructive way or unsustainable exploitation of using plants because the whole plants are destroyed or uprooted in the process. The most frequently cited modes of plant used are as juice (36%), raw (22%), paste (14%), extract (8%), infusion (7%), syrup (5%), powder (3%), bath (3%), decoction (1%) and tablet (1%). In preparing plants into herbal medicine, parts are turned in the form of extract or paste by mixing them in a variety of food, spices or oil. Both external and internal methods of application of herbal medicine have been prescribed. In most cases, the community was found to practice oral application of herbal medicine. In most cases, local herbalists prescribed fresh plant materials as a source of herbal medicine. Most times, they do not store the herbal preparation. Plants are mostly used for the treatment of various types of pain (11), cough (10), rheumatism (10), fever (6), jaundice (5), dysentery (4), skin disease (4), haemorrhages (4), tiredness (4), digestive (3), sexual weakness (3), hair tonic (3), diarrhoea (2), gastritis (2), high blood pressure (2), diabetes (1), heart disease (1), fracture (1) and other illnesses (11). Plants which are used in different parts of the world for the treatment of similar diseases may be considered to be pharmacologically efficient. The most commonly used plant species in the study area are *Azadirachta indica*, *Blumea lacera*, *Calotropis gigantea*, *Centella asiatica*, *Coccinia grandis*, *Eclipta prostrata*, *Glycosmis pentaphylla*, *Kalanchoe pinnata*, *Leucas aspera*, *Mikania micrantha*, *Psidium guajava*, *Ricinus communis*, *Senna alata*, *Spilanthes acmella*, *Stephania japonica*, *Terminalia arjuna* and *Zingiber officinale*. About 40% of the rural people in the study area depend on traditional medicine for their primary health care. Among the informants, 55% are males and 45% are females. The main occupations of the informants are herbal medicine practitioner, agriculture, agricultural labourer and wage labourer. Most of the villagers in the study are poor and they largely depend on plants for food, medicine, fuel and other daily necessities.

There are various ethnomedicinal plant records of the indigenous community of Bangladesh. Rahman *et al.* (2007) reported 198 species and Roy *et al.* (2008) reported 90 species from the Chakma community of the hill tract districts. Yusuf *et al.* (2006) reported ethnomedicinal uses of 34 species from Kaukhali proper and Betbunia of Rangamati district, whereas Yousuf and his co-workers reported 69 species from the Chittagong hill tracts in 2007. Faruque and Uddin (2011) recorded 43 species which are used by the Tripura Community of Hazarikhil for the treatment of various

diseases/ailments, followed by Mohiuddin *et al*. (2012) with 70 species from the Bandarban district, Uddin *et al.* (2006) with 86 species from the Phulbari of Dinajpur district, Uddin *et al.* (2011) with 84 species from Subarnachar of Noakhali, Uddin *et al.* (2012) with 56 species from Lawachara, and Sajib and Uddin (2013) with 111 species from Sandwip island. The presently recorded species have been previously indicated as the ethnomedicinal species from the different areas of Bangladesh. The present study, however, recorded six ethnomedicinal information which is new to the ethnobotany of Bangladesh. The modes of application for *Ipomoea aquatica* include the treatment of removal of infertility in women, the leaf extract of *Lannea coromandelica* to treat jaundice, the bark of *Mangifera indica* to treat jaundice, *Polyalthia longifolia* in rheumatism, *Schumannianthus dichotomus* in earache and the roots of *Urena lobata* to treat abdominal pain.

Establishment of modern health care centres is in progress in many rural areas; this may gradually change the existing pattern of indigenous knowledge system of healthcare (Sajib & Uddin, 2013). Field observations and discussions with the locals demonstrated that the diversity of ethnomedicinal plant species and traditional knowledge of the area are at great risk because of the many threats that include exotic monoculture plantation, agricultural progression, unsustainable collection of medicinal plants, industrialization, urbanization, hill cultivation, degradation of forests and modern lifestyle. Due to illegal logging and forest fires, the undergrowth is greatly affected and has become threatened. The ethnomedicinal plants of the study area grow in various habitats such as hilly lands, cultivated lands, homestead areas, scrub jungles, fallow lands and wetland. The study area is located near the city. As a result, the habitat of some medicinal plants is decreasing day by day due to building up of commercial farms, mills and factories. Moreover, the new generation is losing interest in continuing their parents' profession because it does not provide them proper financial supports for their livelihood. Thus, documentation of these plant uses is indispensable before they disappear permanently.

CONCLUSION

The findings of the current work are most likely the first recorded ethno-medicinal knowledge of Hathazari using standard research methods, focusing on medicinal plants and their local uses for primary health care. This health care knowledge has been passed down from one generation to another through informal education. The study has also suggested that the currently gathered information on medicinal uses of plants by the local people may be used in any ethno-pharmacological research in future for the discovery of new drugs.

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TABLE 1
Medicinal plants used by the rural community of Hathazari

Botanical name	Family	Local name	Voucher Number	Habit	Occurrence	Source	Modes of Application
1. *Achyranthes aspera* L.	Amaranthaceae	Apang	S67	H	W	FL	A necklace prepared from root and thread is tied to head to treat jaundice.
2. *Aegle marmelos* (L.) Corr. Serr.	Rutaceae	Bel	S345	T	C	HL	Juice prepared from the pulp of fruit is taken as tonic to relieve from tiredness.
3. *Aerva sanguinolenta* (L.) Blume	Amaranthaceae	Raktapata	S346	H	C	CuL	Paste prepared from crushed leaves is applied to affected areas to stop haemorrhage.
4. *Allium cepa* L.	Liliaceae	Piaz	S347	H	C	CuL	Juice prepared from the crushed bulb of *Allium cepa* and *Allium sativu*m is mixed with honey and warmed. It is taken to treat cough.
5. *Allium sativum* L.	Liliaceae	Rasun	S348	H	C	CuL	Paste prepared from the crushed bulb is taken to treat rheumatism.
6. *Aloe vera* (L.) Burm f.	Aloeaceae	Gritokumari	S349	H	C	HA	Juice prepared from crushed leaves is taken to get rid of tiredness.
7. *Amaranthus spinosus* L.	Amaranthaceae	Kantamaris	S258	H	W	HL	Extract prepared from crushed leaves is taken to relieve physical weakness or tiredness.
8. *Ananas comosus* (L.) Merr.	Bromeliaceae	Anaros	S350	H	C	HL	Juice prepared from crushed leaves is taken two times daily for the treatment of cough and applied to ear cure infection.
9. *Andrographis paniculata* (Burm.f.) Wall. *ex* Nees	Acanthaceae	Kalomegh	S351	H	C	HA	Juice prepared from crushed leaves is taken at least thrice daily to treat fever.
10. *Artemisia nilagirica* (C.B. Clarke) Pamp.	Asteraceae	Nagmoni	S352	H	W	HL	One centimetre of stem of *Artemisia nilagirica* and half centimetre of *Mimosa pudica* stem are applied to affected area to treat joint pain and rheumatism.
11. *Artocarpus heterophyllus* Lam.	Moraceae	Kanthal	S352	T	C	HL	Latex is applied to affected area for the treatment of skin disease.

cont'd Table 1

12.	*Asparagus racemosus* Willd.	Liliaceae	Shotomuli	S353	Cl	C	HA	Syrup prepared from crushed root of *Asparagus racemosus* and husk of *Plantago ovata* is taken in the morning as tonic.
13.	*Azadirachta indica* A.Juss.	Meliaceae	Nim	S354	T	C	HL	Leaves boiled in water are used to take bath to treat skin disease.
14.	*Berberis asiatica* Roxb.	Berberidaceae	Daruhoridra	S355	S	W	HL	Paste prepared from crushed stem is taken with one spoonful of honey thrice daily for the treatment of rheumatism.
15.	*Blumea lacera* (Burm.f.) DC.	Asteraceae	Kormuta	S356	H	W	SJ	Paste prepared from crushed leaves is applied to stop haemorrhages.
16.	*Bombax ceiba* L.	Bombacaceae	Simul	S357	T	C	HA	Root (removing the root bark) is taken to treat gastritis and sexual weakness.
17.	*Cajanus cajan* (L.) Millsp.	Fabaceae	Arol	S358	S	C	CuL	Juice prepared from crushed leaves is taken in dog bite.
18.	*Calotropis gigantea* (L.) Ait.f.	Asclepiadaceae	Orpata	S359	S	W	FL	Warm leaves are applied to affected area to treat rheumatism.
19.	*Catharanthus roseus* (L.) G.Don	Apocynaceae	Nayantara	S360	H	C	HA	Juice prepared from crushed leaves is taken to treat high blood pressure.
20.	*Centella asiatica* (L.) Urban	Apiaceae	Adagunguni	S361	H	W	FL	Juice prepared from crushed leaves is taken to treat dysentery.
21.	*Cissus qudrangularis* L.	Vitaceae	Harjora	S362	Cl	C	HA	Paste prepared from crushed leaves and stem is applied to affected areas to cure fracture.
22.	*Citrus aurantifolia* (Christm.) Swingle	Rutaceae	Lebu	S363	T	C	HL	Hot infusion of fruits is taken with salt to treat cough.
23.	*Citrus maxima* (Burm. f.) Merr.	Rutaceae	Jambura	S364	T	C	HL	Fruit juice is taken to treat jaundice.
24.	*Clerodendrum viscosum* Vent.	Verbenaceae	Vaitbar	S365	S	W	SJ	Leaves are applied to breast to prevent breast feeding of children.
25.	*Coccinia grandis* (L.) Voigt	Cucurbitaceae	Kalakachi	S58	Cl	W	SJ	Juice prepared from crushed leaves is taken at least thrice daily for the treatment of diarrhoea and diabetes.

cont'd Table 1

26.	*Curcuma longa* L.	Zingiberaceae	Halud	S366	H	C	HL	Paste prepared from crushed rhizome is applied to treat skin disease.
27.	*Cynodon dactylon* (L.) Pers.	Poaceae	Durba	S274	H	W	FL	Juice prepared from crushed leaves is applied to stop haemorrhage.
28.	*Datura metel* L.	Solanaceae	Dutra	S367	H	W	HA	Leaf juice mixed with mustard oil and warmed, and rubbed to affected area to treat rheumatism.
29.	*Eclipta prostrata* (L.) L.	Asteraceae	Kelaona	S77	H	W	FL	Paste prepared from crushed leaves is applied as hair tonic and to treat scuff infection.
30.	*Ficus hispida* L.f.	Moraceae	Dongula	S368	T	W	SJ	Young fruit are taken to treat dysentery.
31.	*Glycosmis pentaphylla* (Retz.) A.DC	Rutaceae	Kawatunipata	S101	T	W	SJ	Juice prepared from crushed leaves is taken thrice daily for the treatment of cough, fever, abdominal pain, and vomiting.
32.	*Hibiscus rosa-sinensis* L.	Malvaceae	Jaba	S198	T	C	HA	Paste prepared from crushed flowers is applied to hair as hair tonic before bath.
33.	*Hibiscus schizopetalus* (Mast.) Hook.f.	Malvaceae	Joba	S369	T	C	HA	Paste prepared from crushed flowers is applied to hair as hair tonic before bath.
34.	*Hyptis suaveolens* (L.) Poit.	Lamiaceae	Tokma	S370	H	W	HA	Twenty seeds are steeped in a glass of water and mixed with one spoon of sugar. Then, infusion is taken to relieve from tiredness and as blood purifier.
35.	*Ipomoea aquatica* Forsk	Convolvulaceae	Kalmi	S87	Cl	W	WL	Standing pond water during the sunset seven pieces of stem of *Ipomoea aquatic* and root of *Clerodendrum viscosum* is taken, followed by taking three glasses of water for five or seven days after menstrual cycle of women to boost fertility.
36.	*Justicia adhatoda* L.	Acanthaceae	Basak	S272	S	W	HA	Juice prepared from crushed leaves is taken for the treatment of fever and cough.
37.	*Kalanchoe pinnata* (Lam.) Pers.	Crassulaceae	Kofpata	S371	H	C	HA	Hot infusion of leaves is taken to treat cough.

cont'd Table 1

38.	*Lannea coromandelica* (Houtt.) Merr.	Anacardiaceae	Badi	S298	T	W	HL	Barks are steeped in water for overnight and the prepared extract is taken in the morning to treat jaundice.
39.	*Lawsonia inermis* L.	Lythraceae	Methi	S372	T	C	HA	Paste prepared from crushed leaves is applied to hair as hair tonic.
40.	*Leucas aspera* (Willd.) Link	Lamiaceae	Gaisshaderos	S115	H	W	FL	Paste prepared from crushed leaves is applied to treat tonsillitis.
41.	*Litsea monopetala* (Roxb.) Pers.	Lauraceae	Medapata	S89	T	W	HL	Extract prepared from crushed barks is taken to relieve from bladder problem.
42.	*Mangifera indica* L.	Anacardiaceae	Aam	S373	T	C	HA	Mango bark is steeped in lime water and used to take bath to treat jaundice.
43.	*Mikania micrantha* Kunth	Asteraceae	Tuhainnalata	S374	Cl	W	SJ	Juice prepared from crushed leaves is taken to treat gastritis. Paste prepared from crushed leaves is applied to stop haemorrhage.
44.	*Mimosa himalayna* Gamble	Mimosaceae	Sadasarmida	S271	S	W	FL	Juice prepared from crushed roots is taken in sexual weakness.
45.	*Mimosa pudica* L.	Mimosaceae	Lajjabati	S375	H	W	FL	Two centimetre stem of male plant is tied in hand to relieve from rheumatism.
46.	*Moringa oleifera* Lam.	Moringaceae	Sajina	S376	T	C	HL	Cooked young fruit are taken to relieve rheumatism and as anthelmintic.
47.	*Musa paradisiaca* L.	Musaceae	Aittakola	S377	H	C	HA	Root is applied to throat to ease fever.
48.	*Ocimum americanum* L.	Lamiaceae	Bontulsi	S378	H	W	HA	Extract prepared from crushed leaves is taken with honey to treat cough.
49.	*Ocimum tenuiflorum* L.	Lamiaceae	Tulsi	S379	H	W	HA	Extract prepared from crushed leaves is taken with honey to treat cough.
50.	*Phyllanthus reticulatus* Poir.	Euphorbiaceae	Horba	S380	S	W	HL	Root is applied to breast to produce sufficient milk for children.
51.	*Piper betle* L.	Piperaceae	Pan	S381	Cl	C	CuL	Leaves are chewed with crushed fruit of *Areca catechu* to improve digestive system.
52.	*Piper nigrum* L.	Piperaceae	Golmorich	S382	Cl	C	CuL	Powder prepared from fruits of black piper and linseed is taken with honey to treat sexual weakness.

cont'd Table 1

53.	*Plumbago indica* L.	Plumbaginaceae	LalChita	S383	H	W	HL	Roots are applied to vagina for easy delivery of baby.
54.	*Polyalthia longifolia* (Sonn.) Thwaites	Annonaceae	Debdaru	S233	T	C	HA	Tablets prepared from the crushed stem of *Polyalthia longifolia* and *Berberis asiatica* are mixed with honey and taken to treat rheumatism.
55.	*Psidium guajava* L.	Myrtaceae	Peyara	S384	T	C	HL	Young leaves and salt are boiled in water and strained. The extract is taken to relieve dental pain.
56.	*Rauvolfia serpentina* (L.) Benth. *ex* Kurz	Apocynaceae	Sarpagondha	S385	S	C	HL	Juice prepared from crushed root is taken to control high blood pressure.
57.	*Ricinus communis* L.	Euphorbiaceae	Verenda	S386	S	W	HL	Juice prepared from crushed bark is taken with honey to treat rheumatism.
58.	*Schumannianthus dichotomus* (Roxb.) Gagnep.	Marantaceae	Sitolpati	S166	S	W	HA	Decoction of stem is applied to ear for the treatment of earache.
59.	*Scoparia dulcis* L.	Scrophulariaceae	Bondone	S387	H	W	FL	Juice prepared from crushed leaves is taken thrice daily for the treatment of dysentery.
60.	*Senna alata* (L.) Roxb.	Caesalpiniaceae	Daud	S388	S	C	HL	Juice prepared from crushed leaves is taken as digestive. Paste prepared from crushed leaves and root is applied to treat skin disease.
61.	*Solanum sisymbrifolium* Lam.	Solanaceae	Kantikari	S389	S	W	FL	Juice prepared from crushed leaves is taken thrice times daily for the treatment of fever and cough.
62.	*Spilanthes acmella* (L.) L.	Asteraceae	Nakpul	S390	H	W	HL	Flowers are applied to teeth to relieve pain.
63.	*Stephania japonica* (Thunb.) Miers	Menispermaceae	Musonipata	S391	Cl	W	HL	Juice prepared from crushed leaves is taken to treat abdominal pain. Paste prepared from crushed leaves is applied to treat eczema.
64.	*Swertia chirata* (Wall.) C.B.Clarke.	Gentianaceae	Chirata	S392	H	W	HA	Juice prepared from crushed leaves is taken to treat fever.

cont'd Table 1

65.	*Terminalia arjuna* (Roxb. *ex* DC.) Wight & Arn.	Combretaceae	Arjun	S393	T	C	HL	Barks are steeped in water overnight and the infusion is taken in the morning to treat heart disease.
66.	*Terminalia bellirica* (Gaertn.) Roxb.	Combretaceae	Bahera	S394	T	C	HL	Syrup prepared from crushed fruit of *Terminalia bellirica*, *Terminalia chebula*, *Phyllanthus emblica* and rhizome of *Zingiber officinale* is taken to treat diarrhoea, dysentery and to improve digestive system.
67.	*Terminalia chebula* (Gaertn.) Retz.	Combretaceae	Horitoki	S395	T	C	HL	Mixture of ten grams crushed fruit of *Terminalia chebula*, ten grams of crushed leaves of *Datura metel*, rhizome juice of Zinger and honey is taken to treat body pain and fever.
68.	*Tinospora cordifolia* (Willd.) Miers	Menispermaceae	Gulanchi	S396	Cl	W	SJ	Juice prepared from crushed leaves is taken to treat jaundice. Stem is applied to waist to treat jaundice.
69.	*Trachyspermum ammi* (L.) Sprague	Apiaceae	Joan	S397	H	W	HA	Powder prepared from seeds is mixed with a bit of salt and taken to treat abdominal pain.
70.	*Tribulus terrestris* L.	Zygophyllaceae	Gokkhur	S398	H	W	HL	Hot infusion prepared from thorn is taken to treat urinary problem.
71.	*Urena lobata* L.	Malvaceae	Bailboli	S399	S	W	FL	Juice prepared from crushed roots is taken to treat abdominal pain.
72.	*Vitex negundo* L.	Verbenaceae	Nishinda	S273	T	W	HA	Juice prepared from crushed leaves is taken to treat rheumatism and applied as hair tonic.
73.	*Withania somnifera* (L.) Dunal	Solanaceae	Aswagandha	S400	S	C	HA	Syrup prepared from crushed root of *Withania somnifera*, bulb of *Allium cepa*, leaves of *Eclipta prostrata* is taken as antioxidant.
74.	*Xanthosoma sagittifolium* (L.) Schott	Araceae	Dudhkachu	S401	H	W	HL	Cooked petiole is taken to increase production of breast milk.
75.	*Zingiber officinale* Roscoe	Zingiberaceae	Ada	S402	H	C	HL	Juice prepared from crushed rhizome is taken to treat cough.

Legend: H- Herb, S- Shrub, T- Tree, Cl- Climber, C- Cultivate, W- Wild, HL- Hilly land, CuL- Cultivated land, HA- Homestead area, SJ- Scrub jungles, FL- Fallow land, WL- Wetland.

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Length-Weight Relationship and Relative Condition Factor of *Parapenaeopsis sculptilis* (Heller, 1862) from the Coastal Waters of Perak, Peninsular Malaysia

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ABSTRACT

Length-weight relationship (LWR) parameters and relative condition factor (K_n) of marine shrimp, *Parapenaeopsis sculptilis* (Heller, 1862) were estimated using length-weight data collected between February 2012 and January 2013 from the coastal waters of Terong, Perak, Peninsular Malaysia. The estimated length-weight relationship of *P. sculptilis* for both sexes was $W = 0.00027TL^{2.80}$. Meanwhile, the estimated relative growth coefficient (b) was 2.80 for both sexes, indicating a negative allometric growth pattern of *P. sculptilis* in the investigated area. Relative condition factor (K_n) values ranged from 0.99 to 1.064 (1.013±0.005, mean ±SD). K_n value changes in various months: the highest peak was in March-April, indicating the spawning period and the trough and small peaks indicating the cycle gonadal development.

Keywords: Parapenaeopsis sculptilis, length-weight relationship, condition factor, coastal waters

INTRODUCTION

The penaeid prawn of the genus *Parapenaeopsis* belongs to family Penaeidae of the decapod groups. *Parapenaeopsis sculptilis*, commonly called 'rainbow prawn', is also known locally as 'udang kulit keras' in Malay (Ong & Weber, 1977). The genus *Parapenaeopsis* is widely distributed from the west and east coasts of India to Hong Kong through Malaysian and Indonesian waters to tropical Australia and New Guinea (Dall, 1957; Lee, 1972). Most of the species are found in the Indo-Pacific

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region, from Persian Gulf Indian region and east coast of Africa to Japan and Australia (Rao, 1969), and inhabit shallow inshore waters where spawning adults are often found and also mostly fished in shallow coastal areas. The juveniles and young prawns live mainly in coastal mud flats or sand flats near to mangroves. The latest information on annual landing of *P. sculptilis* in Malaysia was 3.526 tonnes in 2012 (DOF, 2013). This important commercial fisheries commodity being marketed fresh, frozen, peeled and cooked or canned, and also used as ingredients in the making of shrimp meal or shrimp paste in Malaysia and Singapore (Tham, 1968; Hall, 1962; Kubo, 1949). Studies on the biology of this species are still scarce and the resources continue to be exploited without proper attentive measures. Thus, the objectives of the present study were to estimate the length-weight relationship (LWR) and relative condition factor of *P. sculptilis* collected from the coastal waters of Perak, Peninsular Malaysia. The information gathered would be an added information to the Malaysian penaeid biology database and significant for the management of the resources.

MATERIALS AND METHODS

Study Area and Sampling

The sampling was conducted in the coastal waters of Terong in Perak (N 4°43'0 and E 100°43'60), Peninsular Malaysia, from February 2012 to January 2013 (see Fig.1). The fresh samples were collected from fishermen. The fishermen used special push net (mesh size 0.5 cm) that is fixed in front of the fishing boat. Identification of *P. sculptilis* specimens was carried out based on the works of Dall (1957) and Hall (1962). Total length was measured to the

Fig.1: Geographical location of the study site in Perak, west coast of Peninsular Malaysia (Terong)

nearest 0.1 cm from the tip of the rostrum to the tip of the telson, while body weight was measured to the nearest 0.01 g using an electronic balance.

Data Analysis

For LWR, a total of 660 specimens were analysed. Length-weight relationship was determined by using the relationship of $W = a L^b$ applied by Ricker (1975); where, W is the total weight (g), L is the total length (cm) of the shrimp, 'a' is intercept (condition factor) and 'b' is the slope (growth coefficient). The equation was transformed into logarithmic form as Log W = Log a + b Log L. The estimated b value of *P. sculptilis* was tested by using t-test to verify that it was significantly different from the isometric growth (b = 3) (Froese, 2006). Additionally, 95% of the confidence limits of the parameter b and the statistical significance level of r^2 were estimated (Scherrer, 1984). The monthly relative condition factor (K_n) of *P. sculptilis* was calculated according to $K_n = W/ aL^b$, equation adapted as $K_n = W / w$ (Le Cren, 1951), where, W = observed weight of shrimp (g) and w = calculated weight of shrimp (g). K_n values were estimated for different months and length sizes.

RESULTS

Length-Weight Relationships

Total lengths and total weights of *P. sculptilis* ranged from 7.75-16.65 cm and 2.84-39.89 g for both sexes. The monthly descriptive statistic and estimated parameters of length weight regressions for both sexes of *P. sculptilis* are given in Table 1. The LWR of *P. sculptilis* was found to be $W = 0.00027TL^{2.80}$ or Log W = 2.80 Log TL -4.63 for both sexes. It was found that the estimated b value was significantly lower than the isometric value (3) at 5% level for the population of *P. sculptilis* in the coastal waters of Terong, Perak. Therefore, it could be concluded that the growth pattern of *P. sculptilis* was negative allometric in the study area.

Relative Condition Factor

Relative condition factor (K_n) is a ratio between observed and calculated mean weights. K_n was calculated for different months and for each size class. The K_n values of *P. sculptilis* in the coastal waters of Terong varied from 0.99 to 1.064, while the mean value was 1.013 ±0.005SD. The mean values of K_n for different months and for each size class at 0.5 cm are presented in Fig.2 and Fig.3, respectively. The highest of K_n value was in March-April, indicating reproductive maturity, and the lowest value was in May-June (Fig.2). A marked decline in K_n at 9.30 cm of the total length and a subsequent recovery after 9.30 cm were also observed (Fig.3). Therefore, the first sexual maturity of *P. sculptilis* was attained at 9.30 cm in total length and the peak reproductive maturity was in April.

