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Review Article

Net Primary Productivity of Forest Trees: A Review of Current Issue

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ABSTRACT

Forest growth is important both economically and ecologically and it follows a predictable general trend with age. Generally, the growth of all forests accelerates as canopies develop in young forests and declines substantially soon after the maximum leaf area is attained. The causes of this decline trend are multiple. Initially, age- and size-related declines were attributed to photosynthesis-respiration imbalance. Subsequently, several competing hypotheses have been proposed over the years, although nutrient and hydraulic limitation hypotheses appear to be the most likely to have caused it. In this paper, the authors attempt to review these hypotheses and concentrate on one related aspect, as this can set the scene for further examining the issues of age-related versus size-related processes.

Keywords: Forest growth, canopies, photosynthesis – respiration imbalance

GENERAL BACKGROUND

The rate of production of the biomass by both individual trees and forest stands decreases substantially with age (Assmann, 1970). In particular, age-related reduction in biomass accumulation is an important consideration in the mechanistic models which predict the forest growth and determine the capacity of the forests to act as carbon sinks. With the current impetus towards silvicultural approaches that employ uneven-aged systems and extended rotations, understanding the physiological basis for the age-related decline in productivity of dominant species has become increasingly important. Understanding this component of productivity is crucial to quantifying and manipulating carbon fluxes in forest ecosystems and their influence on the global CO_2 cycles.

Despite many studies attempting to explain the age-related decline of forest productivity through a number of theories, the age-related regulation still remains unclear. There is little direct evidence which explains that old forests assimilate less carbon than the young forests. This makes model-based predictions of the carbon sequestration suspect, as most physiological process models predict carbon assimilation were based on the measurements of photosynthesis on young trees (Ryan *et al.*, 1997a). For example, Bond (2000) provided a list of studies exhibiting two contrasting results in the changes in the net photosynthesis with age of trees and shrubs (Table 1).

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TABLE 1
Studies reporting a comparison of the net photosynthesis in differently aged trees and shrubs

Decreased net photosynthesis (A_{net}) with increased ages of trees and shrubs		
Species	Comparison	Reference
Chrysothamnus nauseosus	Maximum A_{net} in summer. Juvenile *versus* mature.	Donovan and Ehleringer (1992)*
Juniperus occidentalis	Juvenile *versus* young mature.	Miller *et al.* (1995)*
Larrea tridentate	Daily maximum A_{net}, three summer months. Juvenile *versus* mature.	Franco *et al.* (1994)*
Picea abies	Light-saturated A_{net}, open-grown trees. Juvenile *versus* mature.	Kull and Koppel (1987)*
Picea rubens	Seasonal A_{net}. Mature *versus* relatively old-growth.	Day *et al.* (2001)
Pinus aristata	Light-saturated A_{net}, optimal conditions. Mature *versus* old-growth.	Schoettle (1994)*
Pinus contorta	Light-saturated A_{net}. Mature *versus* old-growth.	Yoder *et al.* (1994)*
Pinus ponderosa	Light-saturated A_{net}. Mature *versus* old-growth.	Yoder *et al.* (1994)*
Pinus ponderosa	Mean morning A_{net}. Juvenile *versus* mature and old-growth.	Kolb and Stone (2000)*
Prosopis glandulosa	Daily maximum A_{net}, after rainfall. Juvenile *versus* mature.	DeSoyza *et al.* (1996)*
Prunus serotina	Growing season average A_{net}. Seedling *versus* sapling *versus* mature.	Fredericksen *et al.* (1996)*
Sequoiadendron giganteum	Maximum A_{net}. Seedlings *versus* juvenile, mature and old-growth.	Grulke and Miller (1994)*
No difference or increased net photosynthesis with increased ages of trees and shrubs		
Species	Comparison	Reference
Acer negundo	Maximum A_{net}, during peak of drought period. Juvenile versus mature.	Donovan and Ehleringer (1992)*
Artemesia tridentata	Maximum $A_{net,}$ in mid-summer at a dry site. Juvenile *versus* mature.	Donovan and Ehleringer (1992)*
Chrysothamnus nauseosus	Maximum A_{net}, in mid-summer at a dry site. Juvenile *versus* adult.	Donovan and Ehleringer (1992)*
Prosopis glandulosa	Daily maximum A_{net}. Summer drought. Small (0.5 m) *versus* large (1.1 m).	DeSoyza *et al.* (1996)*
Pseudotsuga menziesii	No clear trend of light-saturated A_{net}. Mature *versus* old growth.	McDowell *et al.* (2002)
Quercus rubra	Light-saturated A_{net}, during growing season. Seedling *versus* mature.	Hanson *et al.* (1994)*

Note: * = cited from Bond (2000).

The causes of this age-related decline are likely multiple, but the most important potential causes which can explain a decline in forest productivity with stand development are nutrient limitation, hydraulic limitation and maturation (genetic programming) hypotheses. According to some authors, the so called "age-related" decline in the forest productivity is primarily a "size-related" decline (Weiner and Thomas, 2001). Ideally, a test should be conducted on the trees of similar size, but different ages or *vice versa*; thereby separating the confounded factors of size and age. An alternative to obtain trees with similar size but with different ages is to graft scions of the different ages onto young rootstocks.

Studies using grafting have been conducted by a few researchers (summarised in Table 2) in relation to the effect of age on the growth, morphology and physiology of grafted scions. However, these studies still yielded contrasting outcomes with the age of scion. Despite the presence of these grafting studies, there are some weaknesses which have never been tackled by the previous researchers. For instance, no study has so far simultaneously compared individuals in the field and genetically identical grafted seedlings. Day *et al.* (2001) conducted a similar study, but the individuals selected in the field were not genetically identical with the grafted seedlings (although they came from the same population). Moreover, most of the published evidences on grafting have been obtained from very young trees. In relation to the studies presented in Table 2, for instance, Hutchison *et al.* (1990) used individuals taken from four age classes, ranging from 1 to 45 years; whereas, Rebbeck *et al.* (1993) used only juvenile and mature (>50 years old) scions. In addition, Greenwood (1984) studied shoot development as a function of age on *Picea taeda*, but the comparisons were only made among scions up to 12 years of age. Furthermore, Greenwood *et al.* (1989) also used grafting approach to study the effect of age on the morphological characteristics and DNA methylation of *Larix laricina*, but the scions involved were taken from juvenile and mature trees, ranging from 1 to 74 years of age.

Mencuccini *et al.* (2005) also used propagated material to separate the relative effects of size and age on tree growth and metabolism. These authors selected four different tree species. Three of them (one conifer, Scots pine and two angiosperms with diffuse-porous wood, sycamore and ring-porous wood, ash) were propagated by grafting, whereas the fourth (poplar) was propagated by direct rooting. Poplar was also chosen because of its clonal nature, which allowed a different approach in the separation of age versus size (cf., Mencuccini, 2007 for further details). The approach adopted in Mencuccini *et al.* (2005) avoided some of the potential artefacts presented in the earlier works (Table 2). For instance, the individuals measured in the field were almost always the exact same donors from where the grafted twigs were taken, thereby avoiding genetic differences between the donor trees and the grafted plants. For the conifer in the study by Mencuccini *et al.* (2005) (Scots pine), the measurements took place five to seven years after the initial grafting, to ensure that enough time had elapsed since grafting had taken place. More importantly perhaps, the grafting technique did not leave any room for competition between shoots of the grafted plant and the shoots of the rootstock, as the canopy of the accepting rootstock was completely pruned away after grafting.

Therefore, additional comparative studies are required to determine whether the different conclusions reached by some of the earlier studies were the results of different species-specific developmental events or whether they are determined by the different experimental protocols.

