Pertanika Journal of

TROPICAL AGRICULTURAL SCIENCE

JTAS

VOL. 36 (1) **FEB. 2013**

PERTANIKA JOURNALS

A scientific journal published by Universiti Putra Malaysia Press

Journal of Tropical Agricultural Science

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Editorial Board

2013-2015

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URL: http://www.pertanika.upm.edu.my/editorial_board.htm

Publisher

The UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia
Tel: +603 8946 8855, 8946 8854 • Fax: +603 8941 6172
E-mail: penerbit@putra.upm.edu.my
URL : http://penerbit.upm.edu.my

Editorial[i]

Climate Change and Tropical Agriculture: an Uncertain Picture

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The first quantitative predictions of the "hothouse effect" were made by in Sweden by Svante Arrhenius in 1896. Arrhenius correctly predicted a temperature rise of 4°C with a doubling of carbon dioxide concentrations, but, based on the low carbon emissions of the time, thought that this would take many centuries to achieve. Moreover, influenced no doubt by Sweden's cool climate, he thought that this warming would be good for agriculture, writing that "we may hope to enjoy ages with more equable and better climates…when the earth will bring forth much more abundant crops than at present, for the benefit of rapidly propagating mankind."

Despite the accuracy of Arrhenius's calculations, it was not until the 1980s that a global scientific consensus on anthropogenic global warning was established. As climate models have improved since then, increasingly sophisticated projections have been made about the likely impacts on agricultural production. As Arrhenius foresaw, some temperate areas would benefit from longer growing seasons and, perhaps, the still-debated CO2-fertilization effect. In other parts of the globe, crops yields are expected to decline as a result of an increasing frequency and intensity of droughts and other extreme events. Overall, however, it is still a very fuzzy picture, with no agreement on overall global trends.

The uncertainties are greatest in the tropics, particularly in the 'maritime continent' of Southeast Asia, where the intimate mix of sea and land plays havoc with climate models. Here, as in much of the tropics, different climate models give such different predictions for future changes in rainfall that no reliable conclusions can be drawn. Temperature predictions are more robust, but the impacts of warming on tropical crop yields are less well understood than those of drought. Farmers in England need only look south to see their agricultural future, since analogues to their expected future climate already exist in southern France or Spain, but there is nowhere warmer than the lowland tropics to serve as an example. By 2100, and probably by 2050, these areas will experience climates that exist nowhere on Earth today. The best way to assess the likely

impacts of these novel climates on crop yields is by field experiments, but while it is relatively easy to make a growing crop wetter or drier, it is very difficult to create a warmer climate on a realistic scale. Greenhouse experiments are more practical, at least for small plants, but less easy to interpret in terms of final yields.

Clearly we need better climate models, with more accurate representations of tropical rainfall. However, the accuracy of even the best models is constrained by uncertainties in the future emissions of greenhouse gases. The idea that we should try to keep global warming below 2°C seems to have been quietly shelved, with most observers convinced that squabbling politicians will make this target unattainable (if, indeed, it ever was). Most scientists working on impacts and adaptation assume a 3-4°C rise, but these figures are only a little less arbitrary. If we do not stabilize emissions over the next couple of decades, global temperatures will continue to rise indefinitely, with an increasing risk that positive feedbacks will make the process self-sustaining. It is hard to imagine the lowland tropics 6°C warmer than it is now, but that is within the confidence limits of the current climate models and the greenhouse gas scenarios.

Uncertainty is part of being a farmer, with good years and bad years averaging out over time, and high yields in one area compensating for low yields in another. But uncertainty on a global scale is harder to live with. What happens when climate change is unidirectional, rather than fluctuating, and correlated across vast areas? This year's droughts in the United States and their impacts on global food prices may be an example of things to come. Or, perhaps Arrhenius was right, and with intelligent adaptation on the part of farmers and agricultural scientists, "the earth will bring forth much more abundant crops". I hope it is true, but I would not want to bet my children's future on it. As scientists, we need to work on three levels: understanding the impacts of the projected climate changes on tropical crops; adapting agricultural crops and practices to the climate change that now appears inevitable; and doing whatever is necessary to hasten a binding global agreement on limiting future greenhouse gas emissions.

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January 2013

Professor Richard Corlett obtained his first degree from the University of Cambridge in 1974, followed by a PhD in plant ecology at the Australian National University, with fieldwork in the highlands of Papua New Guinea. He has subsequently held teaching posts at the University of Chiang Mai (1980-82), National University of Singapore (1982-87), University of Hong Kong (1988-2008) and National University of Singapore (2008-12). In July 2012, he moved to the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences in Yunnan, to take charge of a new Centre for Integrative Conservation. His major research interests include terrestrial ecology and biodiversity conservation in tropical East Asia, plant-animal interactions, urban ecology, invasive species, and the impacts of climate change. In addition to numerous scientific papers, he is the author or co-author of several books, including "The Ecology of Tropical East Asia", published in 2009 by Oxford University Press, and "Tropical Rain Forests: an Ecological and Biogeographical Comparison", which he co-authored with Richard Primack, with a second edition published by Wiley in 2011. He is a Lead Author for Chapter 24, "Asia", in the Working Group II contribution to the Fifth (2014) Assessment Report of the Intergovernmental Panel on Climate Change (IPCC) and a member of the Steering Committee of the IUCN Species Survival Commission Climate Change Taskforce. He was elected President of the Association for Tropical Biology and Conservation (ATBC) for 2012.

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Pertanika Journal of Tropical Agricultural Science
Vol. 36 (1) Feb. 2013

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PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Review Article

Micropropagation: An Important Tool for Conserving Forest Trees

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ABSTRACT

Forest trees are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. The ever increasing human and livestock populations have put heavy demands for plant products, resulting in over exploitation of forest trees. Therefore, there is an urgent need for conservation of germplasm and also for propagation of a sustainable utilization of forest trees. Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and conservation of elite and rare germplasm. This review provides an overview of the success achieved on *in vitro* work done for a number of important forest trees.

Keywords: Micropropagation, multipurpose, *Albizia lebbeck*, *Leucaena leucocephala*, *Prosopis cineraria*

INTRODUCTION

In the past, forests spread over half of the land surface but due to large scale changes in land use, forests covered only 30 per cent of the Earth land area. In particular, the forest and tree cover in India has been reduced by 21 per cent and hence, the forest policy emphasizes on conserving the natural heritage of the country by preserving the remaining natural forests. Forest trees are renewable sources of food, fodder, fuel wood, timber and other valuable non-timber products. Due to the rapid growth in the population and the human desire to progress, there has been a tremendous reduction in the forest and tree cover from the earth's surface, and thus, the increasing demands for biomass fuel wood, timber and pulp for paper industry can no longer be met from the existing natural resources. Consequently, there is an urgent requirement for a large number of improved

ARTICLE INFO

Article history:
Received: 26 March 2012
Accepted: 12 June 2012

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ISSN: 0128-7702

fast growing trees in shortest duration suitable for agro forestry, fuel, timber, and fodder. In order to maintain and sustain forest vegetation, conventional approaches like grafting, layering and cutting have been used for propagation. Nonetheless, these conventional methods of plant propagation and improvement have limited applicability (Yadav & Singh, 2011a; Yadav *et al*., 2012). In general, trees are slow growing, long-lived, sexually self-incompatible and highly heterozygous plants, and limit the use of traditional breeding methods (Williams & Savolainen, 1996). The major constraint with the conventional methods of tree breeding is that these methods are slow, often lead to virus infected material and are less productive, and hence, they cannot be used efficiently for the propagation of trees.

Forest tree biotechnology emerged during the 1980s, and it encompasses a developing collection of tools for modifying tree physiology and genetics to aid breeding, propagation and research (Burdon & Libby, 2006). Advanced biotechnological methods of culturing plant cells and tissues should provide new means for conserving and rapidly propagating valuable, rare and endangered forest tree species. Over the past two decades, various components have been established individually, but all are still considered under the large umbrella of forest tree biotechnology (FAO, 2004). Micropropagation offers a rapid means of aforestation, multiplying woody biomass, conservation of elite and rare germplasm (Bajaj, 1986; Karp, 1994), regeneration of plantlets from both callus cultures and organ cultures (Chalupa, 1987), shortening germination period and developing single cells into callus (Muir *et al*., 1958). The application of micropropagation techniques as an alternative mean of asexual propagation of important trees has increased the interest of workers in various fields. The technique of cell and tissue culture, under controlled and defined conditions, has contributed in raising new plants, manipulation of plant without conventional breeding mechanism and methods, shortening germination and developmental phase of plants. It thus holds a place of unique importance in today's world among plant biologists (Batra *et al*., 2000). Plant tissue culture is also employed in haploid production, production of disease free and resistant plants, elimination of breeding barriers, biosynthesis of secondary metabolites, generation of variability, germplasm conservation and selection of desirable traits (Pijut *et al*., 1990; Karp, 1994; Roja & Rao, 1998). While the main use of propagation technologies has been for forest establishment or clones, there is also a conservation use for those species that are at risk, rare, endangered or of special cultural, economic or ecological value (Benson, 2003). In general, woody trees are difficult to regenerate under *in vitro* conditions. The sticking constraint in the propagation of trees under *in vitro* conditions is the comparatively poor success with mature explants from adult trees. Most of the trees can be propagated by vegetative means during the juvenile phase. As trees grow and attain maturity, the ability of vegetative propagules to root declines. It is well

established that juvenile tissues facilitate propagation of mature trees. Hence, in order to circumvent these impediments, clonal or vegetative propagation has been deployed. Up to 1975, micropropagation involved regeneration of plantlets from callus cultures only, but later organ culture also became quite popular (Chalupa, 1987). The potential benefits of the micropropagation of elite genotypes for production of clonal planting stock for aforestation/reforestation have long been recognized. Plantlets have been regenerated from both juvenile and mature trees (Dunstan, 1988; Thorpe *et al*., 1991). The current extent of the world's plantation forest area is about 187 million hectares (mha) with the annual planting of 4.5 mha. India is one of the largest hardwood plantation resources comprising about 32.5 mha, with *Eucalyptus*, *Acacia* and *Teak* as major species. The annual planting target of India is about 3.0 million hac.

Commercial applications of micropropagation are however generating increasing interest. The potential is huge although, up to now, only several thousand hectares seem to have been established globally using micropropagated material. An overview of the work carried out earlier on the different woody species of forests is given in the following section.

AN OVERVIEW

During the past decade, major advances have been made in this field and now it has become an industrial technology. Great advances in micropropagation have occurred since Harberlandt's exploration of the concept presented in his landmark paper published in 1902. The pioneering experiments were initiated by the father of tissue culture Gottlieb Haberlandt in 1898, in which he chose single cell isolated from the palisade tissue of leaves, epidermis and epidermal hairs of different plants. He grew them on Knop's (1865) salt solution with sucrose and observed the growth in the palisade cells but could not succeed because of handling with highly differentiated cells and lack of proper techniques. Haberlandt had also perceived the concept of growth hormones, which he called "growth enzymes" and felt that these are released from one type of cells and stimulated growth and developments in other cells. From the time Haberlandt presented his paper in 1902 until about 1934, there was hardly any progress was made in the field of plant tissue culture as conceived by Haberlandt. His pioneering experiments inspired other botanists to conduct further work on the morphogenetic potentialities of the living cells and abilities of tissue and organ to develop into complete plant. Kotte (1922), a student of Haberlandt, in Germany and Robbins (1922) were successful in the establishment of excised plant root tips under *in vitro* conditions. Meanwhile, success in the continuously growing cultures of tomato root tips using sucrose, inorganic salts and yeast extract was achieved by White (1943).

Gautheret (1934) observed the proliferation of callus by culturing cambium cells of *Salix* and *Populous* on Knops' solution. Van Overbeek *et al*. (1942) studied

the stimulatory effect of coconut milk on the embryo development and callus formation in *Datura*. These findings set the stage for the large increase in research for tissue culture from this period, advances such as the eradication of viruses through meristem culture (Morel & Martin, 1952), cultivation of single cells and suspension cultures (Muir *et al*. 1954), auxin and cytokinin basis of organogenesis (Skoog & Miller, 1957), somatic embryogenesis (Reinert, 1959), large scale culture of cells (Tulecke & Nickell, 1960), regeneration of plants from single cell (Vasil & Hildebrandt, 1966), uptake of DNA by cells (Ledoux, 1965) and variability of cells in culture (Lutz, 1969) were made. During *in vitro* culture, various intrinsic and extrinsic factors like culture medium (carbohydrates, growth regulators, agar concentration, pH, etc.), culture conditions (photoperiod, temperature), type of explants and their interactions affect the successful growth and development of plant.

This technology of plant tissue culture offers advantages over conventional methods of propagation for a rapid and large scale multiplication of important plants under *in vitro* conditions, irrespective of the season with conservation of space and time (Nehra & Kartha, 1994; Rao *et al*., 1996). The propagation of some commercial plants, which are difficult to reproduce conventionally by seed or vegetative propagules, is realized by *in vitro* tissue culture technique. Thus, advances in biotechnological research have opened new avenues for rapid multiplication of forest trees. Consequently, a large number of horticultural, plantation and forest species, numbers of important fruit trees and medicinal plant are being propagated *in vitro* on commercial scale (Arumugam & Bhojwani, 1990). A large number of woody trees species have been successfully cultured *in vitro* (Table 1).

For micropropagation, beside proper techniques and requirements, various experimental conditions have also been maintained; these are briefly reviewed as follows.

Nutrition

Growth of plants under *in vitro* conditions is largely determined by the composition of the culture medium. The importance of nutrition in plant tissue culture has been reported by Gautheret (1955). The main components of most plants tissue culture media are mineral salts and sugar as carbon source and water. Other components may include organic supplements, growth regulators and gelling agent (Gamborg *et al*., 1968; Gamborg & Phillips, 1995). Although the amounts of the various ingredients in the medium vary for different stages of culture and plant species, the basic MS (Murashige & Skoog, 1962) and LS (Linsmaier & Skoog, 1965) are most widely used media. Media compositions have been formulated for the specific plants and tissues (Nitsch & Nitsch, 1969). Some tissues respond much better on solid media while others on liquid media. As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. Different culture media

TABLE 1
Woody trees list

Plant species	Explants used	Medium + PGR's + additives used	Reference
Acacia melanoxylon	Node	MS + BAP (1.0 mg/l) + NAA (0.5 mg/l)	Jones & Smith (1988)
Eucalyptus tereticornis	Node	MS + BAP (1.0 mg/l) + NAA (0.1 mg/l)	Das & Mitra (1990)
Acacia nilotica	Cotyledonary node	B5 + BAP (1.5 mg/l)	Dewan *et al*. (1992)
Euonymus europaeus	Seedling cotyledone	MS + IAA (22.8 µM) + Kn (0.046 µM)	Bonneau *et al*. (1994)
Acacia senegal	Axillary bud	MS + BAP (4.0 mg/l) + NAA (0.5 mg/l) + Ads (25.0 mg/l) + AA (25.0 mg/l)	Gupta *et al*. (1994)
Acacia tortilis	Cotyledonary node	MS + BAP (5.0 mg/l) + NAA (0.1 mg/l)	Macreae (1994)
Fraxinus angustifolia	Shoot tip and node	DKW + BAP (4.4 µM) + IBA (0.98 µM)	Perez-parron *et al*. (1994)
Stryphnodendron polyphythum	Cotyledonary node	MS + BAP (13.3 µM)	Franca *et al*. (1995)
Dalbergia sissoo	Node	MS + BAP (4.4 x 10^{-6} M) + NOA (4.4 × 10^{-7} M)	Gulati & Jaiwal (1996)
Anogeissus latifolia	Cotyledonary node and epicotyl	MS + IAA (0.1 mg/l) + BAP (1.5 mg/l) + Ads (25 mg/l) + L-arginine (25 mg/l) + AA (25 mg/l) + citric acid (25 mg/l) + L-asparagine (1.0 mM) + 200 µM (Fe-EDTA)	Shekhawat *et al*. (2000)
Cardiospermum halicacabum	Cotyledon, hypocotyl, cotyledonary node, leaf, internode and node	MS + BAP (17.9 µM)	Babber *et al*. (2001)
Anogeissus pendula	Cotyledonary node	MS + BAP (4.4 µM) + IAA (5.7 µM) + casein hydrolysate (100 mg/1) + AA (50 mg /1)	Saxena & Dhawan (2001)
Tectona grandis	Apical shoot	MS + BAP (0.28 µM) + Kn (0.46 µM) + Ads (0.27 mM)	Gangopadhyay *et al*. (2003)
Acacia sinuata	Node	MS + BAP (8.9 µM) + TDZ (2.5 µM) + Ads (135.7 µM)	Vengadesan *et al*. (2003)
Prosopis laevigata	Cotyledonary node	MS + 2,4-D (9.05 µM) + BAP (6.62 µM)	Gonzalez *et al*. (2007)

Table 1 (*continued*)

Acacia senegal	Cotyledonary node	MS + BAP (1.0 mg /l)	Khalafalla & Daffalla (2008)
Acacia chundra	Shoot tip and node	MS + BAP (1.5 mg/l) + IAA (0.05 mg/l) + Ads 50 mg/l	Rout *et al*. (2008)
Wrightia tomentosa	Cotyledonary node	MS + BAP (5.0 mg /l)	Joshi *et al*. (2009)
Melia azedarach	Node	MS + BAP (5µM)	Husain & Anis (2009)
Spondias mangifera	Node	MS + BAP (1.0 mg /l)	Tripathi & Kumari (2010)
Michelia champaca	Seedling cotyledone	MS + NAA (2.0 mg /l)	Armiyanti *et al*. (2010)
Acacia auriculiformis	Axillary bud	B5 + coconut milk (10%) + BAP (10^{-6}M)	Girijashankar (2011)
Streblus asper	Node	MS + Kn (4.60 µM) + BAP (4.44 µM)	Gadidasu *et al*. (2011)
Terminalia catappa	Node	MS + BAP (2.0 mg /l)	Phulwaria *et al*. (2012)

proposed by the different scientists from time to time vary from each other in terms of their salt concentrations. Some of the earliest plant tissue culture media were developed by White (1943) and Gautheret (1939). All the subsequent media formulations are based on White's and Gautheret's media. The pH of the medium is also an important factor for tissue culture. The pH of the medium is usually adjusted to between 5 and 5.8 before autoclaving and extremes of pH are avoided. Each plant species has different optimized conditions both for growth of the cells and for production of useful products, so it is necessary to optimize the conditions in each case. Humidity in the culture vessel and osmotic potential of the medium affects the growth and development of plantlets *in vitro* in different ways (Brown *et al*., 1976; Ziv *et al*., 1983).

The MS medium was used either as described originally or with little variation and combination of phytohormones and vitamins, such as *Dalbergia latifolia* (Raghavaswamy *et al*., 1992), *Terminalia arjuna* (Kumari *et al*., 1998), *Sapindus mukorossi* (Philomina and Rao, 1999), *Melia azedarach* (Shahzad and Siddique, 2001), *Azadirachta indica* (Shekhawat *et al*. 2002). Raghavaswamy *et al*. (1992) observed that axillary bud initiation in *Dalbergia latifolia* was better on the MS medium while multiple shoot induction was better on Woody Plant Medium (*WPM*) or MS (reduced major salts) medium. Bhargava *et al*. (2003) reported that globular proembryonic mass of callus was formed on the MS medium after 40-50 days of incubation, and then transferred to B_5 medium for fragile snowy callus in *Phoenix dactylifera*. Sharada *et al*. (2003) used the MS

medium for shoot induction and B_5 or WPM medium for root development in *Celastrus paniculatus*.

There are some complex substances like coconut milk (CM), casein hydrolysate (CH), adenine sulphate (Ads), activated charcoal (AC), which are sometimes required in addition to growth hormones for callus induction and regeneration. For instance, the coconut milk of green nut is very effective in providing an undefined mixture of organic nutrients and growth factors (Gamborg & Phillips, 1995). Raghavaswami *et al*. (1992) reported that the growth adjuvants, like coconut milk, casein hydrolysate and adenine sulphate were also supplemented to the media for direct organogenesis and somatic embryogenesis in *Dalbergia latifolia*. Deb (2001) used 200 mg/l of casein hydrolysate for the induction of embryogenic callus from 3-4 days imbibed seeds of *Melia azedarach*.

Fridberg *et al*. (1978) reported that charcoal had an important role during culture by absorbing toxic compounds released by inoculated explants. Pierik (1987) showed that the addition of AC often has a promoting effect on growth and organogenesis in plant species. Charcoal has been used in regeneration medium for trees like *Dalbergia sissoo* (Gulati & Jaiwal, 1996) and *Areca catechu* (Mathew & Philip, 2000) to prevent browning of culture due to phenolic exudation released by the explants. The beneficial effects of activated charcoal were also found on multiple shoot induction from nodal explants of *Wattakaka volubilis* (Chakradhar & Pullaiah, 2006).

During culture, carbohydrates play an important role and act as an energy source required for growth, maintenance and for synthesis of cell constituents. The most commonly used carbohydrate source is sucrose, but other sugar like glucose, fructose, dextrose, mannitol, sorbitol etc. are also occasionally used. Meanwhile, sucrose also has an important role as it serves as a source of carbon and energy. Sucrose is also required for differentiation of xylem and phloem elements in the cultured cells (Aloni, 1980). Glucose and fructose are also known to support good growth of some tissues and are occasionally used. Sucrose represents the major osmotic component of the medium and is necessary for various metabolic activities. In most plants, 2-3% sucrose is found very effective for optimal growth and morphogenesis. MS medium with 2% sucrose was optimal for culturing of shoot tips in *Tamarindus indica* (Kopp & Nataraja, 1990). In *Eucalyptus sideroxylon*, however, it was observed that 4 to 6% sucrose caused more callus formation during culturing of axillary shoots, while 2-6 per cent sucrose in the MS medium supported roots development (Cheng *et al*., 1992). It was found that 3% sucrose is effective for shoot initiation from cotyledonary node explants in *Stryphnodendron polyphythum* (Franca *et al*., 1995). Twenty per cent sucrose concentration is more effective for the development of globular embryos of *Terminalia arjuna* (Kumari *et al*., 1998). In

Alnus nepalensis, 1.5 % sucrose in WPM medium was optimal for shoot proliferation from terminal axillary buds (Thakur *et al*., 2001). Shahzad and Siddiqui (2001) reported that 3% sucrose was required for callus as well as for shoot proliferation in *Melia azedarach*. Similarly, Shekhawat *et al*. (2002) also advocated the use of 2-3% sugar to obtain multiple shoots in *Azadirachta indica*. Likewise, Chakradhar and Pullaiah (2006) reported that 1.0 per cent of sucrose was necessary for the rooting of regenerated plantlets in *Wattakaka volubili*.

Agar-agar is used as a solidifying agent and assumed to be that of neutral support for callus growth and multiplication. Normally, 0.8 percent agar is used for culture medium. Pasqualatto *et al*. (1986) reported a higher concentration of solidifying agent in the medium reduced vitrification, but in certain cases, an increase in agar amount causes adverse effect, as observed by Lal and Singh (1995).

Plant Growth Regulators

Plant growth regulators directly or indirectly affect the growth and differentiation of plant tissues. Different plant growth regulators have different effects and they vary with the type and quantity to be applied. There are five known major classes of compounds with plant growth regulatory activity. These are auxins, cytokinins, gibberellins, abscisic acid and ethylene. Among various growth regulators, auxins (NAA, IAA, IBA and 2, 4-D), cytokinins (BAP, Kinetin, Zeatin), ABA, gibberellins and ethylene are very important. The nature of organogenic differentiation is determined by the relative concentration of auxins and cytokinins. Higher cytokinins to auxin ratio promote shoot formation, while higher auxins to cytokinins ratio favours root differentiation. Therefore, an auxin/cytokinin ratio plays a critical role in the induction of roots and shoots (Skoog & Miller, 1957). Auxins have an essential role in shoot induction and plant regeneration in most plant species. Auxins also induce somatic embryogenesis from the callus of *Citrus sinensis* (Kochba & Roy, 1973). Cytokinins alone, or in combination with auxins has been generally used in tissue culture. Cytokinins has been used in range of (0.5-30mg/l) and higher concentrations bring about morphological abnormalities and cause hyper hydration. Among cytokinins, BAP is the most commonly used in a variety of explants for shoot regeneration. Goyal and Arya (1979) observed the regeneration in *Prosopis cineraria* on MS medium with different concentrations and combinations of Kinetin, IAA, IBA and BAP. Gamborg's medium was used by Mukhopadhayay and Mohan (1981) for culturing of *Dalbergia sissoo*. Meanwhile, Rumary and Thorpe (1984) reported that in some cases, mixed cytokinins have beneficial role. Multiple shoots were obtained in *Eucalyptus grandis* on the MS medium supplemented with additional thiamine (Lakshmi Sita & Shobha Rani, 1985). It has been reported that the decrease in NAA ensures shoots formation (Rao *et al*., 1984; Sudha Devi & Natreja, 1987). Mittal *et al*. (1989) obtained multiple shoots from the axillary buds of

Acacia auriculiformis on Gamborg's (B_5) basal medium supplemented with coconut milk and BAP. Kopp and Nataraj (1990) regenerated plantlets by supplementing 2.0 mg/l BAP in *Tamarindus indica*. Multiple shoots were obtained from cotyledonary nodes of *Dalbergia latifolia* on MS medium fortified with (2.0 mg/l) BAP (Lakshmi Sita & Raghavaswamy, 1992). BAP also produced longer shoot as compared to kinetin in *Prosopis cineraria* and *Aegle marmelos* (Kumar & Singh, 2009; Yadav & Singh, 2011b), as shown in Fig.1b, 1c, and 1d.

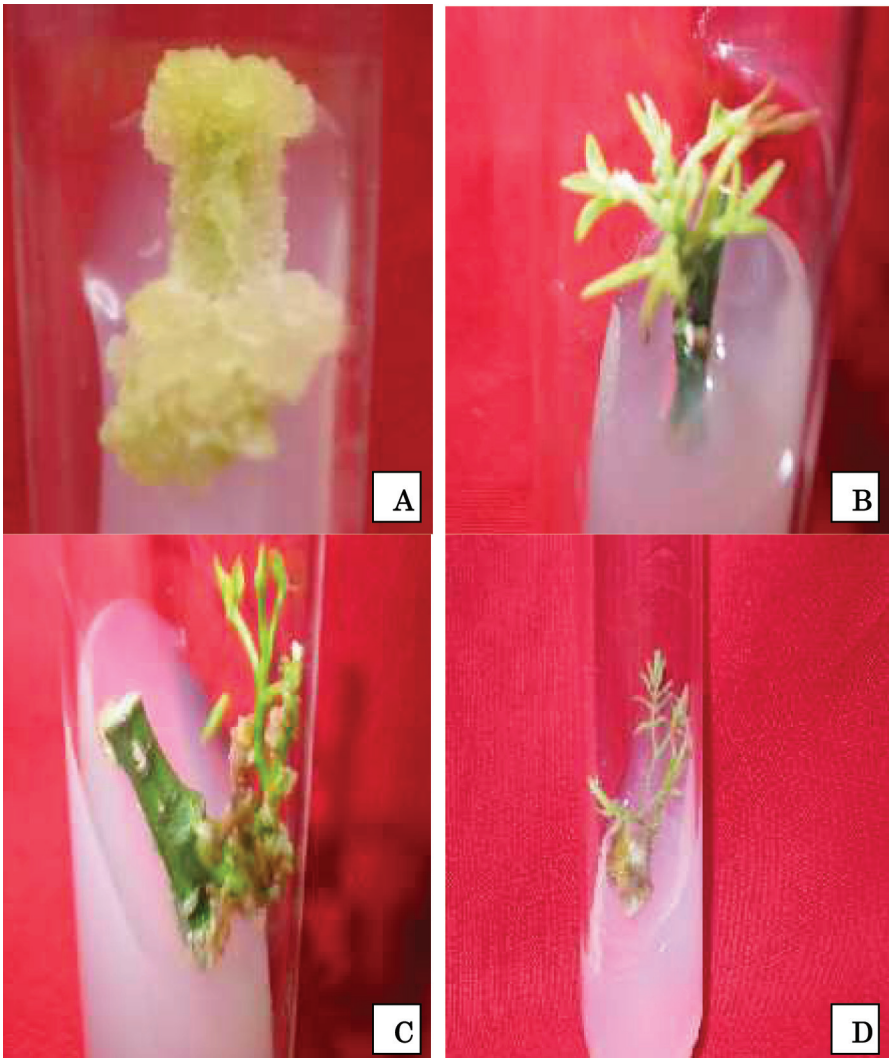

Fig.1: A. Callus induction from internodal segment of *Albizia lebbeck* on MS medium + BAP (2.0 mg/l) + NAA (0.5 mg/l); B. Shoot bud initiation on MS medium + BAP (2.0 mg/l) in nodal explants of *Aegle marmaelos*; C. Shoot formation from nodal explants of *Aegle marmelos* on MS medium with 2.0 mg/l BAP + 1.0 mg/l IAA; D. Callus growth and shoots proliferation from nodal explants of *Prosopis cineraria* on MS medium supplemented with BAP (2.0mg/l).

The MS media supplemented with BAP in combination with NAA supported the highest percentage of callus induction in *Leucaena leucocephala* (Singh & Lal, 2007) and *Albizia lebbeck* (Yadav & Singh, 2011a) (see Fig.1a). In *Eucalyptus camaldulensis*, the highest frequency of somatic embryos were produced from the callus obtained on the MS medium supplemented with 0.5 mg/l of BAP and 0.1 mg/l NAA from mature zygotic embryos (Prakash & Gurumurthi, 2010).