DISCUSSION

The growth coefficient 'b' of the length-weight relationship generally lies between 2.50 and 3.50 and the relation is said to be

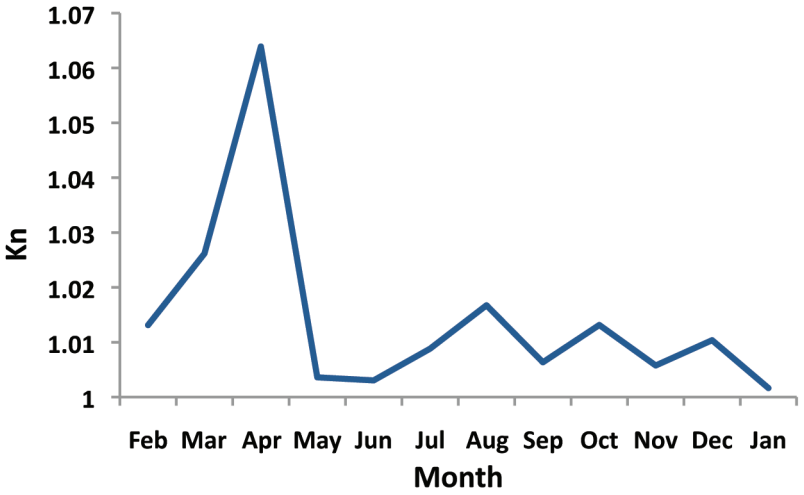

Fig.2: Monthly variation of K_n of females *Parapenaeopsis sculptilis*

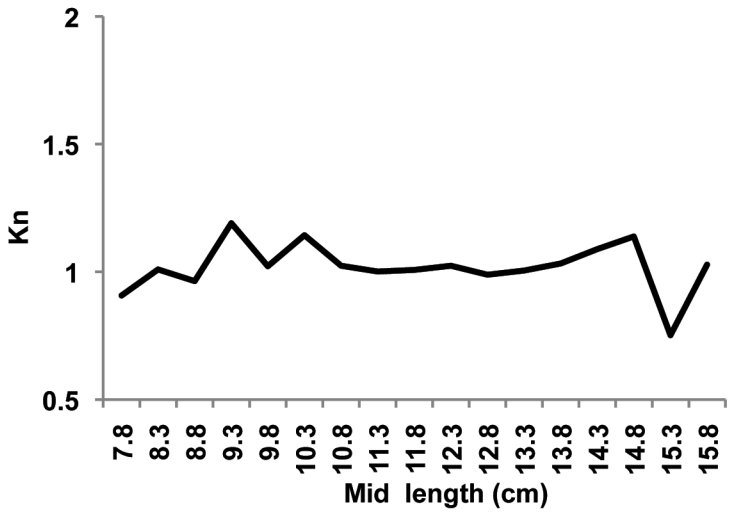

Fig.3: Mean K_n values per each 0.5 cm length for females *Parapenaeopsis sculptilis*

TABLE 1
Monthly descriptive statistic and length-weight relationship parameters for both sexes of *Parapenaeopsis sculptilis* in the coastal waters of Perak, Peninsular Malaysia

Month	Range TL	Range TW	Mean TL ±SD	a	b	95% CI of b	r^2
Feb	10.53 -15.36	8.73 -31.96	12.68±1.08	0.00000339	3.17	2.93-3.41	0.85 ($p < 0.05$)
Mar	9.70 -14.70	8.11-32.57	11.94±1.34	0.00050119	2.13	1.94-2.32	0.81 ($p < 0.05$)
Apr	10.41 -15.60	8.47 -31.59	12.34±1.08	0.00001230	2.90	2.63-3.18	0.79 ($p < 0.05$)
May	9.12 -16.61	8.85 -39.89	12.21±1.16	0.00010000	2.48	2.13-2.83	0.63 ($p < 0.05$)
Jun	8.44 -16.65	8.35 -38.59	12.70±1.41	0.00257040	2.00	1.53-2.12	0.56 ($p < 0.05$)
Jul	9.70 -16.61	8.87 -39.89	12.45±1.08	0.00000912	2.97	2.76-3.18	0.87 ($p < 0.05$)
Aug	10.85 -15.55	9.21 -39.31	12.69±0.88	0.00000045	3.60	3.36-3.83	0.88 ($p < 0.05$)
Sept	7.68 -15.29	2.84 -30.22	11.86±1.71	0.00001549	2.87	2.73-3.01	0.93 ($p < 0.05$)
Oct	9.90 - 14.52	6.45 -26.75	11.83±0.66	0.00000309	3.19	2.90-3.47	0.80 ($p < 0.05$)
Nov	10.27 -15.01	8.72 -26.96	12.01±0.80	0.00001514	2.86	2.58-3.14	0.78 ($p < 0.05$)
Dec	9.85 -14.30	7.02 -27.16	12.06±0.62	0.00000603	3.06	2.75-3.37	0.76 ($p < 0.05$)
Jan	7.75 - 15.00	3.31 - 28.93	11.60±1.27	0.00000331	3.18	3.04-3.32	0.94 ($p < 0.05$)
Overall	7.75- 16.65	2.84-39.89	12.19±1.09	0.000270	2.80	2.70-2.90	0.81 ($p < 0.05$)

Note:
TL= total length (cm); TW= total weight (g); mean ±SD; a and b, parameters of the length-weight relationship; r^2, coefficient of regression

isometric when it is equal to 3, as reported for most aquatic organisms (Ecoutin *et al*., 2005). In the present study, the estimated b value was 2.80 for both sexes. The finding indicated that growth of the *P. sculptilis* is negative allometric because the b value in both sexes is significantly less than the isometric value of 3 at 5% level. Meanwhile, the regression co-efficient (r^2) value for both sexes was 0.81. The present findings seem to be more or less consistent with those of other researchers such as Kirkegaard and Walker (1970) who showed that the exponent 'b' for females was 2.95 and this was 2.94 for males, indicating a negative allometric growth for *P. sculptilis* in Australian waters. Similarly, Thangaraj (2000) also reported negative allometric growth with similar

values for the females and for males of *P. stylifera* from Madras coast, India. Other researchers found higher exponents b of 4.13 and 4.09 for the females and males of *P. stylifera* from Kerala coast (Suseelan & Raj, 1989). Masitah and Chong (2002) reported that the combined sexes of *P. sculptilis* exhibited a negative allometric growth (b = 2.52) from Selangor waters. In addition, Fatima (2001) in *P. sculptilis* from Korangi Fish Harbour, Karachi, showed a negative allometric growth (b = 2.55). In contrast, Hall (1962) reported that this shrimp from Penang waters had isometric growth (b = 2.99). This finding is further supported by researchers from India and Bangladesh, whose results also showed isometric growth (2.98) for *P. sculptilis* (Bhimachar, 1963; Zafar *et al*., 1997; Amin & Zafar, 2003).

There were a fluctuations in the monthly K_n values during the study period. The highest value was obtained in the month of April, while the lowest was in June. The results obtained by Thomas (1975) also showed fluctuations in the monthly K_n values for *Penaeus semisulcatus*. Similar, changes in K_n have been observed for the giant Malaysian freshwater prawn, *Macrobrachium rosenbergii* (Rao, 1969). The K_n value of *Palaemon malcolmsonii* showed the maximum value in September and the minimum value in November, which coincide with the spawning period from September to November (Patel *et al*., 1984). Lack of literature on *P. sculptilis* has prevented comparison of the present study with other works of the same species from Malaysia.

In the present study, the size at sexual maturity of female *P. sculptilis* was 9.30 cm TL (carapace length 2 cm). The highest peak was obtained at 21-22 mm carapace length, which indicated an increase in K_n when the prawn attained maturity, whereas the subsequent peaks represented cyclic gonad development and spawning for *Penaeus semisulcatus* (Thomas, 1975).

CONCLUSION

Parapenaeopsis sculptilis in the coastal waters of Perak showed a negative allometric growth, reaching its first sexual maturity at 9.30 cm of the total length and peak reproductive maturity in March-April. The outcome of the present study has provided basic information on the length–weight relationship and relative condition factors of *P. sculptilis*, which is important for fishery management.

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Dissemination of Natural Resource Management Technology for Irrigated Rice in the Philippines: On-Farm Validation to National Extension

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ABSTRACT

Natural Resource Management (NRM) technologies, some of which include Site-Specific Nutrient Management (SSNM), Ecologically-Based Rodent Management (EBRM) and Alternate Wetting and Drying (AWD) for increased farm productivity and income in the irrigated rice ecosystem, have recently been generated through research. Guaranteeing that these technologies are properly disseminated and utilized by farmers is one of the challenges not only for researchers but for policymakers and various stakeholders as well. These need to be tested and evaluated on-farm to determine their appropriateness for wide-scale adoption of farmers, and the factors that may hinder their diffusion. In 2006, the Philippine Rice Research Institute (PhilRice) forged partnership with the International Rice Research Institute (IRRI) to implement a project enhancing the delivery of NRM technologies for irrigated rice ecosystem from adaptive research to nationwide implementation. Activities of the collaborative project included training of partners, stakeholders' workshop, establishment of demo farm cum learning field, information campaign and on-farm evaluation. Results revealed yield increases of 28.3% and 12.6% in the SSNM demo plots for DS and WS 2007, respectively; 3.17% yield increase in the EBRM field; and 18.8% reduction in production cost by utilizing the AWD technologies. These results encouraged farmers to continue using the NRM technologies and sharing them to others. Eventually, the NRM technologies were integrated into the National Rice Program through the *PalayCheck* system. Overall, the PhilRice-IRRI joint partnership has been proven to be a powerful instrument in facilitating delivery and adoption of NRM technologies, thus, improving the quality of life of Filipino farmers.

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INTRODUCTION

Rice is the staple food of about 100 million Filipinos. It is the agricultural commodity with foremost political and economic significance in the Philippines. Rice accounts for 45% of the calorie intake of the population and around 20% of the typical household's budget (FSSP, 2012). Rice farming is the primary source of income and employment of more than two million households.

Due to the high significance of rice in the life of the Filipino people, a continued increase in rice productivity is critically important for enhancing food security, reducing poverty and hunger, and enhancing environmental sustainability. Unfortunately, in the irrigated rice ecosystem, the road toward achieving growth in agricultural productivity is limited by diminishing natural resources, labor shortage, land conversion and climate change (IRRC News, 2010; Palis *et al*., 2010).

Given the critical role of agriculture to the overall development of the Philippine economy, ensuring that natural resource management (NRM) technologies and best practices in the irrigated lowland rice ecosystem are disseminated and utilized by farmers is imperative and should be done in a fast manner on a wider scale for farmer adoption. Campbell *et al*. (2001) define NRM as a conscious process of incorporating the multiple aspects of natural resource use into a system of sustainable management to meet the production goals of farmers and other direct users, as well as the goals of the wider community. It means sustainable utilization of major natural resources such as land, water, forests, etc. for the welfare of future generations. Some of these NRM technologies include site-specific nutrient management (SSNM), alternate wetting and drying (AWD), and ecologically-based rodent management (EBRM).

SSNM is a plant-based approach that provides principles for effective management of Nitrogen (N), Phosphorus (P) and Potassium (K). It provides guidelines, tools, and strategies that allow farmers to determine when and how much nutrients they need to apply to their rice fields under actual growing conditions in a specific season and location. On the other hand, Singleton *et al*. (2004) describe EBRM as a management strategy developed for rodent pests, which is anchored on environmental and socio-economic dimensions in the community. It means integration of different management actions based on understanding the ecology of specific rodent species and also the integration of ecology, sociology, and economics in rodent management. Moreover, AWD is a water-saving technology that farmers can apply to reduce their irrigation water use without reduction in yield (www.knowledgebank.irri.org). In AWD, the field is alternately flooded and non-flooded depending on a number of factors such as soil type, weather, and crop growth stage.

These NRM technologies need to be validated on-farm to determine whether they are appropriate for wide-scale adoption of farmers. The issue of adoption in most of the NRM technologies, however, is complicated since these are in the forms of knowledge and information, which are made accessible to end users in a less tangible form than physical products such as seed or machinery (Price & Balasubramanian, 1998). Most NRM technologies are considered knowledge intensive technologies because they are techniques to fine-tune farmer's management and cater for farmer's adaptation. Moreover, factors such as low information dissemination and lack of linkage and coordination among different extension agencies, among others, hinder the diffusion of sustainable agriculture innovations (Johnson & Lybecker, 2009). In reality, technology transfer and adoption has not met the needs of target beneficiaries despite the efforts in research and development (FAO, 2000). Adoption of NRM technologies by farmers, especially those with limited holdings as in the case of most Filipino rice farmers, is therefore a great challenge in order to realize the goals of sustainability, increasing profitability and environmental integrity.

Various stake holders work together in disseminating NRM technologies such as the Philippine Rice Research Institute (PhilRice), a government-owned and controlled corporation under the Department of Agriculture (DA) and the International Rice Research Institute (IRRI), one of the main providers of rice-related research in the country.

In 2006, PhilRice-IRRI forged partnership to enhance the delivery of NRM technologies and R&D exchange of information. Through this collaboration, the challenge of achieving food security at lesser cost and in a sustainable manner is expected to be better addressed.

OBJECTIVES

The main goal of this paper is to show the process of disseminating NRM technologies for irrigated rice production from adaptive research to nationwide implementation. In more specific terms, it aims to:

a. Describe the initial stage of disseminating the NRM technologies through on-farm testing and validation;

b. Illustrate the upscaling process in promoting the NRM technologies;

c. Demonstrate the strategies and approaches that facilitate delivery of the NRM technologies; and

d. Discuss the challenges encountered in the pathway toward nation-wide extension.

METHODOLOGY

The PhilRice-IRRI project was conceptualized because the two agencies felt the need to enhance extension mechanisms by which the NRM technologies can be disseminated for wide-scale adoption by farmers. At the project onset in 2006, a workshop on "Implementing Component

Technologies for Irrigated Rice" was conducted. The workshop aimed to orient some selected project stakeholders on the NRM technologies. Scientists from IRRI and PhilRice served as resource persons during the activity. The workshop ended with a work plan by identifying the NRM technologies that the stakeholders would be able to evaluate.

A. Capacity Enhancement

There were two levels of trainings conducted for partners. The first level was for stakeholders and partner agencies such as the Agricultural Training Institute (ATI), National Irrigation Administration (NIA) and Department of Agriculture-Regional Field Office (DA-RFO). After the training, these partners were the ones who did the community orientation about the project. The training duration was usually conducted 1-3 days. The other level of training was for farmers, which was done for one cropping season following the Farmer Field School (FFS) approach.

B. Defining technologies for testing and validation

The technologies to be tested and validated in the different localities were first identified by PhilRice Technology Promotion Project leaders who participated in the January 2006 workshop.

In order to determine whether the technology to be validated matched the needs of farmers, a focus group discussion (FGD) was conducted at each site. Through the FGD, the rice production practices and key production constraints of farmers were identified. The FGD results and information from LGU partners and farmers were used as bases on which NRM technologies would be deployed.

C. Establishment of demo farm cum learning field

Demonstration sites, owned by farmer-cooperators, were established in which about 20-25 farmer-participants observe and monitor. Seeds, sign boards and technical advice were provided to the farmer-cooperators. In return, he followed the recommended technologies of the project on his demo farm measuring about 1,000-2,500 sq m. Farmer-participants, on the other hand, were not compelled to change their farm practices but could freely adapt any that they deem applicable on their farms. There were no seeds provided to farmer-participants; only their regular attendance and participation in the discussion and sharing of knowledge and experiences to the season-long training were required.

The demo farm served as learning field and venue for training exercises and joint field monitoring. Half-day regular farmers' classes/meetings were conducted to facilitate farmer group discussions, sharing and learning. The meetings were centered on the farming practices that would help increase farmers' yield and served as venues to formulate or give immediate action to whatever problems or issues that cropped up during project implementation. The municipal agricultural technologists (AT) served as facilitators, while PhilRice experts

served as resource persons and technical assistance providers.

The identification of a specific site as to where to test and validate the technologies was made by the project leaders of PhilRice in consultation with the officials and personnel of the Office of the Provincial Agriculturist (OPAg) and their respective municipal agriculturists. The site must be irrigated, visible and accessible so that more people, especially farmers will learn and know about the technology.

The criteria used in selecting farmer-cooperators were as follows: 1) they own and till their land to ensure that they are the decision-makers, 2) they commit to share what they have learned about the technology so it is diffused, and 3) they are capable of leading and persuading other fellow farmers within their social network and sphere of influence to try the technology.

D. Performance evaluation of NRM technologies

Farmers and facilitators from PhilRice and LGU conducted demo farm visits before each meeting to determine the performance of the introduced technology as compared to their farming practice. The field observations served as discussion points in the meeting where the strengths and weaknesses of the technology were highlighted.

Assessment meetings were also held at the end of every cropping season to report on progresses and feedbacks on the performance of technology and constraints in implementation. It was during these meetings that technology dissemination strategies and approaches were refined, and mechanisms developed to enhance impacts and sustainability and ensure that technologies were adapted to the locality and to farmers' circumstances. These were usually attended by field implementers from LGUs and PhilRice, some members of the IRRC team and selected farmers.

Also, field days and forums were held at maturity stage to inform the neighboring farming community about the technology's performance.

RESULTS AND DISCUSSION

A. Case studies for the specific NRM or integration of NRM technologies are presented below:

The following NRM technologies were validated on-farm starting wet season (WS) 2006: Site Specific Nutrient Management in Pangasinan, Sultan Kudarat, and Cotabato; Ecologically-based Rodent Management in Nueva Ecija; and Alternate Wetting and Drying in Apayao, Ilocos Norte, and Nueva Ecija.

Site-Specific Nutrient Management (SSNM)

In the three pilot provinces (Pangasinan, Sultan Kudarat, and North Cotabato) that participated in the SSNM on-farm testing and evaluation, nutrient management for rice was identified through FGD as one of the major concerns of the local farmers. The farmers usually do not have a scientific basis for their fertilizer application, which is usually done once or twice during the cropping season. Burning of rice straw after

harvest is commonly practiced. Farmers' average yield is below 3 t/ha.

The results generally showed higher yield in the SSNM plots across three locations for both WS and DS (Table 1). The average yield increase was 28.3% during the dry season and 12.6% during the wet season of 2007. In the SSNM plots, the rate of Nitrogen, Phosphorous and Potassium (NPK) fertilizer applied was 60-38-30 and 95-33-20 during the wet and dry seasons, respectively. The Farmer's Practice, on the other hand, applied fertilizer at the rate of 50-13-16 during WS and 48-13-14 during DS. Furthermore, the timing of fertilizer application on the SSNM plots was based on the leaf color chart (LCC) readings, while this was done arbitrarily in the Farmer's Practice plots. The yield increase is consistent with the findings of Pampolino *et al*. (2007) which showed that on-farm research comparing SSNM and farmers' fertilizer practiced showed increased yield with SSNM even with reduced fertilizer Nitrogen rates in some cases due to improved efficiency of fertilizer use.

After two cropping seasons of testing and evaluation, farmers have learned, as indicated in the assessment reports that using the right amount and type of fertilizer when applied at the right time can result in better yields, and that high quantity of fertilizer is not always equivalent to high yield. Seeing the effects of improved yield encouraged them to continue using the technology.

This result inspired most of the farmers to shift from their old farming practices to the new ones being promoted by the partnership project. This also made it easier for them to share the technology with other farmers. Even without much intervention from extension agents, farmers will follow visible example of those farmers whom they perceive as being successful in their farming operations (Genius *et al*., 2006; Siopongco *et al*., 2013). In Rogers' (1995) terminology, farmers learn from their "hemophilic neighbors" which are individuals with

TABLE 1
Yield comparison between farmer's practice (FP) and site-specific nutrient management (SSNM) during the wet season and dry season of 2007

Location	Yield, t/ha at 14%MC DS 2007		Yield, t/ha at 14%MC WS 2007	
	FP	SSNM	FP	SSNM
1. Kabacan, North Cotabato	5.24	7.21	5.48	5.58
2. Lambayong, Sultan Kudarat	4.34	4.08	4.32	4.09
3. Mangatarem, Pangasinan	3.57	5.57	4.51	6.44
Average yield across locations	4.38	5.62	4.77	5.37
Average amount of NPK fertilizer applied	48-13-14	95-33-20	50-13-16	60-38-30

whom farmers have close social ties and share common personal characteristics. Additionally, they have learned not to burn rice straw because of its importance in maintaining soil fertility.

Ecologically-Based Rodent Management

Zaragosa is one of the heavily rat-infested farming areas in Nueva Ecija, a major rice producing province of the Philippines. Local farmers use chemical and physical methods to control rats, which are spontaneous and conducted individually, making them less effective (Corales *et al*., 2010).

The Barangay Development Council (BDC) and the local government unit (LGU) of Zaragosa, Nueva Ecija in partnership with the Office of the Provincial Agriculturist (OPAg) and the Philippine Rice Research Institute (PhilRice) have joined a group of farmers to respond to the notorious rat problem. The interagency team laid down a stream of activities to promote effective rat management strategies. The campaign aimed to instil in the minds of farmers the following messages: community work, right timing to control rats, and the use of EBRM practices.

A 20-hectare contiguous farmland located in Sitio Mabilog served as the campaign's front. Also, a rat management technology called the Community Trap Barrier System (CTBS) was set up in this area. Farmers regularly conducted rat-hunting activities based on those recommended for specific crop stages of the rice crop such as monitoring of the CTBS, burrow digging and night hunting. Rat catches were recorded by the farmer-in-charge. This allowed farmers and local

TABLE 2
Yield data of farmer-cooperators in the EBRM site in Zaragosa, Nueva Ecija

Name of Cooperators	Actual Area (ha)	Yield, t/ha at 14%MC DS 2006	Yield, t/ha at 14%MC DS 2007
1. William Rafael	3.0	7.03	6.76
2. Eduardo Agustin	3.0	7.48	7.39
3. Larry Calderon	2.5	5.08	6.35
4. Angelito Calderon	1.0	5.44	6.58
5. Felipe Balutan	3.0	4.67	7.03
6. Danilo Madonza	1.0	7.89	7.62
7. Isaias Siobal	3.5	6.21	6.30
8. Romeo Tartado	1.25	5.62	5.31
9. Jerry Calderon	0.75	5.99	6.21
10. Arnold Villa	2.50	7.48	5.49
Total	21.5	62.89	65.04
Average	2.20	6.30	6.50

folks to see how the technology worked and provided them benefits.

Results gathered from the 20-ha rice farm in Sitio Mabilog revealed a yield increase of 0.2 t/ha or equivalent to about 5 cavans/ha from the baseline yield of 2006 DS (Table 2). The 10 farmer-cooperators used hybrid varieties in 2006 DS but have shifted back to inbred during the campaign period causing some reductions in yield. From this, it is clear that the reductions in yield were due to varietal difference and that the overall increase in yield can be attributed to the campaign. Likewise, rat damage assessment results showed that damage was down by 5% during the campaign period. This means around 19 cavans of palay were saved in the 20-ha farm due to CTBS, burrow digging, night hunting, and other campaign activities conducted. Results further emphasized the importance of community participation and understanding farmer knowledge, practices, and beliefs regarding rodents. These factors, according to Palis and Singleton (2006) largely influenced the farmers' perceptions on the effectiveness and feasibility of the EBRM technology in terms of economic profitability, social acceptability, and cultural suitability.

Alternate Wetting and Drying

The testing and validation of alternate wetting and drying technology were conducted in three sites in Northwestern Luzon, namely, Luna, Apayao; Flora, Apayao; and Currimao, Ilocos Norte; and one site in Central Luzon – Nueva Ecija. The AWD plots were set up side by side with the continuously irrigated plots.

Results in Northwestern Luzon showed slightly lower yields in the AWD plots as compared to the farmers' practice (Table 3). However, the production cost was higher for the non-AWD sites. Farmers using AWD claimed that the reduction in the production cost was due to more efficient water use but the amount saved cannot be quantified at the field level due to lack of measuring instrument. In this case, the reduction in yield is inconsistent with several studies conducted in the past (Siopongco *et al*., 2013), which showed that the practice of AWD produced no yield penalty despite reduction in irrigation.

The experience in AWD testing in Nueva Ecija, on the other hand, showed that irrigation water reaches the downstream earlier by about 10 days when upstream farmers adopt AWD unlike before when it takes 30 days for the water to reach downstream. Consequently, farms which were traditionally not planted to rice during dry season were cultivated and planted to rice when AWD was adopted thereby increasing cropping intensity within the service area of NIA.

B. Upscaling: Integrating NRM technologies into the National Rice Programme

Evaluation of NRM technologies for two successive seasons revealed some improvements in farmers' productivity and encouraged the partners to upscale the technologies (refer to the tables). Scaling up

TABLE 3
Yield and production cost comparison between AWD and non-AWD practitioners in Ilocos Norte and Apayao

Location	AWD practitioner		Non-AWD practitioner	
	Yield, t/ha at 14%MC	Production cost/ha	Yield, t/ha at 14%MC	Production cost/ha
1. Currimao, Ilocos Norte	5.11	20,613	6.00	26,822
2. Luna, Apayao	4.82	18,550	5.20	20,750
3. Flora, Apayao	5.92	19,870	6.30	22,530
Average across locations	5.28	19,677	5.83	23,367

or upscaling means expanding, replicating, adapting, and sustaining successful policies, programs, or projects to reach a greater number of people; it is part of a broader process of innovation and learning (Linn, 2012). With NRM, it means intensifying dissemination efforts in order to bring the technologies and its benefits to a greater number of beneficiaries.

PhilRice started lobbying for the inclusion of validated NRM component technologies to the *PalayCheck* system, the technology platform being used by the National Rice Program. This was done through meetings with IRRI and key officials in the DA such as with the National Rice Program Director. Also, high ranking officials at the provincial level were informally informed about the PhilRice-IRRI project. The idea was conceived to enhance farmers' access to improved and environment-friendly technologies in rice production through some policy support. This implied putting some financial and manpower resources from the side of the Department of Agriculture in order to improve outreach and coverage of NRM technologies thereby more farmers benefiting from the NRM technologies.

Similarly, a committee for the nationwide promotion of AWD involving the Upper Pampanga River Integrated Irrigation System (UPRIIS), BSWM, NIA, IRRI, and PhilRice was created. The committee, after a series of meetings facilitated by the National Rice Program Coordinator who happened to be a PhilRice key staffer and a member of this collaborative project, was able to convince the office of the DA Secretary of the significance of increasing water-use efficiency. In April 2008, the DA Secretary issued Special Order No. 266 mandating the creation of a Technical Working Group (TWG) to study and formulate guidelines for implementing water-saving practices for irrigated rice areas in the country. The draft guidelines were presented to NIA personnel in the regional and system levels, officials of confederated water users and Irrigators' Associations (IAs), regional and provincial agriculture officials and representatives from state universities and colleges (SUCs) in four selected regions. In November 2009, more than one year after

the TWG was created, Administrative Order No. 25 mandating the nationwide adoption of water-saving technologies in irrigated rice production systems in the country was issued by the DA Secretary. Moreover, a memorandum was also issued by the Operations Manager of UPRIIS directing all NIS personnel to incorporate AWD at all levels in system management. This means adopting AWD in all five divisions of the system and targeting 80% of all farms in the service area to adopt AWD by 2015. After UPRIIS adopted AWD, NIA Regional Offices 1, 2, 3, 12, CAR and the Magat River Integrated Irrigation system (MARIIS) followed suit, thereby expanding the rice areas following AWD practices.

Furthermore, a year after EBRM implementation, upscaling of its activities was conducted in eight other municipalities of Nueva Ecija: Cabanatuan City, Sta. Rosa, San Leonardo, Peñaranda, San Isidro, San Antonio, Cabiao and Jaen. The activity was incorporated in the Farmer Field School program jointly implemented by the LGUs and the Regional Crop Protection Center (RCPC).

Eventually, the *PalayCheck* system was upscaled to the National Rice Program of the Department of Agriculture named FIELDS (Fertilizer, Irrigation, Extension, Loans, Dryers, and Seeds) in 2008. The NRM technologies were eventually included in the *PalayCheck* platform of PhilRice, thus, facilitating upscaling and outscaling.

C. Strategies and approaches that facilitated delivery of NRM technologies

The following strategies and approaches were utilized in improving the dissemination of NRM technologies:

Forged/revitalized partnerships with stakeholders

One of the main goals of the ICOP project was to help in enhancing the productivity of farmers through the use of improved and environment-friendly technologies in rice production. In order to achieve this goal and to ensure that resources were managed well, partnerships with other extension providers such as LGUs, NGOs, farmers' associations, private companies and other government agencies were revived and or forged.

Among these partners included the Office of the Provincial Agriculturist (OPAg), Municipal Agriculture Office (MAO), Agricultural Training Institute (ATI), National Irrigation Administration (NIA), Department of Agriculture-Regional Field Office (DA-RFO) and Alalay sa Kaunlaran, Incorporated (ASKI). Partner organizations shared their resources – farmers provided labor counterpart; LGUs provided staff to oversee field activities; NGO-partners conducted social preparation and credit assistance; and PhilRice provided technical and some seed assistance. The partnership with ASKI, a microfinancing NGO, enabled farmers in Mangatarem, Pangasinan to discover the comparative advantage of SSNM against their fertilizer application practice.