AGE- AND SIZE-RELATED REGULATION OF THE NET PRIMARY PRODUCTION

Observations have showed that aboveground net primary productivity negatively correlates with the age of tree for both individual trees and single

TABLE 2
Summary of the results showing contrasting trends involving in grafted scions

Species	Results	Reference
Hedera helix	Increased light-saturated A_{net} with increased ages of scions. Juvenile *versus* mature scions grafted to juvenile rootstock.	Bauer and Bauer (1980)
Larix laricina	Height and diameter growth decreased with increased ages of scions. Juvenile *versus* mature scions grafted to juvenile rootstock.	Greenwood *et al.* (1989)
Larix laricina	Total chlorophyll increased with increased ages of scions. Juvenile *versus* mature scions grafted to juvenile rootstock.	Greenwood *et al.* (1989)
Larix laricina (indoor-grown trees)	Increased light-saturated A_{net} with increased ages of scions. Juvenile *versus* mature scions grafted to juvenile rootstock.	Hutchison *et al.* (1990)
Larix laricina (outdoor-grown trees)	No trend observed in light-saturated A_{net} with increased ages of scions juvenile *versus* mature scions grafted to juvenile rootstock.	Hutchison *et al.* (1990)
Larix laricina	Increased xylem diameters with increased ages of scions. Juvenile *versus* mature scions grafted to juvenile rootstock.	Takemoto and Greenwood (1993)
Picea rubens	Decreased A_{net} with increased ages of scions. Juvenile *versus* mature scions grafted to juvenile rootstock.	Rebbeck *et al.* (1993)
Picea rubens	Decreased light-saturated A_{net} with increased age of scions. Mature *versus* old scions.	Day *et al.* (2001)
Picea taeda	Diameter and height growth, leaf area, branch numbers and scion biomass decreased with increased scion ages. Juvenile *versus* young scions grafted to juvenile rootstock.	Greenwood (1984)
Pinus radiata	Diameter and height growth and branch numbers decreased with increased scion ages. Juvenile *versus* mature scions.	Sweet (1973)
Pseudotsuga menziesii	Diameter and height growth, branch numbers and branch length decreased with increased scion ages. Juvenile *versus* young scions grafted to juvenile rootstock.	Ritchie and Keeley (1994)

cohort stands (Assmann, 1970; Whittaker, 1975; Bormann and Likens, 1979; Harcombe *et al.*, 1990). Long-term studies on even-aged forests showed that the maximum bole increment rate occurs shortly after crown closure and declines as trees reach maturity (Assmann, 1970). Yoder *et al.* (1994) suggested that age-related declines in photosynthetic rates for lodgepole and ponderosa pine may be a significant cause of net production decreases in old trees. This is supported by several studies conducted on age-related differences in photosynthetic rates in Scots pine (Kull and Koppel, 1987), bristlecone pines (Schoettle, 1994) and hybrid Englemann x white x Sitka spruce (Richardson *et al.*, 2000). However, photosynthetic rates in the eastern

larch have been reported to be higher in grafts from older trees (Hutchison *et al.,* 1990).

Explanations for these age-related differences in the photosynthetic rates are generally centred on the multiple constraints on carbon assimilation. Among the potential constraints on carbon assimilation are genetic-down regulation of photosynthesis, reductions in the whole-tree leaf area per unit biomass, nutrient based limitations on photosynthetic capacity and reductions in hydraulic conductance, together with the changes in micrometeorological factors such as temperature and the availability of light and water. A few hypotheses have been developed based on the aforementioned constraints such as the respiration hypothesis, the nutrient limitation hypothesis, the maturation hypothesis and the hydraulic limitation hypothesis. In the respiration hypothesis, for instance, the allometry of trees must be such to support the vertical mass and resist the bending forces from wind, which increase rapidly with tree height. Nevertheless, these support systems require considerable investment in non-photosynthetic tissues, which increase maintenance respiration. Meanwhile, in the nutrient limitation hypothesis, growth in older trees is limited by nutrient supply with nutrients being tied up in living plants and soil litter. Moreover, the hydraulic limitation hypothesis is based on the increase in tree height, which may increase hydraulic constraints and limit gas exchange in older or taller trees. Another explanation could be the ontogenetic changes (maturation hypothesis) which contribute to reduced growth in older trees. Each of these four (4) hypotheses has been reviewed in greater detail in the on-going discussions.

Respiration Hypothesis

Before the links between respiration and biosynthesis started to become clear in the 1950s, respiration was considered to be an imperfection in the mechanisms which converted substrates into structural dry matters (Lambers, 1985). Since then, a rapid expansion of knowledge has taken place. Gradually, it has become of interest to establish the quantitative relationship between the substrate use and processes, such as the growth and maintenance of plants and plant parts under different environmental conditions (Lambers, 1985). Yoda *et al.* (1965) estimated the total aboveground wood respiration of a tree by sorting all shoot parts into diameter classes and multiplying the biomass in each class with the average respiration rate for that particular class.

For years, many scientists assumed that the most important constraint to large size is the maintenance cost required by living biomass in very large stems and roots systems (Bond, 1998). This respiration consumes the photosynthate and thus leaves less for the new growth in large trees. Hence, this hypothesis is based on the relative importance of various photosynthate sinks and the observed declines in the ratio of photosynthetic to non-photosynthetic tissues as trees and stands age. Over the years, researchers have expressed respiration rates on the basis of different measures of plant size. Rates expressed on a weight (Yoda *et al.,* 1965) or volume (Ryan *et al.,* 1996) bases decline, while surface area based rates increase with increasing diameter.

Carbon may be allocated away from aboveground growth and toward respiration (Yoda *et al.,* 1965) to defend plant against pathogens or insects, reproduction (Ryan *et al.,* 1997b; Becker *et al.,* 2000b) or belowground growth (Grier *et al.,* 1981; Gower *et al.,* 1996; Magnani *et al.,* 2000). However, the tests of the respiration hypothesis in lodgepole pine (Ryan and Waring, 1992) and eucalyptus (Ryan *et al.,* 2004) have failed to support it. This hypothesis has also been weakly supported by modelling studies (Magnani *et al.,* 2000; Mäkelä and Valentine, 2001).

Nutrient Limitation Hypothesis

The nutrient limitation hypothesis has been supported by some previous studies and refuted by others (Ryan *et al.,* 1997a). As forests age, nutrients may become scarce due to the sequestration in biomass and necromass (Gower *et al.,* 1996; Ryan *et al.,* 1997b). Such scarcity

may lead to the reduction in the allocation of nitrogen to thylakoid membranes and enzymes, therefore reduces photosynthetic capacity. This is because photosynthetic capacity is strongly correlated with leaf nitrogen content across a wide range of plant species (Field and Mooney, 1986; Pearcy *et al.*, 1987; Lambers *et al.*, 1990). However, this relationship may be complicated by the partitioning between various photosynthetic systems and non-photosynthetic components (Evans, 1989), as well as the occurrence of nitrogenous osmoregulatory and storage substances (Sarjala *et al.*, 1987; Margolis and Vezina, 1988; Lavoie *et al.*, 1992; Billow *et al.*, 1994). Reich and Schoettle (1988) suggested that photosynthetic response may be more strongly linked to the interaction of nitrogen and phosphorus content than to the former element alone. On the contrary, Ryan and Waring (1992), Yoder *et al.* (1994) and Mencuccini and Grace (1996b) found no significant age-related differences in the total foliar nitrogen content for chronosequences of *Pinus contorta*, *P. ponderosa* and *P. sylvestris*. However, their analyses were limited to the first year foliage. Numerous investigations have shown that the content of foliar nitrogen is inversely related to the age of leaf (Lehto and Grace, 1994; Field, 1983; Field and Mooney, 1983; Matyssek, 1986; Lang *et al.*, 1987). In addition, lower nutrient available may lead to increased allocation of photosynthetic products to root production, particularly in older stands and therefore to decreased allocation to aboveground structures (Ryan *et al.*, 1997a). Grier *et al.* (1981) found that the allocation to fine roots was dramatically greater in an old rather than in an adjacent young *Abies amabilis* forest. Similar results have also been reported for the *Pinus elliottii* stand, whereby fine-root biomass was greater in a mature stand than in a younger stand (Gholz and Fisher, 1982).

Aboveground net primary productivity (ANPP) may decline during stand development due to decreasing availability of nutrients, particularly nitrogen. In general, nitrogen mineralization and nitrification rates decrease during secondary succession of forests (Vitousek *et al.*, 1989). The decline is strongly correlated with litter decomposition, which in turn, is controlled by environmental conditions, together with chemical and physical characteristics of litter (Gower *et al.*, 1996). Stand-age effects may contribute in two ways. First, the ratio of leaf to woody detritus input (with low and high C:N ratios, respectively) gradually decreases during stand development. Second, the accumulation of woody litter as stands age will slow decomposition due to its low surface area:volume ratio, as compared to fine litter (Landsberg and Gower, 1997). The reduction in litter quality during stand development increases nitrogen immobilization during litter decomposition, which in turn, decreases the net nitrogen mineralization as stand ages (Davidson *et al.*, 1992; Hart *et al.*, 1994).