Selection of the Explants

The success in micropropagation from mature plants depends upon the careful selection of explants (Murashige, 1974; Sommer & Caldas, 1981; Williams & Maheswaran, 1986). In *Albizia lebbeck*, stem, root, leaf, rachis, leaflets, hypocotyls and axillary buds were used for regeneration (Arya *et al*., 1978; Gharyal & Maheshwari, 1990). Several factors influence the behaviour of the inoculum in culture (Murashige, 1974). These factors include:

a. The organ that serves as tissue source;
b. The physiological and ontogenic stage of the organ;
c. Season in which explant is obtained;
d. Size of the explants;
e. Overall quality of plant from which explants are to be taken

Huda *et al*. (2007) studied the *in vitro* morphogenic responses of different explants of *Corchorus olitorius*. The different explants viz. leaf segments, internodes and nodal segments showed different morphogenic responses. The nodal segments initiated callus earlier than leaf segments and the internode explants and higher amount of callus were obtained from the leaf segments than internodes and nodal segments. It was also reported by many workers that the quality of explants primarily determines the establishment of *in vitro* culture (Keathley, 1984). The nodal explants from a mature tree of *Heavea brasiliensis* failed to produce plantlets, while the explants taken from 6 to 8 weeks old plant regenerated plantlets (Rehman *et al*., 1981). Similarly, the explants from mature trees of *Eucalyptus citriodora* required pre-treatment for induction of shoot buds but the explants from seedlings did not require any pre-treatment (Gupta *et al*., 1981). Gulati and Jaiwal (1996) reported that the nodal explants taken from coppied shoots of mature *Dalbergia sissoo* exhibited the least phenolic exudation and responded better shoot regeneration, but this was not observed in the explants taken from mature trees. Callus formation and regeneration of plantlets from nodal explants was reported by Nandwani and Ramawat (1991) in *Prosopis juliflora*. Swamy *et al*. (1992) used the nodal explants of *in vitro* grown root suckers from the 60-80 years old tree of *Dalbergia latifolia* for direct organogenesis. In *Fraxinus angustifolia*, shoot tips and nodal segments were used for micropropagation (Perez-parron *et al*., 1994). The maximum number of shoots (9 shoots per explant) in the *Aegle marmelos* from nodal segments were obtained on

the MS medium supplemented with BAP (8.8µM) + IAA (5.7µM) by Pati *et al*. (2008). In *Melia azedarach*, multiple shoots were produced from the nodal segments on the MS medium supplemented with 5 µM of BAP (Husain & Anis, 2009). Tripathi and Kumari (2010) obtained an efficient *in vitro* propagation of *Spondias mangifera* using nodal explants from seedlings.

In addition, season was also found to affect the shoot proliferation and explants contamination. Seasonal conditions at the time of explants collection may influence the *in vitro* growth of explants, phenolics exudation and degree of contamination. The nodal segments of *Eucalyptus tereticornis* collected during July to September were more responsive to micropropagation because of the negligible phenolic exudation from explants as compared to that collected in October-November and May-June due to the high amount of phenolic exudation (Das & Mitra, 1990). Similar effects of season have also been noticed in other plants like *Tactona grandis* (Gupta *et al*., 1980) and *Eucalyptus tereticornis* (Das & Mitra, 1990). Bonneau *et al*. (1994) observed a higher percentage of embryonic callus production from the zygotic embryo explants in *Euonymus europacus* taken during May to September. Similarly, Thakur *et al*. (2001) observed an optimal establishment of axillary and terminal buds of *Alnus nepalensis* cultured during February and March; thereafter, the percentage establishment showed a declining order. Singh and Goyal (2007) reported that the August to October season was the best for explant collection in *Salvadora oleoides* throughout the year. The harvesting time of pods also showed a significant effect on the *in vitro* germination of seeds. Yadav and Singh (2011a) recorded the highest germination (83.3%) for the seeds extracted from dark-yellow pods in *Albizia lebbeck*.

Meanwhile, the size of the explants plays a key role in expressing the morphogenetic potentiality. Among other, Okazava *et al*. (1967) reported that small explants are more likely to form callus while larger explants maintain greater morphogenetic potentiality. This may be due to the available food reserves and growth regulators which have been proven to be useful in the initiation of new growth (Anderson, 1980). The orientation of the explants also plays an important role in giving morphogenic response. The horizontal position of the explants has been reported to promote adventitious shoot formation in many higher plants (Frett & Smagula, 1983; Pierik, 1987).

Cultural Conditions

Light is an important factor for the success of a tissue culture experiment. The intensity, quality and extent of daily exposure of light are the determining factors in the plant tissue culture. Cultures are usually maintained at a constant temperature of 25±2ºC and a photoperiod of 16 hours of light (20 µmol m^{-2} s^{-1} photosynthetic photon flux intensity) and 8 hours of darkness.

Gupta *et al*. (1981) reported multiple shoots production when the terminal buds from twenty years old tree of *Eucalyptus*

citridora were cultured on the MS medium at 15^{0}C in continuous light, followed by culture at 25^{0}C with 16 hours photoperiod. The effects of light and cytokinins interaction on the cultured cotyledon explants of *Radiata pine* were studied by Victor *et al*. (1984). In *Eucalyptus tereticonis*, a high rate of multiplication was achieved on the MS medium at a slightly higher temperature (30-32^{0}C) (Das & Mitra, 1990). Calleberg and Johansson (1993) studied that direct regeneration was mostly stimulated when the anther cultured was incubated at 20^{0}C. The formation of multiple shoots at 25±2^{0}C and 16 hours of light and 8 hours of dark periods under light intensity of 3000-4000 lux has also been reported in *Azadirachta indica* (Shekhawat *et al*., 2002).

Organogenesis

It involves the formation of organized structure like shoot and root from pre-existing structure, i.e. unorganized mass of cells known as callus. The controlled organogenesis under *in vitro* was given by White (1939) who obtained the shoots from the callus of *Nicotiana glauto* and *N. Iongsodorffi* hybrid on a agar-agar solidified medium. Later on a number of reports approved, depicting the formation of shoots and roots either directly from the explants or indirectly, i.e. from the callus.

Organogenesis deals through two pathways, i.e. direct pathway and indirect pathway. Direct pathway occurs through the continuous development of shoot meristems activity from lateral or axillary buds. Indirect pathways deal with the shoot formation *via* callus formation. Indirect regeneration often results in somaclonal variation making the strategy less desirable for large scale clonal multiplication. Therefore, direct regeneration without a callus phase is a reliable method for clone production.

Direct Organogenesis

Direct organogenesis, i.e. without callus formation, has also been reported in many herbeaceous and tree species. Several limitations, such as low shoot proliferation in forest trees, excessive phenolic exudation (Linington, 1991), basal callusing (Marks & Simpson, 1994), vitrification (Monsalud *et al*., 1995), and shoot tip necrosis (Bargchi & Alderson, 1996) are pronounced in tree tissue culture. Further, difficulty in rooting (Harada & Murai, 1996) has also had a negative effect on micropropagation of woody forest tree species.

Micropropagation without an intervening callus phase is advantageous over conventional vegetative propagation in terms of quantity, quality and economics (Altmann & Loberant, 1998). In general, three modes of *in vitro* plant regeneration have been in practice, namely, organogenesis, embryogenesis and axillary proliferation. The difference mainly matters when it relates to the genetic stability of the resulting micropropagated plants, and the obvious option would be the axillary and adventitious shoot proliferation. Meanwhile, *in vitro* micropropagation has been proven in the recent past as a means for supplying planting material for forestry (Ahuja, 1993; Lakshmi Sita & Raghavaswamy,

1998). Multiple shoots could be induced from the nodal segments of *Eucalyptus grandis* (Cresswell & Nitch, 1975). The use of a protocol to promote axillary and apical shoot bud proliferation *in vitro* has been used for the propagation of forest tree species. The multiple shoots of Himalayan oaks were induced from intact embryos and cotyledonary nodes of Himalayan oaks (Purohit *et al*., 2002). A method for adventitious shoot regeneration from leaf explants of micropropagated *Peach* shoots has been developed (Gentile *et al*., 2002). This method involves the utilization of shoot tips, lateral buds and small nodal and internodal cutting as explants and it genetically establishes stable culture without any callus formation. Mittal *et al*. (1989) observed the formation of multiple shoots from the axillary buds from the *in vitro* grown seedlings of *Acacia auriculiformis*. Kopp and Natraraja (1990) regenerated plantlets from shoot tip explants of *in vitro* grown seedlings of *Tamarindus indica.* Das and Mitra (1990) reported 18-22 shoots per explants in *Eucalyptus tereticornis* when nodal explants were inoculated on the MS medium with BAP (1.0 mg/l) and NAA (0.1 mg/l). Singh *et al*. (1993) observed the sprouting of axillary bud in *Acacia nilotica* on MS and WP medium fortified with BAP (1.0 mg/l).

Kumar and Seeni (1998) achieved a rapid clonal multiplication of *Aegle marmelos* by enhancing axillary bud proliferation in single node segment of a twenty five years old tree on the MS medium supplemented with BAP (2.5mg/l) in combination with IAA (1mg/l). Komalavalli and Rao (2000) established the *in vitro* propagation protocol of *Gymnema sylvestre* - a multipurpose plant. The MS medium fortified with growth regulators such as BAP (0.5mg/l) in combination with NAA (0.01mg/l) has been reported to give optimum results in *Utleria salcifolia* (Gangaprasad, 2003). Kumari *et al*. (2005) reported multiple shoot formation from the nodal and shoot tip explants in *Wedelia chilensis* under *in vitro* conditions. Rathore *et al*. (2005) developed protocol for the *in vitro* propagation of *Maerua oblongifolia* using nodal shoots segments on MS medium and achieved a high rate of shoot multiplication. Thidiazuron (TDZ) was used at 1.0 mM and found to be the most effective in inducing bud break and growth, and also in initiating multiple shoot proliferation at the rate of 25 microshoots per nodal explant with axillary buds, after 4 weeks of culture (Ahmad & Anis, 2007). Rathore and Shekhawat (2009) reported 35–40 shoots per culture vessel shoots proliferated on the MS medium supplemented with 4.44 lM BA and 0.57 lM indole acetic acid (IAA) and additives in *Pueraria tuberosa*. The multiple shoot formation was observed to be the highest in the MS fortified with 2 mg/L Benzyl amino purine (BAP) and 0.1 mg/L Naphthalene acetic acid (NAA) in *Acacia auriculiformis* (Girijashankar, 2011).

Indirect Organogenesis

Lakshmi Sita and Vaidyanathan (1979) raised plantlets from the cotyledonary callus of *Eucalyptus citriodora*.

Adventitious shoot regeneration was reported through the callus culture in *Eucalyptus camaldulensis* taken from the shoot culture of mature tree (Murlidharan & Mascarenhas, 1987). Inamdar *et al*. (1990) reported the formation of somatic embryos via callusing from culture of shoot apices of adult *Crataeva nurvala* on MS medium containing 2, 4-D. The callus cultures and plantlet formation *in vitro* were reported in *Prosopis juliflora* by Nandwani and Ramawat (1991). Joshi and Dhar (2003) obtained the maximum shoots from epicotyle explants that were cultured on the MS medium supplemented with (0.25 µm) NAA and (1.0 µm) Kinetin in *Saussurea obvallata*. Thomas and Philip (2005) reported a high frequency shoot organogenesis from the leaf derived callus of *Dalbergia sissoo*. Similarly, Faisal and Anis (2005) developed a protocol for high frequency shoot regeneration and plant establishment of *Tylophora indica* from petiole derived callus. Organogenic Callus was developed from the stem explant of *Ruta graveolens* on the MS medium composed of 2.5 µm BA + 10 µm 2-4-D. Deepa *et al*. (2006) noticed profuse callusing from cotyledon and shoot tip explants in *Pseudarthria viscida* on the MS medium supplemented with 2,4-D (1.5-2 mg/l) and BAP (1-1.5 mg/l). Agrawal and Sardar (2006) described a high frequency shoot regeneration through leaflet and cotyledon derived calli in *Cassia angustifolia*. The MS medium, supplemented with BAP (1.0 mg/l) and IBA (0.5 mg/l), was found to be the most effective combination for shoot bud differentiation in *Jatropha curcus* (Rajore & Batra, 2005). Meanwhile, subsequent shoot regeneration was achieved in the MS medium supplemented with BAP (2mg/l).

The occurrence of genetic variation is a matter of great variation concern where commercial success in micro propagation is dependent mainly on the maintenance of clonal uniformity (Bonga, 1987). However, abnormalities in the tissue culture and the plants produced from them often increase in frequency with the increase in the culture passages.

Rooting of In vitro Regenerated Shoots

For the development of a perfect plantlet, it is essential that the regenerated shoots must develop the roots. The culture medium for rooting varies from tissue to tissue, as well as from species to species. The shoots of certain plant species developed root only on the simple MS medium, i.e. hormone free media, as reported in *Cardiospermum halicacabum* (Babber *et al*., 2001). Among the auxins, IBA with MS medium is the most commonly used to induce rooting (Raghavaswamy *et al*., 1992; Nandwani & Ramawat, 1993; Shahazad & Siddqui, 2001). Ahmed *et al*. (2007) used different concentrations of IBA, NAA and IAA for rooting and the highest rooting percentage (97.66%) was reported on the MS medium with 0.1 mg/l IAA. This shows that IBA is better than NAA and IAA for shoot and root formation. As for the effect of NAA on root formation, data indicated that the percentage of shoots formed roots was 87%

on the MS basal medium without plant growth regulators as compared to the MS basal medium supplemented with NAA at low concentrations of 0.01 and 0.1 mg/l NAA, where the root percentage was 80%, 73% and 53%, respectively. On the other hand, NAA at 1.0 and 1.5 mg/l did not help shoots to form roots. The shoots must be transferred to a rooting medium which is different from the shoot multiplication medium, particularly in its hormonal and salt composition. However, the nutritive medium for rooting varies from tissue to tissue, as well as from species to species. Shoot multiplication was induced on full strength MS medium whereas the salt concentration was reduced to half (Garland & Stoltz, 1981; Zimmerman & Broome, 1981) or a quarter (Skirvin & Chu, 1979) for rooting. Most frequently, IAA, IBA and NAA (0.1-1.0 mg/l) have been used for this purpose; however, IAA and IBA were found to be more effective (George & Sherrington, 1984). In *Moringa pterygosperma,* half strength MS medium supplemented with GA_3 (0.2 mg/l) produced roots within 7 days in 25% cultures. However, a better rooting was developed with 3% sucrose and IBA (0.2 mg/l) only (Mohan *et al*., 1995). Sharma and Padhya (1996) observed root induction in *Crataeva nurvala* within 7 days on the MS medium with a low dose of NAA (0.5 µm). Mustafa and Hariharan (1997) got limited root in the hormone free medium whereas NAA alone was better than the combination of NAA and IBA in *Alpinia galangal.* Sharada *et al*. (2003) achieved 85% rooting on McCown medium (WPM) containing IBA (5 x 10^{-6}M) in *Celastrus paniculatus.* The high percentage of root regeneration in adventitious shoots was obtained on the MS medium supplemented with 0.1 mg/l IAA in *Populus ciliata* (Thakur *et al*., 2005). Sayd *et al*. (2010) reported that all strengths of MS-medium with 1g/l AC produced rooted shootlet but the quarter strength of MS-medium produced the highest rooting, leaf number and shootlet length. *In vitro* developed microshoots were rooted on the MS half strength medium supplemented with 2.46 µM IBA in *Streblus asper* (Gadidasu *et al.,* 2011).

Hardening of Regenerated Plantlets

After rooting, hardening of regenerants prior to transfer in the soil increases the survival rate of transferred plants. So, it is a step which gradually acclimatizes the plant to the harsh natural environment. Spraying, misting and covering with the thin polythene may serve to fulfil the above objective. Various types of substrates have been used during acclimatization such as soil vermiculites mixture sterilized sand and soil (Goyal & Arya, 1981; Gulati & Jaiwal, 1996; Philomina & Rao, 1999; Thakur *et al*., 2001; Sunaina & Goyal, 2000). The *in vitro* developed plantlets of *Dalbergia latifolia* were successfully transferred by Raghavaswamy *et al*. (1992) by keeping the roots in tap water and high humidity. After this, the plantlets were exposed to fresh air for a few hours daily and after 14 days they were transferred into 1:3 courses sand:soil mixture. The plantlets of

4-5 cm long with fresh leaves were slowly transferred to field conditions (Chaudhary *et al*., 2004). Sharma *et al*. (2006) transferred well-developed plantlets with a complete root system into pot containing sterile vermiculite and covered with transferred polythene bags to ensure high humidity. The serving plants were transplanted to field after 2 months. The most crucial step in the micropropagation is the hardening and acclimatization as it is the process which makes the plantlets capable of tolerating the natural environmental conditions. Complete regenerated plantlets with sufficient roots were taken out from the test tubes and washed several times with sterile distilled water to remove traces of the MS medium by putting the roots under continuous slow running water with the help of fine brush. Then, the *in vitro* regenerated plantlets were transplanted in small earthen pots containing sterilized soil and sand mixture (3:1). Each pot was covered with polythene bags with small holes to maintain high humidity and they were kept in the culture room to get acclimatized. The plantlets were initially irrigated with half strength (salts only) MS medium without sucrose on alternate days. The plantlets were exposed to the conditions for natural humidity for 3-4 hours daily to after 10 days of transfer. After about 30 days, the plants were transferred to bigger pots in the greenhouse and were maintained under natural conditions of day length, temperature and humidity. Finally, the plants were transferred to the field conditions. Successful acclimatization and field transfer of *in vitro* regenerated plantlets have also been reported in *Tamarindus indica* (Kopp & Nataraja, 1990), *Eucalyptus tereticornis* (Das & Mitra, 1990), *Prosopis juliflora* (Nandwani & Ramawat, 1991), *Dalbergia latifolia* (Raghavaswamy *et al*., 1992), *Thevetia peruviana* (Kumar, 1992), *Alpinia galangal* (Anand & Hariharan, 1997), *Terminalia arjuna* (Kumari *et al*., 1998), *Sapindus mukorossi* (Philomina & Rao, 2000), *Salvadora persica* (Mathur *et al*., 2002), *Bupleurum disticho-phyllum* (Karuppusamy & Pullaiah, 2007), *Spondias mangifera* (Tripathi & Kumari, 2010), *Acacia auriculiformis* (Girijashankar, 2011), *Streblus asper* (Gadidasu *et al.,* 2011). Hardening and acclimatization of plantlets were done because the plantlets raised under *in vitro* conditions on the synthetic carbohydrate supplemented medium under artificial light failed to abruptly acclimatize to the rigour of the natural environment. So, a careful transfer of the plantlets in the soil after hardening and acclimatization is required.

CONCLUSION

Tissue culture offers unparalleled opportunity for forest tree improvement. Micropropagation techniques have been applied to a wide range of tree species. Successful *in vitro techniques* are dependent upon the strong and intricate interactions between the explant, plant growth regulators, culture conditions, and genotype. The present status of tree tissue culture, however, is adequate to initiate commercialization

programmes. Commercialization has already been proven to be successful in the cases of some trees like Eucalyptus, Rosewood, Poplar and others. In a commercial scale, the improved protocols should be working efficiently with various genotypes, preferably of the mature origin. Meanwhile, the establishment of protocols with reduced steps of developmental pathways may significantly reduce the time and cost. Most of the tree tissue culture research has centred on methods, primarily on the development of culture media and techniques to induce juvenility in trees for micropropagation. The thrust is towards methods, which have commercial use and the potential for patents. Also, unless the cost of plantlet production by tissue culture techniques is brought down considerably to match with or less than the conventional methods of propagation, the efforts are not worthy enough to justify. Nonetheless, clonal fidelity in trees micropropagated by organogenesis has not been adequately tested with many species. Research on micropropagation will stress on the mass propagation of mature trees and reduction of costs. Research on micropropagation by somatic embryogenesis has to be intensified so as to reach the ultimate goal of mass propagation since it also allows automation which will in turn reduce the cost per propagule. Continued research into the use of biotechnology will contribute to improving the vegetative propagation of the species, which will ultimately play an important role in future breeding programmes of forest tree improvement and reforestation programmes.

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Effects of Low Water Input on Rice Yield: Fe and Mn Bioavailability in Soil

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ABSTRACT

Soil fertility and water condition are the main concerns in rice production. In order to determine the effects of low water input on rice production and soil chemical properties, the Fe and Mn contents, and soil pH in soil were measured during rice cultivation. It was found that rice yield and yield parameters obtained were not significantly different under different water levels. Soil pH was moderately acidic to near neutral. Meanwhile, iron (II) in soil extract slowly increased throughout the rice growing period but it increased markedly after the water was drained off. Manganese availability significantly increased after flooding, but it decreased at a similar trend followed after that, followed by a stable level. In addition, weekly data showed no significant differences in Fe(II) and Mn in the soil extract of the different treatments. These results suggest that low water input does not affect rice production as well as soil pH and Fe(II) and Mn bioavailability in soil.

Keywords: Rice, low water irrigation, plant nutrients, soil pH

INTRODUCTION

Rice is the staple food of Asia with nearly 90% of the world's rice is produced and consumed in this region, providing an average of 32% of the total calorie intakes (Maclean *et al*., 2002). In more specific, out of about 576 million tons rice produced globally per year, 90–91% is produced and consumed in Asia (IRRI, 2002). About 75% of the global rice is produced in the irrigated lowlands (Maclean *et al.,* 2002). Nonetheless, water for agriculture is becoming increasingly scarce (Rijsberman,

ARTICLE INFO

Article history:
Received: 1 February 2011
Accepted: 3 March 2012

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ISSN: 1511-3701

2006). It is predicted that by 2025, 15-20 million ha of irrigated rice will suffer from some degree of water scarcity (Tuong & Bouman, 2003; Tuong *et al.,* 2005). In Malaysia, the overall water demand grows at the rate of 4% annually and is projected to be about 20 billion m^3 by 2020 (Keizrul & Azuhan, 1998). The decreasing water availability for agriculture, especially in rice cultivation has threatened the productivity of the irrigated rice ecosystem and thus, ways must be sought to save water and to increase the productivity of rice (Guerra *et al.,* 1998).

The concentration of water-soluble Fe(II), which is negligible in upland soils, increases in flooded rice soils. Thus, wetland rice suffers iron deficiency less frequently than dryland rice. Electrons (e^-) transferred and hydrogen ions (H^+) consumed through biological activity under reduced soil conditions cause Fe(III) and Mn(III, IV) to be reduced to Fe(II) and Mn(II) forms (Patrick & Turner, 1968). Meanwhile, soil pH is one of the main causes that affects and controls Fe and Mn concentrations in flooded rice soil (Ponnamperuma *et al.,* 1973). To date, the influences of flooding on the physical, chemical and electrochemical properties of soil have been comprehensively researched on and reviewed from time to time (Narteh & Sahrawat, 1999; De Datta, 1981), but less attention has been paid on the effects of low water irrigation on the chemical properties of soil in relation to rice production. Therefore, the current study focused on determining the effects of low water input on rice yield, as well as on Fe and Mn bioavailability in soil solution.

METHODS

In this study, rice (variety MR219) plants were grown in a cylindrical culvert (90 cm in diameter and 90 cm in height) having five different water levels, namely, W1 (continuous flooding at 5 cm), W2 (continuous flooding at 1 cm), W3 (continuous flooding at 5 cm in the first 3 weeks followed by 1 cm), W4 (continuous flooding at 5 cm in the first 6 weeks followed by 1 cm), and W5 (continuous flooding at 5 cm in the first 9 weeks, followed by 1 cm), with five replications. These water levels were maintained by a plastic regulator attached to the culvert wall. Meanwhile, seed rates, fertilizer, agronomic practices were applied according to MARDI (2001). The soil was of silty clay in texture, with 1.2% sand, 44.5% silt and 54.3% clay, a soil pH of 6.0, and an organic matter of 4.12%. The soil also contained 113 mg/kg of Fe and 35 mg/kg of Mn. The soil extracts were collected every week using an SPS200 water sampler (TECNO, 2008), and then analyzed for Fe^{2+} and Mn using an atomic absorption spectrophotometer. A portable Mettler Toledo MP120 pH meter was used to measure the soil pH *in situ* every week. In addition, the electrode of pH meter was calibrated each time before using, while soil pH was measured directly from the soil. The means were compared using Duncan's Multiple Range Test (DMRT) at 5% level using the Statistical Analysis System software version 6.12**.**

RESULT AND DISCUSSION

The Effects of Low Water Irrigation on Rice Yield

The results indicated that low water input did not affect rice yield as well as yield parameters (Table 1). Rice yield containing 14% moisture was in the range of 0.98 to 1.10 kg/m^2, and this is consistent with the finding by MARDI (2001). Bouman and Tuong (2001) stated that water savings under saturated soil conditions were on average (23%) with the yield reductions of only 6%. Soil water condition at the saturated level reduced rice yield about 5% and saved about 35% of the total fresh water as compared to the flooded conditions (Tabbal *et al.,* 2002). Recently, Khairi *et al. (*2011) found that rice could be grown on saturated soil condition without affecting rice yield. As compared to the above results, the findings of the current work suggested that continuously maintaining the water level at 1 cm did not affect yield (Table 1) and it was possible to save >30% of fresh water used in continuous 5 cm flooding condition (data not shown). These results suggest that rice can be cultivated under 1 cm flooding condition without affecting rice yield.

Soil pH

Weekly *in situ* soil pH data showed that the different flooding levels showed no significant effect on the soil pH during rice cultivation (see Fig.1). In other words, soil pH remained in the range of 5.4 to 6.6 throughout the rice growing period. Meanwhile, soil pH at lower acidic to neutral conditions makes available most of the plant nutrients for plant uptakes (Jensen, 2010). Application of fertilizer temporarily increased soil acidity for a short period before it decreases soil acidity by the following week (Fig.1). Flooding may initially decrease soil pH due to CO_2 that is formed in aerobic respiration by bacteria

TABLE 1
The effects of different water levels on rice yield and yield components

Treatments	Tiller number /pot	Panicle number /pot	Unfilled grain /panicle	Filled grain /panicle	1000 seeds weight (g)	Dry straw (kg/m^2)	Dry filled grain (kg/m^2)
W1	384a	361a	22a	93a	27.6a	1.3a	1.01a
W2	381a	365a	25a	90a	27.3a	1.3a	0.98a
W3	377a	345a	24a	91a	27.7a	1.2a	1.01a
W4	390a	359a	22a	94a	27.4a	1.2a	0.99a
W5	388a	352a	21a	95a	27.5a	1.2a	1.01a

Means with the same letter are not significantly different in the column at P≤0.05 by DMRT

and increase after the first few weeks to 6.7 - 7.2 (Ponnamperuma, 1972). The subsequent increase in pH over time is due to the consumption of H^+ ions because of Fe (III) reduction (Kirk, 2004). This result indicates that low water input may not affect soil pH in soil.

Effect of low water input on bioavailability of Fe and Mn

After submergence, hydrated Fe^{3+} oxide is reduced to Fe^{2+} (Ponnamporuma, 1977). Fig.2a shows that Fe^{2+} concentration gradually increased in the soil solution

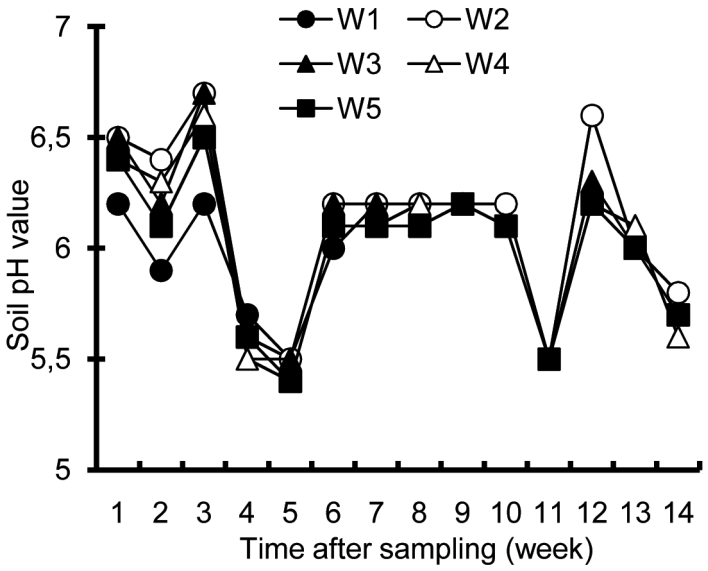

Fig.1: The effects of different flooding levels on *in-situ* soil pH.
A) Soil pH values are more or less similar in all the treatments with time.

TABLE 2
The effects of different water levels on iron and manganese in soil and straw

Treatments	In Straw				In Soil					
	Fe (%)		Mn (mg/kg)		Fe (mg/kg)			Mn (mg/kg)		
	51 DAS	AH	51 DAS	ALP	ALP	51 DAS	AH	ALP	51 DAS	AH
W1	0.115a	0.125b	755a	668a	102a	274a	271ab	271ab	38a	25a
W2	0.12a	0.123b	640a	690a	114a	270a	267ab	267ab	40a	28a
W3	0.113a	0.138ab	711a	740a	128a	279a	275a	275a	38a	26a
W4	0.11a	0.163a	757a	651a	123a	284a	259ab	259ab	38a	27a
W5	0.115a	0.143ab	766a	673a	98a	288a	245b	245b	38a	27a

Means with the same letter are not significantly different in the column at P≤0.05 by DMRT

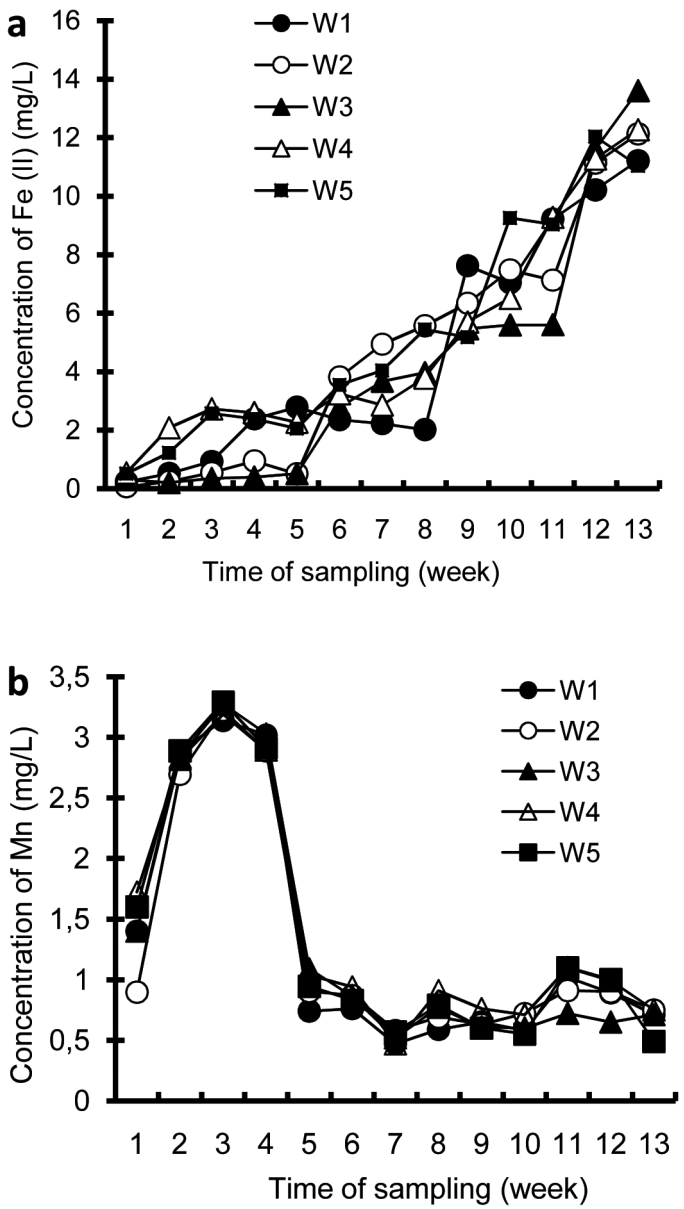

Fig.2: The temporal changes of the iron and manganese concentrations in the soil extract
a) Fe^{2+} concentrations increased slowly with time but increased markedly after water was drained out. Weekly data showed no differences; (b) Mn concentrations increased markedly after flooding but it then decreased again to a stable level.

with increasing time until the ripening stage. This result is consistent with the previous results that the duration of submergence influences iron content in soil as high as 300 mg/L after 4 weeks submergence (Yoshida, 1981). Ponnamperuma *et al.* (1973) also stated that Fe^{2+} concentration increased gradually in the soil with pH 6 but increased markedly after flooding in soil with pH 5.5. This result indicates that soil pH may affect the Fe^{2+} concentration in flooded soil. In this experiment, the Fe^{2+} concentration in the soil extract did not increase markedly after flooding the soil but it gradually increased throughout the rice growing period (Fig.2a). This result suggests that flooding increases Fe^{2+} concentration in soil. In addition, iron content in soil was found to be higher at 51 days after sowing (DAS) and after harvest (AF) than that of after land preparation (ALP), as shown in Table 2. This result also suggests that flooding increases Fe^{2+}concentration in soil with time. Plants accumulate higher content of iron at the reproductive stage than that of the vegetative stage (Table 2). The results of the current study indicate that low water input (i.e. flooding at continuous 1 cm) may not affect the availability of the Fe^{2+} content in the soil solution as compared to the traditional flooding at continuous 5 cm.