Several other partnership projects branched out or were further strengthened owing to the collaborations established. Through these networks, innovation and information sharing on the best practices in rice production were shared, thus, facilitating technology delivery and dissemination. The good relationships among partners were brought about by the knowledge that the project was being done for a common purpose – to enhance the productivity and profitability of farmers through the use of improved and environment-friendly technologies in rice production. Without these partners who have been PhilRice's network in its technology promotion activities, it would have been difficult for the project to accomplish the targeted activities at community, provincial, and national levels. Although the linkage strategy sounds simple, according to Ruaysoongnern (n.d.), the practices were extremely delicate and requires continuous goodwill at all levels.

Established technology demonstrations and employed community-based information campaign

In the wet season of 2006, 23 demonstration farms showcasing NRM technologies were established; these were managed both by farmers and PhilRice. These demonstration farms were maintained and about 50 more were added in 2007. Through the demonstration farms, farmer-cooperators were able to put their newly acquired skills into practice and have seen for themselves the performance of the technologies and how these benefited them.

There were times when technology demonstration is not enough such as rodent management. This was what happened in the promotion of ecologically-based rodent management (EBRM) in Nueva Ecija. The project team members resorted to a community-wide information campaign in order to enhance awareness and improve technology dissemination. Creative campaign materials were developed and distributed not only to farmers but to school teachers, students, and agricultural workers as well using different media. The campaign was featured in a documentary TV program with nationwide network coverage. The Philippine Agricultural Journalists Inc. awarded it the Agricultural Information Campaign of the Year.

Provided assistance in capacity building

Enhancing the capacity of partner researchers and extension workers through trainings and technical briefings was conducted by PhilRice and IRRI at the start of the project to ensure that there was common understanding of the technologies for evaluation and dissemination. Partners were also given chance to attend workshops and exposure trips that provided opportunities for knowledge sharing or meaningful interactions with IRRI scientists. They were also given chances to present their outputs during assessment meetings, all of which contributed in building and strengthening the capacity of partners in doing research and development work, thus, contributing to better project implementation and enhanced technology delivery.

Mobilized local champions to actively participate in the technology testing and evaluation process

The local leaders have been very important in the project for they were responsible in ensuring that farmers were available during the scheduled meeting. The leaders made things easier in the field. Ground working activities were carried out by them together with the LGUs. In other words, through the local leaders, project activities were directly communicated to the community, thus, contributing to enhanced technology delivery.

Utilized participatory approach in project monitoring and evaluation

A project monitoring system was designed to keep track of activities and achievements not only at the project level but in the field as well. For example, in Zaragosa, Nueva Ecija where EBRM was promoted, regular visits to check progress of the campaign activities were done. Farmers monitor EBRM implementation by completing CTBS rat catches monitoring sheets, and through open discussion and games. Knowledge, attitude, and practices monitoring were facilitated by PhilRice through dialogue, quizzes, or fun activities. Program of activities were modified based on feedbacks gathered.

At project level, end-season reviews were conducted to analyze performance and formulate refinements of plans. This also gave key project implementers the chance to share their learning, shortcomings and other accomplishments related to project implementation.

D. Challenges encountered in the pathway towards nationwide extension

The following were some of the challenges met during the project implementation:

1. Documentation. Most often, only the technology performance such as yield was documented by partners. Documenting the constraints and facilitating factors in adoption, the lessons learned in dissemination and the social aspects of the outreach should be emphasized, as well as the project intended to learn the different pathways that lead to nationwide extension. Establishing proper monitoring, evaluation, and documentation of the activities could result in more innovations to improve the system of implementation.
2. Limited capacity of partner-LGUs. Partners, particularly local government units have very limited capacity and resources when it comes to doing development and extension work, as indicated by the weak linkage between research and extension system in the Philippines. They cannot conduct regular monitoring of demo farms and their participation to end-season reviews, meeting, and planning workshops was very limited due to budget constraints.
3. On partnerships. The multi-stakeholder partnerships of farmers and farmer-groups, DA government agencies, PhilRice, IRRI, and LGUs enabled the upscaling and out-scaling of

technologies from the farmer's field through technology demonstration to DA nationwide extension through the *PalayCheck* extension platform. During the initial phase of the project particularly in the conduct of campaign activities in Nueva Ecija, implementation was weak because the roles of partner agencies were not clearly defined. As a result, there were difficulties in achieving project deliverables. A clear hierarchy of roles and responsibilities was then established with partners for effective community campaign. Thereafter, the local government unit took leadership and worked closely with the BDC, farmer groups, and other sectors in the community. This led to an effective campaign implementation.

CONCLUSION

In view of the above results and discussion, the following conclusions are being put forward:

Evaluating the NRM technologies through on-farm testing and evaluation for two successive seasons revealed some improvements in farmers' productivity. This was shown in the yield increases in the SSNM plots due to the application of the right amount of fertilizer when the crops actually needed it, as well as the timing of fertilizer application based on the leaf color chart readings. Similarly, by promoting rodent management technologies, a yield increase of 0.2 t/ha or equivalent to about 5 cav/ha was obtained in the 20-ha demonstration plot due to CTBS, burrow digging, night hunting, and other campaign activities conducted. Rat damage assessment results further showed that damage was down by 5% during the campaign period. Moreover, testing and evaluation of AWD showed yield increases in the upstream and midstream across time but not in the downstream.

The above results as observed by the farmers encouraged them to continue using the NRM technologies as well as sharing it with others. The favourable results paved way to integrating NRM technologies into the National Rice Program by lobbying for their inclusion to the *PalayCheck* system thereby more farmers benefiting from the NRM technologies.

Strategies and approaches that facilitated delivery of NRM technologies included forging partnerships with stakeholders, establishing technology demonstrations and employing community-based information campaign, providing assistance in capacity building, mobilizing local champions in actively participating in the technology testing and evaluation process, and utilizing participatory approach in project monitoring and evaluation. Each approach significantly contributed in the enhanced dissemination of the NRM technologies.

Among the challenges encountered in the pathway towards national extension were documentation, limited capacity of partner-LGUs and role definition. Documentation became an issue since partners usually only managed to document just the technical performance of the project. Roles of partner agencies were not clearly defined at the initial phase of the

EBRM campaign resulting in difficulties in achieving some project deliverables. Limited capacity and resources of partner-LGUs also became a problem since these did not always allow them to conduct regular monitoring of demo farms and to participate in the end-season reviews and meetings. In spite of these challenges, the overall project implementation is deemed successful as it has benefited Filipino farmers and achieved its aim to enhance the delivery of NRM technologies and R&D exchange of information.

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Isolation and Characterization of EgGST, a Glutathione S-transferase Protein Transcript in Oil Palm (*Elaeis guineensis* Jacq.)

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ABSTRACT

The formation of callus and somatic embryos remains one of the major bottlenecks in oil palm tissue culture. Unlike other crops, oil palm tissue culture is a very slow process. In the present study, EgGST (GenBank accession no. AIC33066.1), an oil palm gene coding for a putative glutathione S-transferase protein, has been characterized molecularly. The full length cDNA sequence of EgGST isolated from oil palm cultured leaf explants at the 6th week is 1002 bp in length, with an Open Reading Frame (ORF) of 651 bp. The deduced EgGST encodes a 216-amino-acid protein with a predicted molecular mass of 23.68 kD and a pI value of 6.16. Its protein sequence shares 63% identity with the glutathione s-transferase gstf2 from *Oryza sativa Indica* Group (GenBank accession no. ABR25713.1) and contains thioredoxin fold and chloride channel domain. Real-time RT-PCR results showed that the EgGST transcript was differentially expressed across a time series of fortnightly-cultured leaf explants and had a higher transcript levels in nodular callus (NC) compared to friable callus (FC) for oil palm ortet of clone 4178. EgGST was also found to be preferentially expressed in all tissue culture derived materials except for oil palm cell suspension culture (CSC), whereas there were almost negligible expressions in all the non-tissue culture derived materials, except for root. Hence, it can be suggested that EgGST transcript may possibly be regulated differently at different stages of tissue culture and various tissues. Interestingly, EgGST also displayed a tissue-specific expression pattern via RNA *in situ* hybridization. To our

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knowledge, this is the first reported study on the analysis of the localization of target mRNA transcript of EgGST in different oil palm tissues. We postulated that EgGST might play significant roles at different stages of oil palm callogenesis, and could potentially be a candidate marker for oil palm callogenesis.

Keywords: Oil palm, callogenesis, glutathione S-transferases, full length cDNA, real-time RT-PCR, RNA *in situ* hybridization

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an unbranched monocotyledonous plant of the palm family (Arecaceae) originating from West Africa. The oil palm belongs to the family Palmaceae and the genus *Elaeis*. *Elaeis* is derived from the Greek work *elaion*, which means oil, while the specific name *guineensis* shows that Jacquin attributed its origin to the Guinea coast, West Africa (Corley & Tinker, 2003). At present, there is a potential demand for more than 100 million oil palm (*Elaeis guineensis* Jacq.) tissue cultured plantlets in the world (Corley, 2009; Sharifah & Abu, 2007). In terms of performance, clonal plantlets from selected ortets have out-yielded commercial DxP seedlings by 7%-34% in fresh fruit bunch (Kushairi *et al*., 2010; Sharma, 2006; Simon & Koh, 2005; Zamzuri *et al*., 2005; Khaw & Ng, 1997). The use of clonal palms has been predicted to improve oil production up to 30% (Low *et al*., 2008).

However, unlike other crops, oil palm tissue culture is a very slow process. The regeneration process through oil palm tissue culture takes 2 to 4 years, depending on the genotype. On average, at least 18 months are required to produce complete plants from callus derived from leaf explants, with a callusing rate of only about 20% for young leaf and root explants, and the rate of embryogenesis from proliferating callus culture too was only 3 - 6%, depending on the genotypes (Rajainadu *et al*., 2007; Rohani *et al.*, 2000; Wooi, 1995), making oil palm tissue culture rather inefficient. In an effort to gain insights into oil palm callogenesis, a previous study employed representational difference analysis (RDA) to a pair of cDNA populations from *E. guineensis*; one transcribed from the RNA of the 6th week oil palm cultured leaf explants (where callus initiation occurred) and the other from RNA of 0-day leaf explants, which led to the identification of the abundantly expressed partial glutathione S-transferases gene (Fatihah, 2010).

Plant glutathione S-transferases (GSTs) have been actively investigated during the past decades (Chronopoulou & Labrou, 2009; Basantani & Srivastava, 2007; Moons, 2005; Dixon *et al*., 2002; Edwards *et al*., 2000; Droog, 1997). All the GSTs are reported to be either soluble or loosely membrane-associated dimers with a monomeric size of 15 - 28 kDa, and together they comprise 1 - 3.5% of the total cellular protein (Pairoba & Walbot, 2003; Droog *et al*., 1995). GSTs are a superfamily of multifunctional enzymes in plants, subdivided into eight classes, seven of which (phi, tau, zeta, theta, lambda, dehydroascorbate reductase, and

tetrachlorohydroquinone dehalogenase) are soluble and one is microsomal (Dixon & Edwards, 2010; Lan *et al*., 2009; Basantani & Srivastava, 2007). Since their identification in plants in 1970, these enzymes have been well established as phase II detoxification enzymes that perform several other essential functions in plant growth and development. The GST enzymes have been associated with detoxification of xenobiotics, limiting oxidative damage and other stress responses in plants (Gong *et al*., 2005).

Currently, a large number of the GST genes have been identified or annotated from at least 17 plant species (Chronopoulou & Labrou, 2009; Conn *et al*., 2008; Basantani & Srivastava, 2007). The number of GST-like sequences found in different plant species ranges from 25 in soybean to 42, 53, 59 and 81 in maize, *Arabidopsis* (http://www.arabidopsis.org/browse/genefamily/gst.jsp), rice and *Populus trichocarpa*, respectively (Lan *et al*., 2009; Sappl *et al*., 2009; Sappl *et al*., 2004; Soranzo *et al*., 2004; Dixon *et al*., 2002; Wagner *et al*., 2002; McGonigle *et al*., 2000). Some of the GST genes have been patented (Chronopoulou & Labrou, 2009). Until now, no other data have been reported on the genome-wide identification of the GST family, although at least 20 plant genomes have been completely sequenced (http://www.genomesonline.org/gold.cgi) (Chi *et al*., 2011). To date, the whole family-based expression analyses have been carried out only in *Arabidopsis* (Sappl *et al*., 2009), *P. trichocarpa* (Lan *et al*., 2009) and rice (Jain *et al*., 2010). The transcript profiling of all family members for the other plants, including oil palm, as well as the functional divergence of the GST family, is still not available. Hence, it will be interesting to know the functional role of glutathione S-transferase genes (EgGSTs) in *E. guineensis*. To date, there is no reported full-length cDNA isolation of EgGST from oil palm. Thus, the aims of this study were to isolate a full-length EgGST cDNA from oil palm and perform molecular characterization.

MATERIALS AND METHODS

Plant Material

Tissue cultured materials of *Elaeis guineensis* Jacq. var. tenera were provided by Felda Agricultural Services Sdn. Bhd., Malaysia. For expression analysis, young leaf explants of clone 4178 cultured on Murashige and Skoog (MS) basal culture medium (Murashige & Skoog, 1962) supplemented with auxin, were collected every 2 weeks (day 0 to 26 weeks of culturing). Clone 4178 was selected as the plant material due to the high proliferation ability at the commercial laboratory of Felda Biotechnology Centre, Felda Agricultural Services Sdn. Bhd. (Fatihah, 2010). Meanwhile, the samples for tissue specificity study were provided by Malaysian Palm Oil Board (MPOB), Sime Darby Seeds and Agricultural Services Sdn. Bhd. (SDSAS) and Applied Agricultural Resources Sdn. Bhd. (AAR). The tissue samples were categorised into two groups: tissue culture derived materials from the leaf [embryogenic callus (EC), non-embryogenic callus (NEC), oil palm cell suspension culture (CSC) and the three

different morphologies of oil palm somatic embryos identified during the maturation stage (globular, haustorium and germinating embryo)] and non-tissue culture derived materials [female flower (FF), male flower (MF), apical meristem (M) and root (R)].

Total RNA Extraction

Each plant material (0.1 g) was ground in liquid nitrogen to fine powder using a mortar and pestle. Total RNA extraction was performed by using TRIzol® reagent (Molecular Research Centre, USA) according to the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm (A_{260}) and the purity of the RNA sample was evaluated by A_{260}/A_{280} and A_{260}/A_{230} ratios using NanoPhotometer™ (Implen GmbH, Munich, Germany). The integrity of RNA obtained was determined by visualization on a 1.5% (w/v) formaldehyde agarose gel electrophoresis (Sambrook & Russel, 2001).

Full-Length cDNA Isolation of the EgGST

A partial-length cDNA sequence encoding putative glutathione S-transferase protein was previously identified by Fatihah (2010). This gene was designated as EgGST. EgGST had partial-length sequence lacking the 5'- and 3'-region. Two gene specific primers (5' GSP: 5'-GCATCGCAGA GGTCACCTTCTTGCACGC-3' and 3' GSP: 5'-CCGCATGTTAA GGCATGGTGGGAGG-3') were designed based on the partial-length of the cDNA sequence to isolate the 5' and 3'-region of the putative EgGST, respectively. The SMARTer™ RACE (Rapid Amplification of cDNA Ends) cDNA Amplification Kit (Clontech, USA) and the Advantage 2 Polymerase Mix (Clontech, USA), together with the gene specific primers, were used in the isolation of the 5' and 3'-regions of the gene. The sequences of all the partial-length, 5' and 3'-regions were assembled into contigs by the Contig Assembly Programme (CAP) using the BioEdit Sequence Alignment Editor Version 7.0.9.0 (Hall, 1999) to get the full-length sequence of the EgGST. Based on the full-length sequence, two gene specific primers (ORF forward:5'-AGACGATGGGGGTGAAGGTCTATG-3' and ORF reverse: 5'-ACGCAGATCCAG GCATCGCAGAG-3') were designed to amplify the Open Reading Frame (ORF) region. The ORF of the transcript was isolated by PCR amplification of the 5'-RACE-Ready cDNA template with the two gene specific primers. The PCR product was then cloned into the yT&A cloning vector (Yeastern Biotech, Taiwan) and sequenced at both directions (NHK Bioscience Solutions Sdn. Bhd., Malaysia).

Sequence Analysis

The 5'-RACE PCR product, 3'-RACE PCR product and ORF sequences of the putative EgGST were analyzed using the BLASTN, BLASTX and BLASTP programmes at the National Centre of Biotechnology Information (NCBI). These programmes were used to search for significant similarities between the isolated sequences with NCBI/Genbank databases. BLASTX and BLASTN were

used to search for significant identities in the non-redundant protein sequences (nr) and expressed sequence tags (ESTs) databases, respectively. The clustal W multiple alignment programme in the Bioedit software was used to align the amino acid sequence of the EgGST with homologous sequences from other plants. The acquired sequence data were also analyzed further using the Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences (PSORT) (http://psort.nibb.ac.jp) (Horton *et al*., 2007), plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/) (Kuo-Chen & Hong-Bin, 2010), Biology Workbench Version 3.2 (http://workbench.sdsc.edu) (Subramaniam, 1998), SignalP 4.0 Server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al*., 2011), Compute pI/Mw (Expasy) (http://br.expasy.org/tools/) and FEX Programme (http://www.molquest.com/molquest.phtml?group=index&topic=gfind) (Solovyev *et al*., 1994). The phylogenetic tree of EgGST was constructed using the Mega version 4 software (Tamura *et al*., 2007). In this study, the cut-off score and Expect (E) value were set at 50 and 10^{-5}, respectively; whereby all the matches with BLASTX scores equal to 50 or above and E value equal or less than 10^{-5} were considered significant.

Expression Study by using Real-Time RT-PCR (Reverse Transcriptase-PCR)

One microgram of the total RNA was used for reverse transcription into first-strand cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, USA). Primers for the real-time RT-PCR analysis were designed using the Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on the 3' un-translated regions (UTRs) of the full-length transcript sequence of the EgGST obtained. The suitability of the designed primers was checked by using the Oligonucleotide Properties Calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html). The Brilliant® SYBR Green QPCR Master Mix (Stratagene, USA) was used for real-time RT-PCR reaction. Each PCR mixture contained 1X master mix (comprising SYBR® green I dye, 2.5 mM $MgCl_2$, and dNTPs mixture), 100 nM of gene specific primers (Forward primer: 5'-ATCTGCGTGAGA GGTATCGGTTG-3' and Reverse primer: 5'-ATTACCCACCATCC CACCCTAGA-3'), 2 µL of 10-fold diluted first-strand cDNAs in a total volume of 20 µL. The amplification was performed in the iQ5 Real Time PCR Detection System (BioRad, USA) using the following program: 95°C for 10 minutes; 50 cycles of 95°C for 30 seconds, 60°C for 45 seconds and 72°C for 30 seconds. For each sample, three technical replicates were included. All the experiments contained a non-template control (negative control) and a calibrator. The quantity of the gene expression levels in every experimental tissue was expressed relative to the calibrator, i.e. 0-day leaf explants (clone 4178). Comparative C_T method was used to estimate the relative expression level of EgGST transcripts

(Livak & Schmittgen, 2001). The relative expression were carried out across a time series of fortnightly-cultured leaf explants and in different oil palm tissues and analysed using the geNorm software (Primer-Design, UK) (Vandesompele *et al*., 2002). In the analysis, the relative quantity of the transcripts were normalized with the expression of three endogenous genes including unknown/hypothetical protein (EA 1332; GenBank accession no. EY406625.1), superoxide manganese dismutase (PD 569; GenBank accession no. EL682210.1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; GenBank accession no. DQ267444).

The sequences of the primers are as follow:

EA 1332 sense
5' – TTAAGAATGCTCGGGAAAGG – 3'
EA 1332 antisense
5' – CTACTTCTGTCTGCAATTTTGG – 3'
PD 569 sense
5' – ATCAACCACTCAATCTTCTGG – 3'
PD 569 antisense
5' – CTTCTGCGTTCATCTTTTGC – 3'
GAPDH sense
5' – GCCAGCTTTAACATCATTCCTAGC – 3'
GAPDH antisense
5' – AGCTTTCCATTTAAGGCAGGAAG – 3'

Expression Study by using RNA in situ Hybridisation

One morphology of oil palm somatic embryo identified during the maturation stage (germinating embryo), plus several oil palm *in vitro* cultured-derived samples such as leaf explants (LE), embryogenic callus (macroscopically nodular and friable cultures) and non-embryogenic callus (macroscopically not friable), were used to study the expression of EgGST transcripts. All the plant materials were provided by Felda Agricultural Services Sdn. Bhd., Malaysia. Sense and antisense riboprobes were synthesised by using Ampliscribe™ T3 High Yield and Ampliscribe™ T7 Flash™ Transcription Kit, respectively (Epicentre® Biotechnologies, USA) according to the manufacturer's instructions. Sense and antisense probes were generated by designing the gene specific primers containing the minimum T3 promoter sequence (5'-AATTAACCCTCACTAAAGG-3') and T7 promoter sequence (5'-TAATACGACTCACTATAGG-3'), respectively needed for efficient transcription (as bolded below). The primers were designed at the ORF and 3'UTR region of the EgGST sequence. The sequences of the primers are as follows:

T3F:
5'-**AATTAACCCTCACTAAAGG**GATGTGTGGTTGGAAGTGGAATC-3'
T7R:
5'-**TAATACGACTCACTATAGG**CTCAATAGACAGGGACTCACAGC-3'

In situ hybridisation (ISH) was performed according to the method described by Ooi *et al*. (2012). All the images were then viewed and photographed with a camera attached to the LEICA DM6000 B light microscope (Leica, Germany) and processed with the Progress Research Pro software (Leica, Germany).

RESULTS AND DISCUSSION

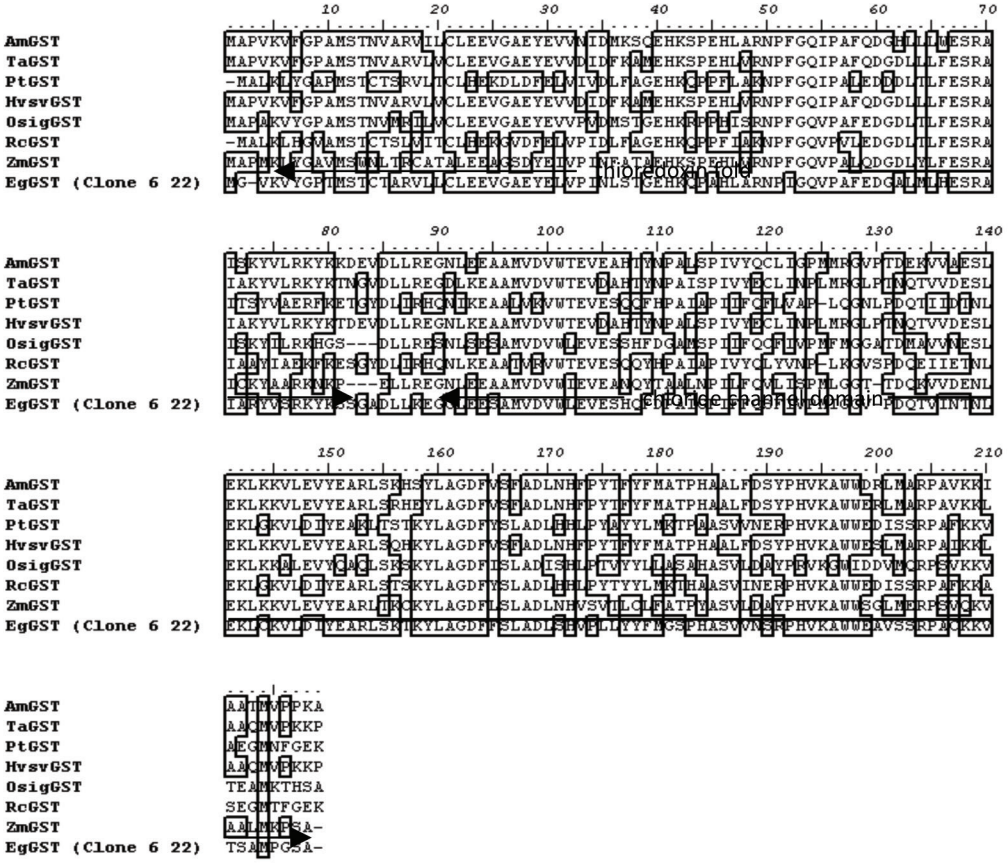
Sequence Analysis of the Full-Length cDNA of EgGST

The full-length cDNA sequence of EgGST (1002 bp) was predicted to encode a polypeptide of 216 amino acid residues with 78 bp of 5' UTR, 651 bp of open reading frame and 273 bp of 3' UTR including a poly-A tail (GenBank accession no. AIC33066.1). By using Compute pI/Mw (Expasy tools software), the EgGST has a predicted molecular mass of 23.68 kD, with a pI value of 6.16. The BLASTX analysis (NCBI) showed that the deduced amino acid sequence of EgGST was 63% identical to glutathione s-transferase gstf2 from *Oryza sativa Indica* Group and 62% identical to glutathione transferase from *Alopecurus myosuroides*. This was followed by phi class glutathione transferase GSTF3 from *Populus trichocarpa* (61%), glutathione transferase from *Hordeum vulgare subsp. vulgare* (61%), glutathione-S-transferase 19E50 from *Triticum aestivum* (61%), glutathione-s-transferase theta, gst, putative from *Ricinus communis* (60%), and glutathione S-transferase 1 from *Zea mays* (56%). The deduced amino acid sequence of EgGST has a thioredoxin fold domain located at the positions of 4 - 82 and a chloride channel domain located at the positions of 90 - 216 (see Fig.1).

Glutathione (GSH) is the tripeptide γ-glutamyl-cysteinyl-glycine and plays a central role in the processes of detoxification and redox buffering (Noctor & Foyer, 1998). GST proteins consist of two well-defined domains, the N-terminal domain that binds the primary substrate GSH and the C-terminal domain that binds the secondary substrate (Edwards *et al*., 2000). Plant GSTs acted by catalyzing nucleophilic conjugation of the reduced form of the tripeptide GSH to a wide variety of hydrophobic, electrophilic, and usually cytotoxic substrates. The toxic molecule, GSH conjugate can then be transported to the vacuole or apoplast and metabolised to a non-toxic compound such as peptide derivatives (Dixon & Edwards, 2010; Edwards *et al*., 2000).

Most GSTs are active as dimers, composed of either homogeneous (the most prevalent form) or heterogeneous subunits (Edwards *et al*., 2000). The thioredoxin-like N-terminal domain (4 - 82 amino acids), as shown in Figure 1, binds to GSH, and is conserved in all classes of GSTs (Dixon *et al*., 2002). By contrast, the C-terminal chloride channel domain (90 - 216 amino acids) (Figure 1) is the domain that provides structural elements for the recognition of xenobiotic substrates, which tends to exhibit much more diversity within and among classes of GSTs (Basantani & Srivastava, 2007; Edwards *et al*., 2000). In 2009, Lan *et al*. suggested that the C-terminal domain could lead to diversification in substrate selectivity and specificity among the members of *Populus trichocarpa* tau GSTs, while preserving the enzymes' primary function and thus, enhance the metabolism of substances encountered in the environment.