Hydraulic Limitation Hypothesis

The hydraulic limitation hypothesis proposes that leaf-specific hydraulic conductance (K_L) declines as trees grow taller, resulting in decreased carbon assimilation (Ryan and Yoder, 1997; Bond and Ryan, 2000). Yoder *et al.* (1994) and Ryan and Yoder (1997) proposed this hypothesis to explain the results of their study on ponderosa pine, which indicated that photosynthesis was limited in older trees relative to younger individuals by reduction in midday stomatal conductance (G_S). This hypothesis was based on older trees having lower hydraulic conductivity in the water path between the roots and shoots, due to longer or more complex hydraulic pathways. Due to this lower conductivity, stomata of older trees show greater sensitivity to evaporative demand and more tightly regulate transpiration to minimize the potential for xylem embolism (Ryan and Yoder, 1997). The rate of xylem water flow is determined by Darcy's law (Tyree and Ewers, 1991; Margolis *et al.*, 1995), in which the flow rate is directly proportional to cross-sectional area of the transmitting structure (sapwood xylem), its permeability and the water potential gradient. As conductivity is equivalent to the combination of area and permeability terms, a decrease in this combined parameter requires an increase in water potential gradient to maintain

a constant flow. If xylem conductance is lower in older (larger) trees, a critical water potential for stomatal closure will be reached more rapidly than in younger (smaller) trees, as evaporative demand increases.

Studies of tree hydraulic architecture provide evidences that potential xylem flux decreases with tree size. Darcy's law further states that flow is inversely related to pathway length. Thus, maintaining an equal flow to leaves at greater distance from a root absorption point, as in larger trees, requires either an increase in conductivity or water potential gradient. Leaf specific conductivity (*LSC*) is commonly used as a measure of the ability of a particular section of stem, or a branch to supply water to more distal leaves and values for this parameter have been found to be relatively constant along the length of tree stems (Tyree and Ewers, 1991). However, Ewers and Zimmerman (1984a, b) found that leaf specific conductivity values are much lower in branches, strongly influenced by branch diameter and order, and subject to significant restriction at branch-stem junctions. Although stem leaf specific conductivity values, if strictly height-determined, cannot be expected to differ greatly between younger and older trees of the same height, the longer branches typical of older crowns may provide significantly reduced leaf specific conductivity values.

McDowell *et al.* (2002) found that hydraulic conductance decreased by 44% as tree height increased from 15 to > 32 m and showed a further decline of 6% with the increase in height. The analyses on sensitivity, based on Darcy's Law to quantify the extent to which compensating mechanisms buffer hydraulic limitations to gas exchange, indicated that without the observed increases in the soil-to-leaf water potential differential ($\Delta\Psi$) and decreases in the leaf area/ sapwood area ratio, K_L would have been reduced by more than 70% in the 60-m trees, as compared to 15-m trees, instead of the observed decrease of 44%. However, compensation may have a cost; for example, the greater $\Delta\Psi$ of the largest trees is associated with smaller tracheid diameters and increased sapwood cavitation, which may also have a negative feedback on K_L and G_S.

Several studies indicate that larger trees are capable of altering their hydraulic architecture to compensate for the longer, more complex pathways between roots and foliage (Becker *et al.*, 2000b; Bond and Ryan, 2000; Mencuccini and Magnani 2000). These were summarized in a critique of the hydraulic limitation hypothesis by Becker *et al.* (2000b). Critics of the hydraulic limitation hypothesis have pointed out the various mechanisms, by which plants compensate for hydraulic limitation, as it is evident that the increased height and structural complexity of old trees are unlikely to constrain hydraulic conductance and hence assimilation. Although tapered xylem conduits may buffer hydraulic resistance from path length effects (West *et al.*, 1999; Becker *et al.*, 2000a) and such buffering appears to be overdriven by hydraulic constraints in tall trees. Furthermore, other buffering factors, such as greater water storage in sapwood (Becker *et al*., 2000b) and increased xylem permeability to water flow during tree ageing (Mencuccini and Magnani, 2000) may to some extent, compensate for the long path lengths for water movement in tall trees.

In addition, several attempts to provide direct evidence in support of the hydraulic limitation hypothesis by experimental manipulation have been unsuccessful. Hubbard *et al*. (1999) girdled young lodgepole pine trees to reduce leaf specific conductivity and remove foliage from older trees to increase it, but found that manipulation could neither significantly change stomatal conductance nor photosynthetic rates. A similar study (in which foliage of old Douglas-fir was enclosed in plastic bags to reduce transpiration and leaf specific conductivity) was also unsuccessful at increasing gas exchange rates of uncovered foliage on the same branches (Brooks *et al.*, 2000).

Maturation Hypothesis

Genetic regulation of photosynthesis has not been specifically proposed in the literature, nor are there data to test this hypothesis. However, dramatic changes in morphological and physiological attributes of foliage, including

photosynthetic capacity, have been described for numerous species during their early development and have been attributed to different challenges to growth and survival, at various life-stages (Greenwood, 1984; Hackett, 1985; Rebbeck *et al.,* 1993; Greenwood and Hutchison, 1993). Although little is known about age-related trends in foliar attributes beyond reproductive maturity or mid-age, there is some evidence which supports the concept of continuous change. Richardson *et al.* (2000) reported that the changes in foliar morphology and gas exchange attributes continue past mid-age in hybrid Englemann x white x Sitka spruce. Similar trends in foliar attributes have been described for Norway spruce (Kull and Koppel, 1987) and in needle morphology of Sitka spruce (Steele *et al.,* 1989). However, none of these studies has directly addressed the potential for ontogenetic changes in meristematic tissue, as a contributing mechanism to age-related declines in productivity, nor do they experimentally separate age from size.

It is important to highlight that the contribution of maturation-related changes in meristem behaviour to age-related decline in forest productivity is still poorly understood. While changes in the morphological and physiological attributes, associated with the transition from juvenile to reproductively-mature phases, have been described for many woody species, only a few studies have examined the maturational changes which occur after the onset of reproductive development (Greenwood *et al*., 1989; Day *et al.,* 2001; Day *et al.,* 2002). In any case, common rootstock studies, in which scions are donated by juvenile and mature trees, hint at the possibility that the differences in morphological and physiological traits associated with those life-stages are inherent in meristems. Such studies have also been carried out on loblolly pine (Greenwood, 1984), eastern larch (Greenwood *et al*., 1989) and red spruce (Rebbeck *et al.,* 1993; Day *et al.,* 2001).

A field study, conducted by Day *et al.* (2001) on *Picea rubens* Sarg., demonstrated significant age-related trends in foliar morphology, including decreasing specific leaf area (*SLA*) and increasing needle width, projected needle area and needle width-to-length ratio. Similar trends were also apparent in foliage from the grafted trees with different scion ages. Both *in situ* foliage and shoots resulting from grafted scions from the oldest cohort showed significantly lower photosynthetic rates than their counterparts from younger trees; however, the differences in stomatal conductance and internal CO_2 concentrations were not significant. They concluded that the lower rates of photosynthesis had contributed to the age-related decline in the productivity of older red spruce and that the decline in photosynthetic rates was resulted from non-stomatal limitations. In addition, a study where scions from juvenile and mature red spruce were grafted onto common rootstock (Rebbeck *et al.*, 1993) clearly showed that maturation-related changes in meristems of red spruce persisted for at least two years after grafting. In their study, the results on scions from mature trees exhibited lower stomatal conductance and net photosynthetic rates than those from the juvenile donors.

In addition, Emebiri *et al.* (1998) and Hutchison *et al.* (1990) have implicated a genetic basis for ontogenetic changes by identifying differential patterns of gene expression related to life-stages. However, the pathways by which genetic changes in meristems and/or the foliage they produce affecting photosynthetic rates have not been described. A possible pathway, by which ontogenetic changes in meristems may affect photosynthetic rates, is by producing tissue in older trees which have an inherently lower growth rate. Shoots resulting from the grafts of meristems from older trees have lower growth rates as than scions from young trees. This was shown for radiata pine (Sweet, 1972), loblolly pine (Greenwood, 1984), eastern larch (Greenwood *et al.,* 1989, Takemoto and Greenwood 1993) and red spruce (Rebbeck *et al.,* 1993). Takemoto and Greenwood (1993) speculated that the older meristems might be weak sinks for resources as compared to shoots arising from the scions taken from younger trees. Stitt (1990) stated that weak sinks for carbohydrate, due to reduced growth, could

result in feedback limitations to photosynthesis. Meanwhile, Leverenz (1981) suggested that sink strength limitations might explain differential photosythetic capacity among the branches in the crowns of mature Douglas-fir.