In the soil extract, the Mn concentration increased markedly after submergence, followed by an equally rapid decline to a stable level throughout the growing period (Fig.2b). This result is consistent with the previous result of Jugsujinda and Patrick (1977), Redman and Patrick (1965) and Cho and Ponnamperuma (1971). This may due to the effects of flooding and poor aeration that increase the availability of manganese (Chen *et al.,* 2005). Fig.2b also shows that different water inputs did not affect Mn content in the weekly data. In soil, Mn concentration increased further after flooding than that of after ripening and the plants also accumulated higher content of Mn at the vegetative stage than the ripening stage (Table 2). The current study showed that no deficiency of Mn was found in soil and this could be due to the effect of soil pH which was above 5.5, and this finding is consistent with that of Bolan *et al.* (2003). The finding of this study also suggests that low water irrigation did not affect availability of Mn in soil.

CONCLUSION

In conclusion, the above results confirmed that flooding at continuous 1 cm did not affect rice yield and yield components, soil pH and Fe and Mn bioavailability. The authors recently stated that maintaining saturated condition throughout the growing period did not show effect on rice yield. Therefore, low water input rice production could be implemented to save fresh water to be used for other sectors and to increase the country's rice production. Nonetheless, further research is needed to justify the field research with low water input and to cope with plants under soil and environmental stress conditions.

ACKNOWLEDGEMENTS

This work was supported by the Ministry of Science, Technology and Innovation, Malaysia.

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Pertanika J. Trop. Agric. Sci. 36 (1): 35 - 42 (2013)

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TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

A Preliminary Chemical and Structural Analysis on the Albumen Gland of Three Snail Species Found in Abeokuta, Ogun State, Nigeria

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ABSTRACT

The function of the albumen gland is to produce albumen or perivitelline fluid for the egg. A preliminary chemical and structural analysis of the albumen gland of three common land snail species (*Archachatina marginata, Achatina achatina and Achatina fulica*) found in Abeokuta (Nigeria) was carried out. The presence of Na^+, K^+, Ca^{2+} Cl^- and PO_4^{2-} was detected in the gland with the *A.marginata* having the highest chemical concentrations, followed by *A. fulica* and *A. achatina*. Ca^{2+} with the highest concentration in the gland and the protein content was similarly high. The gland contains secretory granules, epithelial lining and tissues which are similar in the three species, indicating that they are structurally similar. Meanwhile, the relevance of these results to the egg production in snails is discussed.

Keywords: Albumen gland, giant land snails, egg, histology

INTRODUCTION

According to Yoloye (1994), they are the largest group of Mollusc constituting the largest animal group after the Arthropods. They are gastropods and differ from others in that their respiration is highly modified for terrestrial life.

ARTICLE INFO

Article history:
Received: 8 February 2011
Accepted: 3 May 2012

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Imevbore and Ademosun (1988) reported that snails commonly live in the high forest and in the derived savanna regions of West Africa. The species of African land snail such as *Archachatina marginata, Achatina achatina, Achatina fulica, Helix pomatia* and *Limicolaria aurora* are consumed in many countries of the world (Amusan & Omidiji, 1998). They are highly favoured in Nigeria and Africa, where they constitute the most conspicuous terrestrial mollusc (Odaibo, 1997). Snail meat is high in protein, low in fat and a

ISSN: 1511-3701

very rich source of iron (Akinnusi, 2002; Ademolu *et al*., 2007). Nevertheless, the nutritive value of these three species varies. Idowu *et al.* (2008) reported 16.08, 13.16 and 10.24 g/100g crude protein contents for the flesh of *A. marginata, A.achatina* and *A.fulica,* respectively.

Reproduction in snail is sexual, although they are hermaphrodite. The ovotesis which is the reproductive organ of the snail is a complex organ that produces both the sperm and egg. The albumen gland is a compound tubular exocrine gland found in the female reproductive tract. It contains a fertilization chamber where eggs are fertilized and secretes perivitelline fluid that is composed of protein and polysaccharide complex which coat each fertilized egg.

Idokogi and Osinowo (1998) reported that the reproductive system of *Archachatina marginata* has a high correlation with the live weight of the snail, that is, as the snail matures, the reproductive organ also increases in size. Ademolu *et al.* (2008) reported that *A. marginata* has the biggest reproductive structures, and this is followed by *A. fulica,* while *A. achatina* has the smallest size.

Not much has been done on the comparative physiology of some common African land snails, especially those found in Abeokuta. The only comparative work on the African land snails was that of Idowu and Akinnusi (2006) on the morphology and histology of the ovotestis of African land snails found in Abeokuta. The size and total number of eggs laid differ from species to species, and this is related to the size of hatchlings (Odaibo, 1997), while *A. fulica* laid 5.4 eggs/week, 3.75 and 1.4 eggs/week were laid by *A.achatina* and *A. marginata,* respectively (Ebenebe *et al*., 2011; Okon *et al*., 2011). However, little information is available on how the eggs are produced in African land snails and the likely causes of the variations in the size and number of eggs.

The focus of this study was to analyze the chemical composition and the structures of the albumen gland of three common land snails found in Abeokuta.

MATERIALS AND METHODS

Experimental Site

The study was carried out in the Laboratory of the Department of Biological Sciences, University of Agriculture Abeokuta, Ogun state, in Nigeria.

Experimental Snails

Forty-five mature snails of three different species *(A. marginata, A. achatina,* and *A. fulica)* were used (fifteen snails per species). The snails were identified based on the descriptions of Akinnusi (2002) and Amusan and Omidiji (1998).

Data Collection

The live weight of the snails was taken using the sensitive electronic weighing scale (Mettler DM 11-k) and the morphometric data such as the length and width of the body were measured using a vernier caliper.

Preparation of the Samples for Analysis

The snails were dissected as described by Segun (1975) and modified by Idowu and Akinnusi (2006). The digestive system was dissected and the albumen gland was carefully removed from the hermaphroditic duct with a pair of scissors onto a sterile Petri dish. The length and width of the albumen gland was measured using a vernier caliper. The albumen gland was subsequently weighed using the sensitive electronic weighing scale (Mettler DM 11-k).

Proximate Analysis of the Sample

The crude fibre, ash, crude protein and carbohydrate content of the samples were determined using the methods of Association of Official Analytical Chemsit (A.O.A.C.) (1990). Similarly, the minerals (Mg^{2+}, Na^{+}, Ca^{2+}, K^{+}, Cl and PO_4^{2-}) were determined using Flame Photometer and Atomic Absoption Spectrphotomer (AAS).

Histology

The albumen gland from the three snails species were fixed in 10 % formalin. The fixed glands were dehydrated and embedded in paraffin wax (melting point 58-60%). Sections were cut at 10µm and stained with haematoxylin and eosin. Slides were viewed under a microscope at x400.

Data Analysis

All the collected data were analyzed using a simple one-way analysis of variance (ANOVA) and means separation was done by using the Student Newman Kuel's test.

RESULTS

The mean weight, length and width of the albumen gland of different land snails are presented in Table 1. The results presented in the following table show that the mean weight, length and width of the albumen followed the trend- *A. marginata*> *A. achatina* > *A. fulica.*

Mineral Analysis

The mineral analysis of the albumen gland of different land snails is shown in Table 2. The results depicted in the following table show that Ca^{2+} has the highest concentration of all the minerals analysed, and *A. marginata* has the highest concentration, followed by *A. fulica* while *A. achatina* has the lowest.

TABLE 1
Measurement of the Albumen Gland of the Different Land Snails.

	SNAIL SPECIES		
Albumen gland parameter	*Archachatina marginata*	*Achatina achatina*	*Achatina fulica*
Mean weight (g)	7.96± 0.52^{a}	2.60± 0.16^{b}	2.10± 0.16^{c}
Mean length (cm)	2.03± 0.15^{a}	1.85± 0.05^{b}	1.45± 0.05^{c}
Mean width (cm)	0.51± 0.09^{a}	0.43± 0.07^{a}	0.27±0.07^{b}

Values (mean±SD) within row followed by different superscript were significantly different (P<0.05)

TABLE 2
The mineral analysis of the albumen gland of three land snails species

	SNAIL SPECIES		
Mineral composition (mg/100g)	*Archachatina marginata*	*Achatina achatina*	*Achatina fulica*
Magnesium	0.07±0.017[b]	0.06±0.017[b]	0.09±0.017[a]
Sodium	0.58±0.25[a]	1.00±0.25[a]	0.85±0.25[a]
Potassium	1.75±0.58[c]	3.00±0.58[a]	2.50±0.58[b]
Calcium	82.50±6.76[a]	67.50±6.76[c]	77.50±6.76[b]
Chlorine	0.88±0.075[b]	1.03±0.075[a]	0.95±0.075[b]
Phosphorus	0.120±0.007[b]	0.126±0.007[a]	0.109±0.007[c]

Values (mean±SD) within row followed by different superscript were significantly different (P<0.05).

TABLE 3
Proximate analysis of the albumen gland of different land snails

	SNAIL SPECIES		
PARAMETER(g/100g)	*Archachatina marginata*	*Achatina achatina*	*Achatina fulica*
Moisture content	58.16±1.64[c]	61.54±1.64[a]	60.32±1.64[b]
Fat content	0.88±0.034[a]	0.82±0.034[a]	0.86±0.034[a]
Ash content	1.36±0.043[a]	1.31±0.043[b]	1.27±0.043[c]
Crude fibre content	0.72±0.20[a]	0.70±0.69[b]	0.71±0.20[b]
Crude protein content	38.26±2.20[a]	33.22±2.20[c]	36.26±2.20[b]
Carbohydrate content	0.62±0.094[a]	0.41±0.094[c]	0.51±0.094[b]

Values (mean±SD) within row followed by different superscript were significantly different (P<0.05).

Proximate Analysis

The proximate analysis of the albumen gland of different land snails are shown in Table 3. The results reveal that the moisture content is relatively high in the three species, while the crude protein and ash content values are the highest in *A. marginata.*

Histology

The structures of the albumen gland of the three snail species are shown in Fig.1 to Fig.3. The structures are made up of epithelial lining, secretory granules, and connective tissues. An examination of the structures revealed that the albumen gland of the three species are not different.

DISCUSSION

A. marginata recorded the largest size and weight of albumen gland. Furthermore, the present study revealed that a strong correlation exists between the body parameters and the weight, length and width of the albumen gland of the different land snails. This observation agrees with the findings of Idokogi and Osinowo (1998) and also Ademolu *et al.* (2008) that the reproductive tract of *A. marginata* has a high correlation with the live weight of the snail.

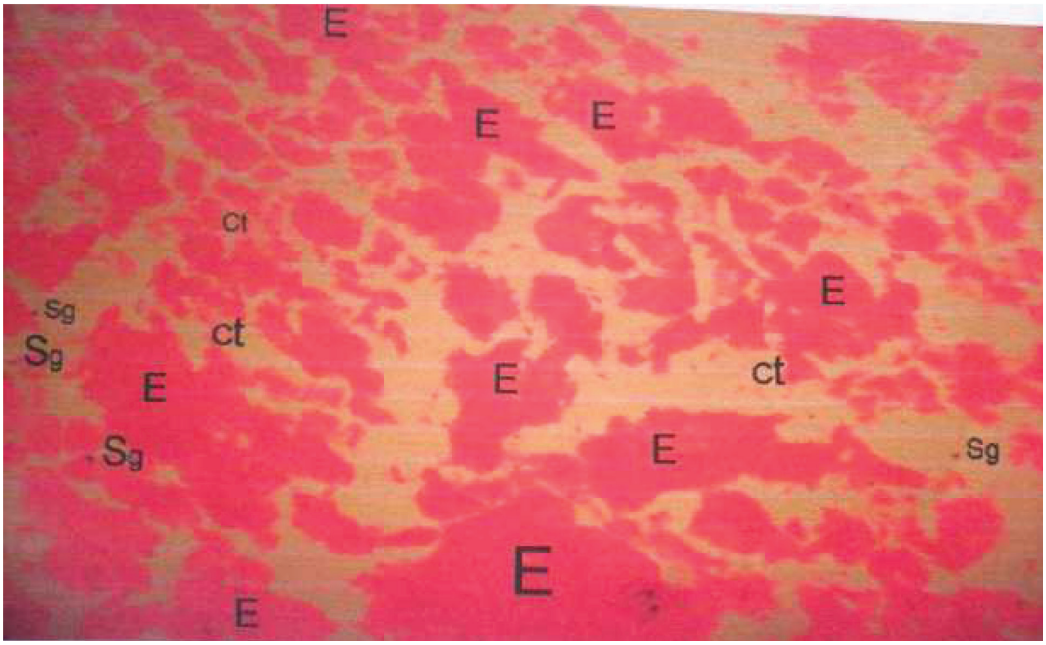

Fig.1: Transverse Section of Albumen gland of *Archachatina marginata* (E-epithelial lining,Sg-secretory granules, ct- connective tissues)

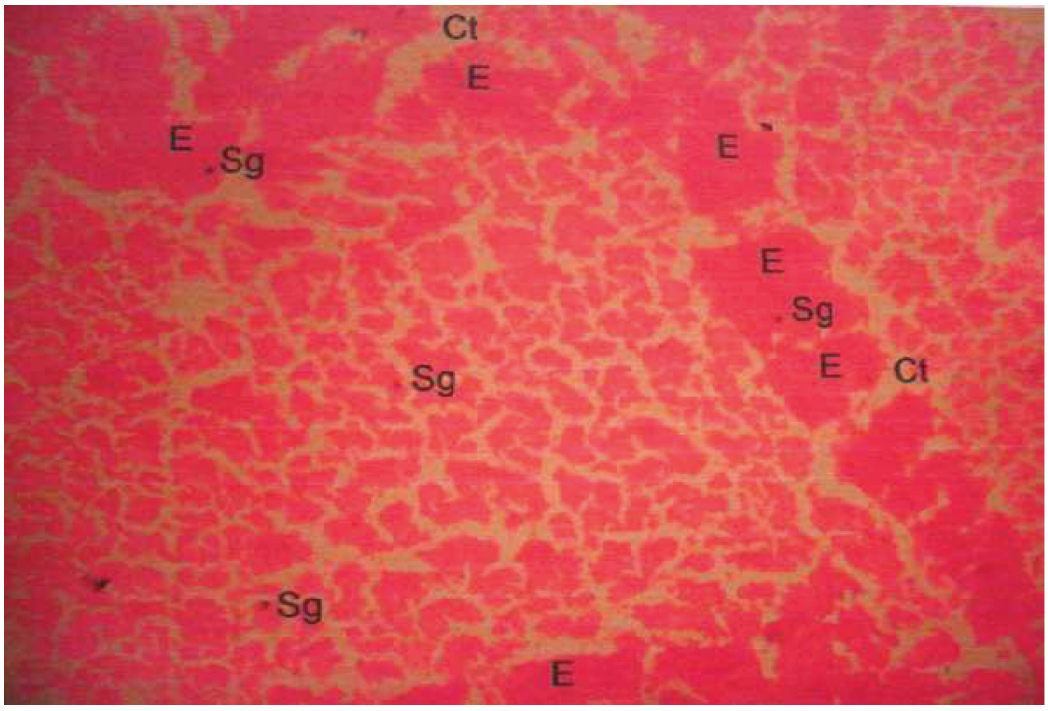

Fig.2: Transverse Section of Albumen gland of *Achatina achatina* (E - epithelial lining, Sg - secretory granules, ct - connective tissues)

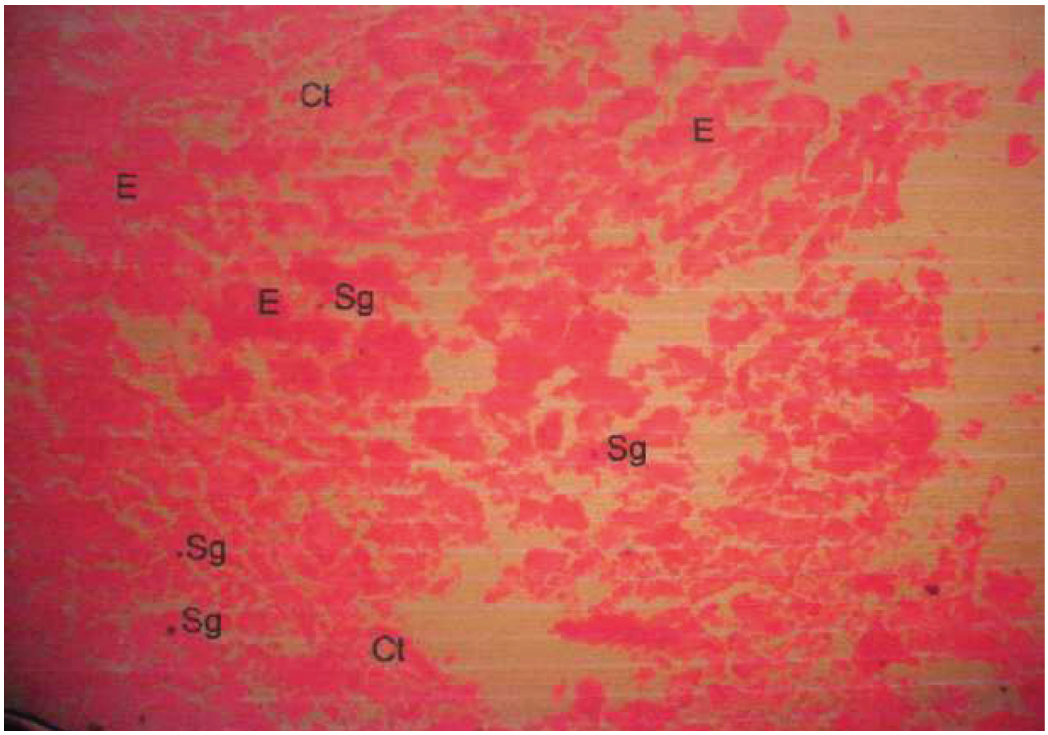

Fig.3: Transverse Section of Albumen gland of *Achatina fulica* (E - epithelial lining, Sg - secretory granules, ct - connective tissues)

Nonetheless, little information is available on the chemical composition of the albumen gland of giant land snails. However, the values recorded in this study can compare the composition of the snail flesh well. Idowu *et al.* (2008) and Ademolu *et al.* (2007) recorded the ash content value of 1.45 -2.78 g/100g and the fat content of 1.18-1.78 g/100g. This seemingly similarity in the values noticed might be due to its open circulatory system, where the haemolymph bathes the tissues and organs, and thereby diffusing the same substances throughout the snail body, as opined by Ademolu *et al* (2008) earlier on.

The presence of the tested minerals in the albumen gland of each species shows how important the organ is in the egg production in snail. Odaibo (1997) earlier highlighted the importance of calcium in the shell formation and that the lack of calcium in snails could lead to the formation of thin and transparent egg shell, as well as stunted growth and infertility in snails. This implies that the highest value of calcium observed in the albumen gland of *A. marginata* might be responsible for its eggs' large sizes as calcium affects the coating of the fertilized eggs, and thus increasing the thickness of the eggs produced. Furthermore, higher concentrations of Ca^{2+} in both *A.marginata* and *A. fulica* might be responsible for the better thickness of their egg shells as compared to that of *A. achatina* which is

thin and fragile, as observed by Chapman and Berker (1972).

Udoh (1994) reported that the ash content is a reflection of the amount of minerals present in such sample. The high ash and protein contents in the albumen gland of *A. marginata* revealed that it has a high nutritive value which could be passed to the egg in the process of egg coating, and thereby increased the nutritive value of the eggs which affects the size and survivability of the hatchings produced by this species. Similarly, high protein values were also recorded in the albumen gland of the fresh water snail *Helisoma duryi* (Morishita *et al.,* 1998). Nubuhiro and Makoto (1999) also discovered a high level of soluble protein in the albumen gland of land snail, *Euhadra peliompha,* which are seasonally influenced. Proteins are regulators of the physiological processes such as growth and reproduction (South, 1992). The eggs and hatchings of *A. marginata* have high hatchability and survival rate, respectively (Akinnusi, 2002) and this may likely be due to the transfer of nutrients from the albumen gland to the eggs during their formation. Albumen provides a major source of nourishment for the fertilized eggs (Yoloye, 1994); the more nutrients it contains, the better the egg produced by the snails.

The transverse section of the albumen gland of the three snail species showed the presence of epithelial lining, secretory granules and connective tissues. Similarly, no marked difference was observed in the structures of the three snails. In a study by Abiona *et al.* (2007), no significant different was recorded in the size of the ova and spermatozoa found in the albumen gland of two giant land snails, namely, *A. marginata* and *A. achacha*. These similarities in the structures may not be unconnected to their being from the same family, i.e. *Achatinidae*. Meanwhile, Idowu and Akinnusi (2006) also observed a resemblance in the structures of the ovotestis of the giant land snails found in Abeokuta, Nigeria. In addition, Egonwon (2007) also reported that the albumen gland of *A. marginata* is made up of tubules with are closely packed columnar secretory cells. The secretory cells produce secretions which are passively stored in their cytoplasm.

CONCLUSION

This study has given a clue or an insight into a better reproductive performance of *A. marginata* as it possesses higher nutrient values and minerals in the albumen gland compared to other land snail species.

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Pertanika J. Trop. Agric. Sci. 36 (1): 43 - 50 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Microscopic Observation of 'Gaharu' Wood from *Aquilaria malaccensis*

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ABSTRACT

Aquilaria produces fragrant wood known as 'gaharu' in its stem and branches, often in mature and damaged trees. In this study, anatomical characteristics in juvenile and mature trees were investigated by comparing their anatomical structures after various staining methods and direct observations under a light microscope. Juvenile and mature wood share similar anatomical structures. No major differences were observed other than the percentage of area covered by included phloem in juvenile was 2.16 times more than that of the mature wood. Microscopic observation revealed that in mature resinous wood, brownish bodies were found in ray and axial parenchyma, included phloem, xylem vessels and fibres, and this finding indicates that these are important elements for 'gaharu' depositing. Thus, it was concluded that juvenile tree possess the anatomical features of that of mature wood in producing 'gaharu'.

Keywords: Gaharu, agarwood, anatomy, *Aquilaria,* included phloem

INTRODUCTION

Aquilaria is a member of the Thymelaeaceae family from the order Myrtales. Members of this genus are known to produce the prized 'gaharu' (which is also known as 'agarwood', 'aloeswood', 'eaglewood', 'jinkoh', and 'agalloch'). 'Gaharu' is highly valuable for its fragrance and medicinal values and has been widely used for perfumery, incense and religious purposes, especially in the Middle East and Asian countries (Chakrabarty *et al*., 1994). 'Gaharu' contains resin and its biosynthesis is commonly associated with the tree's defence system. Mechanical wounding has been shown to be the primary effect to

ARTICLE INFO

Article history:
Received: 27 April 2011
Accepted: 19 October 2011

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ISSN: 1511-3701

commence 'gaharu' formation (Nobuchi & Mohd. Hamami, 2008; Pojanagaroon & Kaewrak, 2003; Rahman & Khisa, 1984) followed by fungal infection. Many varieties or genetic strains of fungi have been identified from 'gaharu'-containing stems (Mohamed *et al*., 2010; Tabata *et al*., 2003).

The stem and branches of *Aquilaria* tree are the main organs where 'gaharu' is deposited. Meanwhile, sections on the wood reveal the existence of the 'included phloem', which is characterized by the strands or layers of the phloem tissue embedded in the xylem (Esau, 2006). In *Aquilaria*, included phloem is produced internally by the cambium (Thouvenin, 1892; Van Tieghem, 1892) and the resin is concentrated mainly in the included phloem (Rao & Dayal, 1992). A few have reported the anatomical structures and changes on mature *Aquilaria* tree due to wounding damages. Among other, parenchyma cells have been shown to take part in resin formation as the cells showed abrupt changes in sapwood after mechanical wounding. Changes include the decrease and disappearance of starch grains in parenchyma cells, vacuolization, and the

Fig.1: Photographs of *Aquilaria malaccensis* trees used in the experiments; (a) A three-year old juvenile tree growing in a polybag in the nursery, and (b) a mature tree from the wild

appearance of brownish droplets following the disappearance of starch grains (Nobuchi & Mohd. Hamami, 2008). However, no report has been made on the anatomy of juvenile wood and its comparison to mature wood. In this work, key anatomical structures in the formation of 'gaharu' in *A. malaccensis* were identified and compared.

MATERIALS AND METHODS

A Comparison between Juvenile and Mature Wood

For the purpose of comparing between juvenile and mature wood, the juvenile wood samples of 3 cm thick (tangential) were taken from a 3-year old sapling (Fig.1a) that grew in a polybag at the nursery of the Faculty of Forestry, Universiti Putra Malaysia (UPM). The sapling was collected from natural population of Jeli, Kelantan. The tree was 2 m tall and had a diameter of 4 cm when measured at 5 cm above ground. Meanwhile, the samples of the mature wood were collected from a wild tree that was growing in Temerloh, Pahang (see Fig.1b). The tree had a diameter at breast height of about 1 m. The wood samples of 3 cm thick (tangential) were collected from parts of the trunk, which had wounding marks. The wood samples were subjected to sectioning and microscopic observations.

Fixation and Sectioning

The samples were fixed in 3% gluteraldehyde. For light microscopy (LM), serial transverse, radial and tangential sections of 20 µm thick were cut using a microtome knife on Leica SM 2000R sliding microtome (Leica, Germany). Two staining methods were applied on the sections, namely, periodic acid-Shiff's (PAS) staining and toluidine blue. For the PAS staining, the sections were oxidized with 1% periodic acid for 30 min, washed with three changes of distilled water, and then treated with Schiff's reagent for 30 min in darkness. After washing with three changes of distilled water, the sections were dehydrated with a series of ethanol (once at 30% and 50%, followed by twice at 70%, and twice at 95%). The sections were then cleared in clove oil and mounted with Di-n-butylPhthalate in Xylene (DPX). For toluidine blue staining, the sections were stained for 3 min with 1% toluidine blue dissolved in distilled water. After washing with three changes of distilled water, the sections were mounted with glycerine. These were observed under the light microscope using 10x and 40x objective lenses and microphotographed using Leitz DMRB research microscope (Leica, Germany).

Measurements of the Included Phloem

The length and width of the included phloem were measured directly from the transverse sections using an image analyzer (Leica, Germany). One hundred measurements were chosen randomly to calculate the mean and standard deviation. Statistical analysis was conducted using the SPSS software version 16.0.2 (IBM Corporation) with significance judge at $P < 0.05$ in independent sample t-test. The percentage of included phloem coverage (area of included phloem) over the total transverse section area was calculated.

RESULTS AND DISCUSSION

Microscopic Observation of Juvenile and Mature Wood from Aquilaria malaccensis

Juvenile wood that was used to characterize the anatomy of *A. malaccensis* was stained with toluidine blue. Transverse, radial and tangential sections of juvenile *A. malaccensis* are shown in Fig.2. Sections of the juvenile wood samples revealed the unique characteristic of the genus *Aquilaria*, which is the existence of included phloem (Fig.2a), when observed under the light microscope. The microscopic survey revealed element constituting xylem parts as vessel elements, axial and ray parenchyma cells and wood fibres. The distribution of axial parenchyma is scanty paratracheal type and occasionally the parenchyma cells are associated with the vessels or form an incomplete sheath around the vessels (Fig.2b), and the ray parenchyma cells are uniseriate and multiseriate (Fig.2c). Observations described here are similar to that reported on *A. crassna* (Nobuchi & Siripatanadilok, 1991).