Plant GSTs are classified based on amino acid sequence identity and conservation of gene structure (i.e., exon/intron numbers),



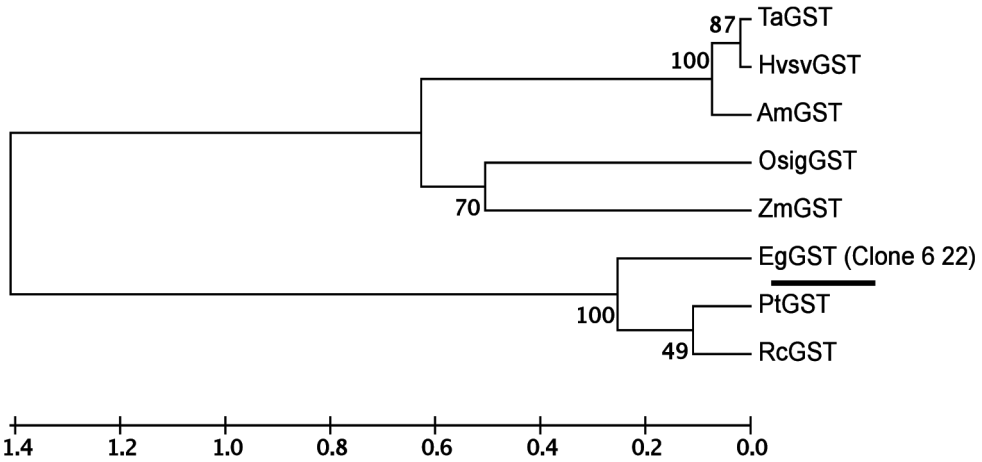
The identical amino acids are boxed. The sequences were downloaded from Genbank: AmGST, glutathione transferase of Alopecurus myosuroides (Acc. No. CAA09191.1); TaGST, glutathione-S-transferase 19E50 of Triticum aestivum (Acc. No. AAL47688.1); PtGST, phi class glutathione transferase GSTF3 of Populus trichocarpa (Acc. No. ADB11382.1); HvsvGST, glutathione transferase of Hordeum vulgare subsp. vulgare (Acc. No. AAL73394.1); OsigGST, glutathione s-transferase gstf2 of Oryza sativa Indica Group (Acc. No. ABR25713.1); RcGST, glutathione-s-transferase theta, gst, putative of Ricinus communis (Acc. No. XP_002531867.1); ZmGST, glutathione S-transferase 1 of Zea mays (Acc. No. NP_001105412.1). The thioredoxin fold domain (4 – 82 amino acids) and chloride channel domain (90 – 216 amino acids) are as shown above.

Fig.1: Alignment of Deduced Amino Acid Sequences of EgGST with *Alopecurus myosuroides, Triticum aestivum, Populus trichocarpa, Hordeum vulgare subsp. vulgare, Oryza sativa Indica Group, Ricinus communis* and *Zea mays* sequences.

in which the number of exons is different for each class (Licciardello *et al*., 2014; Mohsenzadeh *et al*., 2011). For example, phi class of GST genes contains three exons, tau class contains two exons and zeta class contains ten exons in their genes. In the FEX Program (Prediction of internal, 5'- and 3'- exons) analysis, EgGST was predicted to have five potential exons. Conserved Domain Database (CDD) analysis (NCBI) on the deduced amino acid sequence of EgGST (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Marchler-Bauer *et al*., 2013) showed that EgGST belongs

to phi class GSTs. However, this is only a prediction by bioinformatics data and the exact classification of EgGST can only be confirmed after resolving the crystal structures of EgGST through X-ray crystallography. The tau and phi class GSTs are the most represented ones, plant-specific and chiefly involved in xenobiotic metabolism (Basantani & Srivastava, 2007; Moons, 2005). Genome-wide analysis of biochemical characteristics of *Arabidopsis thaliana* and *Populus trichocarpa* tau and phi GSTs found that these two classes of GSTs have broad substrate specificities (Dixon *et al*., 2009; Lan *et al*., 2009), which may be related to the high tolerance to abiotic stresses, especially to a broad spectrum of xenobiotics such as herbicides, salt and UV stressors (Jha *et al*., 2011). Zeta- and theta-class GSTs have very restricted activities towards xenobiotics. Theta-class GSTs are glutathione peroxidases and involved in oxidative-stress metabolism, whereas zeta-class GSTs act as glutathione-dependent isomerases and catalyse the glutathione-dependent conversion of maleylacetoacetate to fumarylacetoacetate. Zeta-class GSTs participate in tyrosine catabolism. Dehydroascorbate reductase- and lambda-class GSTs function as thioltransferases (Basantani & Srivastava, 2007). Dehydroascorbate reductase is also a key enzyme in the ascorbate-glutathione cycle that maintains reduced pools of ascorbic acid, which serves as an important antioxidant (Moons, 2005). Microsomal-class GSTs are members of the MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism) superfamily (Basantani & Srivastava, 2007).

In the WoLF PSORT and Plant-mPLoc analysis, EgGST was predicted to be located in the cytosol. The GSTs reported so far are mostly soluble cytosolic enzymes, and have been classified in classes ranging from mammals, plants, insects, parasites, fungus, to bacteria (Mohsenzadeh *et al*., 2011; Wongsantichon & Ketterman, 2005). By using SignalP, EgGST was predicted to not having any signal peptide, implying that it is located in the cytosol. A sequence comparison between the deduced EgGST with GST protein of other plants revealed that GST proteins are indeed well conserved across monocot and eudicot plants (Figure 1). The similarities are almost evenly distributed throughout the sequence, in the thioredoxin fold domain (4 - 82 amino acids) and the chloride channel domain (90 - 216 amino acids). A phylogenetic tree was plotted to estimate the relationship between the sequences of EgGST with other sequences of plant glutathione transferase proteins (Figure 2). The resulting phylogenetic tree (Figure 2) is organized into two clades. Interestingly, EgGST that encodes a putative glutathione transferase protein appeared to be belonging to clade 2, together with the putative PtGST sequence from *Populus trichocarpa* and RcGST sequence from *Ricinus communis*. Hence, EgGST might belong to either phi- or theta-class GSTs. Therefore, the comparison of monocot and eudicot GST proteins in this study revealed that during evolution eudicot, members of the GST family have



An unrooted neighbour-joining tree generated from the multiple alignment of EgGST protein (underlined) with GST proteins of other plant's proteins, with repeat verification for 5000 times by Bootstrap. The number on the branch means the percentage of repeat verification credibility. Bootstrap values are indicated for branches supported by more than 50% of 5000 replicates. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The length of the branches is proportional to the number of amino acid substitutions per residue. MEGA (Molecular Evolutionary Genetic Analysis version 4) was used to construct the phylogenetic tree based on neighbour-joining method.

Fig.2: Phylogenetic Relationship of EgGST and Different Plant Species Based on the Deduced Amino Acid Sequences

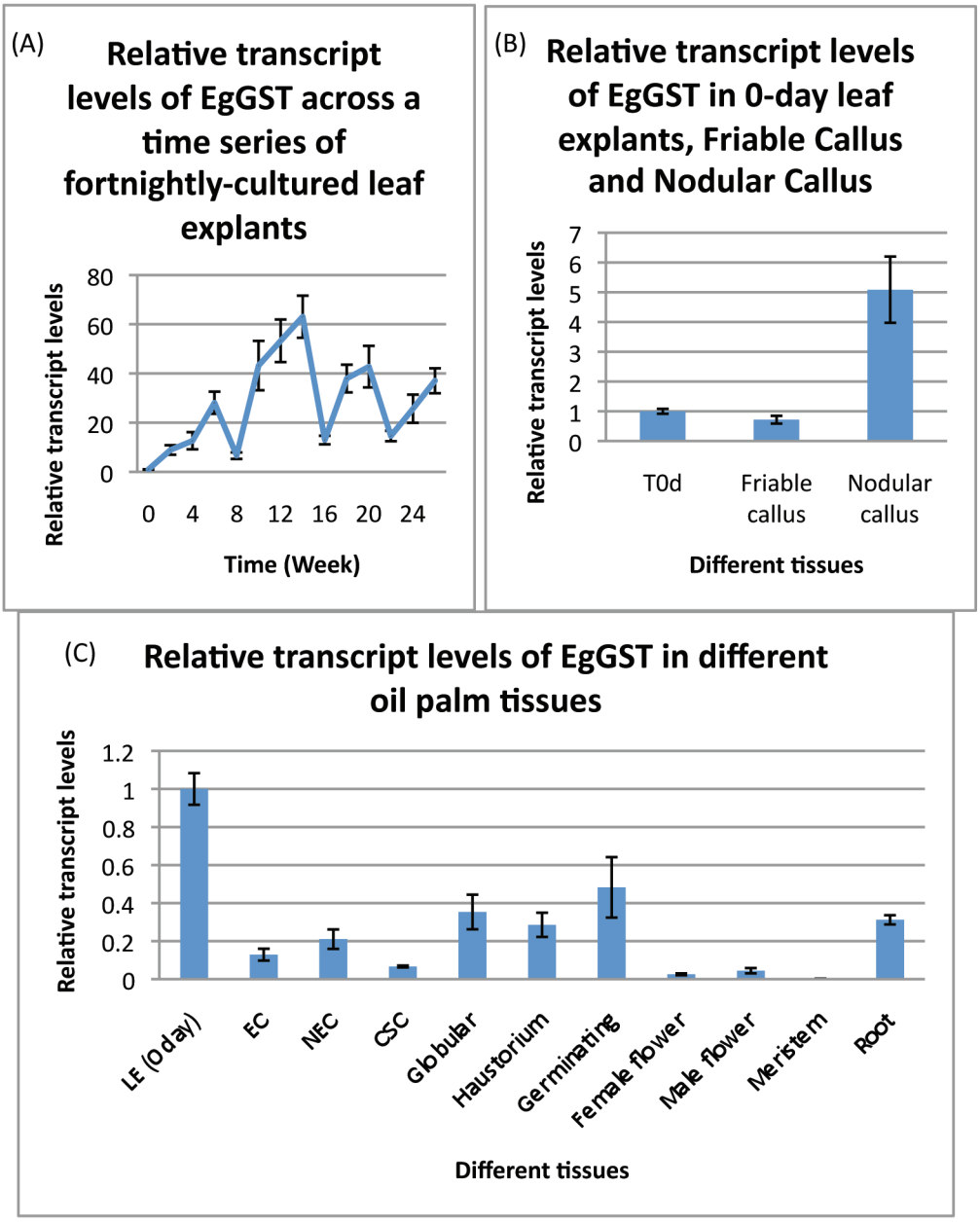
developed along two different directions. Hence, understanding the genomic and functional evolution of gene families is essential for understanding the phenotypic diversification of organisms and their genetic systems. Lan *et al*. (2009) revealed the complex history of genome duplications and chromosomal rearrangements in *Populus* through the course of genome evolution, which is thought to occur in order to supply raw genetic material, allowing functional divergence and rapid biological evolution. The genome duplications and chromosomal rearrangements process could have probably been taking place in oil palm too through the course of evolution as GSTs are evolutionarily ancient proteins. Hence, EgGST might also be descendant from gene duplication. However, to date, limited information is available on the patterns of functional diversification governing the evolution of most classes of gene families in the plant kingdom (Lan *et al*., 2009). Previous phylogenetic analysis had suggested that Theta, Zeta and Omega GSTs as the most ancestral classes in plants (Chi *et al*., 2011; da Fonseca *et al*., 2010). GSTs were also thought to have evolved from a thioredoxin-like ancestor in response to the development of oxidative stress (Martin, 1995; Koonin *et al*., 1994).

Real-Time RT-PCR Analysis of EgGST

The EgGST transcript was shown to be differentially expressed across a time

series of fortnightly-cultured leaf explants. The expression showed an increase from 0-day leaf explants to 6th week cultured leaf explant and dropped at 8th week but started to peak again from 10th week until 14th week cultured leaf explants (see Figure 3A). The expression profile of the EgGST transcript was similar to that reported previously by Che *et al*. (2006) who observed that the frequency of stress response genes increased with time during callus development in *Arabidopsis* tissue culture. However, it dropped again at 16th week, followed by a rhythmic pattern of expression at different time points, peaking at 20th week with a subsequent decline at 22nd week, and a slight increase from 24th week to 26th week cultured leaf explants. The result, as illustrated in Figure 3A, is also in agreement with the previous finding by Fatihah (2010), where the relative transcript level of EgGST was found higher in the cultured leaf explants at 6th week in comparison to the 0-day leaf explants. FC is embryogenic callus and has the capacity to produce somatic embryo of oil palm but the frequently produced callus is NC, which is non-embryogenic callus. An expression comparison was done in both stages of the oil palm callus development, which might help to enhance our understanding on the embryogenic callus production. EgGST had higher transcript levels in nodular callus (NC) compared to friable callus (FC) for oil palm ortet of clone 4178 (Figure 3B), with 5.08764 and 0.71569 folds, respectively, compared to that of the expression level in 0-day leaf explants. The expression level of EgGST transcript in 0-day leaf explants was used as a reference point (calibrator). The result shown in Figure 3B is in agreement with that of Low *et al*. (2008). GST was reported to be up-regulated in non-embryogenic callus of oil palm compared to embryogenic callus and embryoid via northern blot analysis, but was to some extent genotype-dependent (Low *et al*., 2008). Nevertheless, Legrand *et al*. (2007) had also reported that two GSTs were preferentially expressed in the cultured explants from a non-embryogenic genotype of *Cichorium intybus* L. via in silico EST data analysis and real-time RT-PCR experiments.

Evidence showed that the transcript of plant GST genes was regulated by various abiotic and biotic stresses, as well as hormones including xenobiotic-type stresses such as herbicide application (Edwards *et al*., 2000), chilling (Seppänen *et al*., 2000), dehydration (Bianchi *et al*., 2002; Kiyosue *et al*., 1993), hypoxic stress (Moons, 2003), wounding (Vollenweider *et al*., 2000), pathogen attack (Mauch & Dudler, 1993), ethylene (Zhou & Goldsbrough, 1993), auxin (Chen & Singh, 1999), 2,4,6-trinitrotoluene (TNT) (Brentner *et al*., 2008), hydrogen peroxide (H_2O_2) and the defence signal salicylic acid (SA) (Chen *et al*., 1996). Callus is often induced in or upon contact of the wounded part of the explants with the media. During tissue culture, mechanical wounding, osmotic shock, hormonal imbalances and environmental cues such as exogenous auxin induction and cutting may cause significant stress effects that can trigger the somatic cells to



Relative amounts of EgGST transcripts were normalized to the geometric mean of the three endogenous references (EA 1332, PD 569 and GAPDH) by using the Comparative C_T method and were then rescaled to the expression values in 0-day leaf explants (clone 4178). The error bars represent mean ± SD of three technical replicates. LE, leaf explant; EC, embryogenic callus; NEC, non-embryogenic callus; CSC, cell suspension culture.

Fig.3: Expression Profiles of EgGST. (A) Relative transcript levels of EgGST in fortnightly-cultured leaf explants, (B) Relative transcript levels of EgGST in 0-day leaf explants, Friable Callus and Nodular Callus, (C) Relative transcript levels of EgGST in different oil palm tissues.

differentiate into embryogenic competent cells (Singla *et al*., 2007; Fehér *et al*., 2003; Dixon *et al*., 2002; Pfeiffer & Höftberger, 2001). The stress effect may produce reactive oxygen species (ROS) such as H_2O_2 that may lead to lipid peroxidation, biological macromolecule deterioration, membrane dismantling, ion leakage, and DNA-strand cleavage and finally death of plants (Rascio & Navari-Izzo, 2011; Hossain *et al*., 2010; Romero-Puertas *et al*., 2002). H_2O_2 is a small, diffusible molecule that is widely considered to be a signal molecule in the regulation of the defense system (Neill *et al*., 2002). H_2O_2 plays a dual role in plants: at low concentration, it acts as a signal that induces the expression of numerous defense genes encoding cellular protectants such as glutathione S-transferase and glutathione peroxidase, and activates multiple defense responses to abiotic stresses, while excessive accumulation leads to cellular oxidative damage and even programmed cell death (Levine *et al*., 1994; Prasad *et al*., 1994). The ability of a plant to express stress-response genes to endure stress and regulate ROS levels can inevitably help the proliferation of culture lines into embryoids. This was supported by Lin *et al*. (2009), who reported that most of the disease- and defense-related ESTs isolated during oil palm somatic embryogenesis code for GST. This is also in line with the finding of a previous study by Fatihah (2010), which showed that EgGST was up-regulated in leaf explants at 6th week as compared to 0-day, which could be stress responsive effect or to initiate callus formation. Besides its plausible role in the initiation of meristematic cells that led to callus formation in 6th week cultured leaf explants, GST was probably involved in the morphogenesis of NC. Stressful environment can also induce morphogenic events *in vitro* (Gong *et al*., 2005). Stress-induced growth is related to the production of ROS that might trigger the expression of GST (Gong *et al*., 2005; Fehér *et al*., 2003; Dixon *et al*., 2002).

In contrast, there are also reports that GSTs were expressed in cultured leaves of *Cichorium* undergoing somatic embryogenesis (Galland *et al*., 2001) and thus, have been linked with somatic embryo formation in carrot (Kitamiya *et al*., 2000). Consistently, GST accumulation has been reported in somatic embryos of *Cyclamen persicum* (Winkelmann *et al*., 2006), *Vitis vinifera* (Marsoni *et al*., 2008), and embryogenic cells of *Medicago truncatula* (Imin *et al*., 2004). This is not surprising as GSTs are represented by a large and diverse gene family in plants which can be divided on the basis of sequence identity into phi, tau, theta, zeta and lambda classes (Dixon *et al*., 2002). This can also be explained by the compensatory potential of other members of the GST family. Hence, it can be suggested that EgGST transcript might possibly be regulated differently at different stages of tissue culture, FC and NC. However, this can only be verified by performing validation tests using a much larger numbers of samples and a wider range of genotypes.

Over all in oil palm, EgGST was found to be preferentially expressed in all tissue

culture derived materials from leaf except for CSC, whereas there were almost negligible expression in all the non-tissue culture derived materials except for root (Figure 3C). In 2005, Gong *et al*. had reported that *BjGSTF2*, a gene homologous to the phi class GSTs, accumulated differentially in mustard organs, where the transcript was most abundant in root. In addition to that, a plant GST from *Arabidopsis thaliana, AtGSTU17* had been reported to be involved in seedling development and root elongation, whereby the loss-of-function mutant of *AtGSTU17* resulted in a reduced biomass of seedlings and number of lateral roots in the presence of auxin (Jiang *et al*., 2010). Since the GST enzymes have long been associated with detoxification of xenobiotics, limiting oxidative damage and other stress responses in plants (Gong *et al*., 2005), a higher expression of EgGST transcript in root is probably to exclude the processes from the sensitive metabolism in shoot. The relative transcript levels of EgGST were fluctuating during the embryoid transition from globular to germinating stages which occurred at the late stages of somatic embryogenesis. During the transition period, EgGST was found to be highly expressed in germinating embryoid followed by globular and haustorium, with 0.48258, 0.35309 and 0.28537 fold, respectively, compared to that of the expression level in 0-day leaf explants. Since the expression of EgGST transcripts were detected in all tissue culture derived materials from leaf (except for CSC) and in root, it can be concluded that EgGST may have a broader roles in oil palm growth and development, in addition to having functions in various stresses as supported by Gong *et al*. (2005) and Moons (2005). Besides, there are also a few other reported roles of GSTs in endogenous plant developmental processes; in the vacuolar sequestration of anthocyanins in maize, petunia and *Arabidopsis* (Kitamura *et al*., 2004; Alfenito *et al*., 1998; Marrs *et al*., 1995), as binding proteins by binding to various hormones including auxin (Smith *et al*., 2003) and cytokinin (Gonneau *et al*., 2001), as well as porphyrin compounds (Lederer & Böger, 2003) to regulate their activities. Recently, *Arabidopsis* GSTF2 was found to selectively bind the indole-derived phytoalexin camalexin as well as the flavonol quercetin-3-O-rhamnoside, suggesting a role in regulating the binding and transport of defense-related compounds in plants (Dixon *et al*., 2011). The fact that plant GSTs can be induced by a wide variety of phytohormones, including ethylene, auxin, methyl jasmonate, salicylic acid, and abscisic acid (ABA) (Moons, 2003; Smith *et al*., 2003; Wagner *et al*., 2002) and that all these hormones regulate many aspects of plant development also supports that plant GSTs may play vital roles in plant growth and development as well. However, evidence to substantiate this role is still limited.

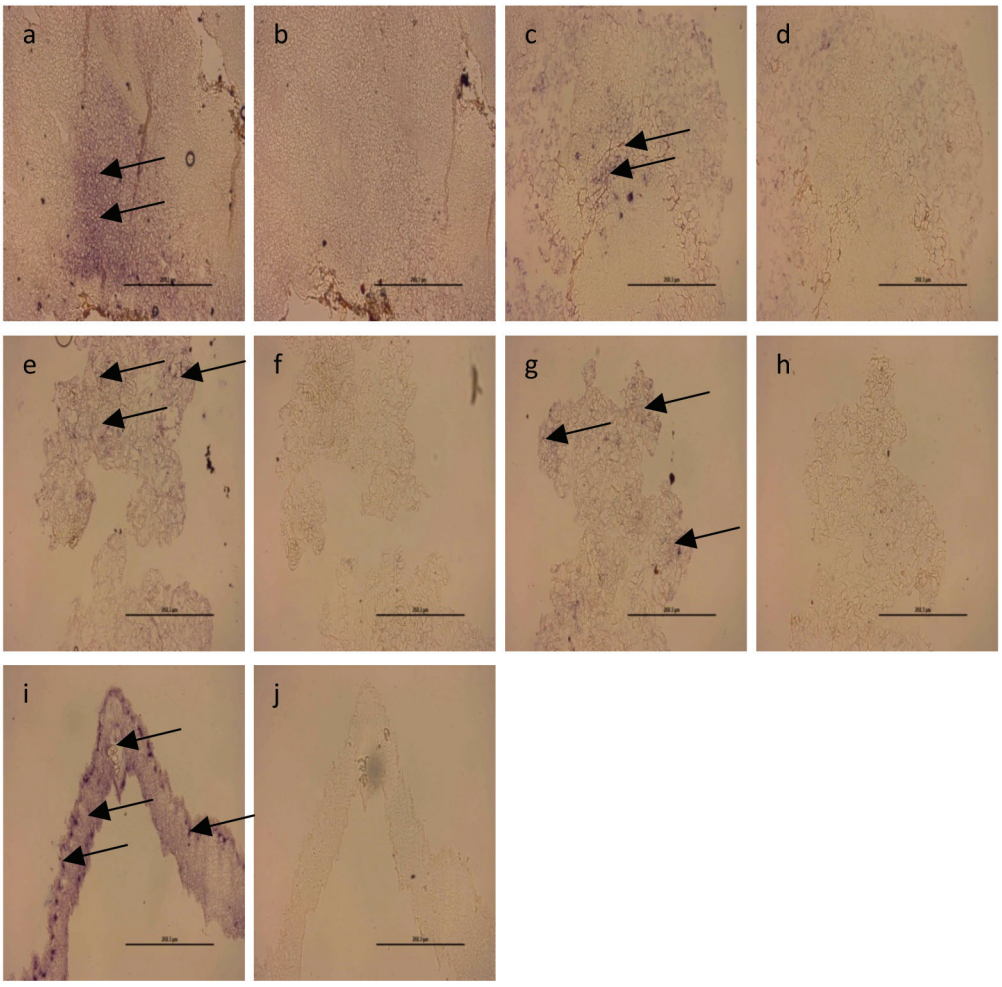
RNA in situ Hybridization Analysis of EgGST

The analysis of the localization of target mRNA transcript of EgGST was performed in germinating embryo, EC, NEC and LE, as the transcripts were found to be preferentially expressed in the respective

tissues of oil palm (Figure 3C). RNA *in situ* hybridization is a widely used method that allows one to analyse the localization of target mRNAs in a preserved tissue section (Bayer *et al*., 2009). The main objective of this part of study is to examine the spatial expression pattern of EgGST in various oil palm tissues at cellular level and to clarify the signals detected in real-time RT-PCR. In addition, eukaryotic cells are highly compartmentalized, and the correct localization of proteins is essential for their function (Boruc *et al*., 2010).

In both germinating embryo and EC hybridized with antisense and sense of riboprobe each, positive signal was detected in the middle of the developing germinating embryo and EC (Figure 4a, 4b, 4c, 4d). No expression was observed in the rest of the germinating embryo and EC tissue. Meanwhile, in NEC hybridized with antisense and sense of riboprobe each, a weak signal was detected at the corner of the developing NEC (Figures 4g & 4h). No expression was observed in the rest of the NEC tissue. When NEC was hybridised with the elongation factor *ELF* antisense probe (positive control), a clear signal was observed that was exclusively localized to the actively-dividing cell layer at the surrounding of NEC (Figures 4e & 4f). Besides its plausible role in the initiation of meristematic cells that led to callus formation in 6th week cultured leaf explants, EgGST is probably involved in the morphogenesis of nodular callus (Fatihah, 2010). Moreover, Galland *et al*. (2001) suggested that the GST transcript accumulation is not only caused by abiotic and biotic stress but might also be involved in cellular proliferation activity. They also reviewed that GST was expressed during transition of G to S phase of mitosis of tobacco mesophyll protoplast and *Arabidopsis* zygotic embryogenesis. During tissue culture, mechanical wounding, osmotic shock, hormonal imbalances and environmental cues such as exogenous auxin induction and cutting may cause significant stress effects that can trigger the somatic cells to differentiate into embryogenic competent cells (Singla *et al*., 2007; Féher *et al*., 2003; Dixon *et al*., 2002; Pfeiffer & Höftberger, 2001). This indicated the role of GST in the initiation of meristematic activity of differentiated cells (Vernoux *et al*., 2000; Takahashi & Nagata, 1992).

On the other hand, strong positive signals were observed in the oil palm leaf explants when hybridised with antisense and sense of EgGST riboprobe each (Figures 4i & 4j). This is not surprising as during tissue culture, mechanical wounding and cutting of oil palm leaf explants may cause significant stress effects that induce the expression of GST. To date, this has been the first reported study on the analysis of the localisation of target mRNA transcript of EgGST in different oil palm tissues. Hence, we proposed that EgGST might play significant roles at different stages of oil palm callogenesis and could potentially be a candidate marker for oil palm callogenesis. As such, EgGST can be used for screening explants with high callusing rates, thus enabling reductions in time and costs in the micropropagation process.



a, b: germinating embryo hybridized with EgGST; c, d: embryogenic callus (EC) hybridized with EgGST; e, f: non-embryogenic callus (NEC) hybridized with elongation factor (*ELF,* positive control); g, h: non-embryogenic callus (NEC) hybridized with EgGST; i, j: oil palm leaf explants (LE) hybridized with EgGST. The a, c, e, g, i are antisense and b, d, f, h, j are sense hybridizations. The presence of purple stain or deposit is regarded as positive signal (black arrows). Scale bar = 260.3 µm.

Fig.4: Localization of mRNA transcript of selective probes (EgGST or *ELF*) on various oil palm tissues.

CONCLUSION

The full length cDNA sequence of EgGST (GenBank accession no. AIC33066.1) isolated from oil palm cultured leaf explants at the 6th week is 1002 bp in length with an Open Reading Frame (ORF) of 651 bp. The deduced EgGST encodes a 216-amino-acid protein and contains thioredoxin fold and chloride channel domain. Based on the real-time RT-PCR results obtained, it can be suggested that EgGST transcript might possibly be regulated differently at different stages of tissue culture and various tissues. EgGST also displayed a tissue-specific expression pattern via RNA

in situ hybridisation. Hence, we postulated that EgGST might play significant roles at different stages of oil palm callogenesis and could potentially be a candidate marker for oil palm callogenesis. It may be interesting to further explore the expression profiles of EgGST across a wider range of oil palm genotypes in order to confirm the suitability as putative marker.