NEW QUESTIONS AND CHALLENGES FOR THE FUTURE

Since 1997, when the idea of 'the hydraulic limitation theory' was first formally presented (Ryan and Yoder, 1997), many steps forward have been made to better characterize the physiological ecology of old and tall trees. Almost every year, a conference session or a workshop is organised around these themes and the attention in the most prestigious scientific journals has also been increased (e.g., Koch *et al.*, 2004; Pennisi, 2005; Penuelas, 2005). The authors' knowledge of the stand and ecosystem development is rapidly expanding and some consensus has emerged over some issues. However, over several other issues, much more work remains to be carried out. Perhaps more importantly, the authors are still not close to the identification of the component or components which are most likely to determine the observed reductions in the GPP and NPP with age. A recent modelling exercise, based on the concept of optimal carbon allocation (Buckley and Roberts, 2005) has clearly highlighted how much work needs to be done to correctly interpret the significance of some of the observed patterns. Therefore, much of the progress thus far could be viewed as useful but marginal to the central question, which was initially posed by Ryan and Waring (1992).

However, on a more positive side, the impetus provided by those initial studies have helped to identify a number of very different research questions that have not been considered at the outset. This review on the issues related to ageing and senescence was attempted to help set the efforts of the stage for future research in this area. An important concept recently emerged has been the demonstration that size per se exerts effects which are clearly independent of age (Mencuccini *et al.*, 2005), a concept which is consistent with the earlier data (e.g. Sweet, 1972). Much of the older literature on ageing in trees confused these two aspects and accepted that characters such as reduced ring width could be taken to indicate senescence processes. Therefore, the newly emerged complexity of tree research, as compared to animal research, is in the current awareness that any symptom or biomarker of ageing must be thought of independently of the size-related effects which occur in large and tall trees. However, other more fundamental questions remain unanswered. These include questions such as: Are trees potentially immortal, but subject to the vagaries of extreme environmental conditions and occasional disturbances, or do they ultimately show senescence processes, like many other life forms? The current evidence points towards a lack of clear senescence processes, but many unclear points still exist and the few investigations on this topic are quite dated. For instance, evidence is required to answer the question on "what are the trade-offs between lifespan, growth and reproduction in long-lived versus short-lived taxa?" At present, the authors have merely had tentative answers to these very fundamental questions. This topic is coming again to the fore of the ecological and environmental sciences debate and the research in this field is likely to yield interesting new information.

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Responses of *Musa* AAA Berangan to 1-methylcyclopropene

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ABSTRACT

1-methylcyclopropene (1-MCP) is an inhibitor of ethylene perception and has been shown to delay the ripening of Cavendish bananas. Nevertheless, no work has reported the delay in the ripening of local bananas using 1-MCP. The objective of this study was to examine the responses of Berangan banana to 1-MCP applied at the ripening stage 1 (mature green) and ripening stage 2 (partially ripe). In Experiment I, 1 µL L^{-1} of 1-MCP was applied to mature green stage banana for 0, 2, 4 and 6 h, followed by ripening initiation using 10 g CaC_2 kg^{-1} fruit for 24 h. Meanwhile, in Experiment II, the fruits were partially ripened using 10 g CaC_2 kg^{-1} fruit for 24 h, followed by exposing them to 1 µL L^{-1} of 1-MCP for 0, 2, 4 and 6 h. Exposing mature green Berangan banana to 4 h of 1-MCP delayed degreening, retained flesh firmness and soluble solids concentration. On the contrary, exposing 1-MCP to the partially ripened banana fruit failed to delay both degreening and ripening processes. Fruit of both experiments ripened normally after the 1-MCP treatment, with more rapid ripening processes in partially ripe than mature green fruit.

Keywords: Peel colour, flesh firmness, soluble solids concentration, titratable acidity, pH

INTRODUCTION

Local bananas such as 'Berangan', 'Mas' and 'Rastali' are popular among Malaysians and ASEAN countries. The consumption and export for these bananas are expected to increase by 30% from 2005 to 2010 (Abbas, 2002). Bananas are climacteric fruit, once induced to ripen with ethylene or ethylene-generated resources, their marketing life is only about 3-5 d, depending on ethylene treatment and holding temperature after treatment. A method to slow down the ethylene-induced ripening process has economical significance for distribution centres, supermarkets and fruit stalls. Recently, 1-methylcyclopropene (1-MCP) has been reported to have inhibitory effects on ethylene action (Sisler *et al*., 1995). In particular, 1-MCP delays senescence of strawberries (Ku *et al*., 1999) and inhibits the degreening of oranges while not suppressing other ethylene-induced effects such as chilling injury (Porat *et al*., 1999).

1-MCP has been shown to delay the ripening of 'Cavendish' banana (Golding *et al*., 1998; Harris *et al*., 2000; Jiang *et al*., 1999a, b; Serek *et al*., 1995; Sisler *et al*., 1999; Sisler and Serek, 1999). Cavendish banana has been found to increase its 'green life' when treated with 1-MCP, where responses are being concentration-exposure time dependent (Jiang *et al*., 1999b; Harris *et al*., 2000; Bagnato *et al*., 2003). Jiang *et al*. (1999a) demonstrated that a 24 h exposure to either 500 or 1000 nL L^{-1} 1-MCP at 20˚C extended the green life

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of Cavendish bananas from 16 to 31 d in the absence of ethylene, as compared with the untreated controls. Jiang *et al*. (1999b) showed that a 1 h exposure to 1000 nL L^{-1} 1-MCP at 20˚C eliminated the ethylene effects for at least 5 d in Cavendish bananas, and that a 12 h exposure to 50 nL L^{-1} 1-MCP was just as effective. Joyce *et al.* (1999) found that banana ripening induced by propylene, an ethylene analogue, could be delayed by exposure to 15 µL L^{-1} of 1-MCP at 20˚C for 12 h. However, the 1-MCP treatment was less effective as propylene-induced ripening progressed, although it was able to maintain the eating-ripe condition of fruits for a longer time than the control treatment. Jiang *et al*. (1999b) found that 1-MCP (in a concentration range 0.01-10 µL L^{-1} at 20˚C for 12 h), applied after 1 d of ethylene treatment, was found to slow the ripening of bananas down, but it was ineffective when applied 3 or 5 d after the ethylene treatment. To the best of authors' knowledge, no study has been carried out to delay the ripening of Berangan bananas using 1-MCP applied at mature green and partially ripe stage of ripening. Cultivar could affect the responses of product to 1-MCP (Abdi *et al*., 1998; Botondi *et al*., 2003; Watkins *et al*., 2000). Therefore, the objective of this study was to examine the responses of mature green and partially ripe Berangan bananas to 1-MCP.

MATERIALS AND METHODS

Plant Material

Hands of mature green bananas (*Musa* AAA Berangan) were obtained from Puchong Wholesale Market, Selangor, Malaysia. The fruits were sorted for freedom from the visual defects and uniformity of weight and shape. Two replicates of three hands, each containing 16-18 fingers per hand, were used in each experiment. Ansip-F® (0.009% a.i.) (Lytone Enterprise, Inc. Taiwan R.O.C.) was used to prepare 1-MCP. Seventeenth point six millilitre of distilled water at 40˚C was added into the beaker containing 1.1 g of crushed Ansip-F® tablets. The beaker was swirled for a few seconds before placing it into a 55 L container, and it was covered up immediately, sealed with Vaseline and tied up in a 0.035 mm thick PE bag to avoid gas leakage. After that, the container was left for 3 h before withdrawing the 1-MCP.

The application of 1-MCP was performed by placing the fruits in a 15 L box, with a 0.035 mm thick polyethylene (PE) bag and exposing them to 1-MCP gas for 0, 2, 4 and 6 h, respectively, at 27˚C with the relative humidity (RH) of 70%.

In Experiment I, 1 µL L^{-1} of 1-MCP was applied to ripening stage (RS) 1 (mature green) fruits. After the various exposure times, the fruits were ventilated and initiated to ripen using 10 g CaC_2 kg^{-1} fruits for 24 h. After 24 h, the fruit were again ventilated and allowed to ripen. In Experiment II, the fruits were initiated to ripen using 10 g CaC_2 kg^{-1} fruits for 24 h to partially ripe or RS 2. The peel of these RS 2 fruit was light green in colour. After exposing the fruits for 0, 2, 4 and 6 h 1-MCP, respectively, they were ventilated and allowed to ripen. Each 15 L box consisting of 24 fruits and analyses were carried out 2 d once until day 6.