Since in natural forest, 'gaharu' is often found in old mature tree, the differences between juvenile and mature wood were

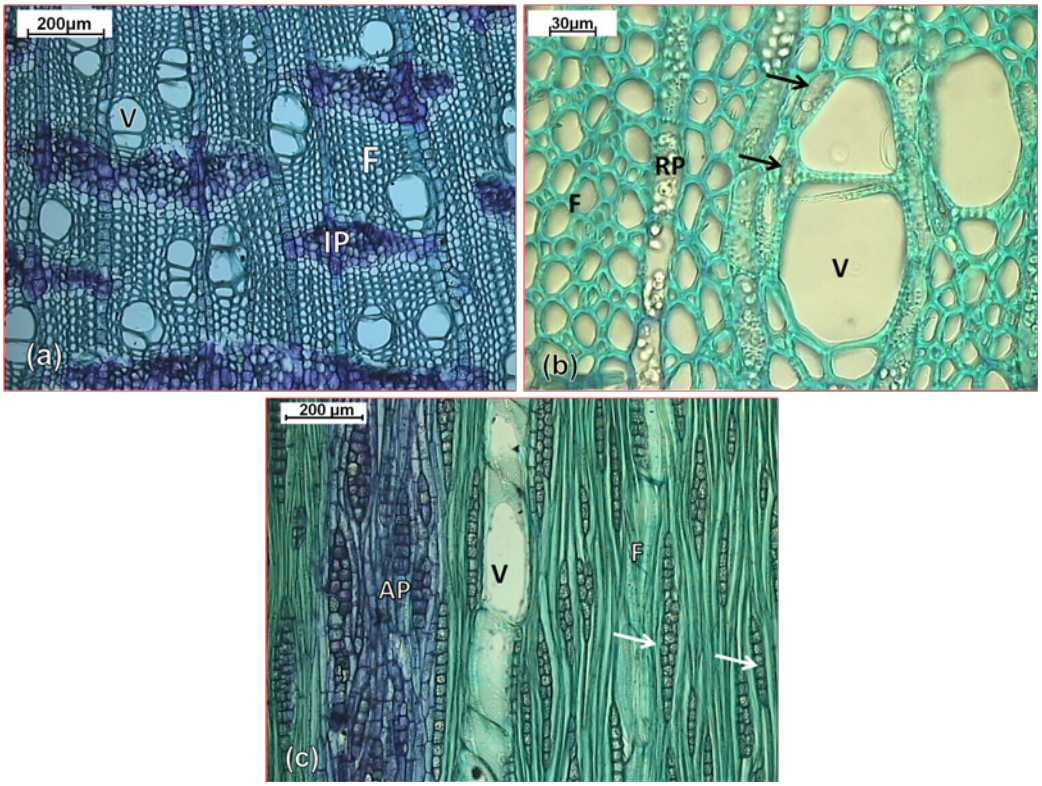

Fig.2: Light micrographs showing the anatomy of unwounded juvenile tree; Top: Transverse sections showing (a) included phloem (Toluidine blue. Scale bar=200 µm), (b) scanty paratracheal axial parenchyma (arrows) (Toluidine blue. Scale bar=30 µm), and (c) tangential section showing uniseriate and multiseriate ray parenchyma cell (arrows) (Toluidine blue. Scale bar=200 µm (arrows). V: vessel, F: fibre, IP: included phloem, AP: axial parenchyma

investigated. For this purpose, the juvenile wood samples were taken from the base stem of a three-year old *A. malaccensis*, while the wood samples were collected from parts of a wild tree trunk which had injured markings and showed the presence of 'gaharu'. The dimensions and distributions of the included phloems in the juvenile and mature woods in the transverse sections were compared under the light microscope. On average, there were no differences between the two types of wood, in terms of the length and width of the included phloem (Table 1; $P_{\alpha = 0.05} = 0.287$ and $P_{\alpha = 0.05} = 0.504$, respectively). However, the only difference found was that the included phloem coverage in the juvenile was two-fold higher than that of the mature wood, which was 36.9% and 17.1%, respectively (Table 2). Meanwhile, the included phloem distribution was found to be denser in the juvenile wood as compared to the mature wood (Fig.3a, Fig.3b). This finding suggests that in *A. malaccensis*, fibre growth increases the distance between included phloem when the tree matures, while the size of the included phloem remains the same from juvenile to mature. Generally in trees, the

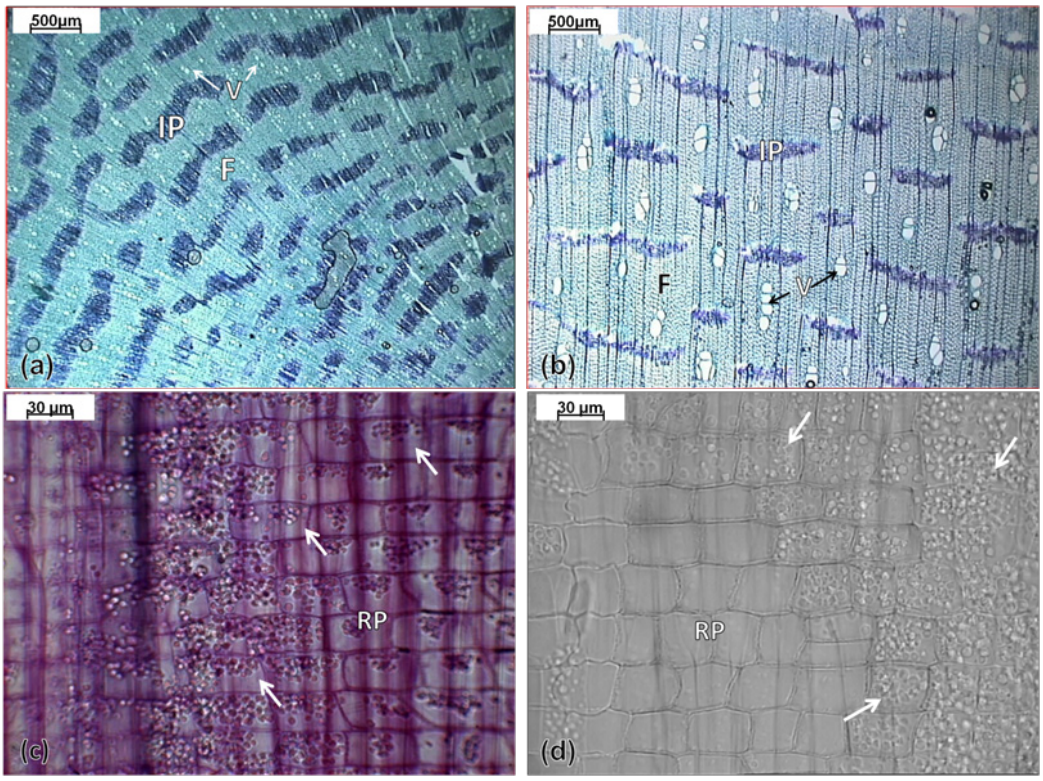

Fig.3: Anatomical comparisons between juvenile and mature wood; Top: Transverse sections showing the distribution of included phloem in (a) juvenile wood and (b) mature wood (Toluidine blue, Scale bar=500 µm). Bottom: Radial sections showing ray parenchyma cells partially filled with starch (arrows) in the (c) juvenile wood, and (d) those fully filled with starch in the mature wood. V: vessel, F: fibre, IP: included phloem, RP: ray parenchyma (PAS staining. Scale bar=30 µm)

vessel area increases gradually while the number of vessels decreases from pith to bark (Leal *et al*., 2003). This is consistent with the vessels but was not the case with the included phloem in *Aquilaria*. We showed that the size of the included phloem in mature wood was only slightly bigger than the one in juvenile wood, although the trees from where the samples obtained had wide physiological differences.

TABLE 1
The average length and width of the included phloem in juvenile and mature wood.

Dimension	Wood Type	Mean (μm)	Std. Error (μm)
Length	Juvenile	620.3	27.8
	Mature	666.4	33.0
Width	Juvenile	188.3	5.6
	Mature	193.3	4.7

Nonetheless, no significant differences were found in the overall anatomical structures of the juvenile and mature wood. Both types of wood had included phloem and parenchyma, which were also observed in other 'gaharu'-producing species of Thymelaeaceae, including *A. crassna* (Nobuchi & Mohd. Hamami, 2008; Nobuchi & Siripatanadilok, 1991) and *Gyrinops versteeghii* (Tabata *et al*., 2003). These two structures are important in *Aquilaria* wood as they are the only living cells that are capable of biosynthesizing chemical compounds in 'gaharu' (Nobuchi & Siripatanadilok, 1991; Tabata *et al*., 2003).

In this study, the radial sections of sapwood were also compared from the juvenile and mature wood using PAS staining. Both types of sapwood were filled with starch grains; however, the ray parenchyma cells in the juvenile wood were only partially filled with starch compared to the mature wood (Fig.3c and Fig.3d). Numerous observations in the closely related *A. crassna* revealed their abundant appearance in the axial parenchyma cells of the outermost sapwood but were scarce in the innermost sapwood; this decrease is accompanied by the increasing amount of lipid (Nobuchi & Siripatanadilok 1991).

When examining unstained transverse sections of the mature wood under the light microscope, brownish substances were detected in the ray parenchyma, included phloem, vessel element and fibre (Fig.4a). As the sections were derived from dark brown wood-block, it was suspected that this brownish substance was the resin 'gaharu'. The presence of 'gaharu' was confirmed from the unique fragrance released upon

TABLE 2
The coverage and dimensional details of the included phloem.

	Coverage of included phloem (%)	Dimensional details	
		Length	Width
		(μm)	
Juvenile wood	36.9	620.35 ± 122.41	188.34 ± 36.41
Mature wood	17.1	663.10 ± 107.09	205.11 ± 37.32

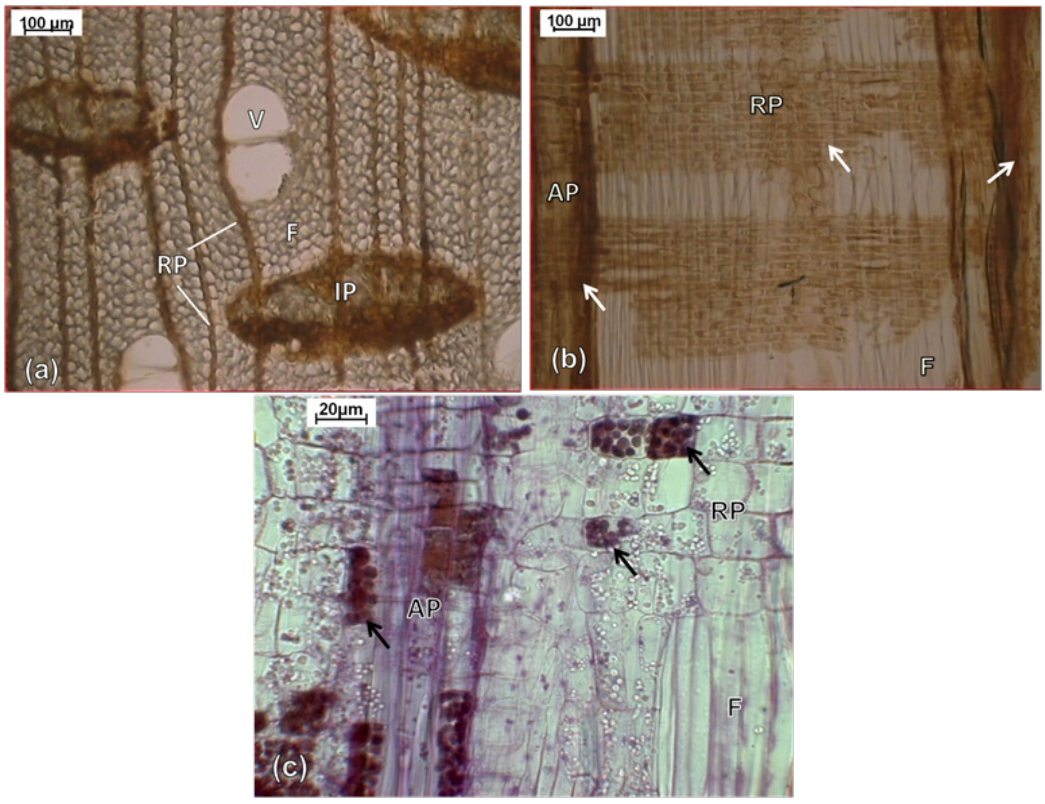

Fig.4: Anatomical structures involved in 'gaharu' synthesis and accumulation. Sections were made from a wood block impregnated with 'gaharu', collected from a wild tree (Fig.1b). All the sections are not stained; (a) Transverse section showing included phloem and ray parenchyma cells containing brownish substances (Scale bar=100 µm), (b) radial section showing axial parenchyma and radial parenchyma filled with brownish substances (arrows) (Scale bar=100 µm) and (c) radial section section showing browning droplets from mature reinous wood (arrows) (Scale bar=20 µm). V: vessel, F: fibre, IP: included phloem, RP: ray parenchyma, AP: axial parenchyma

burning the wood chip. In the transverse section, the ray parenchyma cells were observed to have filled with the brownish substance, while in the included phloem, the substance was dispersed along the inner wall (Fig.4a). The brownish droplets were also found in the axial and ray parenchyma cells (Fig.4b and Fig.4c). The sections made from the control wood-block, where the 'gaharu' was not observed were found free from any brownish substances (data not shown). In *A. crassna*, the brownish droplets appeared after the disappearance of starch grains in the parenchyma cells, and they contained phenolic substances (Nobuchi & Siripatanadilok, 1991). Starch grains were also observed being stored in the ray and axial parenchyma cells of the sapwood and thought as raw precursors in 'gaharu' biosynthesis (Nobuchi & Siripatanadilok, 1991).

CONCLUSION

Little variation was found in the structure and cell compositions of the juvenile and mature trees undertaken in the current study. The distribution of the included phloem was twice more compact in the juvenile wood as compared to mature wood, while the dimensions of the included phloem showed no significant difference at all. The juvenile wood appeared to have the anatomical characteristics necessary in producing 'gaharu' because of the existence of parenchyma cells, and its included phloem were of comparable sizes to the mature wood. The findings of the current study suggest that 'gaharu' can possibly be induced in juvenile trees as young as 3 years old.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support from the Ministry of Higher Education, Malaysia, under the Fundamental Research Grant Scheme (Project No: 01-01-07-069FR). The authors would also like to thank Professor Dr. Tadashi Nobuchi (previously with Kyoto University, Japan, and Universiti Putra Malaysia) for his technical assistance and valuable suggestions.

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Pertanika J. Trop. Agric. Sci. 36 (1): 51 - 60 (2013)

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Biological Performance of *Menochilus sexmaculatus* Fabricius (Coleoptera: Coccinellidae) Upon Exposure to Sublethal Concentration of Imidacloprid

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ABSTRACT

The effects of sublethal exposure to imidacloprid (Kendor® 18.3SL) on the biological performance of the ladybird beetle, *Menochilus sexmaculatus* F., the most common coccinellid beetle found feeding on aphids, were studied under ambient laboratory conditions of 27-32˚C and 50-75% RH. The corn leaf aphid, *Rhopalosiphum maidis*, was offered as prey. The LC_{50} obtained from the contact bioassay at 24 h post-treatment for the regression slope, b=1.08, indicated that imidacloprid was likely to be selective. Sublethal exposure to imidacloprid caused reduction in survival with the females reaching 50% mortality by the 24th day, while that of the control was the 36th day. Meanwhile, demographic parameters including the net reproductive rate (R_o), generation time (T), innate capacity of increase (r_m) and the finite rate of increase (λ) of the treated females were markedly inferior as compared to the untreated females. The R_o value indicated that the control female was capable of producing 17.59 female offspring but treated female could only produce 2.55 female offspring during their generation time of 25.17 and 23.04 days, respectively. The capacity or instantaneous rate of increase (r_c) declined from 0.114 to 0.041, which was parallel with the decrease in the intrinsic rate of increased (r_m) value from 0.125 to 0.041. The values of λ were 1.133 and 1.042 for the control and treated population, respectively. In the meantime, the doubling time (DT) increased sharply to 16.86 days for the treated population, whereas DT for the control was 6.09 days. The sex ratio was biased towards the female and generally the females survived slightly longer (48 days) than the males (46 days), as observed in the control population.

ARTICLE INFO

Article history:
Received: 18 May 2011
Accepted: 15 June 2011

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Keywords: Biological performance, demographic parameters, imidacloprid, *Menochilus sexmaculatus*, sublethal effects

ISSN: 1511-3701

INTRODUCTION

Coccinellid beetles are the most common and intensively studied aphidophagous insects for biological control. In fact, their biology and ecology have been extensively researched (Hodek, 1967; Hagen, 1974). Meanwhile, *Menochilus sexmaculatus* has been reported as the most common of these species feeding on aphids and being the most voracious and has the highest fecundity among the ladybird beetles in Malaysia (Parker & Singh, 1973).

Biological control practitioners usually assume that natural enemies are capable of keeping pest populations below economic thresholds but they have frequently failed to do so. This is because the natural enemies are often harmed by pesticides without regards, especially by non-selective chemicals that can greatly disrupt the stability in an agro-ecosystem (Parker *et al*., 1976). However, it is unthinkable to eliminate the use of insecticides in the near terms since the ladybird beetles alone may not be able to control the aphids below the economic thresholds. Without eliminating the use of pesticides, Integrated Pest Management (IPM) is greatly encouraged, and with the use of predators and parasitoid, the control strategies adopted are ecologically sound and environmentally friendly. To ensure its success, it is essential that natural enemies are not eliminated by insecticides. In this context, imidacloprid has been widely used in Malaysia. At a lower application rate and with fewer applications, imidacloprid has provided a long lasting efficacy; its contact, ingestion and systemic properties and its slightly selective nature, combined with modern application technologies, have in fact made it an important component in the IPM programmes (Iwaya & Kagabu, 1998). In the United States of America (USA), imidacloprid has been used to manage a wide range of insect pests such as aphids, mealybugs, whiteflies and certain scale insects (Ware & Whitacre, 2004), and extensively against the Colorado potato beetle, *Leptinotarsa decemlineata*. However, it was highly toxic to the adults and larvae of the natural enemy of the potato beetle, *Coleomegilla maculate* (Lucas *et at*., 2004). The impacts of neonicotinoid imidacloprid on the natural enemies in greenhouse and interiorscape environments have also been reported by Cloyd *et al*. (2010). This paper examines the sublethal effects of imidacloprid in the laboratory on the demographic performance of *M. sexmaculatus* when fed with corn leaf aphid, *Rhopalosiphum maidis*.

MATERIALS AND METHODS

Chemical Tested

Imidacloprid (Kendor® 18.3SL) was used in this study. Serial dilutions were prepared in distilled water with an initial concentration of 0.2 mg a.i. L^{-1}. For the next concentration of 0.02 mg a.i. L^{-1}, 1 ml was pipetted from the initial concentration and added into 9 ml of distilled water. The procedure was repeated for the subsequent dilutions until the fifth dilution of 0.00002 mg a.i. L^{-1}. The treatments were used immediately after the preparation so as to minimize any decomposition. The control consisted only distilled water.

Insect Culture and Maintenance

The predatory ladybird beetles, *M. sexmaculatus,* were collected from the corn field of Universiti Putra Malaysia (UPM) and mass reared in standard plastic containers (36 x 20 x 30 cm) containing a bouquet preparation of detached brinjal leaves kept fresh in a conical flask as shelters and oviposition substrate. The container was covered with muslin cloth to provide better aeration. Each container contained four pairs of adults. The corn leaf aphids, *R. maidis*, of all stages on detached corn leaves and tassels were provided as prey for the ladybirds. Smaller plastic containers (5 cm diameter x 4 cm) were used for rearing the newly emerged larvae. This culture was used for the experiments as well as for colony maintenance.

Experimental Environment

All the experiments were conducted in a laboratory under an ambient environment of 27-32˚C and 50-75% RH., with 24 h illumination (DURO-Test, 40-W True-Lite, Duro-Test International, Fairfield, New Jersey).

Contact Dose-mortality Bioassay

Filter paper discs, with a standard size of 9 cm in diameter, were used as a testing arena whereby they were dipped in the respective solutions of imidachloprid to complete immersion with light agitation for 5s and left to drip dry. Meanwhile, paper disc treated with distilled water was used as a control. Each of the treated filter paper disc was placed in a plastic container (9 cm diameter x 7.5 cm). Twenty adult female predators were transferred to each of the experimental arenas. Each experiment was replicated six times, and the predators were sustained with unlimited prey.

Mortality was recorded 24 h after the treatments. The beetles, which did not respond when probed with a fine brush, were considered as dead. An estimate of LC_{50} and the regression equation for the dose-mortality line was obtained using a probit programme based on the procedure of Finney (1971) (software: EPA version 1.5, USA). Nonetheless, no mortality was recorded in the control. The LC_{50} value was used for the sub-lethal treatments in the subsequent life-table studies.

The Effects of Sublethal Exposure of Imidacloprid on Survivorship and Pertinent Demographic Parameters

To initiate the study, a cohort of young male and female predators from the stock culture was selected to lay eggs overnight. All demographic data were obtained with these newly deposited eggs beginning with a batch of 15 males and 29 females for the control, and five other subsequent batches producing 121 females for the sublethal treatment, and ended with the death of the last adult. Initially, the eggs were removed the following day and placed in a plastic container (5 cm diameter x 4 cm) which was lined with a filter paper (5 cm diameter) for hatching. Upon emergence, the larvae were individually isolated with a soft brush into a plastic container lined with trimmed filter paper which was moistened daily with a few drops of distilled water. Corn aphids were provided ad libitum every day.

Two days after adult emergence, the control adults were individually transferred into plastic containers (9 cm diameter x 7.5 cm) lined with 9 cm clean filter paper disc, while 121 other females were individually transferred into plastic containers which had been lined with the filter paper disc treated with sublethal concentration of imidacloprid (0.002 mg a.i. L^{-1}) adopted from the bioassay study. In order to maintain continuous residual activity, each beetle was subsequently transferred daily to a freshly treated filter paper disc. Each surviving female was provided with an untreated male from the stock colony. They were fed with corn aphids every day. Daily records for their longevity and fecundity were made at about the same time to minimize any differences in sensitivity that might be associated with diet rhythm. Life table parameters were taken until the death of the last individual.

The demographic parameters were calculated as described by Birch (1948) and Laughlin (1965). A female biased sex ratio of 2:1 obtained from the previous control observation was assumed.

RESULTS AND DISCUSSION

Contact Dose-mortality Bioassay

The mortality of adult females increased with increasing concentration of imidacloprid This was indicated by 0.83% mean mortality with 0.00002 mg a.i. L^{-1} of imidacloprid compared with the highest concentration (0.2 mg a.i. L^{-1}) which resulted in 100% mortality.

No death was observed in the control. Other mortalities were 85.8% at 0.02 mg a.i. L^{-1}, 55.0% at 0.002 mg a.i. L^{-1} and 20.8% at 0.0002 mg a.i. L^{-1}. Cursory observations showed that imidacloprid illicited repellent effect, while disoriented movements away from the treated paper disc were observed. From the probit analysis, a small change in kill for a given variation in the concentration was indicated from the regression equation for dose-mortality line ($y = 8.05 + 1.08x$), and LC_{50} was 0.002 mg a.i. L^{-1} (Table 1). A study by Xue and Li (2001) showed that imidacloprid was relatively safe to *Coccinella septumpunctata*, a coccinellid species that is closely related to *M. sexmaculatus*, and this is thus comparable with the result of the current study. Nonetheless, field studies need to be conducted since it has been reported by Sur and Stork (2003) that imidacloprid is converted into a number of metablites within certain plants and become more water soluble and can be more toxic to natural enemies.

TABLE 1
Results of probit analysis for imidacloprid on females of *Menochilus sexmaculatus.*

No. treated	a	b ±S.E. (mg a.i. L^{-1})	LC_{50}	95% FL
720[a]	8.05	1.08 ± 0.07	0.002	0.001-0.002

[a] 20 females per replicate, with 6 replicates per concentration, 6 series of concentrations per assay

Survivorship

Fig.1 shows the age-specific survivorship for both the treated and untreated cohorts of *M. sexmaculatus* population. Overall, the survivorship was longer for the control than

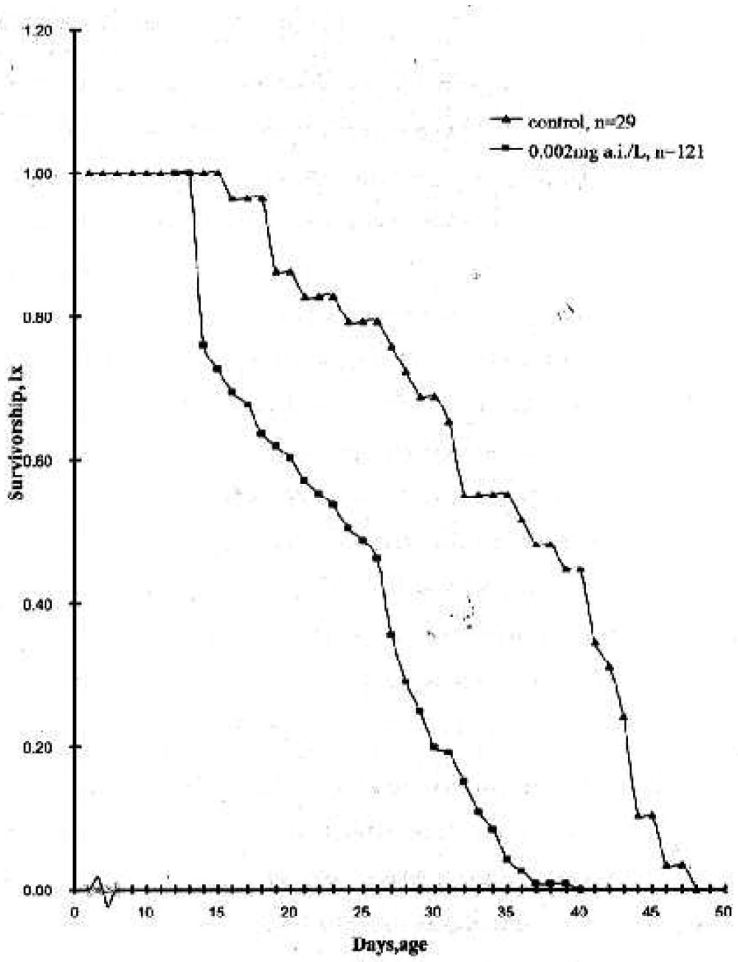

Fig.1: Survivorship of *Menochilus sexmaculatus* upon exposure to sublethal concentration of imidacloprid compared to the control.

with the treated population. Meanwhile, the females in the control group maintained a 100% survivorship for the first fifteen days, declined gradually and continued to survive with the last female to survive up to the 48th day as compared to 40th day for the treated females. On the contrary, the survivorship of the females declined sharply to 76% upon initial exposure to sublethal amount of imidacloprid and steadily declined to reach 50% mortality on the 24th day as compared to a longer survival of up to the 37th day for the control population. This indicates that a continuous exposure to sublethal doses of imidacloprid does not immediately affect the survivorship of *M. sexmaculatus*, and may be acting selectively at sublethal concentration.

Demographic Parameters

It was observed that both the treated and untreated females mated more than

once during their reproductive lives. The females began to lay eggs on the 15th to 17th days, which was 3-5 days after they had emerged. From Fig.2, the peak oviposition period lasted for seven days for the control population and this was five days for the treated population. The peak oviposition fell on the 23rd day for the control, with a maximum of 3.21 eggs per female per day, while that of the treated population fell on the 22nd and 24th day with a maximum of 0.93 eggs and 0.86 eggs female^{-1} day^{-1}, respectively. The oviposition activity for the treated population declined sharply after the peak oviposition with the last egg being oviposited on the 36th day. The second peak oviposition for the control appeared on the 21st day with 2.42 eggs female^{-1} day^{-1}, and the third peak on the final 44th day with 1.33 eggs female^{-1} day^{-1}; the females did not oviposit any more eggs on the remaining three days of survival. For the control population, the total eggs deposited by the 29 females were 765, while those deposited by 121 females of the treated population amounted to 487 eggs. Therefore, each female had deposited an average of 26.4 and 4.0 eggs during its lifetime for both the control and treated populations, respectively. These data clearly showed that although *M. sexmaculatus* was capable of surviving through the sublethal exposure of imidacloropid, the reproduction was apparently and greatly reduced.

A study by Tank and Korat (2007) revealed that *M. sexmaculatus* could lay at least 195 eggs during its lifetime when fed with its preferred host *Aphis gossypii*; however, its mean life span was 34.15 days, and this was comparatively shorter than that recorded in the current study, i.e. 48 days, when fed with corn aphid *R. maidis*.

From the control population, a female biased sex ratio of 2:1 was obtained, while a 1.7:1 sex ratio was obtained from the treated population. It could be deduced that imidacloprid had slightly affected the sex ratio of *M. sexmaculatus* by laying fewer that would become females as compared to the control. The survivorship of the treated females relative to the number of female offspring born per female is shown in Fig.3. The maximum female offspring born per female was 0.63, which was achieved by the 24th day, and this was maintained through the 24th day. Barely half of the females survived up to this point. Thereafter, the number of the female offspring born dropped drastically as the female survivorship declined and reached 100% mortality on the 40th day.

The demographic parameters, including adult longevity and fecundity, showed slight differences between the control and treated populations (Table 2). Even though the treated adults survived to reproduce, the mean fecundity per female declined from 25.16 eggs in the control to 6.10 eggs in the treated population. The net reproductive rate, the intrinsic rate of increase and the finite rate of increase were inferior to the control and these were achieved within a shorter mean generation time of 23.0 days as compared to 25.2 days in the control. With these reductions, the doubling time increased sharply to 19.9 days, which was

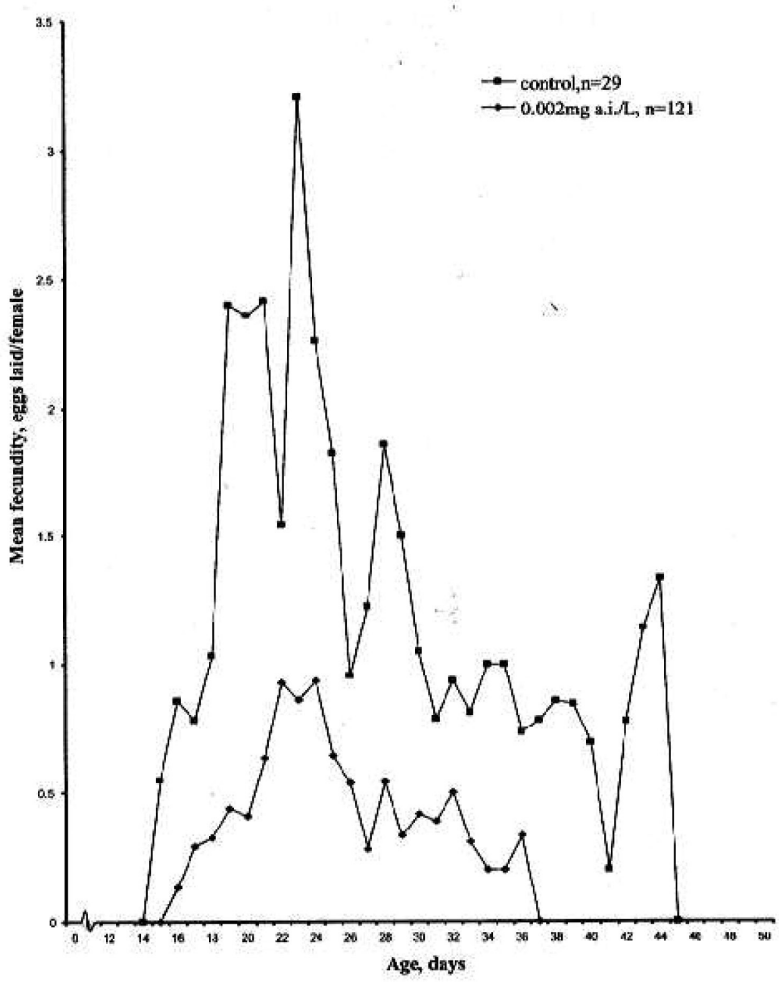

Fig.2: A comparison of the control and imidacloprid treatments on the fecundity of *Menochilus sexmaculatus*

10.8 days longer than that of the control population.