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Optimisation of Solid Liquid Extraction of *Orthosiphon stamineus* Leaves using Response Surface Methodology Technique

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ABSTRACT

Orthosiphon stamineus is one of the popular medicinal plants in Southeast Asia. *O. stamineus* leaves are used in numerous applications related to medicinal purposes and are believed to cure certain health conditions such as hypertension, gout and fever. The aim of this study was to investigate the effect of three parameters involved in extraction process including extraction temperature, extraction duration and solvent to solid ratio on extraction yield, antioxidant activity and referral markers of *O. stamineus* leaves. The optimisation of extraction processes was evaluated with the aid of Design-Expert software using response surface methodology (RSM). The optimum extraction parameter for *O. stamineus* leaves were recorded at the extraction temperature of 60°C, 30:1 (ml:g) solvent to solid ratio and 6 hours extraction duration with 30Wt% extract, 67 and 1 mg/L concentration of Rosmarinic acid and Sinensetin, respectively. Antioxidant activity for optimized extract is 96.56% and 91.51% of SOD and DPPH method, respectively.

Keywords: Optimization process, extraction parameter, antioxidant activity, response surface methodology, *O. stamineus*

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INTRODUCTION

Medicinal plants are promising sources for treating several diseases nowadays due to the phytochemical contents inside them. *O. stamineus* belongs to the Lamiaceae family and has several local names including "Misai

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kucing" in Malaysia, "Kumis kucing" in Indonesia, and "Rau meo" in Vietnam (Awale *et al.,* 2003). *O. stamineus* has been used widely in Southeast Asia for treatment of gallstone, hepatitis, hypertension and renal calculus (Tezuka *et al*., 2000). In Malaysia, the tea prepared from the leaves is taken as a beverage to improve health and also for the treatment of some diseases such as gout and diabetes (Akowuah *et al.,* 2004).

According to Akowuah *et al.* (2004), the separation of bioactive compounds in *O. stamineus* leaves resulted in three methoxylated flavones, namely, sinensetin (SEN), eupatorin (EUP) and 3'-hydroxy-5,6,7,4'- tetramethoxyflavone (TMF) and rosmarinic acid (RA), which are caffeic acid derivatives.

In this study, only RA and SEN were selected as referral markers because of their ability and importance. RA is one of the major phenolic acids that has been reported to possess biological activities such as antibacterial, anti-viral and antioxidant activities (Parnham & Kesselring, 1985) with International Union of Pure and Applied Chemistry (IUPAC) name of 3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl] oxypropanoic acid. Other than that, the antioxidant activity of RA has been reported to be better than Trolox. Trolox is a water-soluble derivative of Vitamin E with an antioxidant activity that is similar to vitamin E, chemically known as 6- hydroxyl- 2, 5, 7, 8- tetramethylchroman- 2- carboxylic acid (Lu & Foo, 2002). In addition, rosmarinic acid has also been claimed as an anti-pyretic agent and has the ability to treat fever similar to paracetamol (Yam *et al.,* 2008).

On the other hand, SEN, with IUPAC name of 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxychromen-4-one, is a flavonoid which belongs to a group of natural substances with variable phenolic structure that is present in vegetables and plant-derived products such as from fruit, grains, bark, roots, stems, and flowers (Middleton & Kandaswami, 1998). Flavonoids are potent antioxidant, free radical scavengers and metal chelators that inhibit lipid peroxidation. SEN present in *O. stamineus* leaves is unique because of a methoxy group at C-5, a rare structural feature in flavonoids (Akowuah *et al.,* 2004). It is also reported that SEN is an important flavonoid having an antioxidant property with high chemosentisising effects, and is used to synthesize multi-drug resistance (MDR) cell for anti-cancer drugs (Choi *et al.,* 2002). The skeletal structures of RA and SEN are shown in Fig.1.

Optimization can be referred as an improvement of performance of a system, process or product to obtain the optimum benefit from it (Araujo *et al.,* 1996). Traditionally, optimization in analytical chemistry was carried out using one-variable-at-a-time method. This method is carried out by changing one parameter while other parameters are kept at constant level (Bezerra *et al.,* 2008). The major disadvantage of this method is that it does not include the interactive effects among the variables studied. Another disadvantage is the increase in number of experiments

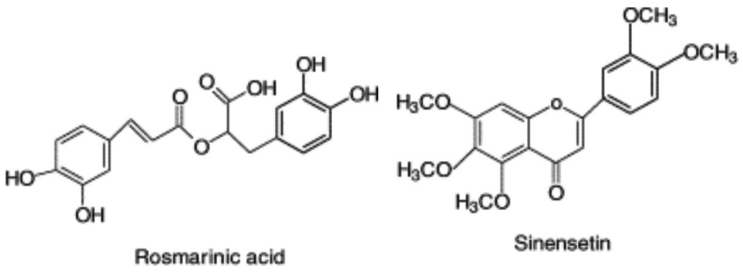

Fig.1: Chemical skeletal structure of RA and SEN (Akowuah *et al.,* 2004)

that are necessary to conduct the research, leading to increased time and expenses required, as well as consumption of reagents and materials (Bezerra *et al.,* 2008).

There are generally four stages involved in optimisation using RSM (Bas & Boyaci, 2007). The first stage is the determination of independent variables with major effects on the system or process. The selection of the independent variables is based on the literature studied. The independent variables are usually selected to cause significant effects on Solid Liquid Extraction (SLE) such as extraction temperature, ratio of solvent to solid, and extraction duration. The second stage is selecting the experimental design according to the selected mathematical matrix. This is followed by selecting of a model as a function of independent variables and the statistical analysis evaluation of the model. Lastly, the response surface plot is used to determine the optimum points.

Yield of extract material, concentration of referral markers and antioxidant activity were strongly dependent on extraction condition. Thus, this study was aimed to investigate the effect of extraction parameters on extraction yield, antioxidant activity, as well as the referral markers of *O. stamineus* leaves. Currently, the information on the optimum extraction of *O. stamineus* by using solvent liquid extraction (SLE) is still limited. Thus, this study will provide a good source of information for developing a new nutraceutical product based on *O. stamineus* leaves in the form of capsule and tablet.

MATERIALS AND METHODS

Materials

O. stamineus leaves were bought from a local supplier in Pulau Pinang. The leaves were kept in Raw Material Storage Room, Herbal Technology Centre, Forest Research Institute Malaysia (FRIM). Then, the samples were ground using a sieve to reduce its particle size.

Optimization of the Extraction Parameter of O. stamineus *Leaves*

The three parameters involved in this study were different extraction temperatures (40°C, 60°C and 80°C), extraction duration (4 – 8 hours) and ratio of solvent to solid (10 – 30 L/kg). The extraction was carried out using water bath (Memmert WNB

45, Germany) and water as the extraction solvent. The extraction processes were conducted in triplicate.

The extracted materials were filtered using filter paper (Whatman No. 1) and stored in a biomedical freezer at -20°C until further use. The samples were frozen dried for four days to remove the water. Yield of the extract material was calculated using the following equation (Pin *et al.*, 2010):

$$Yield\,(Wt\%) = \frac{W_d}{V_e} \times R_{SS} \times 100 \qquad (1)$$

Where, W_d is weight of the dried extract (g), V_e is volume of the aqueous extract used for freeze-drying (ml) and R_{ss} is the solvent to solid ratio (L/kg).

Determination of RA and SEN by Using HPLC

After the freeze-dry process, the sample (10 mg) was diluted with 1 ml of water and sonicated using a sonicator (Hwashin Power Sonic Model 405, Korea) for 10 minutes. Then, the sample was filtered using a syringe filter (Whatman 0.45µm PVDF) prior to injection into HPLC to determine RA and SEN.

The HPLC system consists of Waters 600 System Controller, Waters 2996 Ultra-violet (UV) detector and equipped with Waters 717 Autosampler. Waters 2996 UV detector detects chemical compounds that pass through HPLC column and sends the data to the computer for analysis. Column oven was used to maintain the temperature of column during the analysis.

A Phenomenex Luna C18 100A column (250 mm x 4.6 mm, 5 µm particle size, USA) was used as the stationary phase. The mobile phase was in gradient mode and comprised of 0.1% Orthophosphoric acid, H_3PO_4 and 100% HPLC grade methanol. The mobile phase combinations were selected through optimisation for better separation of compounds and shorter time. The detection wavelength chosen was 330.0 nm because the detection of RA and SEN was sensitive at that particular wavelength (Akowuah *et al*., 2004). The experiments were conducted in triplicate and the results are presented in ppm (mg/L).

Based on the yield and HPLC analysis, prototype extract was developed using designated optimum extraction parameter, and their antioxidant activity was evaluated using Xanthine Oxidase Superoxide Scavenging Assay (SOD) and 1,2-diphenyl-2-picrylhydrazyl (DPPH) assay.

Determination of Antioxidant Activity by using Xanthine Oxidase Superoxide Scavenging Assay (SOD) Method

Stock solutions of the test samples at the concentration of 50 mg/ml were prepared by dissolving the extracts in ethanol. The reaction mixture was prepared by dissolving 0.53 g Na_2CO_3 (pH 10.2), 4.0 mg Ethylene Diamine Tetra Acetic acid (EDTA) and 2.0 mg xanthine in 0.025 mM Nitro Blue Tetrazolium (NBT) solution (100 ml of 4.1 mM/L), and adding 3.15 g Tris HCL, 0.1 g $MgCl_2$, 15.0 mg 5-bromo-4-chloro-3-indolyl phosphate and 34.0 mg 4- nitro blue tetrazolium chloride in 100 ml of distilled water. The mixture was kept refrigerated at 4°C.

The stock solution in 5 µl was mixed with 995 µl of the reaction mixture in a microcuvette. The microcuvette was placed in a cell holder of a spectrophotometer (Lambda 2S, Perkin Elmer, USA) and the reading was set to zero. The reaction was then initiated by the addition of 0.1 µl of XOD ($1x10^{-3}$ U/ml). The absorbance of the resulting mixture was measured at 560 nm for two minutes.

The absorbance of the negative control was obtained by replacing the stock solution with 5 µl of the reaction mixture. SOD was used as a positive control in this assay. The percentage of inhibition was calculated as:

$$\%\ inhibition = \frac{Ab_c - Ab_s}{Ab_c} \times 100 \qquad (2)$$

Where Ab_c and Ab_s are absorbance of the control and samples, respectively.

Determination of Antioxidant Activity by Using 1,2-diphenyl-2-picrylhydrazyl (DPPH) Method

The stock solutions of the test samples were prepared in methanol at a concentration of 0.5 mg/ml. The reaction mixture, which consisted of 4.0 ml of test solution and 1.0 ml of DPPH, was kept in a 5 ml screw cap bottle. The mixture was shaken and left at room temperature for 3 min. The absorbance of the resulting mixture was measured at 520 nm using a spectrophotometer (Lambda 2S, Perkin Elmer, USA). The absorbance of the negative control and positive control was obtained by replacing the test solution with MeOH and ascorbic acid (Vitamin C), respectively. All the reactions were performed in triplicates. The percentage of inhibition was calculated as:

$$\%\ inhibition = \frac{Ab_{c,-ve} - Ab_s}{Ab_{c,-ve} - Ab_{c,+ve}} \times 100 \qquad (3)$$

Where Ab_s is absorbance of the test samples, while $Ab_{c,\text{-ve}}$ and $Ab_{c,+ve}$ are absorbance of the negative and positive control, respectively.

Experimental Design

The extraction parameters were optimized using response surface methodology (RSM). A central composite design (CCD) was employed in this regard. Ratio of solvent to solid (X_1), and extraction duration (X_2) were chosen for the independent variables. Three experiments were carried out for each experimental design point and the mean values were stated as observed responses. The mathematical modelling model corresponding to the composite design is:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{12}X_1X_2 + B_{11}X_1^2B_{22}X_2^2 \qquad (4)$$

where Y is the predicted response variable, B_o is the regression coefficient of intercept term, B_1 and B_2 are linear regression coefficients, B_{11} and B_{22} are squared regression coefficients, and B_{12} is the interaction regression coefficient.

The analysis of variance (ANOVA) was used to evaluate the significance of each variable on the resulted model. The significance was determined statistically by computing the F-value and p-value ($p < 0.05$). The adequacy of the polynomial

model to predict the experiment was determined with correlation coefficient, R^2. The experimental design using CCD is shown in Table 1.

TABLE 1
Central-composite experimental design of the independent variables

Experiment	Independent variables	
	x_1	x_2
1	10.00	4.00
2	30.00	4.00
3	10.00	8.00
4	30.00	8.00
5	10.00	6.00
6	34.14	6.00
7	20.00	3.17
8	20.00	8.83
9	20.00	6.00

RESULTS AND DISCUSSION

Effects of Extraction Temperature

Fig.2 shows the HPLC quantification analysis, whereby the results indicate that the concentration of RA increased from 40 ppm to 140 ppm as the temperature increased from 40°C to 80°C. This could explain that RA is a thermal-stable polyphenol that can withstand a high temperature (80°C). On the other hand, Fig.3 shows the contradicted results for SEN, whereby its concentration started to decrease from 0.6 ppm to 0.3 ppm as the temperature increased from 60°C to 80°C. Fig.4, Fig.5 and Fig.6 represent the HPLC chromatogram for RA and SEN at the temperatures of 40°C, 60°C and 80°C, respectively. It can be deduced that concentration of RA was highest at 80°C but SEN started to denature at the same temperature. Meanwhile, the concentration of RA was higher compared to SEN because RA has four hydroxyl and a carboxylic group, which could effectively form hydrogen-bonding with the water, while SEN has no hydroxyl group at all. This enhances the solubility of RA in the water and results in a higher recovery in the extract compared to SEN.

Based on the figures, the most suitable extraction temperature of *O. stamineus* leaves in this study was 60°C. Akowuah and Zhari (2010) reported that the recovery of SEN from *O. stamineus* leaves extract decreased with the increasing extraction temperature above 60°C because of degradation. A similar finding was also reported; increasing the temperature of the extraction would increase the concentration of RA but decreased the concentration of SEN (Sriyana *et al.,* 2011). Silva *et al.* (2007) reported that temperature was also the most important parameter in extraction of *Inga edulis* leaves because higher temperature increased the solubility and diffusion coefficient of the solute, allowing higher yield and extraction rate. This principle is also applicable in the extraction of *O. stamineus* leaves.

Effects of Solvent to Solid Ratio on Extraction Yield

In order to select the optimum ratio of solvent to solid (X_1) and extraction duration (X_2) towards yield (Y), the quadratic model was solved using RSM and given as:

$$Y = 29.66 + 1.02X_1 + 3.86X_2 + 2.59X_1X_2 + 1.02X_1^2 + 1.61X_2^2 \quad (5)$$

TABLE 2
Turkey test for extraction time and extraction yield

Test	Time	Group a	Group b	Group c
Turkey	3 hours 30 minutes	26.94 Wt %		
	4 hours		29.67Wt%	
	6 hours			31.83Wt%
	8 hours			31.90 Wt%
	8 hours 30 minutes			39.36Wt%

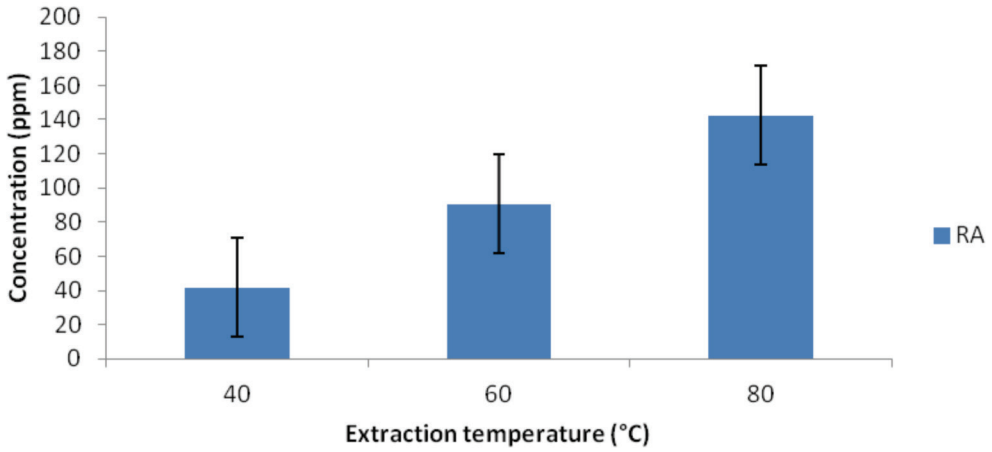

Fig.2: Concentration of RA at different extraction temperatures

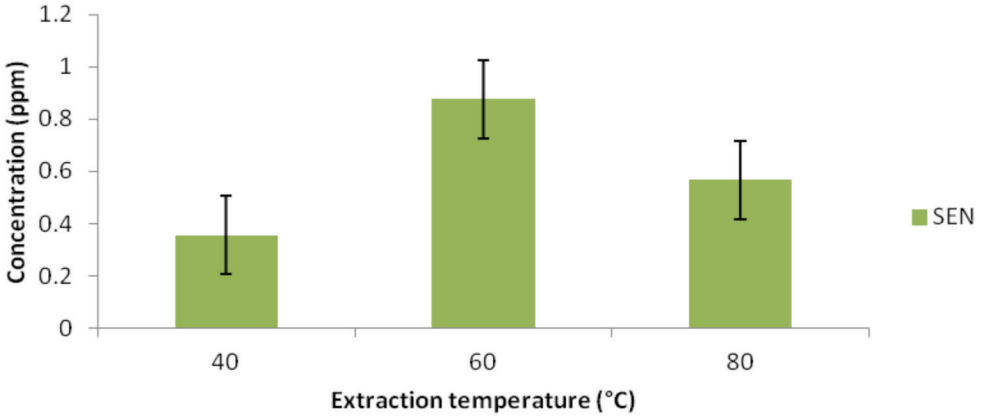

Fig.3: Concentration of SEN at different extraction temperatures

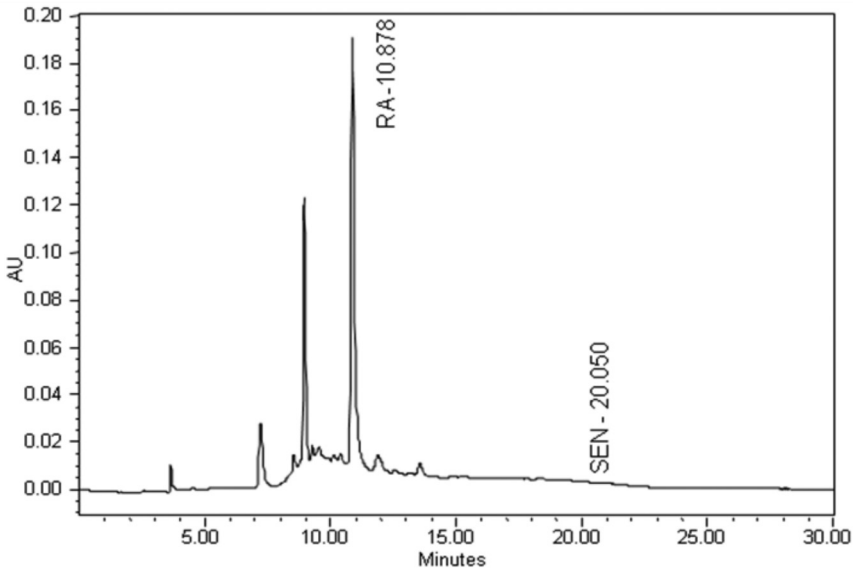

Fig.4: HPLC chromatogram for SLE of *O. stamineus* at 40°C

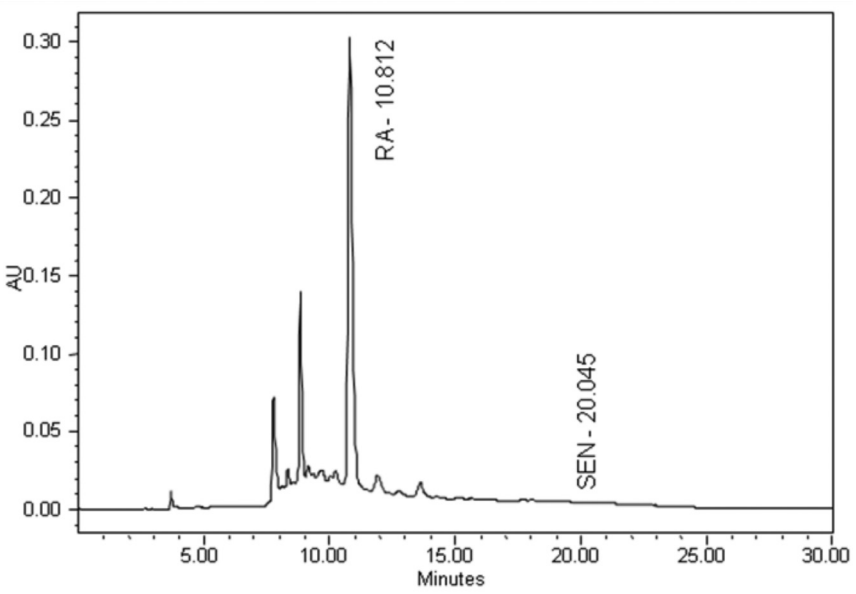

Fig.5: HPLC chromatogram for SLE of *O. stamineus* at 60°C

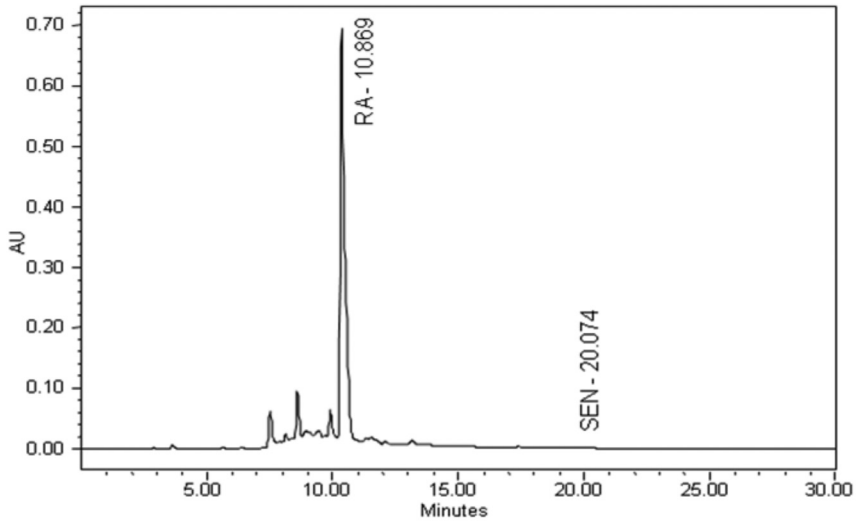

Fig.6: HPLC chromatogram for SLE of *O. stamineus* at 80°C

The model gave a satisfactory fit with the experimental data because R^2 value is 0.9725. This means that the model could explain about 97% of the total variability within the studied range.

Fig.7 shows the three-dimensional response surface graph plotted to illustrate individual and interactive effects of the independent variables on the yield of *O. stamineus* leaves extract. Based on the graph, the extraction yield was found to increase with the increase in extraction duration and ratio of solvent to solid.

Optimization Process

From the RSM analysis, the maximum extraction yield can be obtained by solving the first derivatives of Equation 4, as follows:

$$\frac{\partial Y}{\partial X_1} = 1.02 + 2.59X_2 + 2.04X_1 = 0 \quad (6)$$

$$\frac{\partial Y}{\partial X_2} = 3.86 + 2.59X_1 + 3.22X_2 = 0 \quad (7)$$

The solution of the above equations led to the maximum yield of 39.36 Wt%, with ratio of solvent to solid of 30:1 (ml:g) and the extraction duration of 8 hours and 30 minutes. However, using the SPSS analysis from Table 2, there are no significant differences in terms of the extraction yield between 6 and 8 hours extraction duration. Hence, six hours is selected as the extraction duration for *O. stamineus* leaves. The use of excessive time would lead to the increase of cost for operation. Yin *et al.* (2010) also reported that although the concentration of total phenolic content was highest at 120 min, due to economic aspect, 80 min was selected as the best extraction duration for *Morinda citrifolia.* Besides, after 8 hours of extraction duration, the amounts of RA and SEN decreased, as shown in Table 3.

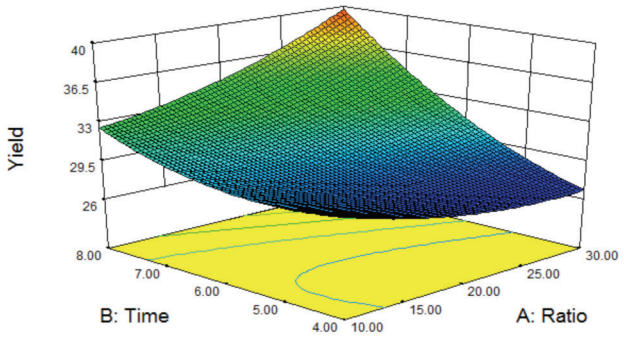

Fig.7: A 3-Dimensional response surface graph between ratio and time towards yield

TABLE 3
Concentration of RA and SEN for different extraction time

Parameter	Yield (Wt%)	RA (ppm)	SEN (ppm)
Time = 8 hours 30 min	39.36	48.67	0.66
Time = 6 hours	30.49	67.09	1.43

Based on those considerations, the optimum extraction conditions for *O. stamineus* leaves are as follows: 60°C of extraction temperature, 6 hours of extraction duration and 30 L/kg ratio of solvent to solid. Although the yield was decreased by 9% (Table 3), both RA and SEN were maintained at that condition. The concentrations of RA and SEN were determined to be 67 mg/L and 1 mg/L, respectively.

Antioxidant activity

Based on the optimised parameter obtained from this study, an antioxidant activity was evaluated and the results are tabulated in Table 4. The results showed a high antioxidant activity (above 70% inhibition) for both SOD and DPPH assays. Akowuah *et al*. (2004) also reported that 84.2% of antioxidant activity was evaluated by using DPPH assays. Thus, this study has provided preliminary data for prototype extracts towards the development of nutraceutical product based on *O. stamineus.*

TABLE 4
Antioxidant activity of prototype extract of *O. stamineus* leaves

No	Sample	% inhibition	
		SOD	DPPH
1	OS R1	96.44	89.97
2	OS R2	96.71	92.20
3	OS R3	96.54	92.37

CONCLUSION

Based on the findings of this present study, it was concluded that when the extraction temperature increased, the yield of RA also increased. However, it will decrease the yield of SEN. In order to to maintain both at the optimum value, 60 °C was selected as suitable temperature.

The best extraction duration was found to be at 6 hours since at 8 hours and above of extraction duration, both RA and SEN were denatured.

The optimum ratio of solvent to solid was selected to be 30 L/kg since the results have shown high values of extraction yield and concentration of RA and SEN at that ratio.

By using this optimum parameters, the concentration of RA and SEN were yield about 67 mg/L and 1 mg/L, respectively, with 31Wt% yield extract.