Determination of Skin Colour

Skin colour was determined using Minolta CR-300 Chroma Meter (Minolta Corp., Osaka, Japan) using the Illuminate C (CIE, 1976) and results were expressed as lightness (L*), chroma (C*) and hue (h˚). The L* values ranged from 0 = black to 100 = white. The h˚ is an angle in a colour wheel of 360˚, with 0, 90, 180 and 270˚ representing red, yellow, green and blue, respectively, while C* is the intensity or the purity of the hue. The measurements at the stem end, mid region and floral end of each face of the peel were made and a mean value was obtained from two fruit per replicate.

Determination of Flesh Firmness

Flesh firmness was evaluated using the Bishop Penetrometer FT 327 (Alfonsine, Italy). The force required for an 11-mm probe to penetrate the cut surface in two opposite locations to a depth of 5 mm was recorded. The penetration force was expressed in kg cm^{-2} according to Ding *et al*. (2007).

Determination of Soluble Solids Concentration (SSC)

Ten g of fruit was macerated and the tissue was homogenised with 40 mL of distilled water, using a kitchen blender. The mixture was filtered with cotton wool. A drop of the filtrate was then placed on the prism glass of refractometer (Model N1, Atago Co., Ltd., Tokyo, Japan) to obtain the % SSC, according to Ding *et al*. (2007). The readings were corrected to a standard temperature of 20˚C by adding 0.28% to obtain % SSC at 27˚C.

Determination of Titratable Acidity (TA) and pH

The remaining juice from the SSC determination was used to measure TA by titrating with 0.1 mol L^{-1} NaOH, using 1% phenolphtalein as an indicator. The results were calculated as a percentage malic acid [(mL NaOH x 0.1 mol L^{-1}/ weight of the sample titrated) x 0.06705 x 100] according to Ding *et al*. (2007).

The pH of the juice was measured using a glass electrode pH meter, model Crison Micro pH 2000 (Crison Instruments, S.A., Barcelona, Spain). The pH meter was calibrated with buffer at pH 4.0 and 7.0, before being used.

Determination of Vitamin C Content

Ten g of banana flesh was well homogenised with 3% cold metaphosphoric acid, using a kitchen blender. The volume was made up to 100 mL and filtered with cotton wool. Then, 5 mL of the aliquot was titrated with 2,6-dichlorophenol-indophenol solution to a pink end-point. The vitamin C content was determined according to Ranganna's (1977) method, as follows:

$$\frac{\text{mg vitamin C}}{100\ \text{g}^{-1}} = \frac{\text{titre} \times \frac{\text{dye}}{\text{factor}} \times \frac{\text{volume}}{\text{made up}} \times 100}{\frac{\text{aliquot used}}{\text{for extraction}} \times \frac{\text{weight of}}{\text{sample taken}}}$$

Statistical Analysis

The experimental design was a completely randomised design with three replications of six fruit per replicate. Data were analysed using the analysis of variance (SAS Institute, Cary, NC) and the means were separated using the Duncan's multiple range test.

RESULTS

Experiment I

There was a significant difference ($P \leq 0.05$) in the L* values of Berangan bananas when exposed to 1-MCP (Table 1). The 1-MCP treated fruit, whether exposed for 2, 4 or 6 h, were found to have significant lower L* values as compared to the control. As ripening day progressed, fruit at day 0 had significant lower L* values, as compared to the other ripening days. By exposing Berangan bananas to 1-MCP for 4 and 6 h, the C* values were significantly lower than the control fruit (Table 1). Nevertheless, there was no significant difference in the C* values of Berangan banana as ripening day progressed (Table 1). The h^o values of Berangan bananas, treated with 4 h of 1-MCP, were significantly ($P \leq 0.05$) higher than 0 and 2 h 1-MCP treated fruit (Table 1). As ripening day progressed, the h^o values of the banana peel decreased significantly ($P \leq 0.05$) to about 101 at day 6 (Table 1). This value is equivalent to yellow in the colour chart, indicating that the fruit ripened as day progressed.

There was a significant difference ($P \leq 0.05$) in flesh firmness when Berangan bananas were treated with 1-MCP (Table 2). The firmness of the fruit flesh was retained by exposing it to 1-MCP for at least 4 h. However, the firmness of the flesh was found to significantly decrease ($P \leq 0.05$) by 34% as the ripening day progressed from 0 to 6 (Table 2). The SSC of Berangan was decreased significantly ($P \leq 0.05$) by exposing the fruit to 1-MCP for at least 4 h (Table 2), indicating that the ripening process was delayed. As ripening day progressed, the SSC of the fruit was increased by 302% (Table 2). The TA of Berangan banana fruit was not affected by 1-MCP treatment and ripening day (Table 2). Similar to the TA, the vitamin C content and pH of Berangan bananas were not affected by 1-MCP treatment and ripening day (Table 2).

TABLE 1
Peel colour (L*, C* and h°) of Berangan bananas, treated with 1 µL L^{-1} 1-MCP for 0, 2, 4 and 6 h, followed by the ripening initiation using CaC_2 for 24 h and the fruits are allowed to ripen for 6 d

Factor	L*	C*	h°
Exposure time, h (E)			
0	62.91 a[z]	38.73 a	100.47 c
2	57.84 b	34.13 ab	106.90 bc
4	56.26 b	29.18 b	115.46 a
6	54.29 b	31.43 b	113.20 ab
Ripening day (D)			
0	53.00 b	30.16 a	120.76 a
2	58.62 a	32.33 a	112.03 b
4	60.33 a	35.50 a	102.19 c
6	58.63 a	34.84 a	101.18 c
Interaction			
E x D	NS	NS	NS

[z] Mean separation within columns and factors by Duncan's at $P \leq 0.05$
[NS] Non significant at $P \leq 0.05$

TABLE 2
Flesh firmness, soluble solids concentration (SSC), titratable acidity (TA), vitamin C and pH of Berangan bananas, treated with 1 µL L-1 1-MCP for 0, 2, 4 and 6 h, followed by the ripening initiation using CaC_2 for 24 h and the fruits are allowed to ripen for 6 d

Factor	Firmness (kg cm^{-2})	SSC (%SSC)	TA (%malic acid)	Vitamin C (mg 100 g^{-1})	pH
Exposure time, h (E)					
0	2.38 b[z]	13.50 a	2.05 a	10.08 a	4.94 a
2	3.50 ab	8.79 a	2.42 a	9.45 a	5.10 a
4	4.51 a	5.54 b	2.17 a	10.55 a	5.18 a
6	4.62 a	5.20 b	2.30 a	9.49 a	5.20 a
Ripening day (D)					
0	4.58 a	3.17 c	2.22 a	10.71 a	5.31 a
2	4.28 ab	7.13 bc	2.45 a	10.16 a	5.00 a
4	3.22 b	9.30 ab	1.81 a	9.52 a	4.94 a
6	3.04 b	12.74 a	2.54 a	9.00 a	5.22 a
Interaction					
E x D	NS	NS	NS	NS	NS

[z] Mean separation within columns and factors by Duncan's at $P \leq 0.05$
[NS] Non-significant or significant at $P \leq 0.05$

Experiment II

Exposing 1-MCP to RS 2 or partially ripe Berangan bananas did not have any effect on the L*, C* and h^o values of the fruit peels, as shown in Table 3. Apparently, once ripening has been initiated, 1-MCP could not inhibit ethylene development. As ripening day progressed, there was a significant difference ($P \leq 0.05$) in the L*, C* and h^o values of the peel colour. The L* values of the peels of Berangan increased significantly ($P \leq 0.05$) towards light colour as ripening day progressed (Table 3). The C* values of the fruit increased significantly from ripening day 0 to 4, and then decreased significantly to ripening day 6 (Table 3). The h˚ values of Berangan were significantly decreased by 31% to yellow-orange at ripening day 6 (Table 3).

The flesh firmness, SSC, TA, vitamin C and pH of Experiment II Berangan bananas did not show any significant differences among 1-MCP exposure times (Table 4). Once again, when the ripening was initiated, 1-MCP could not inhibit ethylene development. The flesh firmness of bananas was decreased significantly by 73% at d 6 of ripening (Table 4). The SSC of bananas was increased by 624% as ripening day progressed from 0 to 6 (Table 4). This is also similar to fruit in Experiment I, whereby TA and vitamin C of Berangan did not show any significant differences as the ripening day progressed (Table 4). The pH of the bananas in Experiment II decreased significantly as the fruit ripened from day 0 to 2, and no significant difference was observed thereafter.