Meanwhile, the conservation of naturally occurring biological control agents is frequently limited by the incompatibility between insecticides and natural enemies. Therefore, knowledge on the sublethal effects of imidacloprid is important in adjusting the predator-prey ratio in an IPM programme. This study showed that although the reproductive performance of *M. sexmaculatus* was inferior to the control, the longevity was not drastically affected and neither was the sex ratio from the eggs laid by the treated females. Maintaining a female biased sex ratio is important since the females actively hunt and serve as reproductive agents. Thus, results from the current population demographic studies should provide sufficient basic

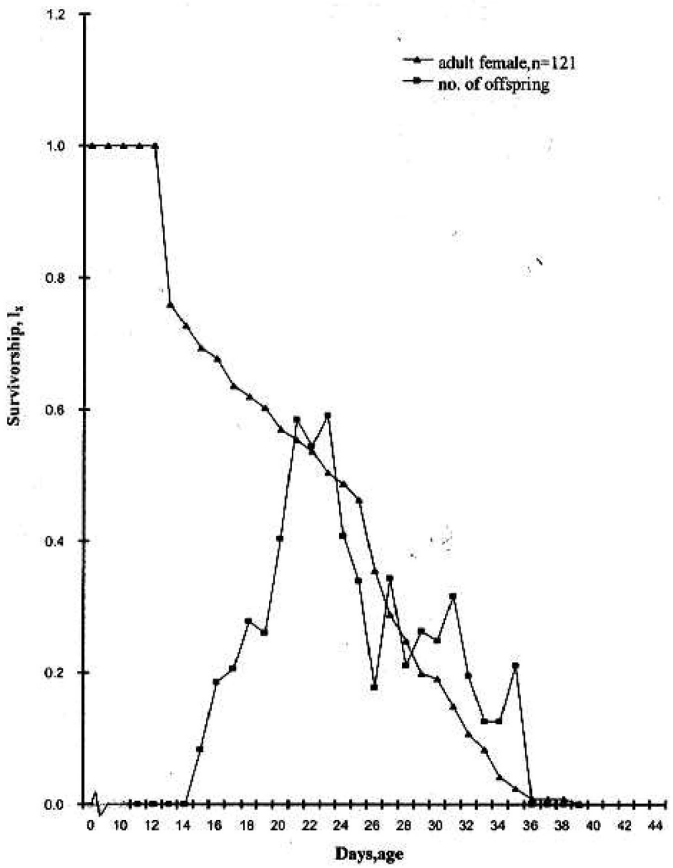

Fig.3: Survivorship of *Menochilus sexmaculatus* relative to the female offspring born per female upon exposure to sublethal concentration of imidacloprid

TABLE 2
A comparison of the demographic parameters of *M. sexmaculatus* upon sublethal exposure to imidacloprid

Parameters	Treatment concentration	
	Control (n=29)	0.002 mg a.i. L^{-1} (n=121)
Life span (days)	48.00	40.00
Fecundity	25.16	6.10
Net reproductive rate, R_0	17.590	2.550
Generation time, T (days)	25.171	23.037
Capacity of increase, r_c	0.114	0.041
Doubling time, DT (days)	6.085	16.855
Intrinsic rate of increase, r_{m*}	0.125	0.041
Finite rate of increase, λ	1.133	1.042

* $r = r_m$ when $\sum e^{-xr} l_x m_x = 1$ was fulfilled.

understanding on whether imidacloprid can be successfully integrated with biological control. As such, IPM studies in the field should follow to determine the full impact of imidacloprid on the bionomics of *M. sexmaculatus* in corn.

ACKNOWLEDGEMENTS

The authors are grateful to Universiti Putra Malaysia for the research facilities and the resources made available by the Department of Plant Protection.

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Pertanika J. Trop. Agric. Sci. 36 (1): 61 - 70 (2013)

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Impact of Indigenous Industrial Compost on the Growth of Coarse and Fine Rice Varieties under Saline Environment

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ABSTRACT

Pakistani rice is popular throughout the globe due to its specific aroma. Rice is categorized as salt-sensitive plant as its growth is significantly reduced under salt toxicity. The effect of exogenous application of indigenous industrial compost (IIC) on the coarse (IRRI-9) and fine (Super basmati-2000) rice varieties under salt stress was investigated in this study. IIC was applied at 0, 0.5 and 1.0% of soil weight, along with recommended chemical fertilizers in respective pots. Twenty-day old rice nursery was transplanted in glazed clay pots filled with normal (EC_e = 1.70 dS m^{-1}) and saline soil (EC_e = 8.0 dS m^{-1}) under flooded condition. Plants were harvested at maturity and different physiochemical parameters were recorded. Salinity stress was found to have significantly (p<0.05) reduced both biological and paddy yield of rice, and the reduction was lower in coarse than fine rice. The compost application significantly improved (p<0.01) dry matter four times as compared with control. In the same way, paddy yield increased three folds both under normal as well as saline growth medium. Na^+ concentration in shoots at 1% IIC in growth medium had a significant negative correlation (r=0.90, p<0.01) but potassium concentration proved a significant positive correlation (r=0.92, p<0.01) in both rice varieties. Enhanced salinity tolerance in rice by IIC application was attributed to increased K^+ uptake, thereby increasing K^+: Na^+ ratio and lower Na^+ translocation towards shoot (sodium exclusion at the shoot level). It was concluded that indigenous industrial compost application improved the growth of rice plant under salt stress.

Keywords: Organo-power, rice, salt stress, sodium and potassium

ARTICLE INFO

Article history:
Received: 18 May 2011
Accepted: 2 November 2011

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ISSN: 1511-3701

INTRODUCTION

In spite of having potential to produce crop, the yield per hectare is very low in Pakistan with an average yield of 2347 kg ha^{-1} (Anonymous, 2010). There are several reasons for it, and these include low organic matter, alkaline soil pH, calcareousness, mining of nutrients with extensive cropping, use of micronutrients free NPK fertilizers and less use of manures. Soils are also being depleted due to inappropriate uses of plant nutrients and cropping sequences. Among the various reasons, soil fertility is the most important. Therefore, restoration and maintenance of fertility status of soil is important, and for which, any addition of organic materials apart from other field practices is important (NFDC, 1998).

Once fertilizer requirement has been evaluated, the nutritional status of soil can be maintained by the addition of organic or inorganic fertilizer or an integrated use of both. However, continued application of inorganic fertilizers may contribute to poor organic matter level of soil and lower crop yields. Some studies have shown that continued use of inorganic fertilizers may results in diminishing soil quality and productive capacity (Doran *et al*, 1996; Bhandari *et al*., 2002; Manna *et al*., 2005). Meanwhile, long-term use of inorganic nutritional sources leads to accumulation of harmful heavy metal ions, thus, polluting the soil with precipitates of hydroxides, carbonates, sulphides and sulphates. Thus, use of organic amendments is necessary for safety of soil environment and health (Choi *et al*., 2002; Ibrahim *et al*., 2011).

In the past, the inherited problems of low fertility, salinity and moisture shortage in semi-arid areas of Pakistan were overcome by applying organic manures like farm manure, as it enhances soil organic matter, humus contents, improve soil water holding capacity, infiltration rate, aeration, porosity, moisture conservation, cation exchange capacity, water stable aggregates, decrease bulk density (Bhagat & Verma, 1991; Sarwar *et al*., 2008). The application of farm manure has been proven to improve crop growth by improving soil physical, chemical and biological properties (Mahmood *et al*., 1997; Ibrahim *et al*., 2008; Iqbal *et al*., 2008).

It was reported by several researchers that an integrated use of organic nutritional sources (farm and green manure), along with inorganic nutritional sources (25:75), produced more yields as compared to inorganic nutritional sources alone in the rice-wheat system (Yadav *et al*., 1996; Ibrahim *et al*., 2010). In Pakistan, several organic nutritional sources like farm and green manures, straws and crop residues are available. However, the shortage of these products and the use of straw as animal feed and burning of crop residues limit the scope for their uses as organic supplement to increase soil organic matter for sustaining crop yield (Korboulewsky *et al*., 2002). Thus, the objective of present study was to evaluate the efficiency of indigenous industrial compost (IIC) to enhance the salinity tolerance in rice crop.

MATERIAL AND METHODS

Soil Characteristics and Growth Conditions

A bulk surface (0-15 cm) sample of a sandy clay loam, hyperthermic Ustalfic Haplargids (Gee & Bauder, 1986) was collected from the University College of Agriculture, Sargodha experimental farms. The samples were dried, ground, and passed through a 2 mm sieve. The prepared soil samples were analyzed for various physicochemical properties, as shown in Table 1). Soil pH was measured in a saturated soil paste by calomel glass electrode assembly using a Beckman pH meter. Soluble salt (EC_e) in the saturated extracts was measured by WTW-Cond 3I 5i EC meter. The $CaCO_3$ in the samples was analyzed by using the acid dissolution method, while the organic matter content was estimated by using the Walkely-Balack method (Nelson & Sommers, 1982). Soil was filled at 10 kg in 36 glazed clay pots. In half of the pots, salinity was artificially induced using sodium chloride salt to maintain soil EC_e level 8 dS m^{-1} and the rest of the pots were kept as control having original soil EC_e of 1.70 dS m^{-1}. This level of EC_e (8 dS m^{-1}) was selected because various earlier researchers have reported a 65% yield reduction in rice at this EC. A basal dose of N at 100 mg kg^{-1} as urea, P at 90 mg kg^{-1} as single super phosphate and K at 120 mg kg^{-1} as potassium sulphate were added prior to transplanting. Indigenous industrial compost (IIC) was applied at 0, 0.5, and 1.0% of soil weight as ICC (Organo-power™). The compost was thoroughly mixed with soil and irrigated using distilled water to field capacity (70%). The average temperature in the greenhouse varied between 35 and 45°C during the night and day, respectively. Meanwhile, the relative humidity in the greenhouse ranged from 27 % to 30% during the day and night, respectively. Light intensity varied between

TABLE 1
Selected physico-chemical properties of the soil used in this experiment

Property	Unit	Values	
Sand	%	55.16	
Silt	%	24.19	
Clay	%	20.65	
Textural class	Sandy clay loam		Hydrometer method
pH of soil paste		7.68	Saturated water extract
EC_e	dS m^{-1}	1.12	Saturated water extract
Organic matter	%	0.78	Walkley and black method
Calcium Carbonate	%	2.01	Extracted with $NaHCO_3$
$NaHCO_3^{-1}$ extractable P	mg kg^{-1} soil	6.05	$CaCO_3$ equivalent by acid dissolution
Available K	mg kg^{-1} soil	220	Extracted with 1N HN_4OAC
Available Si	mg kg^{-1} soil	31	Extracted with $CaCl_2$
Total Si	mg kg^{-1} soil	225	Extracted with sodium acetate buffer

600 and 1600 µmol photon m^2S^{-1}, depending upon day and cloud conditions during the growth period.

Indigenously Industrial Compost (ICC)

The ICC was in the shape of small excluder and the basic raw material of ICC was sugar industry waste, filter cake pressmud, molasses, vegetable market waste, rock phosphate and windrow technique was employed for its preparation. The ICC contained organic matter (35.5%), C:N ratio (20:1), nitrogen (1%), P_2O_5 (2%), sulphur (4%), potassium (1%) and calcium (1%).

Plant Material

The seeds of the two rice varieties used in this study were obtained from Kalashah Kako Rice Research Station, Lahore, Pakistan. IRRI-9 is medium duration, salt-tolerant, short-statured, coarse variety with Philippines origin and Super basmati-2000 is fine with specific aroma, salt-sensitive, and the origin is from Pakistan. For pre-germination, seeds of fine (Super basmati-2000) and coarse (IRRI-9) rice varieties were soaked in petri-dishes for 48 hours using double distilled water. The pre-germinated rice seedlings were sown in pre-washed river bed sand taken in polyethylene lined iron trays. Sand in the trays was kept moistened with distilled water for germination. The seedlings were grown on river bed sand for 21 days before transplanting. Four rice seedlings were sown in each plot under flooded conditions. Plants were grown till maturity and harvested at maturity (116 days in case of IRRI-9 and 130 in case of Super basmati-2000) and grains were separated from the shoots by hands. The samples were then dried in a forced air oven at 65°C for 48 hours. The dried samples were ground in a mechanical mill fitted with stainless steel blades to pass through a 1 mm sieve. For the determination of Na^+ and K^+, fine ground shoot samples were digested in di-acid mixture of nitric and per-chloric acids (3:1) at 60°C for 2 hours and K^+ and Na^+ was determined using a flame-photometer (Jenway PFP-7).

Statistical Analysis

The salinity and compost treatments were arranged in two-factorial, completely randomized design with three replicates. The data were statistically analyzed using PC-based programme MStat-C. Treatment means were calculated and compared by DMR test at 5% probability (Steel *et al*., 1997).

RESULTS

Plant Growth

The shoot dry matter (SDM) of the plants of both the varieties were shown to be significantly lower (p<0.01) when grown in saline soil (EC= 8 dS m^{-1}) than those gown in the normal soil. However, reduction in SDM was lower in IRRI-9 than in Super basmati-2000. Salinity stress reduced the shoot dry matter yield of both coarse and fine rice varieties. Meanwhile, the addition of IIC in the root environment significantly (p<0.01) increased SDM up to 53% and 48%, respectively, at the medium level of

IIC 0.5% of the soil weight and noted 140% and 170% increases at the high level of IIC 1% of soil weight respectively in the coarse and fine rice varieties grown in the saline soil condition. Increase in SDM due to IIC application was shown to be more in Super basmati-2000 when grown in the normal soil (Table 2) but the reverse was true when plants were grown in saline soil.

The salinity stress significantly reduced the paddy yields of both the rice varieties. The application of IIC in the growth medium significantly (p<0.01) enhanced the paddy yield of rice varieties both under the normal and saline soil conditions. Both the varieties differed significantly for the paddy yields as IRRI-9 produced more paddy yields (2.11 and 6.33 g pot^{-1}) under saline and normal soil conditions, respectively.

The IIC amendments enhanced the paddy yield significantly (p<0.01) at the medium salinity level (4.22 g pot^{-1}) and at the high level of ICC application, the increase was found to be 7.16 g pot^{-1} in the case of the coarse rice variety. The paddy yield of fine rice variety on the relative basis increased at the medium and high levels of IIC application under saline environment.

Mineral Content

The application of IIC in the root environment significantly changed the ionic composition of straw under salt stress. The maximum Na^+ concentration (1.98%) was recorded in fine rice at high salinity level under IIC free root environment. On the contrary, the minimum Na^+ concentration (0.37%) was observed in coarse rice under salt free condition, with high level of IIC (Table 3). Similarly, the maximum K^+ (1.75%) concentration was observed in coarse rice shoot in the IIC amended salt free soil condition but the minimum K^+ concentration (0.35%) was recorded in the saline environment without any IIC application in the growth medium. The shoot concentration also changed with the change of IIC levels in the root environment. The fine rice variety showed

TABLE 2
The effects of indigenous industrial compost application on the straw and paddy yields of the rice genotypes grown in the normal and saline soils

ICC per soil volume (%)	IRRI-9	Super basmati 2000	IRRI-9	Super basmati 2000
	Normal Soil (1.70 dS m^{-1})		Saline Soil (8 dS m^{-1})	
	Straw yield (g pot^{-1})			
0.0	21.35±2.97 c	16.65±2.67 cd	8.14±0.99 e	6.87±0.65 ef
0.5	28.49±3.77 b	23.41±3.53 b	12.49±1.71 d	10.21±1.12 d
1.0	37.43±4.87 a	32.16±4.78 ab	20.03±2.92 c	18.58±2.54 c
	Paddy yield (g pot^{-1})			
0.0	6.33±1.76 c	5.06±1.54 cd	2.11±0.19 d	1.93±0.13 d
0.5	9.63±2.21 bc	8.10±1.54 cd	4.22±0.88 c	4.10±0.80 cd
1.0	12.30±2.13 a	11.13±2.33 b	7.16±1.97 c	6.98±1.88 c

ICC = indigenous industrial compost; Means sharing the same letter(s) are similar at P<0.05.

TABLE 3
The effects of ICC application on the ionic composition of rice straw grown under the normal and saline soils

ICC per soil volume (%)	IRRI-9	Super basmati 2000	IRRI-9	Super basmati 2000
	Normal Soil (1.70 dS m^{-1})		Saline Soil (8 dS m^{-1})	
	Na^+ Concentration (%)			
0.0	0.45±0.06 d	0.48±0.06 d	1.72±0.16 a	1.98±0.19 a
0.5	0.40±0.06 d	0.442±0.06 d	1.13±0.10 b	1.23±0.11 b
1.0	0.37±0.06 d	0.38±0.06 d	0.64±0.08 c	0.68±0.08 c
	K^+ Concentration (%)			
0.0	1.49±0.16 a	1.53±0.17 a	0.36±0.05 d	0.35±0.05 d
0.5	1.63±0.17 a	1.67±0.17 a	0.52±0.07 c	0.56±0.07 c
1.0	1.75±0.18 a	1.72±0.18 a	1.17±0.10 b	1.28±0.11 b
	K^+:Na^+ Ratio			
0.0	3.31±0.16 b	3.15±0.17 c	0.21±0.03 f	0.18±0.031 f
0.5	4.08±0.37 b	3.97±0.33 b	0.46±0.06 e	0.46±0.06 e
1.0	4.72±0.51 a	4.52±0.41 a	1.80±0.17 d	1.88±0.18 d

ICC = indigenous industrial compost; Means sharing the same letter(s) are similar at $P<0.05$.

more response to applied IIC as compared with coarse rice. The application of IIC was also found to improve the K^+: Na^+ ratio in the saline, as well as normal field conditions. The maximum K^+: Na^+ ratio was achieved in the plants which were grown with a high level of IIC application, both under normal as well as in saline soil conditions. This finding indicates a possible mechanism of IIC mediated tolerance of salinity in rice plant.

DISCUSSION

Rice is recognized as a salt-sensitive plant; this means salinity affects almost every physiology and biochemical aspect of rice plant and significantly reduces vegetative and reproductive growth of salt-stressed plants (Mass & Hoffman, 1977). The use of organic soil amendments, such as heterogeneous composted organic material, is an important component in sustainable agricultural production under saline environment (Motavalli *et al*., 1994). A number of possible mechanisms have been proposed, whereby IIC can increase resistance of plants against salinity stress, which is a major yield limiting factor in arid and semi-arid areas. The present experiment was an attempt to monitor the beneficial effects under salt stress among two rice genotypes. The application of IIC to growth medium significantly increased dry matter production and gave higher yield in both cultivars when grown under normal as well as in saline environments (Sarwar *et al*., 2010; Sarwar *et al*., 2011). Meanwhile, the increase in dry matter was more pronounced in the saline environments, and this highlighted the beneficial effects of

IIC application in alleviating salinity stress (Sarwar *et al*., 2011). The rice production in the saline environments was very less when compared with non-saline environment. Among major possible mechanism of induced salinity tolerance in rice is increased K^+ uptake (Sarwar *et al*., 2009). The results of the present study also revealed a significant increase in the K^+ uptake in both the genotypes when ICC was added under saline conditions. However, salinity caused a significant decreased in the K^+ concentration when IIC was not applied to the root environment (Ibrahim *et al*., 2007).

Sodium concentration in plants is also a good indicator of salinity tolerance, whereby a lower Na^+ concentration in plants indicates a lower Na^+ uptake as in IRRI-9, suggesting that the variety is relatively salt tolerant. Sodium in higher amounts in plants causes a reduction in shoot dry matter, which is evident from the significant negative correlation between Na^+ concentrations and shoot dry matter in both the genotypes of rice (Fig.1). The findings of the present study also clearly exhibited a reduced uptake of Na^+ in plants when grown with IIC application in soil. The percentage of increase in the Na^+ uptake by salinity treatment was significantly reduced in the plants grown with IIC application (Schlegel *et al*., 1992).

Super basmati-2000 performed better in growth and grain yield under normal conditions; however, under saline conditions, IRRI-9 was found to perform better in terms of growth and K^+ concentration. Potassium is an important nutrient required for normal

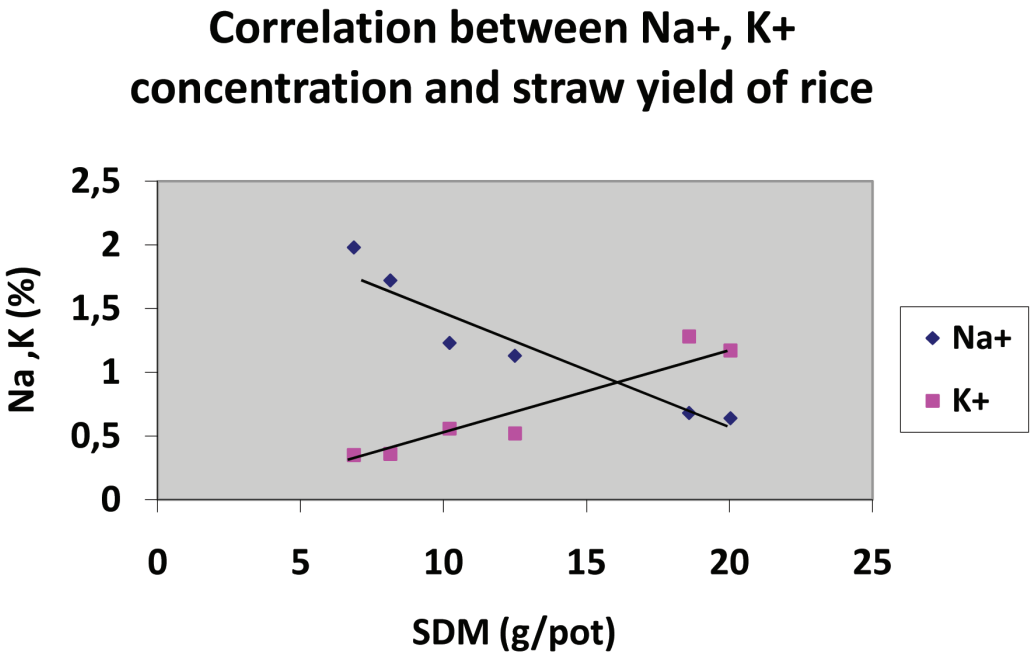

Fig.1: Correlation between Na^+ and K^+ concentrations and straw yield of rice under saline soil root environment

water uptake and transpiration flow. Low potassium uptake under saline and sodic condition further hampers crop production on these soils. In particular, potassium has a significant role in improving plant water status and mitigating the toxic effects of Na^+. The ratio of K^+: Na^+ became significantly low under salt stress when IIC was not applied, and this significantly increased when IIC was added to the root environment. This is an indication of enhanced K^+/Na^+ ratio in rice shoot, which further improved dry matter and paddy yields of both rice genotypes. Increased K^+ uptake resulted in lesser Na^+ uptake by the addition of IIC in rice, which is a major mechanism responsible for better growth of plants under saline soil environment.

CONCLUSION

Rice is an important cash crop of Pakistan and it is also a food source of millions of people around the globe. The indigenous compost application improved straw and paddy yield of both the rice varieties under normal and saline conditions. IIC increased salinity tolerance in rice by reducing Na^+ uptake and its onward translocation to shoots by increasing K^+ uptake and K^+/Na^+ ratio in rice shoot. It may be concluded that the variety of waste materials be used for the production of compost to be used by resource poor farmers for sustainable agricultural production. Hence, the results of the current study have provided an avenue for the studies under field conditions.

ACKNOWLEDGEMENTS

Thanks to Lyallpur Chemical and Fertilizer (Pvt) Ltd, Jaranwala, Pakistan for the financial assistance and Miss Noor-us-Sabah for the technical advice.

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Pertanika J. Trop. Agric. Sci. 36 (1): 71 - 78 (2013)

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Journal homepage: http://www.pertanika.upm.edu.my/

Induced spawning of a river catfish *Hemibagrus nemurus* (Valenciennes, 1840)

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ABSTRACT

There is a developing interest in *Hemibagrus nemurus* culture. Most hatcheries depend on fry from the wild, which is under threat due to human activities. Hatchery production of *H. nemurus* is not well established due to problems associated with artificial propagation. Good egg quality is essential for fry production. This paper reports the findings of research conducted to determine the effects of mammalian gonadotropin releasing hormone analogue (GnRHa) and ovaprim on induced spawning performance and egg quality of *H. nemurus*. Mature female *H. nemurus* were treated with 0.5ml of 0.7% NaCl (control), 5µg/kg, 20µg/kg, 50µg/kg body weight (BW) of fish GnRHa and 0.5ml/kg BW of fish of ovaprim (20µg/ml sGnRHa and 10mg/ml domperidone). The results showed that the administration of 5µg/kg BW GnRHa is not sufficient to induce the spawning of *H. nemurus*. The egg quality of fish treated with GnRHa at doses 20µg/kg and 50µg/kg were numerically different, however, there was no significant difference (P>0.05). Ovaprim treated fish produced the best results. Ovulatory response ranged from 50.0% to 66.7% among all the treatments with high fertilization rate (94-95%). The highest hatching rate (78.3%) and percentage of normal larvae (68.8%) was observed from ovaprim injected fish. The results indicated that 20µg/kg and 50µg/kg BW GnRHa were effective in inducing ovulation and spawning in *H. nemurus* and ovaprim can be recommended to be used by hatcheries to induce *H. nemurus* for spawning. The major significance of this work was 5µg/kg dose of GnRHa is ineffective for ovulation in *H. nemurus*.

ARTICLE INFO

Article history:
Received: 24 May 2011
Accepted: 6 June 2011

Keywords: Catfish, Egg quality, *Hemibagrus nemurus*, Hormone, Induced spawning, Ovulation, GnRHa, Reproduction

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ISSN: 1511-3701

INTRODUCTION

Gonadotropin releasing hormone analogue (GnRHa) has been successfully used in the induced spawning of several fish species (Zohar and Mylonas, 2001; Rainis and Ballestrazzi, 2005). In some fish, dopamine prevents the release of gonadotropin through inhibitory actions on the pituitary in the endocrine system, thereby making it impossible for fish to ovulate and spawn. Pre-spawn fish exhibiting dopaminergic tone were induced to spawn using GnRHa in combination with dopamine antagonist like metoclopramide, haloperidol, pimozide and domperidone. Successful spawning of some fish species were achieved using this technique. GnRHa and metoclopramide (Drori *et al.,*1994), GnRHa and haloperidol (Arabaci and Sari, 2004), GnRHa and pimozide (Tan-Fermin *et al*., 1997; Park *et al*., 2007) and GnRHa and domperidone (Manosroi *et al*., 2004; Sahoo *et al*. 2005) were reported to be successful to induce ovulation and spawning in fish.

Hemibagrus nemurus is a tropical freshwater catfish that belongs to the Family *Bagridae* and widely distributed in Asian countries which include: Thailand, Laos, Cambodia, Vietnam, Indonesia and Malaysia (Rainboth, 1996). It is economically important for aquaculture and fisheries; and nutritionally valued for low cholesterol, high protein and omega-3 poly unsaturated fatty acid (Mesomya *et al*., 2002). Due to the high demand of the fish for food, most of the natural stocks of fish species are being depleted as a result of man's activities and environmental degradation. There are few government and private hatcheries practising artificial propagation of this fish, and the broodstocks are mostly obtained from the wild. The intensive and commercial culture of *H. nemurus* is not well established due to the problems associated with induced spawning, some of which include simultaneous maturation of both male and female fish and availability of high quality broodstocks (Muchlisin *et al.,* 2004). Interest in the aquaculture industry of *H. nemurus* is developing (Khan *et al*., 1990), thus there is a need to intensify studies on the reproductive biology of *H. nemurus* so as to be able to use such information for aquaculture production and management.

Khan *et al*. (1990) provided baseline data for induced breeding of this fish, such as fecundity, gonadosomatic index (GSI) and seasonal variations of oocyte diameters. Besides, Christianus *et al*. (1998, 1999) presented information on the reproductive biology of male *H. nemurus* with respect to gonadal development.

Spawning performance is an important indicator for successful induced breeding programme. Thus, this study was attempted to determine the effect of GnRHa and ovaprim on the spawning performance and egg quality of *H. nemurus*.

MATERIALS AND METHODS

Experimental Animals and Maintenance

One hundred adult male and female *H. nemurus* collected from the wild (Pahang

River, Pahang, Malaysia) were used for this experiment. Fish body weight ranged from 440 - 900g and standard length measured ranged from 30.5 to 39.4cm. Upon arrival the fish were disinfected with potassium permanganate and kept in 40 tonnes flow-through concrete rectangular tanks under natural photoperiod and temperature for acclimatization. Fish were fed at a rate of 3% body weight to satiation with commercial pelleted feed containing 38 - 40% crude protein, 3% crude fat, 6% fiber and 13% moisture. Thirty females and 15 males were randomly selected and transferred into 2 tonnes round fibreglass tanks and were used for the following experiments. Physico-chemical parameters like temperature, dissolved oxygen and pH were monitored and recorded. These parameters were maintained at 27.0 - 27.5°C, 7.72 - 8.02mg/l and 7.74 - 7.80 respectively.

Hormone Preparation and Administration

Hormones (D-Ala^6-Pro^9-NET)-LHRH (GnRHa) and ovaprim (sGnRHa and Domperidone) were purchased from Syndel laboratories, Canada. 1mg GnRHa was dissolved in 10ml of 0.7% NaCl solution. The solution was divided into 10 aliquots and each aliquot was stored in 1ml glass vial. (1ml=100µg GnRHa). The hormone was stored in freezer at -20°C until used. Ovaprim was stored at room temperature. Hormones were administered by intramuscular injections below the dorsal fin of the fish.

Fish Selection and Identification

All fish were anaesthetized with clove oil (0.1ml/l) before handling. Mature fish were used for this experiment and were identified by the genital papillae. Males have elongated genital papillae with reddish tip and released milt upon pressing the abdomen, while the females have round genital papillae and were identified by ovarian biopsy. Eggs were collected and examined under microscope for the germinal vesicle (GV) position and oocyte diameter (OD). Fish having oocytes with the GV migrating towards the periphery were selected. These fish were then tagged with coloured plastic tags for easy identification based on treatment groups.

Experimental Design

Both the male and female fish were injected with hormone to induce spermiation and ovulation. The fish were divided into 5 groups. The first group was injected with 0.5ml of 0.7% saline solution and used as a negative control. The second group received 5µg/kg BW GnRHa hormone treatment, the third group 20µg/kg BW GnRHa, the fourth group 50µg/kg BW GnRHa hormone treatment and the fifth group 0.5ml/kg BW of ovaprim (20µg/ml sGnRHa and 10mg/ml domperidone) as a positive control. Each group comprised of 9 fish (6 females and 3 males).