The antioxidant activity of SOD and DPPH was shown as 97% and 92% inhibition, respectively. Finally, the results from this study can be used to formulate a new nutraceutical product of *O. stamineus* in the form of capsule and tablet.

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Garlic: An Effective Functional Food to Combat the Growing Antimicrobial Resistance

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ABSTRACT

Emerging multidrug resistant bacterial infections are burning public health concerns worldwide. There is an urgent need to explore alternative antimicrobial agents for effective management of bacterial infections. Garlic (*Allium sativum*) has been traditionally used for the treatment of different diseases since ancient times. The present study aims to evaluate the antimicrobial activity of aqueous extract *of Allium sativum* against multidrug resistant clinical isolates of pathogenic bacteria found in human urine in cases of urinary tract infection (UTI). A total of 166 uropathogens were evaluated for antibiotic susceptibility, 56% clinical isolates were found to have high degree of resistance with multiple antibiotic resistance (MAR) index >0.5. About 82% bacterial isolates with MAR index >0.5 were found to be susceptible to crude aqueous garlic extract. Thus, aqueous garlic extract (AGE) was found to possess effective anti-bacterial activity against multidrug resistant clinical pathogens and may be tested further as a natural dietary component to manage drug resistance bacteraemia. Therefore, alternate medicine practices with natural plant extract including garlic may be of great importance in combating public health challenges like UTI.

Keywords: Garlic, Multidrug resistant, antibacterial activity, *Allium sativum*, aqueous garlic extract, urinary tract infections.

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INTRODUCTION

Urinary Tract Infection (UTI) is one of the most common extra intestinal bacterial infections and the second most common infectious disease encountered in community practice. UTI alone poses a serious health problem affecting millions of people each year with total cost for treatment being in

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billions of dollars. Worldwide, about 150 million people are diagnosed with UTI each year (Gupta & Stamm, 2001). Antibiotics have revolutionized medicine in many respects; their discovery was a turning point in medicinal history. Regrettably, the use of these wonder drugs has been accompanied by the rapid appearance of resistant strains (Liu & Pop, 2009). As has been reported earlier on, major UTI causing isolates were identified as *Escherichia coli, Enterobacter* sp., *Klebsiella* sp., *Pseudomonas aeruginosa,* and *Staphylococcus aureus* (Kapur *et al*., 2013). In recent years, several reports from the scientific community have raised concerns that antibacterial drug development at its current pace will not adequately address the problems posed by antibiotic resistance among important bacterial pathogens leading to diseases (Manjunath *et al*., 2011). Emerging antimicrobial resistance compels once again to look back into traditional medicines or herbal products, which may provide appropriate/acceptable alternative solutions. Plants derived products have made large contributions to human health and wellbeing (Karuppiah & Rajaram, 2012). Traditional medicine, if used appropriately shows higher therapeutic efficacy with fewer side effects and cost effective. Hence, there is an imperative need to make judicious use of natural bioactive substances with established safety index to tackle increasing AMR.

Garlic is one of the edible plants which has generated a lot of interest throughout human history as a medicinal cure/remedy for many diseases. Ancient medical texts from Egypt, Greece, Rome, China and India each describe medical applications of garlic. Garlic is one of the earliest documented examples of a plant employed for treatment of disease and maintenance of health (Rivlin, 2001). A wide range of microorganisms including bacteria, fungi, protozoa and viruses have been shown to be sensitive to garlic preparations (Koch & Lawson, 1996). Allicin and other sulphur compounds are thought to be the major antimicrobial factors in garlic. Garlic is effective against a number of gram-negative, gram-positive and acid-fast bacteria including *Staphylococcus, Salmonella, Vibrio, Mycobacteria, Proteus* species and *Helicobacter pylori* along with antifungal, antiparasitic and antiviral activity (Cellini *et al*., 1996; Ankri & Mirelman, 1999). A recent study from the University of East London has shown that aqueous extracts of allicin when formulated into a simple cream are able to kill vast swathes of the so-called "superbug" MRSA (methicillin resistant *Staphylococcus aureus*) (Nummer *et al*., 2011). The present study aims to evaluate the antimicrobial activity of aqueous extract of *Allium sativum* against multi drug resistant clinical isolates of pathogenic bacteria found in human urine with the hope to develop novel treatment regimen for drug resistant UTI.

MATERIALS AND METHODS

Source of Bacterial Test Isolates and Antibiotic Sensitivity Testing

The test organisms were bacterial isolates from the urine samples submitted by patients

having suspected urinary tract infections. Urine samples from 166 patients who had UTI confirmed by positive urine culture reports were used for this study. The cultures were maintained in the laboratory. The antibiotic susceptibility patterns of the test organisms were performed as per standard Kirby–Bauer disc diffusion assay (Bauer *et al*., 1966). All antibiotic discs (Ampicillin 10µg; Gentamicin 30µg; Cefuroxime 30µg; Kanamycin 30µg; Amikacin 30µg; Ciprofloxacin 5µg and Penicillin 2 units) and media used were obtained from Himedia Labs, India. Results were interpreted based on the diameter of the observed zone of inhibition. Following the Clinical and Laboratory Standards Institute Guidelines, the obtained results were categorized into three groups: Sensitive (S); Intermediate (I); Resistant (R) (CLSI, 2006).

Multiple Antibiotic Resistances (MAR) Index of Test Isolates

The antibiotic susceptibility patterns obtained from the standard Kirby–Bauer disc diffusion procedure was used for calculating the MAR index. The MAR index is the ratio of number of antibiotics ineffective against the organisms to the total number of antibiotics exposed (Krumperman, 1983). A MAR value near to 1 indicates that all tested antibiotics are ineffective. The clinical isolates which had MAR>0.5 were used to test the antimicrobial effects of fresh aqueous garlic extract in the liquid form.

Preparations of the Fresh Aqueous Garlic Extract (AGE)

Fresh bulbs of *Allium sativum* were purchased from local vegetable shop in Hyderabad. Individual cloves were separated. Fifty grams of the garlic cloves (weighed after peeling the outer covering) were surface sterilized with 70% ethanol and rinsed thoroughly with sterile water, for 4-5 times. The cloves were sliced, crushed using mortar and pestle and suspended in 50ml of sterile distilled water. The extract was further homogenized using a Waring blender at 2000 rpm for 5mins at room temperature. The froth was allowed to settle down for 30 min at room temperature before filtering through a single layer of muslin cloth and later through pre sterilized Whatman filter paper No. 1. The filtrate was filter sterilized using 0.45µm filter (Millipore, India). The filtrate was collected in sterile tubes. This 1g/ml fresh AGE was stored at 4^0C till further use.

Determination of Minimum Inhibitory Concentration (MIC) of the Fresh Aqueous Garlic Extracts (AGE) Using Macro Broth Dilution Method

The minimum inhibitory concentration (MIC) of the fresh aqueous garlic extract was determined by the broth dilution method. Different concentrations of fresh aqueous garlic extract were prepared ranging from 70mg/ml to 10mg/ml from stock of 1gm/ml in LB broth. The final volume in each tube was made to 5ml by adding LB broth.

Then, 0.1 ml of fresh microbial culture (10^7 cells) was inoculated in all the tubes. For positive control, no fresh aqueous garlic extract (AGE) was added and for negative control only LB broth was taken without garlic extract and bacterial cells.

The tubes were kept for overnight shaking at 120rpm at 37^0C. The Optical Density at 600nm (OD_{600}) was recorded for all the tubes. Bacterial growth kinetics profile was produced with respect to varying concentrations of fresh aqueous garlic extract. The tube which contained the least concentration of fresh AGE and whose OD_{600} value was equivalent to the blank i.e. only LB broth was considered as the Minimum Inhibitory concentration (MIC) of the fresh garlic extract in mg/ml against bacteria after seven days of preparation of fresh aqueous garlic extract.

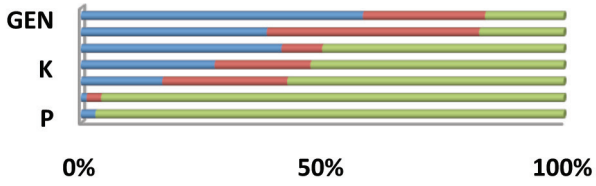
Testing the Bio Efficacy of the Aqueous Garlic Extract (AGE)

Bacterial strains (N=93) which showed resistance to 4 or more antibiotics out of 7 (MAR>0.5) were tested against AGE for growth inhibition. The bacterial strains were inoculated in 1ml LB broth and grown overnight at 37^0C with continuous shaking. The next day before performing the antimicrobial assay, 50µl of overnight grown culture was aseptically transferred in fresh 5ml LB broth (pH 7.2) and placed in shaking incubator at 37^0C for 3-4 hours. Thick LB agar plates were prepared by pouring 30ml of LB agar into sterile petri-dishes. Then, 0.1ml of the fresh bacterial broth culture was spread over the solidified LB agar plates using L-shaped glass spreader inside the laminar air flow chamber. The plates were incubated for 5 min at room temperature for the attachment of bacterial strains over the LB agar surface.

Agar well diffusion technique was used to test anti microbial activity of AGE. Wells (5 mm diameter) were made in the inoculated LB agar plates by using a sterile cork borer. Fresh AGE was (0.7mg /well) dispensed into the wells. The plates were initially kept at room temperature for 1 hour for the diffusion of the extracts in agar and later kept in bacterial growth incubator at 37^0C in an up-side down position. The zones of inhibition were measured after 12-16 hours.

RESULTS

The MAR index value calculated for the test organisms showed that 56% (93 of 166 isolates) of uropathogens had MAR index higher than 0.5, i.e. resistant to four or more antibiotics out of seven antimicrobial agents tested and 24% isolates had MAR $\geq$ 0.7, that is, they were resistant $\geq$ 5 antibiotics. The results also showed a very high incidence of resistance among uropathogens against penicillin and ampicillin, with 97% and 96%, respectively (see Fig.1). About 50% of bacterial isolates were resistant to even third generation antibiotics such as ciprofloxacin. Gentamicin was found to be the most effective drug against all common uropathogens in the studied cohort with only 16% (27/166) of clinical isolates being resistant to it. The next effective antibiotic was amikacin (17% resistance).



	P	A	CXM	K	CIP	AK	GEN
S	3.01	1.2	16.86	27.7	41.56	38.5	58.4
I	0	3.01	25.9	19.87	8.43	43.97	25.3
R	96.98	95.78	57.23	52.41	50	17.47	16.26

Fig.1: Resistance and susceptibility profile of uropathogens to antimicrobial agents (N=166; values expressed in percentage) [P=Penicillin, A=Ampicillin, CXM=Cefuroxime, K=Kanamicin CIP=Ciprofloxacin, AK= Amikacin, GEN=Gentamicin]

In the present investigation, antibacterial activity of AGE was tested for 93 isolates having MAR index > 0.5 and it was observed that 82% (76 out of 93) of the multi drug resistant isolates showed a zone of inhibition larger than 10mm, proving the high degree of growth inhibitory activity of AGE against MDR uropathogens (Table 1). Among the 76 clinical pathogens *S. aureus* and *E. coli* were most susceptible followed by *Enterobacter* sp. and *Klebsiella* sp. AGE showed the highest diameter of zone of inhibition of 28 mm against *E. coli* followed by *S. aureus* (26 mm).

TABLE 1
Range of zone of inhibition with fresh AGE for various groups of uropathogens

Clinical isolate	No. of isolates	ZOI
E. coli	35	17-28 mm
Enterobacter sp	19	13-25 mm
Klebsiella sp	11	14-24 mm
S. aureus	6	16-26mm
P. aeruginosa	5	13-22 mm

Minimum Inhibitory concentration (MIC) of fresh aqueous garlic extract was found to be 35mg/ml where fresh AGE resulted in effective inhibition of bacterial growth, as shown in Table 2 and Fig.2.

TABLE 2
Absorbance at 600nm of varying concentrations of fresh AGE for MIC determination

Tube No	AGE Concentartion mg/ml)	OD_{600}
1	70	0.02
2	50	0.031
3	35	0.033
4	25	0.781
5	15	1.084
6	10	1.102
7	Positive control	1.105
8	Negative control	0.017

One of the representative clinical isolate of *Staphylococcus aureus*, isolated from the infected urine sample and found to be resistant to ampicillin, penicillin, kanamycin and cefuroxime, was tested for

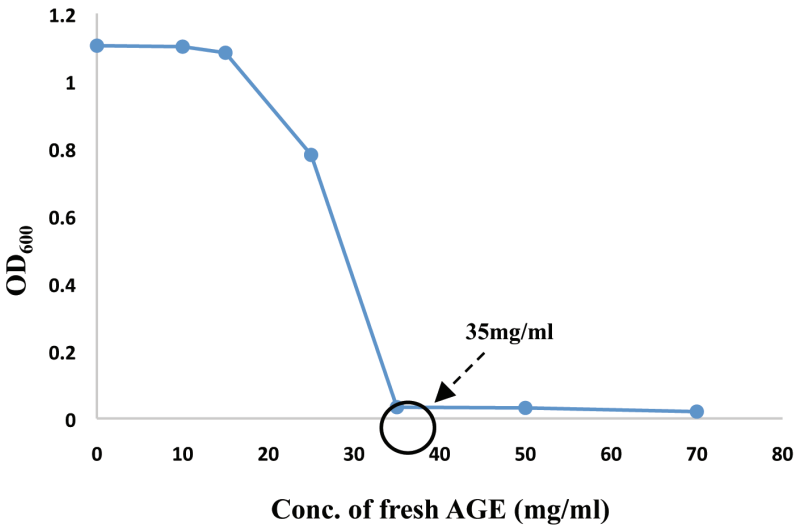


Fig.2: Absorbance at 600nm and concentration of fresh aqueous garlic extract, AGE (mg/ml) after 12 hour of incubation

the antimicrobial effect of AGE using water as the negative control and gentamicin as the positive control. AGE was found to be as effective as gentamicin (see Fig.3).

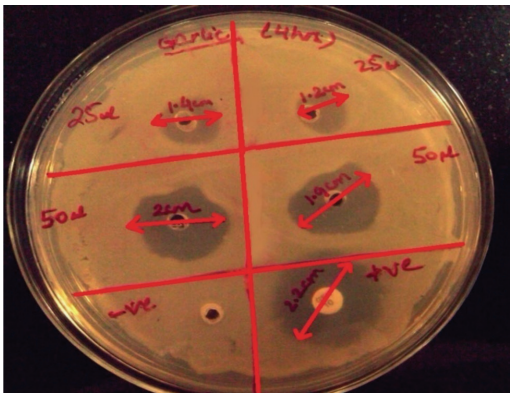


Fig.3: Zone of inhibition obtained with aqueous garlic extract against *Staphylococcus aureus* [+ve control is gentamicin and –ve control is water]

This provides a strong evidence for the antibacterial potential of fresh AGE in case of multi drug resistant infections, where most of the available antibiotics are not effective. AGE depicts the antimicrobial efficacy equivalent to one or the other antibiotics (generally prescribed in UTI cases) in 76 multidrug resistant uropathogens having MAR index > 0.5 (Table 3).

TABLE 3
Comparing the efficacy of AGE with other antibiotics used for uropathogens

Range of ZOI (mm)	No. of isolates	Equivalent effective antibiotic
11-15	25	Gentamicin, Tobramicin, Levofloxacin
16-20	44	Amikacin, Naladixic acid, Trimethoprim, Ofloxacin
>20	07	Cotrimoxazole, Cefturoxime, Piperacillin

DISCUSSION

Garlic has been studied for its antibacterial activity against a wide range of bacteria

but limited data is available for its efficacy in case of infectious diseases like UTI. The results of this work provide evidence that the fresh aqueous garlic extract possesses significant antibacterial activity. Similar results were obtained by Sharma *et al* (2009) in India (Sharma & Patel, 2009), where they showed a strong antimicrobial activity of *Allium sativum* against *Vibrio cholera*. The results of the present study support the use of natural products as affordable effective substitutes and/or additives for reducing the disease burden and overall cost of disease management. Garlic has recognized traditional medicinal applications (Ross *et al*., 2001); similar to other organosulphur and phenolic compounds have been reported to be involved in the antimicrobial activity (Raja *et al*., 2011; Johnson *et al*., 2011; Nweze *et al*., 2012; Aboaba & Efuwape, 2001). Several studies have reported *in vitro* evidence of the antimicrobial activity of fresh and freeze dried garlic extracts against many bacteria (Rees *et al*., 1993), fungi and viruses (Weber *et al*., 1992). However, ours is the first study to report the antibacterial activity of AGE against MDR bacterial isolates from the infected urine samples leading to UTI.

It is interesting to note that even crude extracts of this plant showed good activity against multidrug resistant strains where antibiotic therapy had limited or no effect. This provides hope for developing alternative drugs which may be of help in fighting the menace of growing antibacterial resistance. To conclude, there is evidence that garlic has potential in the treatment of UTI and may be other microbial infections. Considering the morbidity rate and economic burden of infectious diseases like UTI, use of garlic in an appropriate form might contribute substantially to public health. However, it is necessary to determine the bio availability, side effects and pharmaco-kinetic properties in more details.

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Comparison of Protein Extraction Protocols for Proteomic Analysis of Red Algae, *Eucheuma cottonii*

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ABSTRACT

Eucheuma cottonii is a red seaweed of the family *Rhodophyceae*, which is mainly harvested in South East Asia. *E. cottonii* has long been explored as a major source of kappa carrageenan, which is of great economic and industrial importance. The proteomics of marine macroalgae is always a tough task due to low protein concentration and a greater possibility for the co-extraction of contaminants such as anionic polysaccharides, polyphenols and salts, which may massively deteriorate the resolving power of SDS-PAGE and current proteomic tools. The main objective of this study is to compare three different phenol based protein extraction protocols: (i) Phenol/lysis buffer extraction, (ii) TRI reagent/ chloroform extraction, and (iii) Phenol/SDS buffer extraction in terms of total protein yields and resolving patterns of single dimension SDS-PAGE. Among the three phenol based extraction protocols, the phenol/lysis buffer produced slightly higher protein yields (0.027±0.000 mg/g), followed by the phenol/SDS buffer (0.024±0.002 mg/g) and the TRI reagent/chloroform (0.018±0.001 mg/g). In SDS-PAGE, all the three extraction protocols showed clear protein profiles, with several intense protein bands observed from 27 kDa to 158 kDa. The phenol/SDS buffer extraction protocol was recommended for proteome study of *E. cottonii* as it is the cheapest and relatively easy in preparation as compared to the other two protocols in this study. This study represents an initial attempt to study the proteome of *E. cottonii*. Further proteomic works, such as two-dimensional gel electrophoresis, mass spectrometry and protein crystallography, can be carried out in the near future. A successful establishment of *E. cottonii* proteome is important to facilitate the exploration of *E. cottonii* and other carrageenan rich seaweed species due to its agricultural significance.

Keywords: Eucheuma cottonii, 1DE, Macroalgae proteomics, Phenol based protein extraction

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INTRODUCTION

Eucheuma cottonii is an eye-catching element of the marine flora in Southeast Asia. It is abundantly cultivated as a commercial source of carrageenan (Bixler, 1996). Carrageenans are widely used in the food and pharmaceutical industries due to their precious value as stabilizers, viscosity modifiers and gelling agents. *E. cottonii* is a good source of kappa-carrageenans, which can form strong gels in the presence of potassium ions and are widely employed as a suspending, thickening and gelling agent in certain food such as chocolate, milk, ice cream, convenience foods, puddings, etc. Pharmaceutical applications of kappa-carrageenan include suspension of barium sulfate and antibiotics (Parker, 1974). It also contains high amounts of dietary fibres, minerals, vitamins, antioxidants, polyphenols, phytochemicals, proteins and polyunsaturated fatty acids (Matanjun *et al.*, 2009).

E. cottonii belongs to the genus that is known in commerce as "*cottonii*'. The term "*cottonii*" refers to the seaweed that produces kappa carrageenan (Santos, 1989). *Eucheuma* species are hardy, multibranched, non-rooting algae which grow attached by holdfasts to dead coral. *Eucheuma* species reproduce both vegetatively and by sporulating. They can be sectioned anywhere and re-growth will occur in all sections (Parker, 1974). This feature makes *Eucheuma* species adaptable to different environments in various forms. Due to morphological plasticity and lack of defined morphological characteristics, the taxonomic classification of the *Eucheuma* species is confused with the *Kappaphycus* species, with the expansion of commercial cultivation that triggered the misapplication of commercial and scientific names. Until very recently, Liu *et al*. (2013) showed that the 18S rDNA sequence can separate *Kappaphycus* from *Eucheuma* species. Nevertheless, further identification of *Eucheuma* and *Kappaphycus* at the inter-species level is still unclear.

Genomics concerns the generation and analyses of nucleotide sequences of the full or near-full genome, as well as cDNA collections. One step beyond the raw sequence data which is provided by genomics, transcriptomics, offers a better view of the active components in the cell by providing information on the presence and relative abundance of RNA transcripts. Proteomics is complementary to genomics and transcriptomics as it provides additional information on protein expression, protein–protein interaction, organization of proteins in multi-protein complexes and their localisation in tissues (Jamers *et al*., 2009).

The earlier proteome studies mostly revolved around the microbial and animal world. The proteomics of the kingdom Plantae is relatively rare. This is particularly true for the proteome study of marine algae, which is presumably due to the difficulty in the extraction of algae proteins. The cell walls of algae, especially those of multicellular macroalgae, are often heavily impregnated with polysaccharides such as cellulose, pectin, mannan, glucomannan, xyloglucan, carrageenan, etc. (Popper *et*

al., 2011). This makes protein extraction of macroalgae extremely tough, as co-extraction of polysaccharides may result in a smearing effect that deteriorates the resolution of gel analysis. Furthermore, due to the inherent endogenous proteolytic activity of macroalgae, the protein yields of macroalgae are often very low, resulting in a poor resolution gel image and insufficient protein samples for subsequent analysis.

In any proteome study, the most critical step is sample preparation and extraction. As mentioned above, proteomics analysis of algae tissues is typically more problematic and troublesome than of other biological systems. In this regard, several protocols have been developed for protein extraction from macroalgae, which included urea extraction (Contreras *et al*., 2008), Tris-buffer extraction, TCA/acetone extraction and phenol extraction (Wong *et al*., 2006; Contreras *et al*., 2008; Nagai *et al*., 2008). Among these, phenol extraction proved to be the most efficient protocol for protein extraction from algae tissues (Wong *et al*., 2006; Contreras *et al*., 2008; Nagai *et al*., 2008).

In the present study, three phenol based extraction protocols; (i) phenol/ lysis buffer extraction, (ii) phenol (TRI reagent)/chloroform extraction, and (iii) phenol/dense SDS extraction were utilised for protein extraction from *E. cottonii*. The aim of this study was to compare the effectiveness of protein extraction protocols by producing a good quality one dimensional gel electrophoresis (1DE) profile of *E. cottonii* and to enhance protein yields of *E. cottonii*. A high quality gel based protein profile produces a gel image with clear protein bands and without significant smearing. As of this writing, no proteomic work has been carried out on *E. cottonii*. Hence, this study is important in assisting in the choice of a suitable protein extraction protocol for up-scaling the proteomic analysis of *E. cottonii* and other carrageenan-rich seaweed species in the near future.

MATERIALS AND METHODS

Sample Preparation

Seaweed samples (*E. cottonii*) were delivered in dried form from the east coast of Sabah to UCSI University. In order to minimise possible variations in protein contents in different samples, all of the seaweed samples were harvested at the same time from the ocean. Within 48 hours of harvest, all the seaweed samples were air-dried at 35ºC for about three days. The dried *E. cottonii* samples were kept in a cabinet until used. Prior to protein extraction, the seaweed samples were carefully chosen and those with suspected endoparasitic infections and others with unknown defects or infections were thrown. Then, each seaweed sample was rinsed with tap water thrice and with ultrapure water (Merck Millipore) another three times. By using a pestle and a mortar, the seaweed sample (whole plant) was ground into fine powder in liquid nitrogen. Finally, about 5 g of the resulting powder was placed in a 50mL falcon tube for each subsequent extraction.

Protein Extraction Protocols

In this study, a total of three protein extraction protocols; (i) phenol/lysis buffer extraction, (ii) phenol (TRI reagent)/ chloroform extraction and (iii) phenol/ dense SDS extraction, were carried out to compare 1DE protein profiles of *E. cottonii*. Each protein extraction protocol was done in triplicates.

Phenol/lysis Buffer Extraction

This protocol was based on Contreras *et al.* (2008) with some modifications. About 5g of *E. cottonii* powders were suspended in 15 mL of lysis buffer (1.5% w/v polyvinylpyrrolidone [PVP], 0.7 M sucrose, 0.1 M potassium chloride, 0.5 M Tris-HCl pH 7.5, 250 mM EDTA, 20 µL/ mL complete protease inhibitor cocktail, 2% v/v 2-mercaptoethanol and 0.5% w/v CHAPS) and homogenised for 15 minutes. An equal volume of Tris-HCl saturated phenol, pH 7.9, was added, and the mixture was re-homogenised for another 15 minutes. The mixture was centrifuged at 10000 *g* for 15 minutes and the upper phenol phase was transferred into a new 50 mL falcon tube. The lower phase was re-extracted using an equal volume of Tris-HCl saturated phenol, pH7.9. The mixture was centrifuged at 10000 *g* for 15 minutes, and again the upper phenol phase was transferred into the previous 50 mL falcon tube. For protein precipitation, five volumes of 0.1 M methanolic ammonium acetate were added to the phenol phase and the mixture was incubated at -20^{0}C overnight. After that, the mixture was centrifuged at 10000 *g* for 15 minutes, the supernatant was discarded, and the protein pellet was rinsed in 0.1 M methanolic ammonium acetate at -20^{0}C for 20 minutes. The resulting protein pellet was rinsed with 4 volumes of 80% v/v ice-cold acetone and once in cold acetone containing 20 mM DTT.

TRI Reagent / Chloroform Extraction

This protocol was based on Wong *et al.* (2006) and the manufacturer's recommendation with some modifications. About 5 g of seaweed powders was added into 15 mL of TRI reagent. The mixture was homogenised at room temperature for 10 minutes. Three (3) mL of chloroform was added. The mixture was shaken vigorously for 15 seconds and incubated at room temperature for 10 minutes. The mixture was centrifuged at 10000 g for 15 minutes. The upper aqueous phase was discarded. Six (6) mL of 100% ethanol was then added. The mixture was mixed by inversion and incubated at room temperature for 5 minutes. The mixture was centrifuged at 8000 g, 4 ºC for 5 minutes. The pinkish organic layer was transferred into a new 50 mL falcon tube and 22.5 mL of isopropanol was then added for protein precipitation. The organic layer-isopropanol mixture was incubated at room temperature for about 45 minutes. The mixture was centrifuged at 10000 g, 4ºC for 15 minutes. The supernatant was removed and the protein pellet was washed with 25 mL of 95% ethanol containing 0.3 M guanidine hydrochloride and 2.5% v/v glycerol and centrifuged at 10000 g, 4ºC for 2 minutes. The washing process was

repeated twice. Finally, the protein pellet was rinsed once in 25 mL of 100% ethanol.