DISCUSSION

The application of 1-MCP to mature green stage fruit has retained its peel colour and some quality characteristics of Berangan. Subjectively, the fruit treated with 1-MCP, either in Experiment I or II, showed a better visual appearance than the control fruit (data not presented). In specific, the L*, C* and h^o values of the fruit peels obtained in these two experiments reflected the yellow peel fruit for Experiment I, and yellow-orange

TABLE 3
Peel colour (L*, C* and h^o) of partially ripened Berangan bananas, using CaC_2 for 24 h, followed by the treatment of 1 µL L^{-1} of 1-MCP for 0, 2, 4 and 6 h, and the fruits are allowed to ripen for 6 d

Factor	L*	C*	h^o
Exposure time, h (E)			
0	63.78 a^z	40.17 a	98.50 a
2	61.10 a	38.19 a	99.68 a
4	61.86 a	38.25 a	99.26 a
6	61.85 a	38.14 a	98.75 a
Ripening day, (D)			
0	53.43 c	30.21 d	120.79 a
2	62.38 b	37.10 c	104.09 b
4	67.17 a	45.65 a	86.66 c
6	65.76 a	41.95 b	83.38 d
Interaction			
E x D	NS	NS	NS

^{z} Mean separation within columns and factors by Duncan's at $P \leq 0.05$
^{NS} Non-significant at $P \leq 0.05$

TABLE 4
Flesh firmness, soluble solids concentration (SSC), titratable acidity (TA), SSC to TA ratio, vitamin C and pH of partially ripened Berangan bananas using CaC_2 for 24 h, followed by the treatment of 1 µL L^{-1} of for 0, 2, 4 and 6 h and the fruits are allowed to ripen for 6 d

Factor	Firmness (kg cm^{-2})	SSC (%SSC)	TA (%malic acid)	Vitamin C (mg 100 g^{-1})	pH
Exposure time, h (E)					
0	2.44 a[z]	13.41 a	2.34 a	8.01 a	4.81 a
2	3.01 a	13.63 a	2.28 a	7.72 a	4.80 a
4	3.11 a	14.25 a	2.49 a	7.70 a	4.83 a
6	3.21 a	14.17 a	2.51 a	7.68 a	4.75 a
Ripening day, (D)					
0	5.40 a	2.83 c	2.21 a	7.39 a	5.26 a
2	3.32 a	13.54 b	2.00 a	9.11 a	4.63 b
4	1.53 c	19.17 a	2.35 a	7.43 a	4.51 b
6	1.44 c	20.50 a	3.12 a	7.11 a	4.79 b
Interaction					
E x D	NS	NS	NS	NS	NS

[z] Mean separation within columns and factors by Duncan's at $P \leq 0.05$
[NS] Non-significant at $P \leq 0.05$

peel fruit for Experiment II by ripening day 6. The colour of the peel implied the ripeness of Berangan (Ding *et al*., 2006). The post-harvest life for the partial ripe fruit (Experiment II) was 6 d, while the fruit in Experiment I could last for 7 d (data not presented). This result is in agreement with the findings by Golding *et al*. (1998) and Jiang *et al*. (1999b) who found that the application of 1-MCP at mature green stage had delayed the colour changes in Cavendish peel. However, incomplete and uneven ripening of 1-MCP treated Cavendish banana fruit were reported by these authors. Nevertheless, this disorder did not happen to Berangan banana fruit in both experiments of this study. Harris *et al*. (2000) found that the uneven ripening of Cavendish banana was due to the variation in fruit maturity.

The result of this study also showed that by exposing Berangan bananas to 1 µL L^{-1} 1-MCP 4 h, at mature green stage, could sustain the flesh firmness and SSC of fruit (Tables 2 and 4). The flesh firmness and SSC of partially ripe fruit in Experiment II (Table 4) showed a tremendous change as compared to the ones in Experiment I (Table 2) when ripening progressed from day 0 to 6. In the study by Pelayo *et al*. (2003), the 1-MCP treatment was found to delay the colour changes in the peel and flesh firmness of partially ripe Cavendish banana. Jiang *et al*. (1999b) also reported that 1-MCP (in a concentration range 0.01-10 µL L^{-1} at 20˚C for 12 h), which was applied after 1 d of ethylene treatment, had slowed down the ripening of bananas. However, the subsequent experiments in Pelayo *et al*. (2003), the 1-MCP treatments were much less effective in retarding these partially ripe banana fruit. Therefore, it was concluded that the efficacy of 1-MCP, in delaying ripening of partially ripe Cavendish bananas, was too inconsistent for commercial application.

The finding of the present study indicated that once ripening of Berangan was initiated with the commencement of ethylene autocalatyitc,

the 1-MCP treatment could not inhibit ethylene development and its related ripening process. This study also showed that the peel colour changes, flesh firmness and SSC of Berangan banana were dependent on the functioning ethylene receptors during the first 24 h of CaC_2 treatment, as well as the number of active receptors rapidly increased after the initiation of ripening. Titratable acidity, vitamin C and the pH of Berangan banana in both experiments were not affected by the 1-MCP treatment. This showed that TA, vitamin C and pH of this banana type were not dependent on ethylene autocatalytic. Hence, the result gathered in the current study is inadequate to enable the researchers to draw a clear conclusion about the efficacy of 1-MCP in delaying the ripening of Berangan bananas. Further study, with broader resources and maturity of fruit, is therefore needed to draw a better conclusion.

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Juvenile Stem Volume Equations for Planted *Azadirachta excelsa* in the State of Johore, Peninsular Malaysia

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ABSTRACT

Azadirachta excelsa or sentang is one of the important tree species grown in Malaysia. Equations for the predicting stem volume, from the ground to the tip for this species, are not available and hence this paper attempts to apply and examine the suitability of the existing volume equations for the species which can be used by plantation growers in Malaysia. Furthermore, the performances of available volume equations in Malaysia are seldom investigated. Evaluation is important to determine the usefulness and increase the confidence of using a model. Three commonly used models were compared using the data derived from 36 harvested trees to fit the equations. The addition of the weight function of $1/(d^2h)$ was used to fit the Spurr's combined variable volume equation. Then, the performances of the models were evaluated based on R^2, PRESS, bias and RMSE values. The Schumacher and Hall's equation was found to be the most appropriate for determining the total and merchantable underbark stem volume of *A. excelsa*, whereas the Spurr's equation was indicated as more suitable to determine the merchantable overbark volume.

Keywords: *Azadiractha excelsa*, height and diameter, model evaluation, Schumacher and Hall's Volume Equation, Spurr's Volume Equation

INTRODUCTION

The Malaysian government has been promoting the planting of *Azadirachta excelsa* (Jack) Jacobs since 1996. In 2000, it was estimated that 5000 ha of *A. excelsa* plantation has been establishedw in Peninsular Malaysia with most planted at a small scale of less than 20 ha. In the state of Johore alone, a total of 653 ha of the plant have been established (T.H. Ong, *pers. comm.*). The shift took place rather quickly and many fundamental management issues were not properly addressed.

The ability to estimate the current yield production of a stand is one of the most important issues in the forest plantation management, in order to plan for proper silvicultural activities. In forestry, volume is perhaps one of the most common used parameters to estimate stand output. In order to have a good forecast on the forest growth and yield, equations which provide accurate tree volume prediction are therefore needed. The conversion of volume predictions into biomass, for example, can be used as the basis for calculating carbon storage and sequestration rates of the forest stands.

Although a good estimation of volume can be achieved by integrating the taper function, commonly used volume equations, related to the stem or total wood volume to the diameter at breast height (d) and height (h), are also useful.

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Logarithmic volume equation (Schumacher and Hall, 1933) and Spurr's (1952) combined variable equation are two most commonly used forms of volume equations. However, the performances of available volume equations in Malaysia have limitedly been investigated. Further investigation is therefore necessary to determine the usefulness of a model and to increase the confidence level in using it for an intended purpose and in larger areas (Reynolds *et al*., 1981; Reynolds and Chung, 1986). Thus, the objective of this study was to apply and examine the performance of the existing volume equations in an *A. excelsa* stand.

MATERIALS AND METHODS

Site Description

A small plantation covering an area of 2.9 ha was selected for this study. The plantation was located about 9 km south of Labis (2˚21'N and 103˚02'E) in the state of Johore, Peninsular Malaysia, and it is owned by a small-holder. The average monthly temperature varies from 24.3 to 27.3˚C. The rainfall data, over a period of 10 years (1990-1999), suggested a bimodal distribution (March – May and October – December), with a mean annual rainfall of 2181 mm. The area has an average elevation of less than 50 m a.s.l. and the slopes ranging from 1 to 50%. The landform can be grouped into three slope classes, namely (1) nearly level – 50%, (2) undulating – 25%, and (3) steep – 25%.