Ovulation rate was measured as the percentage of females that ovulated in a treatment group. Ovulated eggs were checked under the microscope for germinal

vesicle breakdown. Artificial fertilization was carried out using the dry method. Artificial fertilization of spawn from each fish was carried out in triplicates for statistical reasons. Fertilized eggs were incubated in 5 litres plastic tanks. To determine the fertilization rate, 3 subsamples were collected (200 eggs per sample) and embryo at 4-cell division stage was characterized as fertilized. To determine the hatching rate, the subsamples of the fertilized eggs were collected, placed in 1000ml beakers with aeration and observed until hatching. Hatching occurred around 24-28 hours after fertilization. Normal larvae (Fig.1) and abnormal larvae (larvae with deformed body, curved spine, round yolk sac, short and thick body, enlarged fin folds, big yolk sac not proportional to the body) were counted and recorded. Survival rate was determined by counting the number of larvae after 4 days of hatching.

Fig.1. Normal Larvae of *H. nemurus*

Statistical Analysis

All data analyses were carried out using a computer Statistical Analysis Software (SAS). Data were expressed as mean ± standard error of mean. One way analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test (DMRT) was used for the variation in treatment means and $P < 0.05$ was selected as the level of significance.

The following formulas were used to calculate the egg quality parameters:

$$\text{Ovulatory response} = \frac{\text{Number that spawn}}{\text{Number induced}} \times 100\%$$

$$\text{Fertilization rate} = \frac{\text{Number of fertilized eggs}}{\text{Number of eggs incubated}} \times 100\%$$

$$\text{Hatching rate} = \frac{\text{Number of hatched eggs}}{\text{Number of fertilized eggs}} \times 100\%$$

$$\text{Percentage of normal larvae} = \frac{\text{Number of normal larvae}}{\text{Number of hatched eggs}} \times 100\%$$

$$\text{Percentage of abnormal larvae} = \frac{\text{Number of abnormal larvae}}{\text{Number of hatched eggs}} \times 100\%$$

$$\text{Survival rate} = \frac{\text{Number of larvae after 4 days}}{\text{Number of hatched eggs}} \times 100\%$$

RESULTS AND DISCUSSION

Table 1 shows the number of fertilized eggs, hatched eggs, normal larvae, abnormal larvae and survived larvae of induced spawn *H. nemurus*.

There was no ovulatory response in the control and 5µg/kg BW GnRHa groups. This indicated that for a successful induce spawning using GnRHa, higher doses are required. Hence, based on these results, the major significance of this study was that 5µg/kg BW GnRHa dose is ineffective for ovulation in *H. nemurus*. Ovulatory

TABLE 1
Number of fertilized eggs, hatched eggs, normal, abnormal and survived larvae of induced spawn *H. nemurus*.

Treatments	Fertilized Eggs	Hatched Eggs	Normal Larvae	Abnormal Larvae	Survived Larvae
Control	0.00± 0.00 [b]	0.00± 0.00 [c]	0.00± 0.00 [c]	0.00 ±0.00 [c]	0.00±0.00 [c]
5µg/kg BW GnRHa	0.00± 0.00 [b]	0.00± 0.00 [c]	0.00 ±0.00 [c]	0.00 ±0.00 [c]	0.00±0.00 [c]
20µg/kg BW GnRHa	188.33±4.41[a]	107.72±1.28 [b]	58.54±0.72 [b]	49.15±0.59 [a]	46.00±1.06 [b]
50µg/kg BW GnRHa	187.91±0.80 [a]	100.96±0.55 [b]	58.69± 0.43 [b]	42.25±0.17 [b]	40.68±0.33 [b]
Ovaprim	189.99±0.96 [a]	148.76±0.66 [a]	102.30±0.48 [a]	46.41±0.29 [ab]	62.23±0.11 [a]

[a]Data are mean ± SEM and based on subsamples of 200 eggs.
[b]Numbers with the same superscripts in a column are not significantly different ($p > 0.05$)
Number of female fish used per treatment = 6

responses of 50% were observed in fish injected with 20µg/kg BW GnRHa and ovaprim which is the positive control group. When compared to the ovulatory response of wild catfish *Silurus asotus,* GnRHa at doses of 0.01 - 0.02µg/g induced a low ovulation response of 25.0 - 37.5% (Wen and Lin, 2004), but in catfish *Pangasius hypophthalmus*, a higher ovulatory response (88%) was obtained using ovaprim (Legendre *et al.,* 2000). Ovaprim (sGnRHa (20µg/kg) and domperidone (10mg/kg) is ideal for induction of spawning in the Asian catfish *Clarias batrachus* for high ovulatory response (Sahoo *et al*., 2005). In this study, *H. nemurus* produced a high ovulatory response of 66.7% when treated with 50µg/kg BW GnRHa. On the contrary, 50µg/kg BW GnRHa could not induce ovulation in the Asian catfish *Clarias macrocephalus* (Tan-Fermin *et al*., 1997) and in the stinging catfish *Heteropneustes fossilis* (Tharakan and Joy, 1996).

A high fertilization rate (94-95%) was obtained with fish injected with 20µg/kg GnRHa, 50µg/kg GnRHa and ovaprim; and fertilization rates were not significantly different ($p > 0.05$) among these treatments. In this study, ovaprim resulted in the highest fertilization rate (95%). Ovaprim was found to produce high fertilization rate of 98.31% in African catfish *Heterobranchus bidorsalis* (Nwokoye *et al.,* 2007) and 73% in *Pangasius sutchi* (Chand *et al*., 2011). Thus, indicating that, GnRHa is capable of producing high fertilization rates when used to induce spawn fish.

Hatching rate was significantly different ($P<0.05$) among all the treatments. A high hatching rate (78.3%) was obtained from fish treated with ovaprim. Ovaprim injected catfish *Pangasius hypophthalmus* resulted in 72 ± 25% hatching rate (Legendre *et al*., 2000). A high hatching rate (98.35%) was also reported by Nwokoye *et al*. (2007) in ovaprim treated African catfish

Heterobranchus bidorsalis. The same hormone produced hatching rate of 60% in Asian catfish *Heteropneustes fossilis* (Haniffa and Sridhar, 2002). This signifies that ovaprim has a high potential for induced breeding of catfishes producing high hatching rates. In this study, 20µg/kg GnRHa and 50µg/kg GnRHa resulted in hatching rates of 57.2% and 53.3% respectively and there was no significant difference ($P > 0.05$) in the hatching rate of *H. nemurus* treated with 20µg/kg and 50µg/kg GnRHa. Some catfishes require higher doses of GnRHa to spawn. A higher dose of 100µg/kg GnRHa was used in channel catfish *Ictalurus punctatus,* and hatching rate of 69±5% was recorded (Silverstein *et al*.,1999).

The percentage of normal larvae obtained from the different treatments was significantly different ($P<0.05$). The highest percentage of normal larvae (68.8%) was obtained using ovaprim, followed by 50µg/kg GnRHa (58.1%) and 20µg/kg GnRHa (53.4%). Results of 20µg/kg GnRHa and 50µg/kg GnRHa were not significantly different ($P>0.05$). This study showed that ovaprim produced the lowest percentage of abnormal larvae. A larval survival rate of 40 - 43% was observed for all the treatments. Similarly, a low survival rate (38%) was observed in ovaprim treated Asian catfish *Heteropneustes fossilis* (Haniffa and Sridhar, 2002). The low survival rate might be due to some external factors such as water quality, temperature and feeding of larvae. Further studies are needed to investigate the effect of these factors on larvae survival. Thus, based on the findings of this study, it is obvious that in terms of egg quality, ovaprim injected *H. nemurus* gave the best results. GnRHa injected fish at a dose of 5µg/kg, is not effective in inducing ovulation and spawning, and the results showed that there was no significant difference ($P>0.05$) in the egg quality of induced spawn *H. nemurus* using GnRHa at doses of 20µg/kg and 50µg/kg.

CONCLUSION

This study confirms that GnRHa is effective in inducing ovulation and spawning in *H. nemurus* and can be used in hatcheries for induced breeding. However, a low dose of GnRHa (5µg/kg) may not induce the fish to spawn. Thus, the significance of this research was that 5µg/kg GnRHa can not bring about ovulation in *H. nemurus*. Induced breeding of this fish in hatcheries will help to reduce pressure on the wild population due to over-harvesting. At the same time, *H. nemurus* fry produced in hatcheries can be used for wild restocking. Ovaprim injected *H. nemurus* produced the best spawning performance and egg quality and can be recommended to hatcheries. The spawning performance and egg quality of *H. nemurus* treated with 20µg/kg GnRHa and 50µg/kg GnRHa were not significantly different. Therefore, it is economical and cost effective to use 20µg/kg GnRHa.

ACKNOWLEDGEMENTS

This research was supported by internal grant of UPM. The authors are also grateful to the staff of UPM Aquaculture Research

Station, Puchong (Mr. Azmi Yaacob, Mr. Syah Rizan, Mr. Zainal Abidin, and Mr. Jasni Yusuf) for their assistance.

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Pertanika J. Trop. Agric. Sci. 36 (1): 79 - 88 (2013)

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TROPICAL AGRICULTURAL SCIENCE

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Inhibitory Property of Metabolite Combinations Produced from *Lactobacillus plantarum* Strains

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ABSTRACT

The inhibitory property of metabolite combinations produced from six different strains of *Lactobacillus plantarum* were evaluated in this study. The final pH of culture media and cell population of each strain were determined and the antimicrobial activity of the metabolite from individual strains and combinations were conducted against *Pediococcus acidilactici* as indicator microorganism. The lowest pH was observed in the media which were cultured with *L. plantarum* RG11 and RG14 ($P < 0.05$), while the highest cell population was found in *L. plantarum* RI11 and RG11. There was no significant difference in the inhibitory activity among the six individual strains. However, when the three strains of *L. plantarum* were combined, the combination of strains (TL1-RI11-RG11, strains RS5-RI11-RG11 and strains RI11-RG14-RG11) showed a higher level of inhibition. The metabolite combinations from *L. plantarum* strains could be potential pathogen inhibition compound in the food and feed industry.

Keywords: Lactobacillus plantarum, metabolite combination, inhibitory activity

ARTICLE INFO

Article history:
Received: 24 May 2011
Accepted: 20 January 2012

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INTRODUCTION

Over the past fifty years, antibiotics have been extensively used in the industrial animal production. The widespread use of antibiotics to accelerate growth among livestock and to compensate for the unhealthy conditions found in

ISSN: 1511-3701

confined animal feeding operations has led to an increase in virulent strains of pathogenic bacteria and occurrence of drug residues in animal products. Therefore, recommendations to restrict and limit the use of antibiotics in feed have been made gradually.

New approach, such as probiotics, has been suggested as an alternative to growth promoting antibiotics in animal feed. Beneficial or commensal bacteria, such as *Lactobacillus* spp., function as a Competitive Exclusion (CE) culture. When supplemented into the diet of animals, it could reside and colonize the intestinal tract, thereby reducing the colonization of pathogens in the gastrointestinal tract (Harvey *et al*., 2005). In addition, the metabolite produced by the probiotics contains inhibitory compounds such as bacteriocins and organic acids (Thanh *et al*., 2009; Thu *et al.*, 2011) which further restrict the survival of pathogens.

Various recent studies have shown the benefits of bacterial metabolite as a feed additive in farm animals. Reductions in faecal *Enterobacteriaceae* (ENT) and cholesterol level in the plasma were observed when the metabolite produced from *L. plantarum* was supplemented into the feed of broiler chickens (Thanh *et al*., 2009) and pigs (Thu *et al*., 2010). Similarly, the bacterial metabolite also exhibits a wide range of inhibitory activities against pathogens such as *Escherichia coli*, *Salmonella typhimurium*, *Listeria monocytogenes*, *vancomycin resistant enterococci* (VRE) due to the presence of bacteriocins and organic acids (Gaggia *et al*., 2010; Thanh *et al*., 2010). Therefore, the purpose of this study was to determine the inhibitory activity of the individual of cell free extracts and select the best metabolite combination produced by 6 strains of *L. plantarum* based on the inhibitory activity against an indicator bacterium. In this study, *Pediococcus acidilactici* was chosen as the indicator because it is a common food spoilage bacterium in both animal and human food products (Waite *et al*., 2009).

MATERIALS AND METHODS

Preparation of the Bacterial Cultures

Six different strains of bacteriocin producing *Lactobacillus plantarum* (UL4, TL1, RS5, RI11, RG11 and RG14) were used in this study. These *L. plantarum* were previously isolated from Malaysian fermented food by Dr. Foo Hooi Ling from the Department of Biotechnology, Universiti Putra Malaysia, Malaysia (Foo *et al*., 2003).

Revival of the Bacterial Cultures and Collection of Metabolite

The media used to revive and cultivate *L. plantarum* were MRS (Man ROGOSA and SHAPE) broth, MRS agar and soft agar (Merck, Darmstadt, Germany). The stock cultures of these strains were prepared by inoculating *L. plantarum* (2%, v/v) into MRS broth (Merck, Darmstadt, Germany) and incubating at 30°C anaerobically for 24 hours before glycerol was added to a final concentration of 20% (v/v). The stock cultures were kept at -20°C until required. Before each use, the stock cultures were

revived twice by transferring them into 10 mL MRS broth and were incubated overnight at 30°C anaerobically. Plate spreading was then conducted for the revived cultures, followed by 48 hours of incubation. A single colony of *L. plantarum* was picked and inoculated into 10 mL MRS broth and incubated anaerobically for 24 hours, followed by re-subculturing into 10 mL MRS broth and incubating anaerobically for another 24 hours. 2% (v/v) of overnight culture was inoculated into 1 L MRS broth and incubated overnight at 30°C. The metabolites were collected by separating the bacterial cells by centrifugation at 10,000 xg for 15 min. The crude cell-free extracts were then kept at 4°C until further usage, as described by Foo *et al*. (2003) and Loh *et al.* (2010). A total of 20 different metabolite combinations were established from these strains, with each combination (COM) containing 3 strains (Table 1).

Total Viable Plate Count

The population of LAB was determined by carrying out a 10-fold serial dilution of bacterial culture using sterile distilled water. A total of 0.1 mL aliquot of diluted bacterial suspension was spread on MRS agar using spread plate method. The number of colony forming unit (CFU) was enumerated and recorded after 48 h of incubation at 37°C. The CFU per millilitre of the sample was calculated as follows (Thu *et al.,* 2010):

$$\text{Total viable plate count (CFU/mL)} = \frac{\text{Number of colonies X Dilution factor}}{\text{Volume of Sample (mL)}}$$

Optical Density and pH Determination

Optical density is a means of numeric expression of the turbidity of a suspension, such as a bacterial culture. A total of 100 µL metabolite was mixed with 900 µL of MRS broth. The optical density for each strain was adjusted and measured

TABLE 1
Twenty different metabolite combinations established from 6 strains of *L. plantarum*, each combination (COM) containing 3 strains

COM	strains	COM	strains
COM123	UL4, TL1 and RS5	COM234	TL1, RS5 and RI11
COM124	UL4, TL1 and RI11	COM235	TL1,RS5 and RG14
COM125	UL4, TL1 and RG14	COM236	TL1,RS5 and RG11
COM126	UL4, TL1 and RG11	COM245	TL1, RI11 and RG14
COM134	UL4, RS5 and RI11	COM246	TL1, RI11 and RG11
COM135	UL4, RS5 and RG14	COM256	TL1, RG14 and RG11
COM136	UL4, RS5 and RG11	COM345	RS5, RI11 and RG14
COM145	UL4, RI11 and RG14	COM346	RS5, RI11 and RG11
COM146	UL4, RI11 and RG11	COM356	RS5, RG14 and RG11
COM156	UL4,RG14 and RG11	COM456	RI11, RG14 and RG11

using a spectrophotometer (Novaspec III, Biochrom, Cambridge, UK) at 600 nm wavelength. The pH of each strains of *L. plantarum* was determined using Mettler-Toledo pH meter with a glass electrode (Mettle-Toledo., England).

Inhibitory Assay

The inhibitory assay against an indicator bacterial (*Pseudococcus acidilactici*) was determined by using the Agar-well diffusion method (Tagg & McGiven, 1971). The cell-free supernatant (CFS) containing bacteriocins was collected and diluted 2-fold until level 5 with normal saline (0.85% w/v), followed by dispensing 20 µL of each diluted CFS into MRS agar wells (5.5 mm diameter). The diluted CFS was allowed to diffuse for about 2 hours at room temperature before overlaid with 3 mL of MRS soft agar containing 1% (v/v) indicator strain of *P. acidilactici*. The plates were then sealed with parafilm and incubated at 30°C for 24 hours under anaerobic condition. The inhibitory activity was examined for a clear inhibition zone surrounding each agar well. The diameter of the inhibition zone was measured to indicate the magnitude of the inhibitory activity.

The antimicrobial activity of the cell-free extract was calculated from the reciprocal of the highest dilution producing a clear zone of growth inhibition on the indicator media under standardized condition, and it is expressed as Arbitrary Unit (AU) as described by Thanh *et al.* (2010).

$$\text{Inhibitory activity unit} = \frac{\text{Highest dilution with clear zone AU/mL}}{\text{Volume of CFS in each well}}$$

Statistical Analysis

The data were analyzed using one-way analysis of variance (ANOVA) and the General Linear Model procedure by SAS 1998 (SAS Inst., Inc., Cary, NC). Meanwhile, the Duncan's Multiple Range Test System was used to compare the significant differences of the treatments at $P < 0.05$. The data were presented as the mean ± standard error of the mean (SEM).

RESULTS

Characteristics of the Metabolites Produced by L. plantarum Strains

The optical density, final pH and cell population are shown in Table 2. Nonetheless, there is no significant difference ($P > 0.05$)

TABLE 2
Optical density (OD), pH and LAB counts of *L. plantarum* strains

	UL4	TL1	RS5	RI11	RG14	RG11
OD_{600nm}	9.64±0.12^{c}	9.85±0.08^{ab}	9.92±0.11^{a}	9.91±0.18^{a}	9.89±0.14^{ab}	9.91±0.15^{a}
pH	4.34±0.22^{a}	4.16±0.18^{ab}	4.27±0.12^{a}	4.12±0.16^{b}	3.83±0.16^{c}	3.85±0.12^{c}
LAB counts	8.14±0.05^{d}	8.79±0.03^{c}	8.85±0.03^{b}	8.95±0.02^{a}	8.82±0.04^{b}	8.92±0.03^{a}

[1] The results are presented as mean values ± SEM.
[2] $^{a-d}$ Means in the same row not sharing a common superscript are significantly different ($P < 0.05$)

in OD_{600nm} between TL1, RS5, RI11, RG14 and RG11. The mean optical density ranges from 9.64 to 9.91, with the highest optical density observed in RS5, RI11 and RG11 as compared with the other strains. In contrast, the lowest OD was observed in UL4 compared with the other strains. Meanwhile, the pH value was significantly lower ($P < 0.05$) in the culture media of RG14 and RG11 compared to the other strains. The highest pH value was observed in the medium which had been cultured with UL4. In general, the mean pH value ranges from 3.83 to 4.34. Among the six strains, RI11 and RG11 had the highest LAB counts ($P < 0.05$), and these were followed by TL1, RG14 and RS5. UL4 produced the lowest cell count ($P < 0.05$) compared with other strains.

The Inhibitory Activity of Individual L. plantarum Strains

The inhibitory activity of the metabolite produced by *L. plantarum* strains against *P. acidilactici* is shown in Table 3. As shown, there is no significant difference ($P > 0.05$) in the bacteriocin inhibitory activity among the strains. However, the largest diameter of inhibitory zone was observed in RG11 and RG14, followed by RS5, TL1 and RI11. The smallest diameter of the inhibitory zone was found in UL4.

The Inhibitory Activity of Different Metabolite Combinations

The inhibitory activity of the metabolite combinations against *P. acidilactici* is shown in Table 4. Two levels of inhibitory activities were observed in this experiment. The highest inhibitory activity (1600 AU/mL; $P < 0.05$) was observed in COM246, COM345 and COM456. Among the 3 combinations, COM456 showed the largest diameter of inhibitory zone. The remaining 17 metabolite combinations produced a lower level of inhibitory activity (800 AU/mL).

DISCUSSION

Optical Density, pH and LAB Counts

The optical density is an expression of transmittance of an optical element, under a known wavelength. The turbidity or optical density of a suspension of cells is directly related to cell mass or cell number. In this experiment, the OD was adjusted prior to the production of the active cultures where metabolite is harvested. The observation is

TABLE 3
The inhibitory activity and diameter of inhibitory zone exhibited by different *L. plantarum* strains

	UL4	TL1	RS5	RI11	RG14	RG11
Inhibitory activity (AU/mL)	800	800	800	800	800	800
Inhibitory zone (mm)	8.52±0.14[d]	8.85.±0.21[c]	9.17±0.17[b]	8.85±0.18[c]	9.57±0.23[a]	9.61±0.28[a]

[1] The results are presented as mean values ± SEM.
[2 a-d] Means in the same row not sharing a common superscript are significantly different ($P < 0.05$).

TABLE 4
The inhibitory activity and mean diameter of bacteriocin inhibitory zone exhibited by different metabolite combinations of *L. plantarum* strains

Combinations	Inhibitory activity (AU/mL)	Inhibitory zone	Combinations	Inhibitory activity (AU/mL)	Inhibitory zone
COM123	800[b]	9.23±0.18[ab]	COM246	1600[a]	8.26±0.23[b]
COM124	800[b]	9.34±0.10[a]	COM345	1600[a]	8.28±0.29[b]
COM125	800[b]	9.15±0.13[b]	COM456	1600[a]	9.35±0.32[a]
COM126	800[b]	9.27±0.11[ab]			
COM134	800[b]	8.86±0.13[bc]			
COM135	800[b]	8.70±0.14[bc]			
COM136	800[b]	9.28±0.15[ab]			
COM145	800[b]	9.17±0.13[b]			
COM146	800[b]	9.45±0.16[a]			
COM156	800[b]	8.83±0.15[bc]			
COM234	800[b]	9.49±0.21[a]			
COM235	800[b]	8.84±0.04[bc]			
COM236	800[b]	8.72±0.14[bc]			
COM245	800[b]	8.69±0.12[bc]			
COM256	800[b]	8.54±0.15[c]			
COM346	800[b]	9.14±0.11[b]			
COM356	800[b]	9.34±0.12[a]			

[1 a-b] Means in the same column not sharing a common superscript are significantly different ($P < 0.05$)

coincided with that of Thu *et al.* (2011) who found a significant difference in the final OD among the different strains of *L. plantarum*. This is mainly because of the variation in the biochemical and physiological properties between the different strains of *L. plantarum.* Under a similar condition, different strains of bacteria tend to grow and produce varied levels of metabolite which may affect the reading of the OD.

Meanwhile, the differences in the LAB counts and pH among the strains of *L. plantarum* were due to the production of metabolic compounds such as organic acids. In general, the increase in the active cell population leads to a higher production of metabolite. The metabolite production rate is correlated with a lot of factors, including growth, pH of culturing media, and temperature of incubation (Foo *et al.*, 2005; Barugue-Ramos *et al.*, 2006).

Inhibitory Activity of Metabolite and Combinations

There was no significant difference in the inhibitory activity of the single strain *L. plantarum.* However, a higher inhibitory activity was observed in COM246, COM345 and COM456 when the metabolite combination from 3 strains of *L. plantarum*

was produced and used against the indicator (*P. acidilactici)*. This is in agreement with the finding of Thu *et al*. (2011), whereby a higher inhibitory activity was observed in some of the 3 strains metabolite combinations which derived from *L plantarum.* The ability of the metabolite in expressing antimicrobial activity is mainly due to the presence of the compounds such as bacteriocins, organic acids and hydrogen peroxide (Reid, 2001). Bacteriocin from *L. plantarum* is an antimicrobial peptide in nature, and it is capable of inhibiting the growth of pathogens at cellular and molecular levels (Drider *et al*., 2006).

In addition, organic acid acts as an acidifying agent, reducing the pH of surrounding and survivability of non-acid-tolerant pathogens. Besides, organic acid could retard the enzymatic activity of the pathogens and force the bacterial cell to use the remaining energy to expel the excess of protons H^+, which would ultimately result in death by starvation (Holyoak *et al.*, 1998).

According to Yeaman and Yount (2003), the combination of metabolites from the different strains of bacteria enhanced the inhibitory activity against the pathogens due to the synergistic effects of the different antimicrobial peptides. This coincides well with the study by Luders *et al*. (2003), where a stronger inhibitory effect was observed when mixed bacteriocin from LAB was used against *E. coli*. Another study showed the inhibitory potential of *L. plantarum* where pathogens, such as *E.coli*, *L. monocytogenes*, *S. typhimurium* and vancomycin resistant *Enterococcus,* could be effectively inhibited when metabolites from all the six strains used in the study were mixed (Thanh *et al.,* 2010). Meanwhile, Bouttefroy and Milliere (2000) induced greater inhibitory effects against *L. monocytogenes* when niacin and curvacin were used.

The presence of other beneficial compounds in the metabolites, such as organic acid and hydrogen peroxide, could further potentiate the inhibitory activity of bacteriocin (Jack *et al*., 1995; Stiles, 1996). The acidification of organic acid may increase the solubility of bacteriocins and facilitate the translocation of the molecule through the cell wall. Buncic *et al*. (1995) showed that the sensitivity of *L. monocytogenes* to niacin increased when lactic acid was added.

CONCLUSION

This experiment concluded that the variations in OD, pH and LAB counts were observed in single strains. However, they produced a similar level of inhibitory activity. On the contrary, when three different strains of *L. plantarum* strains were combined, some combinations produced higher inhibitory effects. Among the 20 combinations of metabolites, the combination of strains TL1-RI11-RG11, strains RS5-RI11-RG11 and strains RI11-RG14-RG11 showed a higher level of inhibitory activity. The full potential of the metabolites in the single strains or even in combinations was still undetermined. However, this experiment could serve as a basis to show the enormous potential of the metabolites produced by *L. plantarum* strains, which could then be applied to the animal food and feed industry.

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Pertanika J. Trop. Agric. Sci. 36 (1): 89 - 98 (2013)

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Micropropagation of Asam Karanda (*Carissa carandas* Linn)

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ABSTRACT

An efficient micropropagation protocol was developed for *C. carandas* using nodal segment as the explants. Murashige and Skoog medium (MS), supplemented with 2.0 – 4.0 mg/L Benzylaminopurine (BA), induced an average of 3.6 - 5.5 shoots/explants but the number of multiple shoots formed was not significantly different. However, the addition of IBA combined with BA had shown a reducing trend in the number of shoot being formed. The MS medium supplemented with 2.0 mg/L 3-Indolebutyric acid (IBA) was found to be effective for rooting of micro-shoots. After the acclimatization process, 84% ± 4.3% of the *in vitro* plantlets survived but there was a reduction in the number of survived plantlets (59 ± 3.7%) after 4 weeks of incubation in the green house condition.

Keywords: Acclimatization, nodal segment, shoot multiplication, root formation

INTRODUCTION

Carissa carandas Linn. (Family – Apocynaceae) is an important medicinal perennial shrub growing to a height of 2 to 3 meters. The species is native to India and distributed in Sri Lanka, Indonesia, Malaysia, Myanmar and Pakistan (Hegde *et al.,* 2009). The fruit are traditionally used in the treatments of malaria, epilepsy, nerve disorder, relieve of pain and headache, fever, blood purifier, myopatic spams, dog bite, cough, colds, itches and leprosy (Rahmatullah *et al.,* 2009; Warrier *et al.,* 1993). The ripe fruit are used as appetizer for the prevention of scurvy and in the treatments of anorexia, burning sensation, skin diseases and pruritus. Meanwhile, the roots are used as anthelmintic, stomachic and antiscorbutic agents and for the treatments of intestinal worm, scabies and pruritus (Warrier *et al.,* 1993). The green fruit of *C. carandas* are also used for making pickles in India (Sturock, 1959).

ARTICLE INFO

Article history:
Received: 20 June 2011
Accepted: 5 December 2011

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ISSN: 1511-3701

The plant extract of *C. carandas* has been reported to possess cardioprotective, antipyretic and antiviral activities (Dhawan & Patnaik, 1985; Taylor *et al.,* 1996; Rajasekaran *et al.,* 1999). It is also a potential source of anthocyanin and used as a natural colouring agent for products that require mild processing treatment and low temperature storage (Iyer &Dubash, 1993).

Conventionally, *C. carandas* can be propagated through seeds, cuttings, grafting, air layering, and stooling (Misra & Jaiswal, 1993; Misra & Singh, 1990; Tyagi *et al.,* 1999). However, these methods are season-specific and require a long time to propagate. In the recent years, there has been an increased interest in the use of *in vitro* culture technique as a viable tool for mass multiplication and germplasm conservation of rare, endangered, aromatic and medicinal plants (Arora & Bhojwani, 1989; Sharma *et al.*, 1991; Sudha & Seeni, 1994). Attempt has been made for the micropropagation of *C. carandas* by using shoot tips as explants (Rai & Misra, 2005) and our experiences on shoot culture involved severe contamination problem and difficulty in establishing aseptic cultures. Hence, the present study reports on the establishment of an efficient micropropagation protocol of *C. carandas* using nodal segment as the explants.

MATERIALS AND METHODS

Establishment of Aseptic Seedlings

Mature fruit of *C. carandas* were collected from Gelugor area, Penang, Malaysia. They were washed with a combination of detergent and Clorox®, a commercial bleach containing 5.3% Sodium hypochlorite, and also rinsed under running tap water for 30 minutes to remove any soil contaminants. Two different methods of surface sterilization were used. The fruit were immersed in 95% ethanol for 2 sec and then flamed for 10 sec. This process was repeated twice. In another method, the fruit were immersed with continuous agitation in 20% Clorox® for 20 minutes, followed by rinsing them three times with sterile distilled water. The fruit were subsequently surface-sterilized with 10 % Clorox® solution for 10 minutes, followed by rinsing them three times with sterile distilled water.

The seeds were removed from the fruits and inoculated in 350 ml culture bottle (with 5 seeds in each culture bottle) containing basic Murashige and Skoog (1962) medium (MS) for germination. Seven replicates (experimental units) were used for each sterilization treatment and the experiment was repeated four times. The percentages of seed germination and survival were determined after 4 weeks of culture and the data were analyzed using Student t-test at $p \leq 0.05$. The best sterilization method was selected for subsequent experiments.

The nodal segments of the six–week old seedlings were used as the explants for the subsequent studies. All the cultures were maintained at 25 ± 2°C in a culture room with a continuous lighting provided from cool white fluorescent tubes at 35µmol m^{-2} s^{-1}.