Phenol/SDS Buffer Extraction

This protocol was based on Nagai *et al*. (2008) with some modifications. About 5 g of seaweed powders was added to 15 mL of SDS buffer (0.1M Tris-HCl buffer, pH8.0, 30% w/v sucrose, 2% w/v SDS and 5% v/v 2-mercaptoethanol). The mixture was homogenised for about 15 minutes. An equal volume of Tris-HCl saturated phenol (pH7.9) was added, and the mixture was re-homogenised for another 15 minutes. The mixture was centrifuged at 1000 g for 15 minutes, and the upper phenol phase was transferred into a new 50 mL falcon tube. The lower phase was re-extracted using an equal volume of Tris-HCl saturated phenol (pH7.9). The mixture was centrifuged at 10000 g for 15 minutes, and once again, the upper phenol phase was transferred into the previous 50 mL falcon tube. For protein precipitation, five volumes of 0.1 M methanolic ammonium acetate were added to the phenol phase and the mixture was incubated at -20^{0}C overnight. After that, the mixture was centrifuged at 10000 g for 15 minutes, the supernatant was discarded, and the protein pellet was rinsed in 0.1 M methanolic ammonium acetate at -20°C for 20 minutes. The resulting protein pellet was rinsed with 4 volumes of 80% v/v ice-cold acetone and once in cold acetone containing 20 mM DTT.

Protein Quantification

All the protein extracts were solubilised in 40 mM Tris-HCl. The concentration of each sample was estimated using the Bradford microassay (Quick Start Bradford Protein Assay, Biorad). In this study, bovine serum albumin (BSA) was used as a protein standard in the estimation of protein content in different extracts of *E. cottonii*. A series of protein standards (1.25 - 10µg/mL) was prepared in triplicate. Each protein sample was diluted 100 fold (triplicates): 1.5µL stored sample with 148.5µL ultrapure water. Then, 150µL of each protein sample and the BSA standard were mixed with 150µL of Bradford reagent (Biorad) in a final volume of 300µL. All the mixtures were incubated at room temperature for about five minutes. The absorbance value for each mixture was measured at 595nm using a spectrophotometer (Techne 6320D). Table 1 shows the absorbance values of BSA standards in five different concentrations.

TABLE 1
Absorbance value of BSA standards at 595nm

BSA protein standards	
Concentration (µg/mL)	Absorbance value at 595nm (A_{595})
1.25	0.054±0.002
2.5	0.100±0.003
5	0.206±0.002
7.5	0.294±0.002
10	0.370±0.002

Results are means ± SD (n = 3)

A calibration curve was plotted using Microsoft Excel. Using the calibration curve, the protein concentration of each sample was determined. The absorbance value at 595 nm is directly proportional to the amount of solubilised protein in each sample. For protein quantification, 100-fold dilution was carried out for each of the protein sample.

Fig.1 presents a calibration graph obtained using a series of BSA standards. BSA is commonly used as a protein standard because it is inexpensive and readily available in pure form (Kruger, 2002). Due to the ubiquity of BSA as a protein standard, it allows the results of this study to be compared directly to those of many previous studies.

One Dimensional Gel Electrophoresis (1DE)

The gel unit with glass sandwich set was assembled in casting mode with 1.0 mm spacers in place. 13% separating gel (3.1 mL of distilled water, 4.3 mL of 30% acrylamide /bis, 2.5 mL of 1.5 M Tris-HCl, pH8.8, 0.1 mL of 10% w/v SDS, 50 µL of 10% w/v ammonium persulfate and 5 µL of TEMED) was prepared. The solution was gently swirled in the beaker for proper mixing, and applied into the glass sandwich as soon as possible. One (1) mL of ultrapure water was immediately applied on top of the gel to prevent the formation of curved meniscus. The ultrapure water was then decanted when the separating gel polymerised. A 4% stacking gel (6.1 mL of distilled water, 1.3 mL of 30% acrylamide/bis, 2.5 mL of 0.5 M Tris-HCl, pH6.8, 0.1 mL of 10% w/v SDS, 50 µL of 10% w/v ammonium persulfate and 10 µL of TEMED) was prepared. The solution was gently swirled for proper mixing and was then applied into the glass sandwich as soon as possible. The Teflon comb was inserted carefully and time was allowed for gel polymerization. When the gel

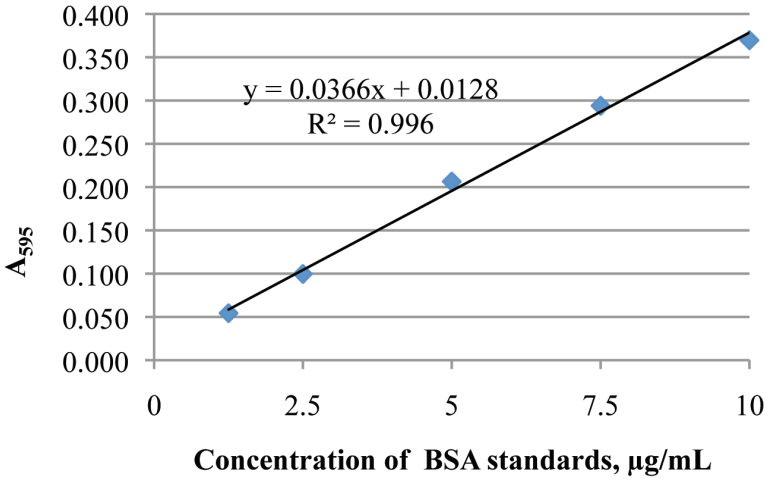

Fig.1: A calibration graph of BSA standards for protein quantification of *E. cottonii* extracts

polymerised, the Teflon comb was removed. The wells were immediately rinsed with running buffer. The gel was removed from its casting stand and was assembled into the gel electrophoresis slab unit. The wells and chamber were filled with running buffer. 10 µg of each protein sample was mixed with 25µL of SDS sample buffer. Then, all of the samples and 7µL of NEB protein marker (2-212 kDa) were heated at 95ºC for four (4) minutes. About 40µL of sample mixture and 7µL of protein marker were loaded into the wells. The gel electrophoresis system was connected to a power source, and a constant voltage of 200 V was applied for about 45 minutes until the tracking dye reached the end of the separating gel. The gel was removed carefully and rinsed with distilled water thrice. The gel was then soaked in 50 mL of Bio-Safe Coomassie staining solution (Biorad) and gently rocked on a shaker for 1 hour. Finally, the gel was rinsed several times with 200 mL of distilled water for 30 minutes each until the background was clear. The gel image was viewed and captured using GS-800™ Calibrated Densitometer (Biorad).

RESULTS AND DISCUSSION

Comparison of Protein Yields

Bradford microassay (Kruger, 2002) was utilised to determine total protein contents in various extracts of *E. cottonii* with three extraction methods. Bradford assay is a rapid and relatively accurate colorimetric method to estimate protein concentration in proteomic studies. This assay was first described by Bradford in 1975 (Bradford, 1976) and it is still widely used as a key protocol in protein quantification. In general, the Bradford assay relies on the binding of the dye Coomassie blue G250 to protein. The dye exists in three appearances: it is red in colour when in cationic forms, remains green in colour when it is neutral, and turns blue when it is in anionic form. Ordinarily, the dye was purchased in protonated red cationic form with maximal absorption of 470nm. However, when the dye bound to proteins in protein assay, it was converted to a stable unprotonated blue complex with a maximal absorption of 595nm (Georgiou *et al*., 2008). Thus, the protein concentration in a particular sample can be quantified by measuring the absorbance of the solution at 595 nm.

All of the protein pellets, obtained through the three different extraction methods, were dissolved in 40 mM Tris-HCl, pH7.5 buffers, as mentioned by Contreras *et al*. (2008). This was performed because certain buffering components such as mercaptoethanol, tris, SDS and others are known to be incompatible with Bradford reagent (Campion *et al*., 2011). Table 2 reveals the total protein yields of extracts from *E. cottonii*, using three different protein extraction methods: (a) Phenol/ lysis buffer extraction, (b) TRI reagent/ chloroform extraction, and (c) Phenol/SDS buffer extraction.

Among the three phenol based extraction protocols with different combinations of chemicals, the phenol/ lysis buffer gave slightly higher protein yields (0.027±0.000 mg/g) than the phenol/

TABLE 2
The concentration (µg/mL), mass (µg) and total protein yields (mg/g) of *E. cottonii* extracted with three different methods.

Protein extraction from *E. cottonii*			
	Concentration (µg/mL)	Mass (µg)	Total protein yields (mg/g)
Phenol/lysis buffer extraction	675.410±11.375	135.082±2.275	0.027±0.000[a]
TRI reagent/chloroform extraction	448.330±22.793	89.666±4.559	0.018±0.001[c]
Phenol/SDS buffer extraction	597.086±38.067	119.417±7.613	0.024±0.002[b]

Results are means ± SD (n = 3). For total protein yields, values followed by the same letter (a–c) are not statistically different at $P < 0.05$.

SDS buffer (0.024±0.002 mg/g) (Table 2). The TRI reagent/chloroform extraction showed the lowest protein yields, i.e. about 0.018±0.001 mg/g (Table 2). TRI reagent is a mixture of phenol and guanidine thiocyanate in a monophase solution. It is a commercial reagent that is used in the simultaneous isolation of RNA, DNA and proteins from different biological samples and most commonly used for the isolation of total RNA. Wong *et al*. (2006) successfully established the proteomics of *Gracilaria changii* (red algae) by using TRI reagent in combination with chloroform. With reference to protein extraction from *G. changii*, the protein yields of *E. cottonii* are slightly higher. As shown in Table 2, the protein yields of *E. cottonii* were 0.018±0.001 mg/g as compared to 0.005 mg/g from *G. changii* (Wong *et al*., 2006). In this study, it was assumed that the protein contents of *E. cottonii* are much higher than in *G. changii*. However, this is not conclusive as more work needs to be carried out in order to verify the claim. Protein contents in seaweeds may also vary due to growing environments, harvesting seasons, etc. (Parker, 1974).

In this study, the phenol/lysis buffer showed the highest protein yields (0.027±0.000 mg/g) of the three protein extraction protocols. This is probably because the inclusion of a protease inhibitor cocktail may have reduced the loss or degradation of seaweed proteins during the extraction process. In addition, the water-soluble properties of polyvinylpyrrolidone (PVP) in the lysis buffer may have enhanced the solubility of seaweed proteins (Wang *et al*., 2006), resulting in higher readings in Bradford's protein quantification. Conversely, the protein extracts of *E. cottonii* from the TRI reagent/chloroform extraction showed relatively weak solubility in 40 mM Tris-HCl and thus contributed to the lowest protein yields (0.018±0.001 mg/g).

Bradford protein assay was employed as a protein quantification tool in this study. The readings relied on the extent that Coomassie Brilliant Blue G-250 dye binds to the proteins. In other words, the spectral change that accompanies protein binding was used to determine the protein concentration. Generally, all dye binding assays suffer from the limitation of potential interference from

any non-protein substance that can also form a complex with a dye, or otherwise, modify the binding interaction between the dye and the protein (Berkelman, 2008). For instance, the guanidine hydrochloride used in the washing steps of the TRI reagent/chloroform extraction may compete with the dyes for the proteins in certain degrees, thus leading to underestimation of the protein content (Kruger, 2002).

Nevertheless, the Bradford assay is commonly applied for protein quantification in many other studies (Berkelman, 2008). This is because the Bradford assay is relatively easy to use and fairly free from interference by most commonly used biochemical reagents, except for certain chaotropic solubilising agents, detergents, reductants, buffers, or carrier ampholytes (Berkelman, 2008). In order to avoid any possible interference by other harsh solubilising agents, all protein pellets obtained from the three extraction protocols were dissolved only in 40 mM Tris-HCl, pH 7.5 buffers (Contreras *et al*., 2008). However, the protein pellets obtained from the TRI reagent/chloroform extraction are partially soluble in 40mM Tris-HCl buffer.

As compared to bacteria, animals and other terrestrial plants, the protein yield of macroalgae like *E. cottonii* was very low. This may due to the nature of low protein content in macroalgae species (Wang *et al*., 2006). Furthermore, BSA, which is a common protein standard in many protein assays suffers from the disadvantage of exhibiting an unusually large dye response in the Bradford assay. This may cause underestimation of the protein content in a sample (Kruger, 2002). These are the two major drawbacks in the protein quantification of this study, but no other studies have addressed these issues.

Comparison of the Three Extraction Protocols Based on the 1DE patterns

One dimensional gel electrophoresis (1DE) is commonly known as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is a relatively easier and cheaper protocol for proteomic analysis, and hence, is widely adopted in different disciplines such as forensic science, molecular biology, biochemistry, ecology, agricultural sciences and others. A SDS-PAGE separates protein based on their electrophoretic mobility, and is commonly used to estimate the relative molecular mass of protein subunits in order to determine the relative abundance of major proteins in a sample and to determine the distribution of proteins among fractions. Fig.2 shows the SDS-PAGE protein profiles of *E. cottonii* with three different protein extraction methods: (a) Phenol/lysis buffer extraction, (b) TRI reagent/chloroform extraction, and (c) Phenol/SDS buffer extraction.

In this study, three different protein extraction protocols were compared on seaweed *E. cottonii* to determine the method that best shows resolved proteins clearly and fewer interfering substances. At the first glance, the qualitative comparisons of protein extracts from *E. cottonii* using the three different extraction methods revealed that all three methods shared almost similar

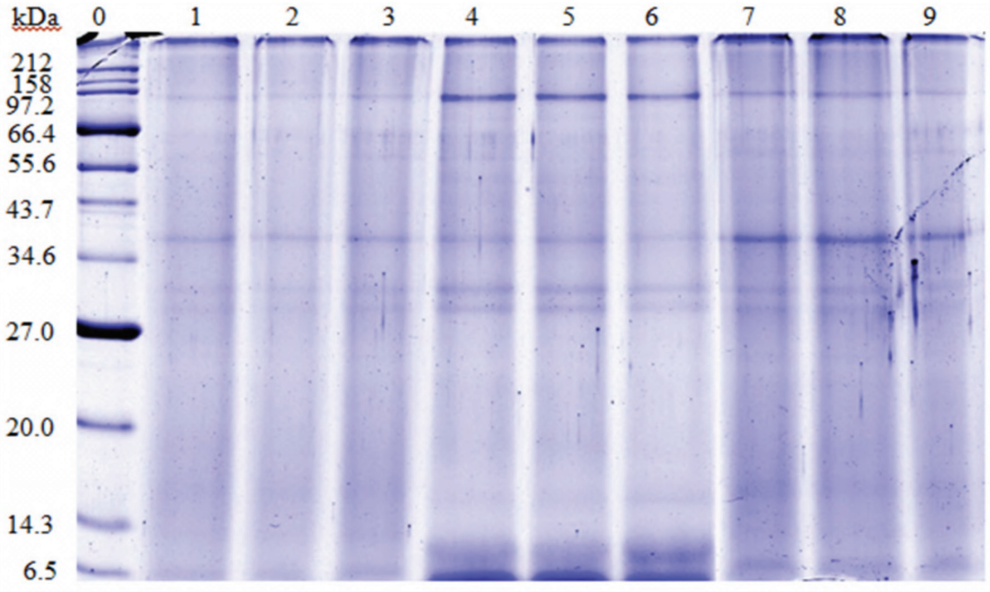

Fig.2: SDS-PAGE separation of *E. cottonii* proteins extracted using different methods. Lane 0: NEB protein marker (2-212 kDa), lanes 1-3: 10 µg of protein sample from phenol/lysis buffer extraction (triplicates), lanes 4-6: 10 µg of protein sample from TRI reagent/chloroform extraction (triplicates), lanes 7-9: 10 µg of protein sample from phenol/SDS buffer extraction (triplicates).

protein profiles. Intense protein bands were observed from 27 kDa to 158 kDa (Fig.2). All three extraction methods utilised phenol as part of their extraction reagents.

However, the different extraction methods produced protein bands in different intensities. TRI reagent/chloroform extraction gave the most intense protein band at about 97.2 kDa. Phenol/SDS buffer extraction produced an intense protein band at about 40 kDa. Phenol/lysis buffer extraction produced diverse protein bands, which resolved in the ranges of 27 kDa to 34.6 kDa, 34.6 kDa to 43.7 kDa, 55.6 kDa to 66.4 kDa and 97.2 kDa (see Fig.2). All the three phenol based extraction methods produced fairly good one dimensional electrophoresis (1DE) protein profiles of *E. cottonii* with almost no smearing observed.

As compared to the higher level terrestrial plants, macroalgae proteomics are relatively less studied. Most of the protein extraction protocols utilised in macroalgae proteomics were adapted from the proteomics of other higher level terrestrial plants. Pavokovic *et al*. (2012) stated that phenol extraction was the most effective method for the removal of unwanted interfering substances. Phenol extraction was utilised in the extraction of other macroalgae species: *Scytosiphon gracilis* and *Ectocarpus siliculosus* (Contreras *et al*., 2008), *Gracilaria changii* (Wong *et al*., 2006) and *Ecklonia kurome* (Nagai *et al*., 2008). By comparing the TCA/acetone and phenol extraction protocols used by Carpentier *et al*. (2005) and Saravanan and Rose (2004), respectively, an observation

that both of the methods were efficient in extracting proteins from recalcitrant tissues was ruled. Phenol extraction was found to be the most efficient method of removing the interfering substances, and it resulted in the highest quality gels with less background and fewer smearing effects. Phenol based extraction minimises protein degradation that is often encountered in sample preparation that occurs because of endogenous proteolytic activity. Saravanan and Rose (2004) also pointed out that the phenol method yielded a greater number of glycoproteins.

Phenol extraction is based on the separation of macromolecules into organic and aqueous phases. Phenol is a simple aromatic alcohol that contains only a polar hydroxyl group that is bound to an aromatic ring. It exhibits weak acidic properties and is partially miscible with water. When saturated with water, the aqueous layer contains about 7% phenol and the organic layer contains about 28% water (Faurobert *et al*., 2007). During the extraction, phenols interact with proteins mainly via hydrogen bonding, which causes protein denaturation and causes the phenols to become solubilised in the organic phenolic phase. The aqueous phase preferentially dissolves nucleic acids, carbohydrates and cell debris, while the phenolic phase carries proteins and lipids. In both phenol/lysis buffer extraction and phenol/SDS buffer extraction, proteins from the phenolic phase were then precipitated with methanol and ammonium acetate.

TRI reagent solution is a combination of phenol and guanidine thiocyanate in a monophasic solution. It was first invented by Chomczynski and Sacchi (1987) for the isolation of RNA from cultured cells in most animal tissues. TRI reagent was then utilised in protein extraction from *Gracilaria changii* (Wong *et al.*, 2006). Chloroform mixed with phenol is more efficient at denaturing proteins than the individual reagent alone (Perry *et al.*, 1972). In this study, the mixtures of phenol to chloroform used were 5:1 (v/v). At acidic pH, a 5:1 ratio caused the absence of DNA from the upper aqueous phase; this further enhanced the purity of protein extracts later. Purified phenol has a density of 1.07 g/cm^3 and formed the lower phase when mixed with water (1.00 g/cm^3) later. Chloroform ensured phase separation of the two liquid because chloroform is miscible with phenol and it has a higher density (1.47 g/cm^3) than phenol. As a result, this procedure facilitated the removal of proteins containing pink coloured organic phase with minimal cross contamination of nucleic acids from the aqueous phase.

According to a general rule, a solute dissolves best in a solvent that shares a similar chemical structure. Therefore, the overall solvation capacity of a solvent depends primarily on its polarity. The protein extracts of *E. cottonii* may contain varying proportions of charged and uncharged domains, which construct hydrophobic and hydrophilic regions. In the presence of phenol in the TRI reagent, the hydrophobic cores interact with phenol, causing precipitation of proteins and glycoproteins and further partitioning at the interphase and

the pink coloured organic phase. This may explain the intense band close to 97.2 kDa (Fig.2) that was observed in the TRI reagent/chloroform extraction, as glycoproteins usually have higher molecular weight than single protein subunits. In other words, TRI reagent/chloroform extraction yielded a higher amount of high molecular weight glycoproteins as compared to the phenol/lysis buffer extraction and phenol/SDS buffer extraction.

As discussed earlier, the protein extracts obtained from the TRI reagent/chloroform extraction were sparingly dissolved in 40 mM Tris-HCl, pH 7.5 buffers. At this stage, it was speculated that the protein extracts obtained with TRI reagent/chloroform were more hydrophobic and hence less likely to dissolve in water soluble Tris buffers. However, with the addition of SDS as a protein denaturation and solvation agent prior to 1DE, the protein extracts of *E. cottonii* from TRI reagent/chloroform extraction were well dissolved in SDS-denaturing buffer. SDS is a long-chain hydrocarbon with a negatively charged hydrophilic head and a long hydrophobic hydrocarbon tail. SDS is an anionic detergent known to dissociate and denature proteins. It acts at a very low concentrations and causes the unfolding of proteins by inducing conformational changes to alpha helices (Rao & Prakash, 1993). As a result, the protein extracts obtained from TRI reagent/chloroform extractions displayed well resolved, distinctive protein bands, resembling the protein profiles obtained from the phenol/lysis buffer extraction and phenol/SDS buffer extraction. Ten (10) µg of the protein extracts were loaded for 1DE for all three protein extraction protocols from *E. cottonii*.

As shown in Fig.2, all the three extraction methods shared similar 1DE protein profiles. Each extraction method succeeded in producing well-resolved protein bands. In other words, there was no smearing or tailing as observed in other non-phenol based extraction protocols in a range of other macroalgae species (Contreras *et al*., 2008; Nagai *et al*., 2008). This proved that the phenol based protein extraction protocols applied in this study had successfully removed other non-protein interfering substances from *E. cottonii*. In addition, all the three phenol based extraction methods resulted in high molecular weight proteins, specifically of protein bands of about 97.2 kDa that were intensely observed in the *E. cottonii* sample with TRI reagent/chloroform extraction and protein bands of about 39 kDa that were strongly observed in the *E. cottonii* sample with phenol/SDS buffer extraction. Overall, three distinctive protein bands in a range of 27 kDa to 43.7 kDa and a protein band of about 97.2 kDa were clearly observed in the *E. cottonii* sample with the three different phenol based protein extraction protocols. Other protein bands in a range of 43.7 kDa to 66.4 kDa were barely detected in the *E. cottonii* sample with the three phenol based protein extraction protocols used in this study. Nonetheless, the limitations of 1DE protein profiles restrict the identification of protein groups or protein types, which are

represented by each of the well resolved protein bands in the protein profile of *E. cottonii* with three different phenol based protein extraction protocols.

CONCLUSION

This study is an initial proteome study of *E. cottonii*. To date, no other proteomics on *E. cottonii* have yet been reported. Three phenol based protein extraction protocols were compared for their suitability in 1DE proteome studies of *E. cottonii*. Of the three extraction methods, the phenol/lysis buffer protocol yielded the highest protein concentration (0.027±0.000 mg/g), followed by the phenol/SDS buffer protocol (0.024±0.002 mg/g), and the TRI reagent/chloroform protocol (0.018±0.001 mg/g). All the three extraction protocols utilised in this study produced good quality 1DE protein profiles of *E. cottonii* with distinctive protein bands. The 1DE profile of *E. cottonii* from the three extraction protocols showed similar patterns, and high molecular weight protein molecules ranging from 27 kDa to 158 kDa in different intensities were observed. Among the three phenol based protein extraction protocol, the phenol/SDS buffer protocol was recommended for preliminary proteome study of *E. cottonii* as it is the cheapest method, relatively easy to start up and yet gave good protein yields, as well as produced comparable good quality 1DE protein profiles of *E. cottonii* as in the other two protein extraction protocols in this study. In conclusion, proteomics is increasingly seen as an important tool in marine biology, and it is particularly important to enhancing a deep understanding of marine seaweed biology. Proteomics is an effective research tool when used in addition to other "omics" technologies. This study may facilitate other downstream studies of *E. cottonii* and other carrageenan rich seaweed species. Seaweed proteomics is particularly valuable to aquaculture sectors. This study shows that "*E. cottonii*" proteomics is vital to reaching the goals of aquaculture of high productivity and a better quality product.

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Relationship between Size of Fish, Temperature and Parasitic Intensity in Snakehead Fish Species from Kepala Batas, Penang, Peninsular Malaysia

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ABSTRACT

Acanthocephala parasite infestation was found to be high in wild Snakehead fish (*Channa striata*) from a Kepala Batas paddy field in Penang, Peninsular Malaysia. The fish parasites collected from 98 out of 100 fish samples were examined. The study showed infestation prevalence rate of 98%. Further analysis was carried out to correlate the parasitic intensity, fish size, and temperature, while statistical model summary was produced using SPSS version 15 statistical software. Statistical model summary concluded that for every increment of 1 cm in length of fish, an extra of 0.5 ≈ 1 number of parasites was found in the fish. This is only true if the effect of mean temperature remained constant. Meanwhile, an increase of 1°C is associated with a decrease of 1.487 ≈ 2 numbers of parasites in fish. This is only true when the effect of fish length remained constant. Therefore, overall model summary is described as: *Number of Acanthocephala parasites = 34.74 + (0.462*Length of Fish) + (-1.487*Mean Temperature)*.

Keywords: Acanthocephala parasite, Predictor, Snakehead fish, Variance Inflation Factor (VIF)

INTRODUCTION

Malaysia is a country which depends on fisheries as one of its economic resources. Fishery not only serves as one of the most important protein supplies in Malaysia but it also helps in increasing its national Gross Domestic Product (GDP) and foreign exchange. Apart from that, the Fishery sector in Malaysia serves as an economic source of job opportunities for many people in this country, especially in rural area either as fisherman or fish farmers (Kabata, 1986; Othman, 1998).

Snake head fish (*Channa* sp.), which is more commonly known as Haruan, has

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been acknowledged in Asian countries as one of the most important fish species. Snake head fish (*Channa* sp.) is commonly found in the wild rice fields, ponds and wells distributed throughout Asia. Moreover, the feeding habits of wild snake head fish are carnivorous as the animal feeds on worms, prawns, frogs and especially other fishes (Mat Jais *et al.*, 1994).

The main reason for the popularity of this species is its medical values with antinociceptive properties. It has been considered as a very good source of healthy food among Asians because of the high levels of amino acids and fatty acids (Mat Jais *et al*., 1994). Although there are numerous advantages of snake head fish, there are also several parasites identified to be present in this fish species. The parasites present may or may not cause illness, and this characteristic is governed by various factors. Parasites have arisen by evolution from free living animals, whereby some of them have developed special organs to be able to live in host organisms (Yalcin *et al*., 2002).

The common disease recorded in snake head fish species throughout Asia is mostly on the wild type of fish from endoparasites of Acanthocephalans that are commonly found in the intestinal of fish. The lifecycle of Acanthocephalans involves invertebrates as an intermediate host and vertebrate as the final host. The phylum of Acanthocephalans consists of 4 classes: Archiacanthocephala, Palaeacanthocephala, Eoacanthocephala and Polyacanthocephala. This classification is based mainly on their morphological features such as the location of the lacunar system (network of cavities in the epidermis), the persistence of ligament sacs in females, the number and shape of cement glands in males, the number and size of proboscis hooks, as well as host taxonomy and ecology (Crompton, 1985).