The plantation was established following a clear cutting of the second rotation rubber trees in March 1998. Debris on the site was burned prior to planting. Seedlings were planted at a spacing of 2.0 × 2.0 m. The seedlings were fertilized with 50 g of 15(N)-15(P)-15(K) at the time of planting. The operational schedules for the application of fertilizers, prior to the beginning of the experiment, are as presented in Table 1. The weeds were cleared by herbicide application each time before fertilization, leaving no ground cover for most of the time. Selective thinning was carried out in December 1999, with approximately 4% of the small trees removed. Thus, the stand was left with 2400 trees ha^{-1} before the starting of the experiment in May 2000 (Table 1).

TABLE 1
Schedule for the application of fertilizers and monitoring of growth and foliar nutrients for *Azadirachta excelsa* at Sungai Karas plantation

Age (months)	Month	Operation	Fertilizer application rate (g $tree^{-1}$)/(kg ha^{-1} elemental)
0	March 1998	Planting	50 g N(15)-P(15)-K(15)
4	July 1998	Routine fertilizer application	50 g N(15)-P(15)-K(15)
8	November 1998	Routine fertilizer application	50 g N(15)-P(15)-K(15)
12	March 1999	Routine fertilizer application	50 g N(15)-P(15)-K(15)
16	July 1999	Routine fertilizer application	50 g N(15)-P(15)-K(15)
20	November 1999	Routine fertilizer application	50 g N(15)-P(15)-K(15)
26	May 2000	Establishment of experimental plots	
		1st fertilizer application	N50 P_{50} K_{50} $Lime_{250}$
30	September 2000	2nd fertilizer application	N_{50} P_{25}
34	January 2001	3rd fertilizer application	N_{50} P_{25}

Fertilizer was broadcasted along planting lines

Measurement

In order to assess the growth performance, six plots of 0.1 ha were established based on the land formation of the area. Three plots were established on the level site, two plots on the undulating site and one plot on the steep site. Prior to the establishment of the plantation, the owner had planted *A. excelsa* seedlings under the existing *Durio zibethinus* trees which covered most of the steep site. Thus, only a single plot could be established in such a site.

The diameter at breast height (*d*) and the total or merchantable height (*h*) of all the trees in the plot were measured every three month. The *d* was measured using a diameter tape, while *h* was determined using a clinometer, at a distance of 10 m. The top of the tree (total height) and its first branch (merchantable height) were determined by shaking the tree briefly prior to the measurement. In August 2002, six trees were selected, based on the mean *d* and their standard deviation from each plot, after the last growth measurement to estimate the tree volume. A total of 36 trees were harvested as close to the ground level as possible, and were therefore used in the development of the volume equations. Each tree was divided into ten equal sections to obtain the sub-samples of the stem, based on the *h*. A 5 cm thick disc was obtained from the base of each section. In addition, a disc was also sampled near the first live branch. The overbark (with bark) diameter of all discs was first measured. Later, the bark of the disc was removed to determine the diameter of the underbark. Smalian's formula (Equation 1) was used to calculate both the overbark and underbark sectional volumes for each tree (in m^3), up to 90% of the total *h* of the tree or up to the first branch to determine the merchantable volume. The volume of the last stem section was calculated as a geometric cone.

$$V = \frac{g_l - g_u}{2} \cdot l \qquad (1)$$

where *V* is sectional volume, g_l is cross-sectional area at the lower end, g_u is cross-sectional area at the upper end, and *l* is length of section log.

Tree volume predictions can be achieved by integrating *d* and *h* as variables. Although the inclusion of the third variable, such as the upper-stem diameter or taper function could further improve the volume estimates, the equations which express stem volume as a power function of *d* and *h* are still commonly employed (Bi and Hamilton, 1998; Fonweban *et al*., 1995; Tewari and Kumar, 2003; Wan Razali *et al*., 1989). The volume equation which relates the stem volume to *d* is also used when *h* is not available (Husch *et al*., 1982), particularly in a dense forest condition. Therefore, three basic equations to predict the stem volume with a constant stem, formed at a given diameter and height, were chosen and examined:

$$V = a + bd^2h \qquad (2)$$

$$V = ad^b \qquad (3)$$

$$V = ad^b h^c \qquad (4)$$

where *V* is stem volume, either overbark or underbark, *d* is diameter at breast height overbark, *h* is tree height, either merchantable or total. Equation (2) is Spurr's (1952) combined variable equation, equation (3) is adopted from Husch *et al*. (1982), while equation (4) is according to Schumacher and Hall's (1933).

Two equations, namely equations (3) and (4), were transformed logarithmically after fitting them with the least squares method to satisfy the assumption of homoscedasticity. A test for heteroscedasticity was carried out, together with equation (2), according to the method by White (1980). The results of the test indicated that heteroscedasticity was present in equation (2). Equation (2) was fitted using the weighted least squares regression, with $1/(d^2h)$ as the weight. The weight was selected by adopting Furnival's (1961) index of fit from $1/(d^2h)$ and $1/(d^2h)^2$. Residuals were evenly spread above and below the zero line, with no systematic trend (*Fig. 1*), suggesting that the weighted least squares are effective in stabilizing the error variance.

A generalized form of R^2 was calculated to compare the percentage of variation in the stem volume, as explained by the regression equations,

due to the use of the logarithmic transformation and weighted least squares regression.

$$R^2 = 1 - \sum_{i=1}^{n} (V_i - V_p)^2 / \sum_{i=1}^{n} (V_i - V_m)^2 \qquad (5)$$

where V_i is the observed volume of tree *I*, V_p is the predicted volume of tree *I*, and V_m is the mean volume of all trees (Bi, 1994).

Snowdon's (1990) bias correction factor, a ratio of the arithmetic sample and back-transformed predicted means from the regression, was calculated to correct the systematic bias brought about by the logarithmic transformation after fitting the equations. The best-fit model was selected based on the precision of the equation on the basis of the following evaluation criteria.

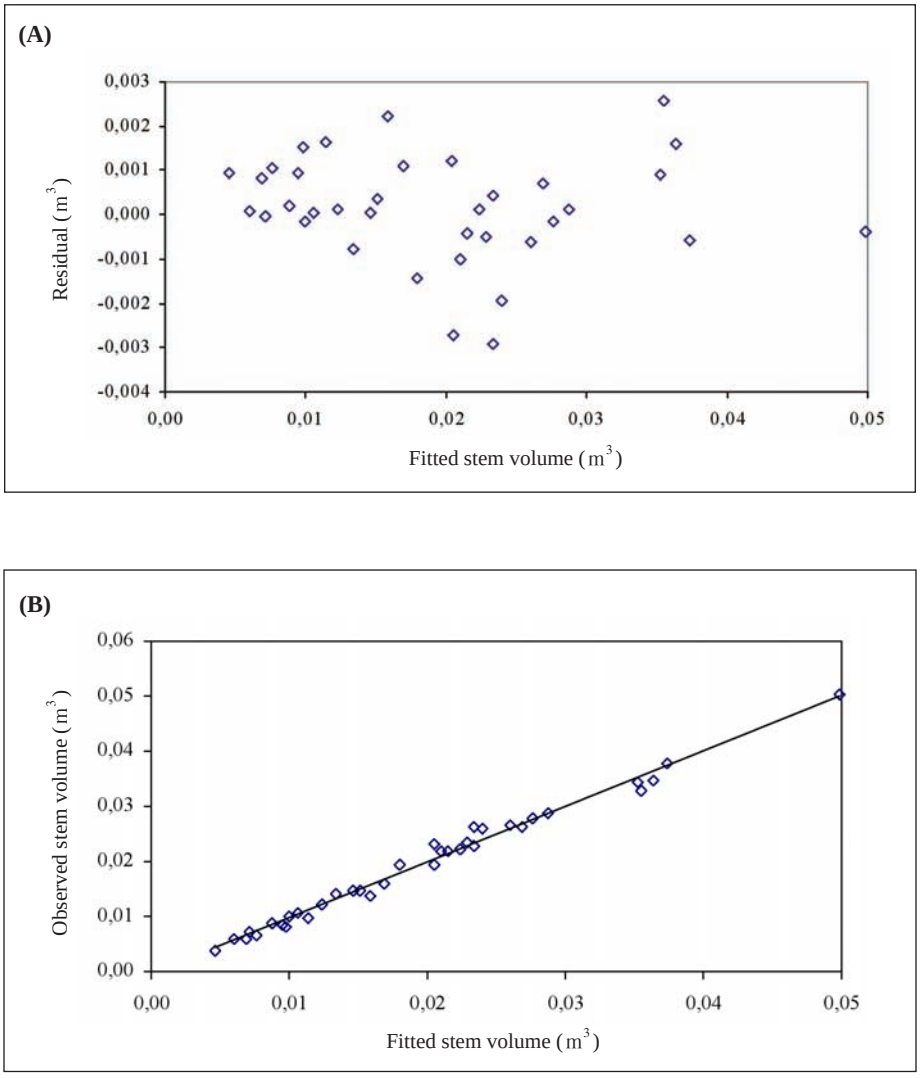

Fig. 1: Residual plot (A), and the relationship between the observed and predicted stem volumes (B) of the weighted least squares regression (Equation 2)

Average residual or prediction bias (B):

$$B = \sum_{i=1}^{n} r_i / n \quad (6)$$

Where, r_i represents the difference between the observed and the predicted volume of tree *i* in the sample data set.