Induction of Multiple Shoot Formation

The nodal segments (1.0 cm) of the aseptic seedlings were inoculated in a 350 ml culture bottle containing MS medium which was supplemented with different concentrations of Benzylaminopurine (BA) (0.0, 1.0, 2.0, 3.0 and 4.0 mg/ L) and 3-Indolebutyric acid (IBA) (0, 1, and 2 mg/L). A total of five nodal segments were inoculated into each culture vessel and three vessels were used for each treatment combination using 5 x 3 factorial with a complete randomized design. The numbers of shoots and roots regenerated from each explant were recorded after 8 weeks of culture. The data for each parameter were analyzed using One-Way ANOVA, and this was followed by mean comparison using Tukey test at $p \leq 0.05$, with the aid of SPSS ver. 17.

In vitro Rooting of Micro-shoots

Multiple shoots of *C. carandas* were separated into single shoot and were sub-cultured on MS medium supplemented with 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/L of IBA. In more specific, five shoots were cultured into each culture vessel and each treatment was done in three replicates with a complete randomized design. The number of roots produced from each micro-shoot was recorded after six weeks of culture and the data were analyzed using the One-Way ANOVA, followed by the mean comparison using Tukey test at $p \leq 0.05$.

Acclimatization

The eight-week old rooted *in vitro* plantlets of *C. carandas* with shoot height of 3-5 cm were removed from the culture bottle and washed thoroughly under running tap water before they were transplanted into a plastic tray (30 cm x 20 cm) containing a mixture of top soil, organic soil and sand (1:1:1). The trays were covered with a plastic sheet to create a relative humidity of 80- 90%, and they were then placed in the green house with a day temperature of 28 ± 2ºC and a night temperature of 24 ± 2ºC. After one week, the plants were transferred into poly-bags and the percentage of the surviving plantlets was recorded after four weeks in the plant house condition. The plantlets were watered with tap water twice a day (morning and evening).

RESULTS AND DISCUSSION

The results obtained in this study indicated that 100% of the aseptic seeds could be established by flaming mature fruit or

TABLE 1
The establishment of the aseptic seedlings of *C. carandas* and their germination percentage

Sterilization Technique	Aseptic seeds (%)	Seed Germination (%) ± s.e
Double surface-sterilization with Clorox®	100 a	81.1 ± 5.5 b
Immersed in 95% ethanol and flame.	100 a	81.1 ± 4.9 b

Mean values within the same column followed by same alphabet are not significantly different (Tukey's HSD test, $p \leq 0.05$)

double surface-sterilization with Clorox®. More than 80% of the aseptic seeds of *C. carandas,* which were germinated using either of methods of sterilization, were found to be insignificantly different (Table 1).

The results also revealed that the shoot proliferation of *C. carandas* was affected by the addition of plant growth regulator auxin and cytokinin such as BA and IBA. The MS medium, which was supplemented with different concentrations of BA, induced multiple shoot formations from the nodal segments of *C. carandas*. The results also indicated that as the amount of BA added into the medium increased, the number of shoots produced would also increase. The MS medium supplemented with 2 – 4.0 mg/L BA induced an average of 3.6-5.5 shoots/explants but the number of the multiple shoots formed was not significantly different. However, the addition of IBA (1.0 and 2.0 mg/L) with the presence of BA showed a reducing trend in the number of the shoots formed from each explant (Table 2). Gomes *et al.* (2010) also found that the addition of NAA into the MS medium in combination with different types of cytokinin was unable to increase the number of shoots of *Arbutus unedo*. Since the MS medium supplemented with 2.0 mg/L BA was sufficient for the induction of multiple

TABLE 2
The effects of BA and IBA supplemented into the MS medium on the *in vitro* growth of *C. carandas* after 8 weeks of culture

Treatment (mg/L)	Number of shoots (n ± s.e)	Length of shoots (cm ± s.e)	Number of roots (n ± s.e)
0 IBA 0 BA	1.6 ± 0.2 cd	0.9 ± 0.1 b	0 ± 0 b
0 IBA 1 BA	2.3 ± 0.2 bcd	2.8 ± 0.7 ab	0 ± 0 b
0 IBA 2 BA	3.6 ± 0.6 abcd	3.3 ± 0.5 a	0 ± 0 b
0 IBA 3 BA	4.2 ± 0.7 ab	2.8 ± 0.5 ab	0 ± 0 b
0 IBA 4 BA	5.5 ± 0.9 a	2.5 ± 0.2 ab	0 ± 0 b
1 IBA 0 BA	1.3 ± 0.2 d	2.2 ± 0.7 ab	2.6 ± 1.2 b
1 IBA 1 BA	2.1 ± 0.4 bcd	3.5 ± 0.4 a	0 ± 0 b
1 IBA 2 BA	3.1 ± 0.7 bcd	3.1 ± 0.3 a	0 ± 0 b
1 IBA 3 BA	2.6 ± 0.3 bcd	2.1 ± 0.2 ab	0 ± 0 b
1 IBA 4 BA	3.7 ± 0.5 abc	1.8 ± 0.3 ab	0 ± 0 b
2 IBA 0 BA	1.3 ± 0.2 d	2.9 ± 0.6 ab	6.6 ± 1.9 a
2 IBA 1 BA	1.5 ± 0.6 cd	1.7 ± 0.5 ab	0 ± 0 b
2 IBA 2 BA	2.7 ± 0.4 bcd	2.3 ± 0.3 ab	0 ± 0 b
2 IBA 3 BA	2.0 ± 0.5 bcd	1.9 ± 0.6 ab	0 ± 0 b
2 IBA 4 BA	3.6 ± 0.6 abcd	2.6 ± 0.3 ab	0 ± 0 b

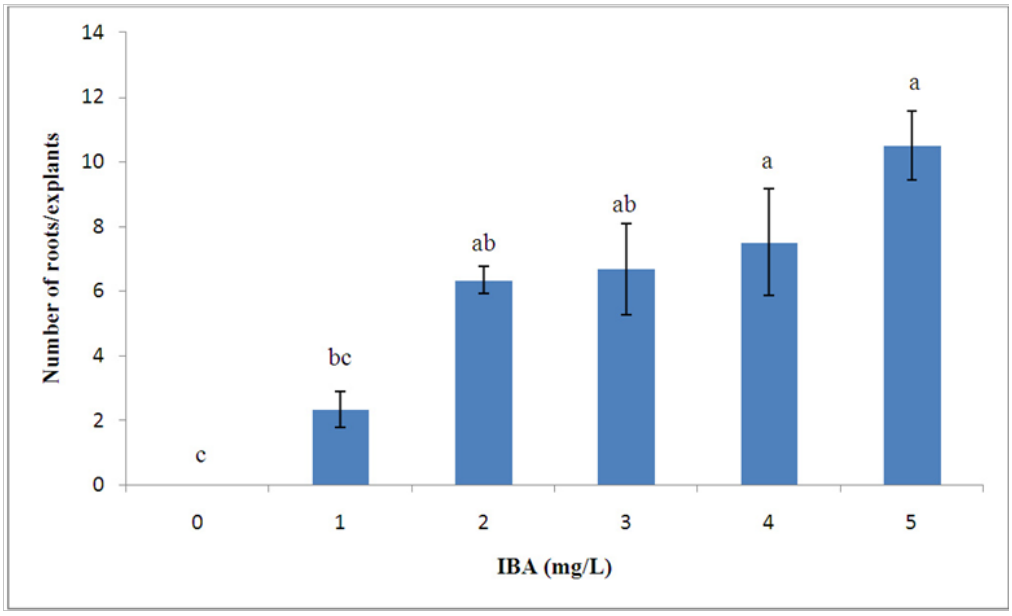
Mean values within the same column, followed by same alphabet, are not significantly different (Tukey's HSD test, $p \leq 0.05$).

shoots formation, it was used as the shoot proliferation medium for mass production of *C. carandas*. Rai and Misra (2005) also found that the presence of only cytokinin (13.33 µM BA) in the MS medium could induce the formation of high number of shoots (12.5 shoot/explants) in *C. carandas* but using shoot tips as the explants. Hence, this finding indicates that the requirement of plant growth regulators is dependent upon the type of explants, genotype and plant species. For instance, Oliveira *et al.* (2010) reported that different plant growth regulators gave different effects on the shoot growth of *Melaleuca alternifolia*.

The results obtained in the current study indicated that the MS medium supplemented with different concentrations of BA and IBA did not affect the height of the shoots. There was a high variation in the shoot height when they were cultured in the BA and IBA combination treatment. Meanwhile, a reasonable good shoot height (3.5 cm) was obtained in the MS medium supplemented with 1.0 mg/L IBA and 1.0 mg/L BA and MS plus 1.0 mg/L IBA and 2.0 mg/L BA (3.1 cm), as shown in Table 2.

The addition of BA in the medium inhibited the root formation in *C. carandas* and callus, and they appeared at the base of

Fig.1: Callus appeared at the base of the micro-shoots of *C. carandas* cultured on the MS medium containing 2.0 mg/L BA



Mean values with the same alphabet are not significantly different (Tukey's HSD test, $p \leq 0.05$)

Fig.2: The effects of different concentrations of IBA supplemented in the MS Medium on the rooting of *C. carandas*

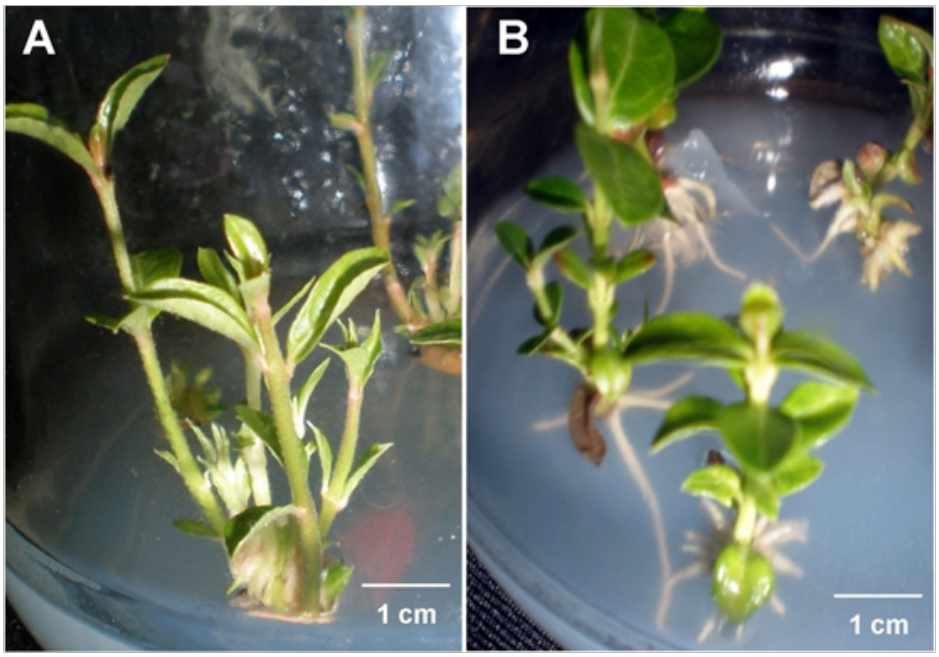


Fig.3: *In vitro* cultures of *C. Carandas;* (A) Multiple shoots on the MS medium supplemented with 3.0 mg/L BA (B) The rooting of micro-shoots on the MS medium supplemented with 2.0 mg/L IBA.

the micro-shoots when cultured in the MS medium containing different concentrations of BA (Fig.1). A similar result was observed for *Dendrobium candidum*, whereby the addition of BA inhibited root formation (Wang *et al.,* 1997). On the other hand, the addition of IBA was found to be effective for the root induction in *C. carandas*. MS medium supplemented with 2 mg/L IBA inducing the highest number of roots (6.6 roots/ shoot) (Table 2). Additional study indicated that the addition of more than 2.0 mg/L (2- 5.0 mg/L) of IBA into the MS medium induced the roots but not significantly different in the number of roots produced (Fig.2). Therefore, MS supplemented with 2 mg/L IBA was chosen as the rooting medium for the micro-shoots separated from the multiple shoots (Fig.3A) for the production of *in vitro* plantlets (Fig.3B).

The *in vitro* plantlets of *C. carandas* could survive well (84 ± 4.3%) when they were acclimatized in the plastic tray covered with transparent plastic sheet. However, only 59 ± 3.7% of the plantlets survived after four weeks of being transferred to the poly-bags and placed in the plant house. These results indicate that plantlets cannot adapt well with the outside environment. Low humidity and full exposure to light may be the main cause for the lower survival rate of *C. carandas* plantlets under green house condition. Nonetheless, the plantlets that survived did not show any morphological abnormalities (Fig.4).

Fig.4: Four-week old acclimatized plantlets of *C. carandas*

CONCLUSION

The present *in vitro* propagation protocol using MS medium supplemented with 3.0 mg/L BA was found to be effective for the mass multiplication of *C. carandas* shoots. However, the addition of 2.0 mg/L IBA into the culture medium was required for the rooting of the micro-shoots. The established *in vitro* propagation protocol could be used as an alternative for the propagation of *C. carandas* plantlets.

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Pertanika J. Trop. Agric. Sci. 36 (1): 99 - 114 (2013)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

The Estimation of Economic Benefits of Urban Trees Using Contingent Valuation Method in Tasik Perdana, Kuala Lumpur

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ABSTRACT

Urban trees provide a multitude of tangible and intangible services which include provisionary, regulatory, as well as cultural and support services to the community. Unfortunately, to set a monetary value on these said services is challenging to say the least. Ignorance of such monetary value is unintentional and this is mainly due to the lack of awareness and the absence of monetary value of the services itself. Hence, the quality of these urban trees degrades over time as no one appreciates its monetary value. In light of this situation, a study was initiated to determine the economic benefits of the urban trees that were planted surrounding Tasik Perdana (TP) area. For this purpose, a total of 313 respondents were interviewed in the TP area using the contingent valuation method (CVM). The objective of this study was to elicit willingness to pay (WTP) for these urban trees. WTP represents the willingness of a person to pay in monetary terms to secure and sustain these urban trees. Hence, seven bid prices were used and distributed to the respondents: RM1.00, RM5.00, RM10.00, RM15.00, RM20.00, RM25.00 and RM30.00. Logit and linear regression models were applied to predict the maximum, mean, and median WTP. The study concludes that the estimated mean WTP is RM10.32 per visit and the estimated median WTP is RM10.08 per visit.

Keywords: Monetary value, urban park, willingness to pay, bid price, logit model

ARTICLE INFO

Article history:
Received: 20 February 2012
Accepted: 28 March 2012

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INTRODUCTION

Urban trees provide a variety of services which include provisioning services such as aesthetic trees, regulating services (e.g., flood control and climate control), cultural services (e.g., historical park, national parks or natural forms of land), and also

ISSN: 1511-3701

supporting services in soil formation, plant growth and oxygen production. Studies have found that there has been an extraordinary disengagement of humans from the natural environment since few hundred years ago (Mazlina & Ismail, 2008; Maller *et al*., 2005; Axelrod & Suedfeld, 1995). Thus, people are encouraged to engage in outdoor activities and have regular contact with nature. Trees are the main feature of nature and urban parks are a commonplace for urban dwellers to conduct their activities and to be close to nature.

Sadly, the same urban trees are slowly but surely moving towards physical degradation. Some of the more seriously degraded trees have to be removed as they pose danger to the general public. One of the factors that has contributed to urban trees degradation is violence. Vandalism is becoming more prominent especially in urban park areas (Abdul Malek & Mariapan, 2009). For example, the Kuala Lumpur City Council (DBKL) and other related government agencies have spent a total of RM2.4 million to replace the facilities that were damaged by vandals (Dalip, 2001). Moreover, improper use of urban trees such as leaving heavy objects under them may cause physical stress. Improper planning during the plantation phase, such as planting the trees in unsuitable locations, will also cause stress and affect their growth. In addition, natural disasters or events such as in severe lighting storms can also cause serious damages to the tress and inevitably pose danger to the public. Often, the maintenance of these trees is seen to be the sole responsibility of the respective agencies that are responsible for landscaping services and not as the responsibilities of the beneficiaries of the services provided by these trees. These trees should be properly managed and conserved to ensure that they will continue to provide the services and at the same time improve the quality of life of the urban dwellers.

A management plan for urban trees should be in place as a guide for related decision-makers, especially for park managers. A well-distributed, open space (green lung) can dramatically improve the quality of life of the people (Federal Department of Town and Country Planning, 2005). Meanwhile, a high quality park service requires huge city government budget to be allocated for personnel, park resources, and for administrative activities (Iamtrakul *et al*., 2005). In order to estimate the amount of budget that is needed for such items, the monetary value of these urban trees should be determined prior to undertaking the economic valuation of urban trees.

However, the monetary value of the urban trees in urban parks is often undefined as there is no available market for them. Therefore, the urban trees which provide intangible benefits and services are often misconstrued as non-market value products and not traded in a real economic market. If the economic value of the urban trees is not known, these trees may never be insured and they will thus remain inadequately managed and subsequently affect the urban dwellers' safety and quality of life.

In addition, one of the consequences for not knowing the real economic value of urban trees is the lack of appreciation towards the services provided by these trees. Therefore, the general public might not be willing to pay if an additional fund is required for urban tree management. Moreover, a park manager may pay less attention on the urban trees management when there is no monetary value given to these urban trees. As a result, these trees are removed without much due consideration to their environmental benefits and the monetary losses that they may have incurred.

One of the methods that can be used to quantify the benefits and services of non-market goods such as urban trees is the contingent valuation method (CVM) (Jim & Chen, 2008; McPherson, 1999). The first CVM survey was proposed by Ciriacy-Wantrup (1947). After seventeen years, Davis (1963) successfully developed a practical application to evaluate the economic value of recreation in the Maine woods. Now, many studies on the non-market goods utilising the CVM method have been conducted such as those by Treiman and Gartner (2005), Togridou *et al*. (2006), Adams *et al*. (2008) and Natalia and Mercedes (2010).

MATERIALS AND METHODS

Study Area

Kuala Lumpur Lake Gardens is also known as *Taman Tasik Perdana* (TTP). It is located right in the middle of Kuala Lumpur city. The whole area is estimated to be 91.6 hectares, which includes the flowering shrubs, shady trees, botanical gardens, and other notable features. This is a recreational park and it is one of the most popular recreational areas among the locals and foreign visitors. The park was established amongst other parks, providing recreational facilities and historical structures – these include the Orchid Garden, Hibiscus Garden, Butterfly Park, Bird Park, Boathouse, Deer Park, Panggung Anniversary, Malaysian National Monument, ASEAN Gardens, Memorial Tun Razak and Carcosa Seri Negara. TTP is the first and oldest public park in Kuala Lumpur. It was the brainchild of Alfred Venning, the British State Treasurer in the 1880s (Malaysia Travel Guide, n.d.). The second Prime Minister of Malaysia, Tun Haji Abdul Razak Hussein officially opened the park on May 1st, 1975.

This research study site is located in the Lake Gardens or Taman Tasik Perdana (TTP), which is close to the artificial lakes. According to Kuala Lumpur City Hall (DBKL), approximately RM5.6 million was spent to manage and maintain this park from 2005 to 2009. Currently, there are five new phases added to the park, which have started in April 2010. To date, there are 753 new trees and shrubs which have been planted in this area (Table 1).

TABLE 1
The newly planted species in Tasik Perdana

No.	Plant	Quantity
1	*Actinodaphne macrophylla*	10
2	*Actinodaphne sesquipedalis*	6
3	*Adansonia digitata*	4
4	*Alerites moluccana*	5
5	*Alstonia scholaris*	5
6	*Amherstia nobilis*	5
7	*Anacardium occidentale*	5
8	*Andira surinamensis*	3
9	*Aquralia malaccensis*	3
10	*Aralidium pinnatifidum*	10
11	*Arfeuillea arborescens*	5
12	*Azadirachta excelsa*	5
13	*Baccaurea parviflora*	5
14	*Bacckia frutescens*	5
15	*Barringtonia racemosa*	5
16	*Bauhinia grafinii*	20
17	*Biringtonea* Spp *(Milingtonia hortensis)*	3
18	*Brachychiton rupestris*	4
19	*Brownea grandices*	3
20	*Buchanania arborescens*	5
21	*Bucida buceras*	5
22	*Caesalpinia ferrea*	10
23	*Calophyllum curtisii*	5
24	*Calophyllum soulattri*	5
25	*Campnosperma auriculatum*	4
26	*Canarium littorale*	3
27	*Cassia rainbow shower*	1
28	*Chempaca alba* (Michelia alba)	5
29	*Chorisia speciosa*	5
30	*Chrysephyllum cainito*	5
31	*Chukrasia tabularis*	5
32	*Cleitanthus malaccensis*	5
33	*Clusia majar* cv var	5
34	*Cola gigantea*	2
35	*Couroupita guianensis*	5
36	*Cratoxylum formosum*	4
37	*Crotoxyllum cochinchinensis*	10
38	*Crotoxylon cochinchinenses*	2
39	*Cycas peetinata*	10
40	*Cycas rumphii*	5
41	*Cynometra malaccensis*	3
42	*Cynometra ramiflora*	3
43	*Delonix regia*	3
44	*Dialium indum*	5
45	*Dillenia reticulata*	4
46	*Diospyros blancoi*	5
47	*Diospyros buxifolia*	5
48	*Diospyros lanceifolius*	12
49	*Diospyros tristis*	5
50	*Diospyros wallichii*	5
51	*Dipterocapus chartacens*	5
52	*Dipterocapus kunstleri*	5
53	*Dyera costulata*	10
54	*Elaeocarpus angustifolius*	10
55	*Eleoocarpus apiculatus*	5
56	*Erythrina glauca*	3
57	*Erythrophleum guineense*	5
58	*Eugenia cerinum*	5
59	*Eugenia clariflorum*	20
60	*Eugenia companulatum* (Fine leaf)	5
61	*Eurycoma longifolia*	20
62	*Fagraea fragrans*	5
63	*Fagraea racemosa*	15
64	*Ficus microcapha*	5
65	*Ficus* Sp (Thai)	10
66	*Firmiana malayana*	5
67	*Flacourtia inermis*	5

Table 1 (*continued*)

68	*Garcinia cowa*	5
69	*Garcinia scortechinii*	20
70	*Gardenia carinata*	5
71	*Gardenia tubifera*	5
72	*Guaiacum officinle*	5
73	*Hopea ferruginea*	3
74	*Horsfieldia superba*	5
75	*Intsia bijuga*	6
76	Japanese round pine	5
77	*Kigelia africana*	6
78	*Knema hookeriana*	5
79	*Koelreuteria formosana*	5
80	*Koomposia excelsa*	5
81	*Lagerstroemia floribunda*	5
82	*Lagerstroemia speciosa*	2
83	*Lagerstromia* sp (Red flower)	5
84	*Lecythis ollaria* (Monkey pot)	10
85	*Lepisanthes alata* (Johore tree)	5
86	*Lopanthera lactescens*	10
87	*Mangifera lagenifera*	4
88	*Maniltoa* sp	5
89	*Moringa thouarsii*	5
90	*Neodypsis lastelliana* (Red neck)	15
91	*Osmosia sumatrana*	5
92	*Pagiantha dichotoma*	5
93	*Pandanus utilis*	5
94	*Parkia javanica*	3
95	*Parkia speciosa*	3
96	*Pentaspadonmotleyi*	5
97	*Phyllanthus pectinatus*	3
98	*Phyllocarpus septrenlrionalis*	4
99	*Pimeleodendron griffithianum*	5
100	*Plumeria* pink	2
101	*Plumeria* red	1
102	*Plumeria tricolor*	1

Table 1 (*continued*)

103	*Plumeria* white/yellow	2
104	*Podocarpus makii*	5
105	*Polyalthia rumphii*	5
106	*Pometia pinnata*	5
107	*Pongamia pinnata*	5
108	*Pritchardia pacifica*	10
109	*Pterocarpus indicus var pendula*	15
110	*Samanea saman*	5
111	*Sandoricum koetjape*	3
112	*Saraca cauliflora*	10
113	*Scorodocarpus borneensis*	5
114	*Sindora* Sp	5
115	*Sterculia cordata*	5
116	*Sterculia parviflora*	5
117	*Sterculia rubiginosa*	5
118	*Streblus elongatus*	5
119	*Suregada multiflora*	5
120	*Syzygium malaccaensis*	4
121	*Tabebuia argentea*	10
122	*Tamarindus indica*	4
123	*Terminalia calamansanai*	5
124	*Tristania obovata* (Multi Stem)	15
125	*Tristania obovata* (single stem)	7
126	*Tristania whitetiana* (single stem)	5
127	*Xanthophyllum eurhynchum*	5
128	*Xanthostemon chrysanthus*	5
	Total	753

Data Collection

The actual survey was carried out from October 2010 to January 2011 in the mornings of many weekends. The survey was authorised by the Dewan Bandaraya Kuala Lumpur (DBKL) who allowed such a

survey to be carried out within the stipulated time period. A total of 313 respondents (park visitors) were randomly selected and successfully interviewed.

Questionnaire Design

The questionnaire was written in Malay and English. Open and close-ended questions were used for this purpose. Open-ended questions are subjective questions that require the respondents to respond in any way they prefer while close-ended questions require responses that limit the subjects to the choices provided to them. The questionnaire was further divided into three separate parts that cover the following subjects:

- Background

  This includes the general background of the study site and the research objectives.

- Visitor's valuation of environment goods

  These questions elicit the respondents' willingness to pay (WTP) and their preferences relevant to the urban trees conservation.

- Demographic questions

  These questions are related to the respondents' personal characteristics (origin, gender, age, marital status, race, working status, level of education, and monthly income).

Model Formulation

Economists define value based on the ideals of rationality and consumer sovereignty (Hanley *et al*., 1997). An individual is assumed to have preferences over urban trees, and thus the utility function formed. Consumer surplus is the money metric of unobservable utility function and can be either willingness to pay (WTP) or willingness to accept (WTA) compensation measure. Hence, preference can be indexed by the utility function and changes in the utility are estimated by consumer surplus.

With appropriate restrictions, individual's WTP or WTA for a change in urban trees is based on a theory of rational choice by consistent estimate of preferences. Logit or logistic regression is normally used to determine WTP (Hanemann, 1984). The answer given by the respondent either is 'Yes' or 'No' in the WTP. Meanwhile, the form of the logit model is as follows:

$$P_i = \left(E(Y_i = \frac{1}{X_i}) \right) = \frac{1}{1 + \exp^{-(\alpha + \beta_i BID + \beta_i x_i + \varepsilon_i)}} [1]$$

This model determines the probability of saying 'yes' to a bid price at different levels of independent. Where P_i is a probability that Y_i =1 (yes response), BID_i is the bid offered, X_i is the vector of independent variable, i is the index of observation, α and β are the intercept and vector of the coefficients to be estimated corresponding to a logistic distribution, and Ɛ is a random error that follows the normal distribution with a mean zero and a common variance σ^2. The linear form of the model [2] is as follows:

$$L_i = \ln\left(\frac{P_i}{1-P_i}\right) = \alpha + \beta_i + \beta_i X_i + \varepsilon_i \quad [2]$$

where, L_i is called logit and it is the log of the odd ratio. The maximum likelihood is the estimation method. The coefficient represents the change in L_i that is associated with a one unit change in X_i when other coefficients are held constant. The estimation of the mean and median WTP for the logit model, using the estimated coefficients from [2], can be estimated as follows (Hanemann *et al*., 1991):

$$Mean \approx \frac{\ln(1+\exp^{\hat{\alpha}+\hat{\beta}_i \ddot{x}_i})}{-\hat{\beta}_i} \quad [3]$$

$$Medium \approx \frac{\hat{\alpha}+\hat{\beta}_i \ddot{\ddot{X}}_i}{-\hat{\beta}_i} \quad [4]$$

where is the coefficient of the estimate on the bid price, is the estimated intercept, and is the mean of the respective explanatory variable. In addition to the logit model, the liner regression model was also estimated using the open-ended WTP question. By using the maximum WTP data (open-ended WTP question) as the dependent variable against the other independent variables, a linear regression model using the ordinary least square technique (OLS) was employed, as follows:

$$\text{Max } (WTP_i) = \alpha + \beta_i X_i + \varepsilon_i \quad [5]$$

where X_i is the vector of the independent variables, and ε_i is an error term which is assumed to be normally distributed with a mean zero and a common variance σ^2, $\varepsilon_i \sim N(0, \sigma^2)$.

RESULTS AND DISCUSSION

Respondents' Profile

Table 2 shows the social-demographic characteristics of the local respondents interviewed. The highest percentage of the respondents was from Kuala Lumpur (53.2%), and this was followed by the respondents from Selangor (43.2%). This also means that the majority of the respondents were from Kuala Lumpur, and this was due to the close proximity of the park to their places of residence. Approximately 50.6% of the respondents were female and 49.4% were male. There is an even distribution of percentage of respondents' gender in the study.

TABLE 2
Respondents' profile

Variable	Percentage (%)
Origin	
Kuala Lumpur	53.2
Selangor	43.2
Negeri Sembilan	0.9
Pahang	0.9
Penang	0.9
Perak	0.9
Gender	
Male	49.4
Female	50.6
Age	
20 or below	9.7
21-30	47.0
31-40	26.0
41-50	13.7

Table 2 (*continued*)

50 Above	3.3
(Minimum = 18, Maximum = 63, Mean = 32)	
Marital Status	
Single	44.6
Married	53.8
Divorced	0.3
Widowed	1.3
Race	
Malay	61.2
Chinese	27.6
Indian	10.6
Other	0.6
Working Status	
Government servant	15.6
Private sector	36.8
Businessmen	16.0
Home duties	12.7
Student	16.3
Retiree	1.6
Unemployed	1.0
Level of Education	
Completed primary school (standard 1 to 6)	1.0
Completed secondary school (form 1 to 5)	24.2
Completed high school (form 6)	10.5
Certificate or diploma education	26.8
First degree	32.4
Master and PHD	5.2
Monthly Income (RM)	
500-1000	1.7
1001-2000	18.8
2001-3000	43.3
3001-4000	25.0
4001-5000	6.8
5001 Above	3.4
(Minimum = RM 500, Maximum = RM 10000, Mean = RM 2896.63)	

The age of the respondents is illustrated and grouped into five categories, as shown in Table 1. The majority of the respondents were between 21 and 30 year old (47.0%), followed by the age group of 31 to 40 year old (26.0%). Meanwhile, the mean age of the respondent was 32 year old.

About 53.8% of the respondents were married, 44.6% were single, and a small percentage was either divorced (0.3%) and widowed (1.3%). In terms of their racial category, 61.2% of the respondents are Malays, 27.6% are Chinese, 10.6% are Indian, and 0.6% indicates other races.