To date, there has been no reference available on the correlations of Acanthocephalans infestations to the fish size and environmental factors such as water temperature in Malaysia. There is an urgent need for this important aspect of research as it will benefit fish farmers for aquaculture industry to predict any Acanthocephalans fish parasite infestation in their farm and to take initiatives to prevent intestinal parasite infections. Thus, the objective of this study was to determine the prevalence and statistical analysis of Acanthocephalans parasite to the fish size and water temperature of wild snakehead fish sampled from paddy field in Kepala Batas, Penang, Peninsular Malaysia.

MATERIALS AND METHODS

The experiment was carried out with 100 specimens of wild *Channa striatus* from Kepala Batas, Penang (latitude 5.51531, longitude 100.41627), Peninsular Malaysia. The length (cm) of each fish was measured prior to parasite examination. Tricaine methane sulfonate (MS 222) (50 mg/L) was used as anesthetics to reduce the stress, and for easy handling. After the fish had been anaesthetized, presence of endoparasite was examined via disection of fish intestine and direct observation under light microscope.

The fish abdominal wall was pierced using a sharp pointed scissor in the mid ventral line, just behind and between pectoral fins and cut along the mid ventral line toward the anal region. Then, the belly flap was removed and this exposes the internal organs. All the internal organs like the heart, liver and gut were carefully taken out as a unit by making a transverse cut from the mouth to the anal region. Then, these organs were separated and each was placed in a different petri dish. These organs were then added with a few drops of saline.

Then, the heart, liver, spleen and kidney were dissected and observed for any abnormalities. Any white spot found was carefully cut to check on the possibilities of the parasite infestation. These organs were cut into thin slice and observed under microscope using 10x and 40x magnification. Then, the heart, liver and kidney squash were prepared and observed under a microscope. Next, the gastrointestinal tract was removed and cut from the posterior end to open it up. The intestine is dissected under dissecting microscope. The intestinal wall is scraped and their content is diluted with saline in a bottle. These diluted intestinal contents were shaken and was checked for the presence of any parasite that had settled at the bottom of the bottle. Then, the intestinal walls were carefully examined under a microscope for the attachment of the parasites (Kabata, 1986).

First, the morphological identification of parasite was done by first staining the parasite with a few drops of lactophenol solutions (200 mL lactic acid, 200 g/L phenol, 400 mL glycerol and 200 mL deionized water). Upon staining, slides were observed under the compound microscope (Leica USA). Second, the morhological identification was done using the Supra 50vp ultra high resolution LEO analytical Fesem, Scanning electron microscope. Parasite found was taken out carefully from the infected area, and the number of parasites obtained from each fish was recorded and preserved with 70% ethanol solution in universal bottle for further examination (Lasee, 2004). After taking pictures of the parasites, identification of the parasites collected was done by morphological observation using identification keys, as suggested by Hoffman (1970).

Electron microscopic sample preparation was done as suggested by the protocol of Supra 50vp ultra high resolution LEO analytical Fesem, Scanning electron microscope guide manual. Firstly, the suspended samples in ethanol were put into serial dilution of 90%, 80% and 70% ethanol. Then, a droplet of the suspension was placed on a carbon film coated with 400mesh copper grid for 1-3 minutes. The droplet was then wicked to dryness using pieces of filter paper. The grid was then placed in a filter paper lined petri dish for preservation in desiccator. Finally, imaging would be carried out after 3 days of preservation.

The statistical analysis in this study was performed using the Statistical Package for Social Sciences software 15.0, SPSS version 15, using the analysis of multiple

regressions. In all cases, the level of significance was set at $p< 0.05$ (Field, 2005). Weather report of Kepala Batas Region in Penang, Peninsular Malaysia was collected from Malaysian Meteorology Department, while the statistical analysis was performed with SPSS version 15, using analysis of multiple regressions. In all cases, the level of signicifance was set at $p< 0.05$ (Field, 2005).

Note:
* Prevalence is defined by:

$$\frac{\text{Number of fish infected}}{\text{Total number of fish}} \times 100\ \%$$

(Poulin *et al*., 1995)

RESULTS AND DISCUSSION

Acanthocephalan parasite was observed with few body segments. Proboscis is a hollow structure filled with fluid. These fluid associates with the proboscis inverter muscles help in invagination of the proboscis into the proboscis receptacles. The movement of proboscis is activated by the apical sensory organ located at the tip of the proboscis (see Fig.1-1.3). These proboscises have a variety of shapes ranging from spherical to cylindrical (Bush *et al*., 2001). Proboscis has a proboscis sheath or septum that separates its cavity from the pseudocoelom. Their proboscis is armed with a set of chitinised pointing hooks that are arranged in horizontal rows piercing the intestinal tissue of the infected animals, resulting in inflammation at the attachment site. These hooks are usually longer and slender at the length of the proboscis but shorter at the base (see Fig.1-1.3). Some of the Acanthocephalans can enter abdominal cavity by inserting their proboscis in the host intestine. The action of this highly pathogenic parasites cause inflammation at the attachment site and severe infection that can lead to death (Kabata, 1985; Crompton & Nickol, 1985; Hoffman, 1970).

Typically, wild animals are exposed to a diversity of parasitic species including nematodes, cestodes, trematodes and acanthocephalans, representing a various group of transmission strategies and effects on host's health (Poulin, 1998b; Morand, 2000; Roberts *et al.*, 2002). An extensive approach is needed to understanding the parasite community diversity because the multiple host characteristics like environment temperature, host length may be correlated with one another, and different host characteristics may be important for understanding the parasite species correlations (Nunn *et al.*, 2003).

In this study, the multiple regression analysis was used to examine the multiple factors that had been predicted to influence the diversity of parasites in wild hosts like their length size, environment temperature and parasites count (Nunn *et al.*, 2003). These variables are predicted to influence host encounter rates with parasites in the wild, and the number of parasite species that can persist in populations. A positive association is expected between host length and parasite diversity because larger hosts represent larger habitats that provide more niches for colonization (Kuris *et al.*, 1980; Poulin, 1995; Gregory *et al.*, 1996).

The multiple regression analysis is incorporated with the aim to produce a model that would best predict on the optimal number of Acanthocephalan parasitic infestation based on the observed values of two independent variables which was the length of the fish and the mean temperature. Along with that, the multiple regression analysis also produces unique contribution on the length of fish and mean temperature variables on the number of Acanthocephalan parasitic infestation in fishes. This determines the effects of the fish's length and the mean temperature on the number of Acanthocephalan parasitic infestation.

A total of 98 out of 100 fish were found to have been infected by Acanthocephala parasite with the prevalence of 98%. Table 1 shows the descriptive statistics of

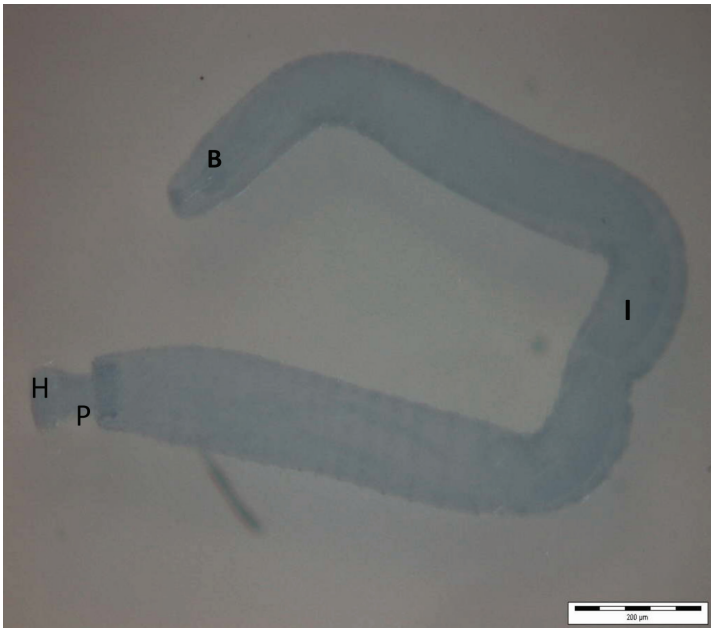

Fig.1: The morphology of Acanthocephala parasite sample collected; B= bursa, I-internal organs, H-hooks,P-proboscis

TABLE 1
Descriptive statistics of variables

	N	Minimum	Maximum	Mean	Std. Deviation
Acanthocephala Parasite	98	0	14	4.82	2.902
Length Fish	98	18.00	38.00	23.0969	4.14844
Mean Temperature	98	25.60	28.80	27.3721	.71921
Valid N (listwise)	98				

TABLE 2
Correlation between the variables

		AcanthocephalaParasite	Length Fish	Mean Temperature
Pearson Correlation	Acanthocephala Parasite	1.000	.850	-.774
	Length Fish	.850	1.000	-.667
	Mean Temperature	-.774	-.667	1.000
Sig. (1-tailed)	Acanthocephala Parasite	.	.000	.000
	Length Fish	.000	.	.000
	Mean Temperature	.000	.000	.
N	Acanthocephala Parasite	98	98	98
	Length Fish	98	98	98
	Mean Temperature	98	98	98

TABLE 3
Regression model summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	Durbin-Watson
1	.894(a)	.800	.793	1.303	2.024

a Predictors: (Constant), Mean Temperature, Length Fish
b Dependent Variable: Acanthocephala Parasite

the dependent variable and explanatory variables involved in this study. The average number of the Acanthocephala parasites found in dissected fishes was approximately 5, with a standard deviation of 2.902. The mean values of the parasite, fish length, and temperature were 4.23, 23.10 and 27.37, respectively.

Table 2 shows the correlation between the pairs of variables. It is evident that high positive correlations exist between the number of Acanthocephala parasite and the length of fish, which is 0.850 at 0.05 significance level, whereby p-value is 0.000. Among all the predictor variables, the length of fish correlates best with the dependent variable and therefore, it is likely that this variable will best predict the number of Acanthocephala parasite in fishes. High negative correlation between the mean temperature and number of Acanthocephala parasite, with a correlation value of -0.774, is significant at 0.05 significance level. Thus, it can be concluded that there is no multicollinearity in this data due to no substantial correlations between the predictors' value higher than 0.9 (Field, 2005).

The Table 3 shows the correlation value between the length of fish and mean temperature with the number of Acanthocephala parasites found in fish, which is 0.894. The value of R Square is 0.800, indicating that the length of fish

TABLE 4
ANOVA

Model		Sum of Squares	Df	Mean Square	F	Sig.
1	Regression	440.206	2	220.103	129.680	.000(a)
	Residual	110.323	65	1.697		
	Total	550.529	67			

a Predictors: (Constant), Mean Temperature, Length Fish
b Dependent Variable: Acanthocephala Parasite

TABLE 5
Coefficients for model

		Model		
		1		
		(Constant)	Length Fish	Mean Temp
Unstandardized Coefficients	B	34.741	.462	-1.487
	Std. Error	9.018	.057	.297
Standardized Coefficients	Beta		.601	-.373
T		3.852	8.063	-5.006
Sig.		.000	.000	.000
95% Confidence Interval for B	Lower Bound	16.730	.348	-2.080
	Upper Bound	52.751	.576	-.894
Correlations	Zero-order		.850	-.774
	Partial		.707	-.528
	Part		.448	-.278
Collinearity Statistics	Tolerance		.555	.555
	VIF		1.002	1.002

a Dependent Variable: Acanthocephala Parasite

and mean temperature account for 80.0% of the variation in the number of parasites. The difference between the adjusted R Square and R Square is small, i.e. about 0.007 or 0.7%; this shrinkage defines that if the model was to be derived from the whole population rather than a sample, it would account for approximately 0.7% less variance in outcome. The Durbin-Watson value is 2.024, which is not greater than the value 3 nor is it lesser than the value 1. This only proves that the assumptions of independent errors are tenable and the assumptions have been met (Field, 2005).

Table 4 shows F-ratio is 129.680, which is very unlikely to happened by chance due to a significant p-value lesser than 0.05 or 5% significance level. This result proves that the model significantly improves the ability to predict the outcome variable. Therefore the regression model overall predicts the number of the Acanthocephala parasites

in fish significantly well. Table 5 presents the estimates of the b-values, which are the parameters in the regression model, and the individual contribution of each predictor to the model. The b-values represent the parameter estimates of the model for each independent variable. For this data, the length of the fish has a positive b-value indicating that there is a positive relationship between the numbers of the Acanthocephala parasite and the length of fish. As the length increases, the number of Acanthocephala parasite also increases. However, the mean temperature has a negative b-value representing a negative relationship between the numbers of Acanthocephala parasite and the mean temperature (Field, 2005).

The overall model can be defined as follows:

$Y=b0+b1x1+b2x2$

Number of Acanthocephala parasites = 34.74 + (0.462*Length of Fish) + (-1.487*Mean Temperature)

The b-value for each predictor affects the outcome variable, if the effects of all other predictors are held constant (Andy, 2005). This is further explained as the length of Fish (b = 0.462) value indicates that as the length of fish increases by one unit, the number of parasites will increase by 0.462 unit. In other words, for every increase of 1 cm in the length of the fish, an extra of 0.5 ≈ 1 number of parasites is found in it. This is only true if the effect of the mean temperature remains constant. Next, the mean temperature (b = -1.487) value indicates that as the mean temperature increases by one unit, and the number of parasites decreases by -1.487 units. Therefore, an increase of 1°C is associated with a decrease of 1.487 ≈ 2 numbers of parasites in the fish. This is only true when the effect of the length of the fish remains constant.

For this model, the t-test associated with a b-value which is significant shows that the explanatory variables are making a significant contribution to the model. The length of fish has the value t-statistics of $t(65) = 8.105$ at a p- value < 0.05 and the mean temperature has the value of $t(65) = -5.006$ at a p-value < 0.05, and they are all significant predictors of the number of parasites in the fish. The magnitude of the t-statistics shows that the length of the fish has a higher impact on the number of parasites compared to the mean temperature. The model does not have confidence intervals that cross the value zero, proving that the model is quite a good model. The 95% confidence intervals for the independent variable mean temperature is small, indicating that the estimates for the current model are likely to be the representative of the true population values.

The confidence interval for the length of the fish is wider, however, did not cross zero, indicating that the parameter estimate for this variable is less representative, but it is significant. From Table 5, it can be concluded that the assumption of no multicollinearity is true due to the values of VIF and the tolerance statistics, whereby the value of largest VIF value does not exceed 10 and tolerance statistics are above 0.2.

Fig.2 shows the histogram of the residuals data. The histogram has bell shaped curve which represents that all residuals are normally distributed. Fig.3 shows the normal P-P plot and it shows that the points lie along a diagonal line indicating residuals are normally distributed. Fig.4 shows that the points are randomly and evenly dispersed throughout the plot. This pattern shows that the assumptions of linearity and homoscedasticity of the residuals have been met (Field, 2005).

CONCLUSION

In conclusion, length of the fish and temperature are the main factors that influence the number of fish parasites present in snake head fish (*Channa* sp.). Eventually, this study has focused on predictions of several parameters so as to discover the closest correlations among the abiotic and biotic factors. The effets of temperature in this study shows a decreasing value when the number of parasites increases accordingly with the length of fish, immunological studies have suggested an immune suppression effect associated with a decrease in water temperature (Bly *et al*., 1992). Several studies have reported that a decrease in water temperature may cause suppression of acquired immunity, with components of innate immunity being relatively independent of water temperature (Magnadottir *et al*., 1992). Furthermore, there are some suggested studies which show that larger fish tend to harbour more parasites as compared to smaller ones (Rahman & Saidin, 2012).

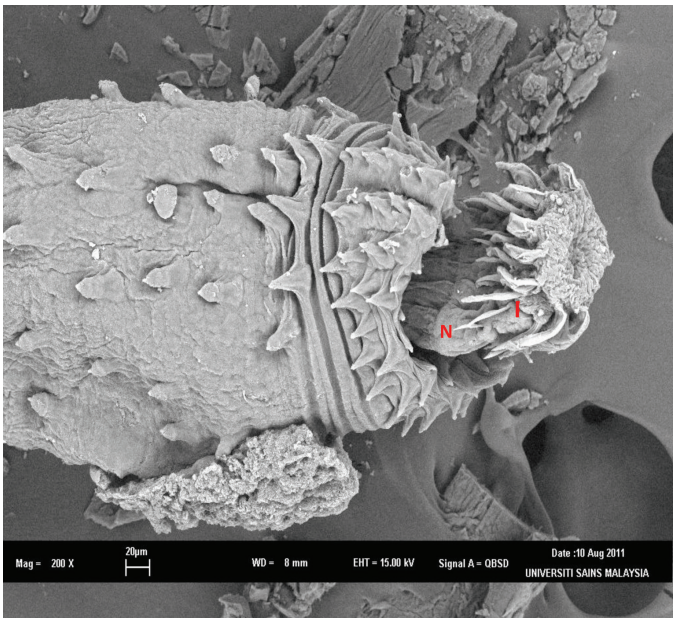

Fig.2: The upper portion view of the Acanthocephala parasite sample collected; P= proboscis, N= neck.

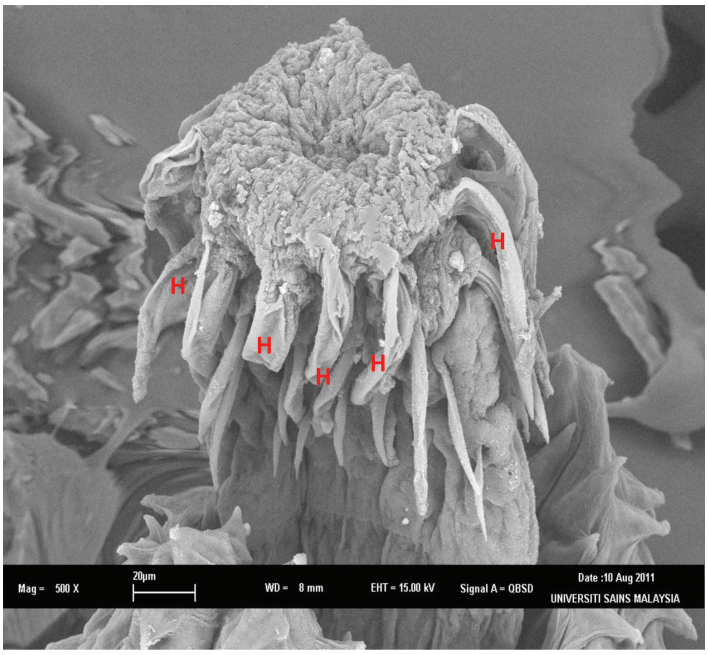

Fig.3: Anterior view of Acanthocephala parasite sample collected; H=hooks

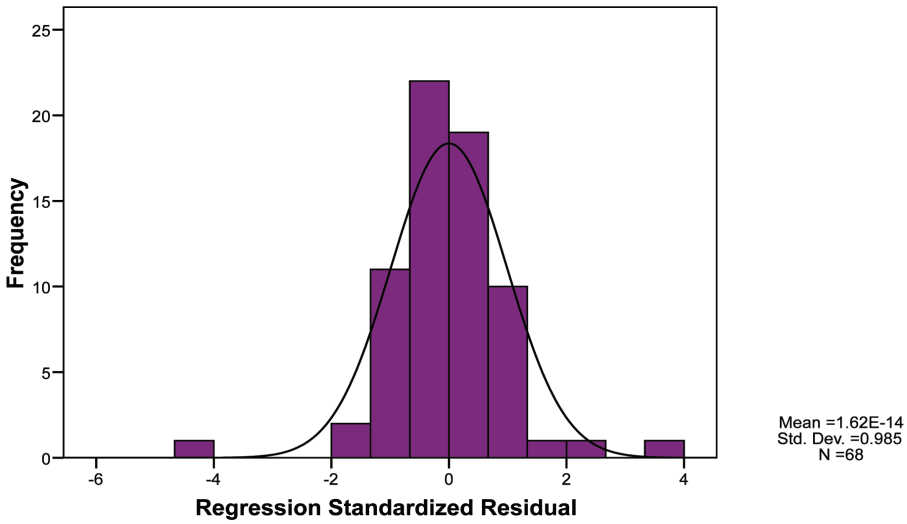

Fig.4: Histogram of the residuals data that has a bell-shaped curve which shows that residuals are normally distributed

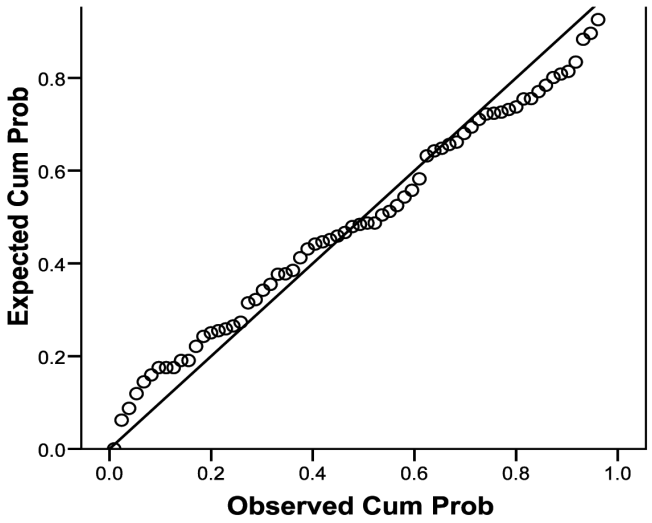

Fig.4: Normal P-P plot shows the points lie along the diagonal line indicating residuals are normally distributed

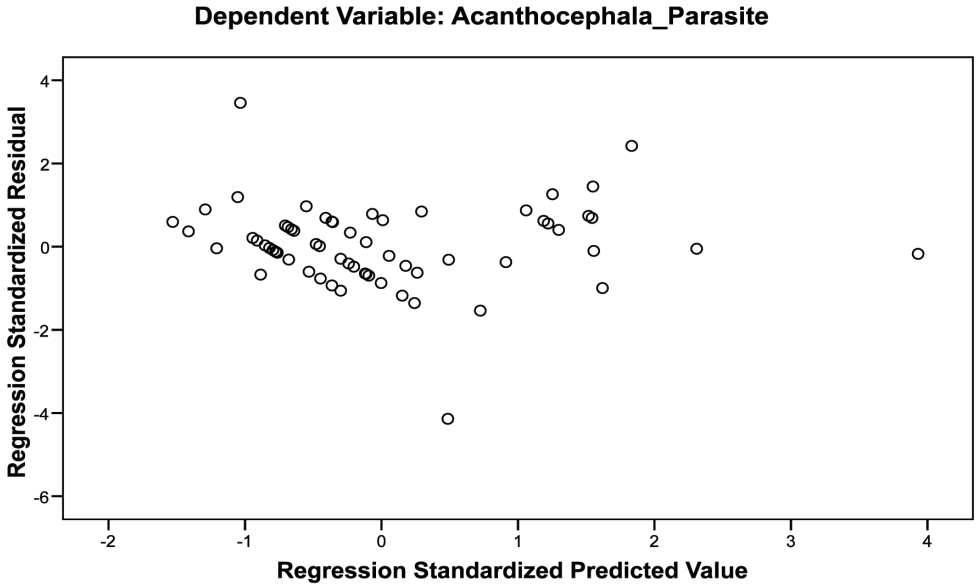

Fig.5: Points were randomly and evenly dispersed throughout the plot. Pattern shows that the assumptions of linearity and homoscedasticity of the residuals have been met

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In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts. Authors are encouraged to indicate in the **Referral form** using the **Manuscript Submission Kit** the names of three potential reviewers, but the editors will make the final choice. The editors are not, however, bound by these suggestions.

Manuscripts should be written so that they are intelligible to the professional reader who is not a specialist in the particular field. They should be written in a clear, concise, direct style. Where contributions are judged as acceptable for publication on the basis of content, the Editor reserves the right to modify the typescripts to eliminate ambiguity and repetition and improve communication between author and reader. If extensive alterations are required, the manuscript will be returned to the author for revision.

The Journal's 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 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 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 executive editor asks them to complete the review in three weeks and encloses two forms: (a) referral form B and (b) reviewer's comment form along with reviewer's guidelines. 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 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 Editorial Board, 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 submit a revised version of the paper to the executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor.

5. The executive editor sends the revised paper out for 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 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, the paper is sent to that Press and 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 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.

English language editing

Pertanika **emphasizes** on the linguistic accuracy of every manuscript published. Thus all authors are required to get their manuscripts edited by **professional English language editors**. Author(s) **must provide a certificate** confirming that their manuscripts have been adequately edited. A proof from a recognised editing service should be submitted together with the cover letter at the time of submitting a manuscript to Pertanika**. All costs will be borne by the author(s)**.

This step, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

Author material archive policy

Authors who require the return of any submitted material that is rejected for publication in the journal should indicate on the cover letter. If no indication is given, that author's material should be returned, the Editorial Office will dispose of all hardcopy and electronic material.

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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.

Hardcopies of the Journals and off prints

Under the Journal's open access initiative, authors can choose to download free material (via PDF link) from any of the journal issues from Pertanika's website. Under "Browse Journals" you will see a link entitled "Current Issues" or "Archives". Here you will get access to all back-issues from 1978 onwards.

The **corresponding author** for all articles will receive one complimentary hardcopy of the journal in which his/her articles is published. In addition, 20 off prints of the full text of their article will also be provided. Additional copies of the journals may be purchased by writing to the executive editor.

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QUALITY: Our journals' reputation for quality is unsurpassed ensuring that the originality, authority and accuracy of your work is fully recognised. Each manuscript submitted to Pertanika undergoes a rigid originality check. Our double-blind peer refereeing procedures are fair and open, and we aim to help authors develop and improve their scientific work. Pertanika is now over 35 years old; this accumulated knowledge has resulted in our journals being indexed in SCOPUS (Elsevier), Thomson (ISI) Web of Knowledge [BIOSIS & CAB Abstracts], EBSCO, DOAJ, Google Scholar, AGRICOLA, ERA, ISC, Citefactor, Rubriq and MyAIS.

AUTHOR SERVICES: We provide a rapid response service to all our authors, with dedicated support staff for each journal, and a point of contact throughout the refereeing and production processes. Our aim is to ensure that the production process is as smooth as possible, is borne out by the high number of authors who prefer to publish with us.

CODE OF ETHICS: Our Journal has adopted a Code of Ethics to ensure that its commitment to integrity is recognized and adhered to by contributors, editors and reviewers. It warns against plagiarism and self-plagiarism, and provides guidelines on authorship, copyright and submission, among others.

PRESS RELEASES: Landmark academic papers that are published in Pertanika journals are converted into press releases as a unique strategy for increasing visibility of the journal as well as to make major findings accessible to non-specialist readers. These press releases are then featured in the university's UK-based research portal, ResearchSEA, for the perusal of journalists all over the world.

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

An **Award-Winning** International-Malaysian Journal —MAY 2014

About the Journal

Pertanika is an international multidisciplinary peer-reviewed leading journal in Malaysia which began publication in 1978. The journal publishes in three different areas — Journal of Tropical Agricultural Science (JTAS); Journal of Science and Technology (JST); and Journal of Social Sciences and Humanities (JSSH).

JTAS is devoted to the publication of original papers that serves as a forum for practical approaches to improving quality in issues pertaining to **tropical agricultural research-** or related fields of study. It is published four times a year in **February**, **May**, **August** and **November**.

JST caters for **science and engineering research-** or related fields of study. It is published twice a year in **January** and **July**.

JSSH deals in **research or theories in social sciences and humanities research**. It aims to develop as a flagship journal with a focus on emerging issues pertaining to the social and behavioural sciences as well as the humanities, particularly in the Asia Pacific region. It is published four times a year in **March**, **June**, **September** and **December**.

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