The majority of the respondents (68.4%) are employed and they work in the private sector (36.8%). The respondents who do not work (31.6%) are categorised as home duties, students, retirees, and unemployed. The majority of the respondents are bachelor degree holders (32.4%), followed by certificate and diploma holders (26.8%) and secondary school leavers (24.2%). These data indicate that the majority of the respondents have completed at least secondary school level.

Meanwhile, the respondents' levels of income are grouped into six categories. The majority of the respondents are with income between RM2,001.00 and RM3,000.00 (43.3%). The highest income recorded is for a respondent who works as a professional (RM10,000) and the lowest income is RM500.00. This result shows that most of the respondents' income status is in the medium class category.

Reasons for WTP and Not WTP for Urban Tree Conservation

Table 3 indicates the reasons of the respondents who are willing to pay and the reasons for those who are not willing to pay for urban tree conservation. Surprisingly, the majority of the respondents indicated that they are willing to pay for urban trees conservation as a means to contribute towards the general maintenance of the park (34.4%), and this is perhaps to have a sense of ownership of the park. They felt that this is the most efficient and general way to improve the urban tree conservation. As for those who are not willing to pay (28.3%), one of the reasons cited is that the bid price recommended is too high.

TABLE 3
Reason for WTP and not WTP for urban tree conservation

Statement	Percentage (%)
Reasons for WTP	
Restore and rehabilitate natural features	33.8
Improve the park to become more attractive	22.3
Reduce the burden of the government	5.2
As a general contribution to maintain the park	34.4
Other reason	4.3
Reasons for not WTP	
Cannot afford to pay	13.8
Would like to pay but not this much	28.3
Support urban tree programme in other ways	27.7
Cost should be borne by the government	25.4
Other reason	4.8

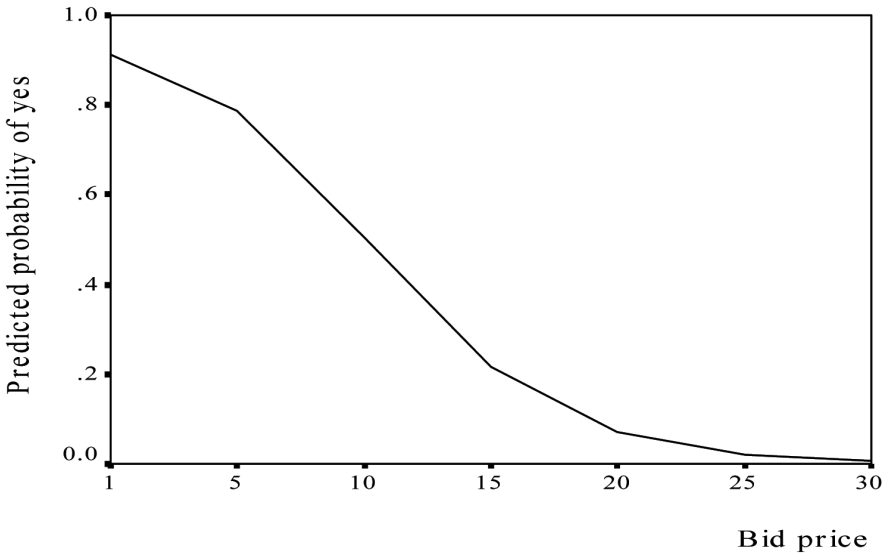

Fig.1: Predicted Probability of Yes on Bid Price

The Estimated WTP

As shown in Table 4, the percentage of the respondents who are not willing to pay increases as the bid level increases, while the percentage of those who are willing to pay decreases as the bid level increases. Figure 1 indicates a negative relationship between the respondents' willingness to pay and the bid price offered. This shows that most of the respondents are willing to pay a lower amount for urban tree conservation as an entrance fee.

TABLE 4
WTP and the mean of maximum WTP at different bid levels

Bid	Number (N)	Not WTP (%)	WTP (%)
1	44	0	100.0
5	44	20.6	79.4
10	46	63.0	37.0
15	45	82.2	17.8
20	45	88.9	11.1
25	44	93.2	6.8
30	45	100.0	0

Table 5 demonstrates the theoretical expected relationship of the willingness to pay and the explanatory variables prior to the survey and the actual relationship after the survey has been completed. It is expected that the bid price has a negative sign, while other variables (except origin, gender, age, marital status, and race have no prior expectation) have positive signs. The positive sign indicates that the variable has a direct relationship with the willingness to pay for the urban tree conservation, and vice versa.

TABLE 5
The expected relationship of the variables

Variable	Expected Relationship	Actual Relationship
Bids	-	-
Origin	+	+
Gender	?	+
Age	?	-
Marital status	?	+
Race	?	-
Working status	+	+
Education level	+	-
Income	+	+

Note: + indicates positive relationship; - indicates negative relationship; ? indicates no prior expectation

In comparison to the expected sign to the actual sign, only the education level is out of the expectation. This signifies that as the education level of the respondent is lower, the higher the respondents' willingness to pay for urban trees conservation. The actual relationship indicates that gender and marital status are positively related to the willingness to pay for the urban tree services. The male respondents (gender) are more likely to pay in comparison to the female respondents, whereas the unmarried respondents (single) are more likely to pay as compared to the married ones. On the other hand, age and race were found to be negatively related to the willingness to pay for the urban tree conservation. Jamal and Shahariah (2003) also found that there is a negative relationship between age and the probability of willingness to pay. They also found that the younger respondents were more likely to pay and the respondents from other races were more likely to pay as compared to the Malay respondents.

TABLE 6
Results of the logit and OLS models

Variable	Model 1		Model 2		Model 3		Model 4
	Coefficient (stand. Error)	$EXP(co)_1$	Coefficient (stand. Error)	$EXP(co)_2$	Coefficient (stand. Error)	$EXP(co)_3$	Coefficient (stand. Error)
Bids	-0.472*** (0.130)	0.624	-0.450*** (0.107)	0.638	-0.442*** (0.100)	0.643	-
Origin	2.588* (1.399)	13.304	2.065** (0.984)	7.887	2.106** (0.961)	8.215	3.053** (1.175)
Gender	2.648** (1.317)	14.120	1.920* (0.992)	6.820	1.814* (0.968)	6.134	0.796 (1.294)
Age	-0.050 (0.082)	0.951	0.016 (0.063)	1.016	0.018 (0.059)	1.108	0.036 (0.103)
Marital status	1.051 (1.379)	2.862	1.573 (1.230)	4.820	1.698 (1.173)	5.461	0.985 (1.589)
Race	-0.945 (1.221)	0.389	-2.288** (1.115)	0.068	-2.436** (1.047)	0.088	-1.265 (1.371)
Working status	1.680 (1.363)	5.367	2.216* (1.153)	9.167	1.814* (1.039)	6.137	1.127 (1.406)
Education level	-	-	0.152 (0.157)	1.164	-	-	-0.002 (0.231)
Income	0.000 (0.001)	1.000	-	-	-	-	0.000 (0.000)
Constant	2.321 (3.599)	10.184	0.263 (3.414)	1.301	2.1291 (2.566)	8.410	2.022 (5.230)
-2 Log likelihood	30.288		45.712		46.990		
Cox & Snell R Square	0.595		0.578		0.578		
Nagelkerke R Square	0.812		0.794		0.794		
R square							0.150
Adjusted R Square							0.040

Note: *** - significant at 0.01 level
** - significant at 0.05 level
* - significant at 0.1 level

Logistic and OLS Model

There were four models used to estimate the WTP for the socio-demographic variables (Table 6). As shown in Table 6, Model 1 indicates the highest R square value compared to that of the other logit models. There is no commonly accepted threshold value for the pseudo R square statistic that denotes a satisfactory or well specified model (Bateman *et al*., 2002). Model 4 is the OLS estimate, with only origin from Kuala Lumpur (0.012) is significant at the 0.05 level. The maximum willingness to pay for urban trees is increased multiplicatively by 3.054 for every unit increased in origin from Kuala Lumpur.

Without the education level variable, only Model 1 has three significant variables, which are bid price (0.01), origin (0.05), and gender (0.1). The odd ratio of the willingness to pay for urban trees is increased multiplicatively by 0.624 for every unit decreased in the bid price; the odd ratio of willingness to pay for urban trees is increased multiplicatively by 13.304 for every unit increased in the origin from Kuala Lumpur; the odd ratio of willingness to pay for urban trees is increased multiplicatively by 14.120 for every unit increased of the male respondents (gender).

Meanwhile, Model 2 also has five significant variables, which are bid price (0.01), origin (0.05), gender (0.05), race (0.05), and working status (0.1). Model 2 shows that the odd ratio of willingness to pay for urban trees is increased multiplicatively by 0.638 for every unit decreased in bid price; the odd ratio of willingness to pay for urban trees is increased multiplicatively by 7.887 for every unit increased in the origin from Kuala Lumpur; the odd ratio of willingness to pay for urban trees is increased multiplicatively by 6.820 for every unit increased in the male respondents (gender); the odd ratio of willingness to pay for urban trees is increased multiplicatively by 0.068 for every unit decreased in the Malay respondents (race); the odd ratio of willingness to pay for urban trees is increased multiplicatively by 1.164 for every unit increased in the government servant (working status).

With the absence of the education level and income variables, Model 3 has the same five significant variables with lower standard errors compared to that of Model 2. Model 2 shows that the odd ratio of willingness to pay for urban trees is increased multiplicatively by 0.643 for every unit decreased in the bid price; the odd ratio of willingness to pay for urban trees is increased multiplicatively by 8.215 for every unit increased in the origin from Kuala Lumpur; the odd ratio of willingness to pay for urban trees is increased multiplicatively by 6.134 for every unit increased in the male respondents (gender); the odd ratio of willingness to pay for urban trees is increased multiplicatively by 0.088 for every unit decreased in the Malay respondents (race); the odd ratio of willingness to pay for urban trees is increased multiplicatively by 6.137 for every unit increased in the government servant (working status).

Mean, Median, and Maximum WTP

The estimated mean WTP for urban trees is approximately RM10.32 per visit, while the estimated median of WTP is approximately RM10.08 per visit. The estimated maximum WTP is RM5.40. These estimates were calculated as follows:

$$E(WTP) = \frac{\ln(1 + e^{3.348 - 1.003 * 0.612})}{-(-0.271)} \quad [6]$$

$$E(WTP) = RM10.32$$

$$Me(WTP) = \frac{3.348 - 1.004 * 0.612}{-(-0.271)} \quad [7]$$

$$Me(WTP) = RM10.08$$

$$Max\ (WTP) = 2.022 + 3.053(0.532) + 0.796(0.494) + 0.036(31.531) + 0.908(0.446) - 1.265(0.612) + 1.127(0.156) - 0.002(13.788) + 0(2896.635) \quad [8]$$

$Max(WTP)$ = RM5.40

The estimated mean WTP is close to the median WTP (Puan, 2005), and the mean WTP is slightly greater than the median WTP (Nik Mustapha, 1993; Amiry, 2009). The median of this study is similar to the median from Alias *et al*. (2002) who found that both the medians are almost RM11.00. As for the estimated mean WTP of this study, it is also similar as the mean of the local respondents from a study by Samdin (2002) and in between the mean range of the local respondents from Amiry (2009), in which those means are almost equivalent to RM10.00. The result indicates that the willingness to pay is at RM10.32 per visit on average, while the majority preferred paying RM10.08 per visit.

CONCLUSION

The payment vehicle in this research was accepted as the WTP as an entrance fee. Once the entrance fee mechanism is implemented, the monetary value of the park is expected to improve. However, one should note that there is a high probability that many of the respondents are willing to pay at a lower bid level for urban tree conservation in TP. The logit model indicates that the mean and median WTP are RM10.32 and RM10.03 per visit, respectively. The linear model indicates the maximum WTP of RM5.40 per visit. Based on the findings, an entrance fee mechanism is recommended to be put in place so that park managers may utilise the additional fund for urban tree management and maintenance work and thus, reduces its dependence on the public fund for this particular purpose.

ACKNOWLEDGEMENTS

The authors wish to extend their appreciation to the Landscaping and Urban Cleaning Control Department of Kuala Lumpur City Council (DBKL) for their cooperation and provision of research data and information for this study.

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Special Acknowledgement

The **JTAS Editorial Board** gratefully *acknowledges* the assistance of **Doreen Dillah**, who served as the English language editor for this issue.

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Please read the guidelines and follow these instructions carefully; doing so will ensure that the publication of your manuscript is as rapid and efficient as possible. The Editorial Board reserves the right to return manuscripts that are not prepared in accordance with these guidelines.

About the Journal

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* began publication in 1978 as a Journal of Tropical Agricultural Science and became a leading agricultural journal in Malaysia. After 29 years as a multidisciplinary journal, the revamped Journal of Tropical Agricultural Science (JTAS) is now focusing on tropical agricultural research. Other *Pertanika* series include Journal of Science and Technology (JST) and Journal of Social Sciences and Humanities (JSSH).

JTAS is published in **English** and it is open to authors around the world regardless of the nationality. It is currently published four times a year, i.e. in **February**, **May**, **August** and **November**.

Goal of *Pertanika*

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

Quality

We aim for excellence, sustained by a responsible and professional approach to journal publishing. Submissions are guaranteed to receive a decision within 12 weeks. The elapsed time from submission to publication for the articles averages 5-6 months.

Indexing of *Pertanika*

Pertanika is now over 33 years old; this accumulated knowledge has resulted in *Pertanika* JTAS being indexed in SCOPUS (Elsevier), Web of Science (BIOSIS), EBSCO, DOAJ, CABI, AGRICOLA and ISC.

Future vision

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

We also have views on the future of our journals. The emergence of the online medium as the predominant vehicle for the 'consumption' and distribution of much academic research will be the ultimate instrument in the dissemination of research news to our scientists and readers.

Aims and scope

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

Editorial Statement

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

Guidelines for Authors

Publication policies

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

Editorial process

Authors are notified on receipt of a manuscript and upon the editorial decision regarding publication.

Manuscript review: Manuscripts deemed suitable for publication are sent to the Editorial Board members and/or other reviewers. We encourage authors to suggest the names of possible reviewers. Notification of the editorial decision is usually provided within to eight to ten weeks from the receipt of manuscript. Publication of solicited manuscripts is not guaranteed. In most cases, manuscripts are accepted conditionally, pending an author's revision of the material.

Author approval: Authors are responsible for all statements in articles, including changes made by editors. The liaison author must be available for consultation with an editor of *The Journal* to answer questions during the editorial process and to approve the edited copy. Authors receive edited typescript (not galley proofs) for final approval. Changes **cannot** be made to the copy after the edited version has been approved.

Manuscript preparation

Pertanika accepts submission of mainly four types of manuscripts. Each manuscript is classified as **regular** or **original** articles, **short communications**, **reviews**, and proposals for **special issues**. Articles must be in **English** and they must be competently written and argued in clear and concise grammatical English. Acceptable English usage and syntax are expected. Do not use slang, jargon, or obscure abbreviations or phrasing. Metric measurement is preferred; equivalent English measurement may be included in parentheses. Always provide the complete form of an acronym/abbreviation the first time it is presented in the text. Contributors are strongly recommended to have the manuscript checked by a colleague with ample experience in writing English manuscripts or an English language editor.

Linguistically hopeless manuscripts will be rejected straightaway (e.g., when the language is so poor that one cannot be sure of what the authors really mean). This process, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

The instructions for authors must be followed. Manuscripts not adhering to the instructions will be returned for revision without review. Authors should prepare manuscripts according to the guidelines of *Pertanika*.

1. Regular article

Definition: Full-length original empirical investigations, consisting of introduction, materials and methods, results and discussion, conclusions. Original work must provide references and an explanation on research findings that contain new and significant findings.

Size: Should not exceed 5000 words or 8-10 printed pages (excluding the abstract, references, tables and/or figures). One printed page is roughly equivalent to 3 type-written pages.

2. Short communications

Definition: Significant new information to readers of the Journal in a short but complete form. It is suitable for the publication of technical advance, bioinformatics or insightful findings of plant and animal development and function.

Size: Should not exceed 2000 words or 4 printed pages, is intended for rapid publication. They are not intended for publishing preliminary results or to be a reduced version of Regular Papers or Rapid Papers.

3. Review article

Definition: Critical evaluation of materials about current research that had already been published by organizing, integrating, and evaluating previously published materials. Re-analyses as meta-analysis and systemic reviews are encouraged. Review articles should aim to provide systemic overviews, evaluations and interpretations of research in a given field.

Size: Should not exceed 4000 words or 7-8 printed pages.

4. Special issues

Definition: Usually papers from research presented at a conference, seminar, congress or a symposium.

Size: Should not exceed 5000 words or 8-10 printed pages.

5. Others

Definition: Brief reports, case studies, comments, Letters to the Editor, and replies on previously published articles may be considered.

Size: Should not exceed 2000 words or up to 4 printed pages.

With few exceptions, original manuscripts should not exceed the recommended length of 6 printed pages (about 18 typed pages, double-spaced and in 12-point font, tables and figures included). Printing is expensive, and, for the Journal, postage doubles when an issue exceeds 80 pages. You can understand then that there is little room for flexibility.

Long articles reduce the Journal's possibility to accept other high-quality contributions because of its 80-page restriction. We would like to publish as many good studies as possible, not only a few lengthy ones. (And, who reads overly long articles anyway?) Therefore, in our competition, short and concise manuscripts have a definite advantage.

Format

The paper should be formatted in one column format with at least 4cm margins and 1.5 line spacing throughout. Authors are advised to use Times New Roman 12-point font. Be especially careful when you are inserting special characters, as those inserted in different fonts may be replaced by different characters when converted to PDF files. It is well known that 'μ' will be replaced by other characters when fonts such as 'Symbol' or 'Mincho' are used.

A maximum of eight keywords should be indicated below the abstract to describe the contents of the manuscript. Leave a blank line between each paragraph and between each entry in the list of bibliographic references. Tables should preferably be placed in the same electronic file as the text. Authors should consult a recent issue of the Journal for table layout.

Every page of the manuscript, including the title page, references, tables, etc. should be numbered. However, no reference should be made to page numbers in the text; if necessary, one may refer to sections. Underline words that should be in italics, and do not underline any other words.

We recommend that authors prepare the text as a **Microsoft Word** file.

1. Manuscripts in general should be organised in the following order:

    - **Page 1: Running title.** (Not to exceed 60 characters, counting letters and spaces). This page should **only** contain the running title of your paper. The running title is an abbreviated title used as the running head on every page of the manuscript.

      In addition, the **Subject areas m**ost relevant to the study must be indicated on this page. Select the appropriate subject areas from the Scope of the Journals provided in the Manuscript Submission Guide

    - A **list** of n**umber of black and white / colour figures and tables** should also be indicated on this page. Figures submitted in color will be printed in colour. See "*5. Figures & Photographs"* for details.

    - **Page 2: Author(s) and Corresponding author information**. This page should contain the **full title** of your paper with name(s) of all the authors, institutions and corresponding author's name, institution and full address (Street address, telephone number (including extension), hand phone number, fax number and e-mail address) for editorial correspondence. The names of the authors **must** be abbreviated following the international naming convention. e.g. Salleh, A.B., Tan, S.G., or Sapuan, S.M.

      Authors' addresses. Multiple authors with different addresses must indicate their respective addresses separately by superscript numbers:

      George Swan[1] and Nayan Kanwal[2]
      [1]Department of Biology, Faculty of Science, Duke University, Durham, North Carolina, USA.
      [2]Office of the Deputy Vice Chancellor (R&I), Universiti Putra Malaysia, Serdang, Malaysia.

    - **Page 3**: This page should **repeat** the **full title** of your paper with only the **Abstract** (the abstract should be less than 250 words for a Regular Paper and up to 100 words for a Short Communication). **Keywords** must also be provided on this page (Not more than eight keywords in alphabetical order).

    - **Page 4 and subsequent pages**: This page should begin with the **Introduction** of your article and the rest of your paper should follow from page 5 onwards.

    Abbreviations. Define alphabetically, other than abbreviations that can be used without definition. Words or phrases that are abbreviated in the introduction and following text should be written out in full the first time that they appear in the text, with each abbreviated form in parenthesis. Include the common name or scientific name, or both, of animal and plant materials.

    Footnotes. Current addresses of authors if different from heading.

2. **Text.** Regular Papers should be prepared with the headings **Introduction**, **Materials and Methods**, **Results and Discussion, Conclusions** in this order. Short Communications should be prepared according to "*8. Short Communications."* below.

3. **Tables.** All tables should be prepared in a form consistent with recent issues of *Pertanika* and should be numbered consecutively with Arabic numerals. Explanatory material should be given in the table legends and footnotes. Each table should be prepared on a separate page. (Note that when a manuscript is accepted for publication, tables must be submitted as data - .doc, .rtf, Excel or PowerPoint file- because tables submitted as image data cannot be edited for publication.)

4. **Equations and Formulae.** These must be set up clearly and should be typed triple spaced. Numbers identifying equations should be in square brackets and placed on the right margin of the text.

5. **Figures & Photographs.** Submit an original figure or photograph. Line drawings must be clear, with high black and white contrast. Each figure or photograph should be prepared on a separate sheet and numbered consecutively with Arabic numerals. Appropriate sized numbers, letters and symbols should be used, no smaller than 2 mm in size after reduction to single column width (85 mm), 1.5-column width (120 mm) or full 2-column width (175 mm). Failure to comply with these specifications will require new figures and delay in publication. For electronic figures, create your figures using applications that are capable of preparing **high resolution** TIFF files acceptable for publication. In general, we require **300 dpi or higher resolution for coloured and half-tone artwork** and **1200 dpi or higher for line drawings** to be submitted in separate electronic files.

   For review, you may attach low-resolution figures, which are still clear enough for reviewing, to keep the file of the manuscript under 5 MB. Illustrations may be produced at no extra cost in colour at the discretion of the Publisher; the author could be charged Malaysian Ringgit 50 for each colour page.

6. **References.** Literature citations in the text should be made by name(s) of author(s) and year. For references with more than two authors, the name of the first author followed by *'et al*.' should be used.

   Swan and Kanwal (2007) reported that …
   The results have been interpreted (Kanwal et al. 2009).

   - References should be listed in alphabetical order, by the authors' last names. For the same author, or for the same set of authors, references should be arranged chronologically. If there is more than one publication in the same year for the same author(s), the letters 'a', 'b', etc., should be added to the year.

   - When the authors are more than 11, list 5 authors and then et al.

   - Do not use indentations in typing References. Use one line of space to separate each reference. The name of the journal should be written in full. For example:

     - Jalaludin, S. (1997a). Metabolizable energy of some local feeding stuff. *Tumbuh, 1,* 21-24.
     - Jalaludin, S. (1997b). The use of different vegetable oil in chicken ration. *Malayan Agriculturist, 11,* 29-31.
     - Tan, S.G., Omar, M.Y., Mahani, K.W., Rahani, M., & Selvaraj, O.S. (1994). Biochemical genetic studies on wild populations of three species of green leafhoppers *Nephotettix* from Peninsular Malaysia. *Biochemical Genetics*, *32,* 415 - 422.

   - In case of citing an author(s) who has published more than one paper in the same year, the papers should be distinguished by addition of a small letter as shown above, e.g. Jalaludin (1997a); Jalaludin (1997b).

   - Unpublished data and personal communications should not be cited as literature citations, but given in the text in parentheses. 'In press' articles that have been accepted for publication may be cited in References. Include in the citation the journal in which the 'in press' article will appear and the publication date, if a date is available.

7. **Examples of other reference citations**:

   Monographs: Turner, H.N., & Yong, S.S.Y. (2006). *Quantitative Genetics in Sheep Breeding*. Ithaca: Cornell University Press.

   Chapter in Book: Kanwal, N.D.S. (1992). Role of plantation crops in Papua New Guinea economy. In Angela R. McLean (Ed.), *Introduction of livestock in the Enga province PNG* (p. 221-250). United Kingdom: Oxford Press.

8. ***Proceedings***: Kanwal, N.D.S. (2001). Assessing the visual impact of degraded land management with landscape design software. In Kanwal, N.D.S., & Lecoustre, P. (Eds.), *International forum for Urban Landscape Technologies* (p. 117-127). Lullier, Geneva, Switzerland: CIRAD Press.

9. **Short Communications** should include **Introduction, Materials and Methods, Results and Discussion, Conclusions** in this order. Headings should only be inserted for Materials and Methods. The abstract should be up to 100 words, as stated above. Short Communications must be 5 printed pages or less, including all references, figures and tables. References should be less than 30. A 5 page paper is usually approximately 3000 words plus four figures or tables (if each figure or table is less than 1/4 page).

   *Authors should state the total number of words (including the Abstract) in the cover letter. Manuscripts that do not fulfill these criteria will be rejected as Short Communications without review.

STYLE OF THE MANUSCRIPT

Manuscripts should follow the style of the latest version of the Publication Manual of the American Psychological Association (APA). The journal uses American or British spelling and authors may follow the latest edition of the Oxford Advanced Learner's Dictionary for British spellings.

SUBMISSION OF MANUSCRIPTS

All articles should be submitted electronically using the ScholarOne web-based system. ScholarOne, a Thomson Reuters product provides comprehensive workflow management systems for scholarly journals. For more information, go to our web page and click "**Online Submission**".

Alternatively, you may submit the electronic files (cover letter, manuscript, and the **Manuscript Submission Kit** comprising *Declaration* and *Referral* forms) via email directly to the Executive Editor. If the files are too large to email, mail a CD containing the files. The **Manuscript Submission Guide** and **Submission Kit** are available from the *Pertanika*'s home page at http://www.pertanika.upm.edu.my/home.php or from the Executive Editor's office upon request.

All articles submitted to the journal **must comply** with these instructions. Failure to do so will result in return of the manuscript and possible delay in publication.

Please do **not** submit manuscripts to the editor-in-chief or to any other office directly. All manuscripts must be **submitted through the executive editor's office** to be properly acknowledged and rapidly processed at the address below:

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Authors should retain copies of submitted manuscripts and correspondence, as materials cannot be returned. Authors are required to inform the Executive Editor of any change of address which occurs whilst their papers are in the process of publication.

Cover letter

All submissions must be accompanied by a cover letter detailing what you are submitting. Papers are accepted for publication in the journal on the understanding that the article is original and the content has not been published or submitted for publication elsewhere. This must be stated in the cover letter.

The cover letter must also contain an acknowledgement that all authors have contributed significantly, and that all authors are in agreement with the content of the manuscript.

The cover letter of the paper should contain (i) the title; (ii) the full names of the authors; (iii) the addresses of the institutions at which the work was carried out together with (iv) the full postal and email address, plus facsimile and telephone numbers of the author to whom correspondence about the manuscript should be sent. The present address of any author, if different from that where the work was carried out, should be supplied in a footnote.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed on a cover sheet.

Peer review

Pertanika follows a **double-blind peer-review** process. Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

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.

Copyright

Authors publishing the Journal will be asked to sign a declaration form. In signing the form, it is assumed that authors have obtained permission to use any copyrighted or previously published material. All authors must read and agree to the conditions

outlined in the form, and must sign the form or agree that the corresponding author can sign on their behalf. Articles cannot be published until a signed form has been received.

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

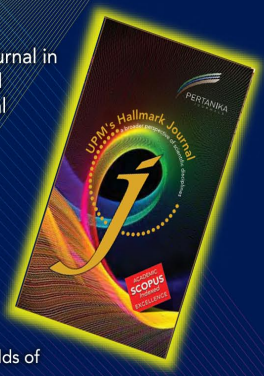
Under the Journal's open access initiative, authors can choose to download free material (via PDF link) from any of the journal issues from Pertanika's website. Under "Browse Journals" you will see a link entitled "Current Issues" or "Archives". Here you will get access to all back-issues from 1978 onwards.

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.

Pertanika

Our goal is to bring high quality research to the widest possible audience

Pertanika is an international 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**.

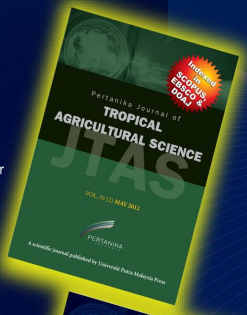
JST caters for science and engineering research or related fields of study. It is published twice a year in **January** and **July**.

JSSH deals in research or theories in social sciences and humanities research 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**.

Why should you publish in *Pertanika* Journals?

Benefits to Authors

PROFILE: Our journals are circulated in large numbers all over Malaysia, and beyond in Southeast Asia. Recently, we have widened our circulation to other overseas countries as well. We will ensure that your work reaches the widest possible audience in print and online, through our wide publicity campaigns held frequently, and through our constantly developing electronic initiatives via *Pertanika* online submission system backed by Thomson Reuters.



QUALITY: Our journals' reputation for quality is unsurpassed ensuring that the originality, authority and accuracy of your work will be fully recognised. Each manuscript submitted to Pertanika undergoesa rigid **originality check**. Our double-blind peer refereeing procedures are **fair and open, and we aim to help authors** develop and improve their work. Pertanika JTAS is now over 33 years old; this accumulated knowledge has resulted in Pertanika being indexed in **SCOPUS** (Elsevier), **Thomson** (Biosis), **EBSCO**, **CABI**, **AGRICOLA**, **Google Scholar**, **MyAIS**, **ISC** and **ERA**.

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 publish with us again and again.

LAG TIME: Submissions are guaranteed to receive a decision within **14 weeks**. The elapsed time from submission to publication for the articles averages 5-6 months. A decision of acceptance of a manuscript is reached in 3 to 4 months (average 14 weeks).

Pertanika is Indexed in SCOPUS, EBSCO, DOAJ & ISC

Call for Papers

Pertanika invites you to explore frontiers from all fields of science and technology to social sciences and humanities. You may contribute your scientific work for publishing in UPM's hallmark journals either as a regular article, short communication, or a review article in our forthcoming issues. Papers submitted to this journal must contain original results and must not be submitted elsewhere while being evaluated for the *Pertanika* Journals.

Submissions in English should be accompanied by an abstract not exceeding 300 words. Your manuscript should be no more than 6,000 words or 10-12 printed pages, including notes and abstract. Submissions should conform to the *Pertanika* style, which is available at www.pertanika.upm.edu.my or by mail or email upon request.

Papers should be double-spaced 12 point type (Times New Roman fonts preferred). The first page should include the title of the article but no author information. Page 2 should repeat the title of the article together with the names and contact information of the corresponding author as well as all the other authors. Page 3 should contain the title of the paper and abstract only. Page 4 and subsequent pages to have the text - Acknowledgments - References - Tables - Legends to figures – Figures, etc.

Questions regarding submissions should only be directed to the Executive Editor, *Pertanika* Journals.

Remember, *Pertanika* is the resource to support you in strengthening research and research management capacity.

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Pertanika Journal of Tropical Agricultural Science
Vol. 36 (1) Feb. 2013

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ISSN 1511-3701
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