The variance:

$$Var(B) = \sum_{i=1}^{n} (r_i - B)^2 / (n - 1) \quad (7)$$

Root mean squared error of the volume prediction (RMSE):

$$RMSE = (B^2 + Var(B))^{1/2} \quad (8)$$

In case of a small sample size, Green (1983) suggested the use of the predicted residual sum of squares (PRESS) statistic, a prediction-oriented statistics.

RESULTS AND DISCUSSION

Data on the stand growth (Lim and Ong, 2005) and biomass (Ong *et al*., 2004) of the same stand have been reported previously. The summary of the data set used in this study (based on the 2060 trees ha^{-1} available before harvesting) are shown in Table 2. The *d* of the sampled trees ranged from 4.8 to 10.4 cm, while the total height ranged from 4.8 to 11.8 m, and the merchantable height ranged from 1.7 to 6.8 m.

The volume equations for the merchantable overbark stem volume and the results of the precision of the equations are presented in Table 3. The Spurr's combined variable equation (Equation 2) has the smallest PRESS, RMSE and average bias than the logarithmic equation. Meanwhile, the Schumacher and Hall's equation (Equation 4) showed the smallest PRESS, average bias and RMSE, but the highest R^2 for both the total overbark stem volumes (Table 4), and the merchantable underbark stem volume equations (Table 5). As for the total underbark stem volume equation (Table 6), the Schumacher and Hall's equation was also found to record the smallest PRESS, but with slightly higher average bias and RMSE than the Spurr's combined variable equation.

The Schumacher and Hall's equation recorded the highest R^2 in all the regression models. However, based on the PRESS statistics, the Spurr's combined variable equation can be considered as the best equation to accurately predict the merchantable overbark stem volume. The combined variable equation has been reported to be the most appropriate equation to determine the total underbark stem volume of *Eucalyptus fastigata* regrowth in New South Wales, Australia (Bi, 1994). Meanwhile, Tewari and Kumar (2003) also indicated that the best equation to estimate the total tree volume of *Eucalyptus camaldulensis*, planted in an arid area in India, was the combined variable equation. Similarly, the same equation was also reported to be the best equation for the total underbark volume prediction for *Eucalyptus paniculata* (Bi and Hamilton, 1998).

As compared to the Husch *et al*.'s equation and the Spurr's combined variable equation, the Schumacher and Hall's equation generally

TABLE 2
A summary of the parameters used

Parameters	Mean	SD	Min	Max
DBH (cm)	7.1	1.5	4.8	10.4
Merchantable height (m)	4.9	1.3	1.7	6.8
Total height (m)	7.9	1.5	4.8	11.2

TABLE 3
Regression coefficients of the merchantable overbark stem volume equations and R^2, PRESS, bias and RMSE for the equations

Equation number	Model	Parameter			R^2	PRESS	Bias	RMSE
		a (±S.E.)	b (±S.E.)	c (±S.E.)				
2	$V = a + bd^2h$	1.42×10^{-3} (8.55×10^{-4})	6.59×10^{-5} (0.17×10^{-5})		0.9879	0.0197	1.08×10^{-4}	1.50×10^{-4}
3	$V = ad^b$	1.34×10^{-4} (1.23×10^{-5})	2.48 (0.1079)		0.7860	0.7435	5.53×10^{-4}	7.85×10^{-4}
4	$V = ad^bh^c$	-1.06×10^{-4} (1.81×10^{-5})	1.75 (0.4281)	1.06 (0.2740)	0.9914	0.0274	5.94×10^{-5}	5.61×10^{-4}

TABLE 4
Regression coefficients of total overbark stem volume equations and R^2, PRESS, bias and RMSE for the equations

Equation number	Model	Parameter			R^2	PRESS	Bias	RMSE
		a (±S.E.)	b (±S.E.)	c (±S.E.)				
2	$V = a + bd^2h$	2.32×10^{-3} (8.38×10^{-4})	5.83×10^{-5} (0.26×10^{-5})		0.9886	0.0626	9.40×10^{-5}	1.37×10^{-4}
3	$V = ad^b$	1.99×10^{-4} (1.40×10^{-5})	2.35 (0.2155)		0.6410	0.6988	1.13×10^{-3}	1.60×10^{-3}
4	$V = ad^bh^c$	9.85×10^{-5} (1.08×10^{-6})	0.97 (0.0423)	1.81 (0.0296)	0.9888	0.0307	6.62×10^{-5}	9.95×10^{-5}

TABLE 5
Regression coefficients of the merchantable underbark stem volume equations and R^2, PRESS, bias and RMSE for the equations

Equation number	Model	Parameter			R^2	PRESS	Bias	RMSE
		a (±S.E.)	b (±S.E.)	c (±S.E.)				
2	$V = a + bd^2h$	2.03×10^{-3} (6.63×10^{-4})	7.92×10^{-5} (0.13×10^{-5})		0.9749	0.0603	1.81×10^{-4}	2.59×10^{-4}
3	$V = ad^b$	1.68×10^{-4} (1.26×10^{-5})	2.46 (0.1194)		0.7570	0.8518	7.79×10^{-4}	1.11×10^{-3}
4	$V = ad^bh^c$	1.01×10^{-4} (1.13×10^{-5})	1.65 (0.0775)	1.11 (0.0849)	0.9829	0.0575	1.05×10^{-4}	1.53×10^{-4}

TABLE 6
Regression coefficients of total underbark stem volume equations and R^2, PRESS, bias and RMSE for the equations

Equation number	Model	Parameter			R^2	PRESS	Bias	RMSE
		a (±S.E.)	b (±S.E.)	c (±S.E.)				
2	$V = a + bd^2h$	2.78×10^{-3} (7.85×10^{-4})	5.92×10^{-5} (0.24×10^{-5})		0.9685	0.1724	1.00×10^{-4}	1.53×10^{-4}
3	$V = ad^b$	2.13×10^{-4} (1.43×10^{-5})	2.33 (0.2275)		0.6764	0.6732	1.13×10^{-3}	1.59×10^{-3}
4	$V = ad^bh^c$	0.91×10^{-4} (0.11×10^{-5})	1.02 (0.0599)	1.72 (0.0420)	0.9717	0.0636	1.28×10^{-4}	1.89×10^{-4}

predicted both, the total overbark and underbark stem volume and merchantable underbark stem volume with higher accuracy and less bias. In Peninsular Malaysia, Wan Razali *et al*. (1989) reported that the logarithmic equation was the best model to estimate both the merchantable overbark and underbark stem volume of a 5-year-old *Acacia mangium* plantation. Fonweban *et al*. (1995) also found the logarithmic equation was the most suitable for predicting merchantable volume of *Eucalyptus saligna* found in two forest reserves in Cameroon. Shiver and Brister (1990) reported similar findings for *E. saligna* stands in Western Kenya.

Spurr's combined variable equation and Schumacher and Hall's equation are the two equations which were most commonly used in the development of the stem volume equations. The superiority of these equations was clearly demonstrated in this study, with the R^2 value above 0.95 for all the prediction models. Generally, based on the R^2 value and the volume prediction, the logarithmic equation was found to be the best in determining the stem volume of *A. excelsa* in this study. However, the combined variable equation seemed to be the most appropriate to determine the merchantable overbark stem volume of *A. excelsa,* based on its overall predictive performance. Thus, the best equations to be used in predicting the total or merchantable stem volume are as follows:

$$V_{mob} = 1.42 \times 10^{-3} + 6.59 \times 10^{-5}(d^2 h_m) \quad (9)$$

$$V_{tob} = 9.85 \times 10^{-5}(d^{0.9737} h_t^{1.8106}) \quad (10)$$

$$V_{mub} = 1.01 \times 10^{-4}(d^{1.6527} h_m^{1.1116}) \quad (11)$$

$$V_{tub} = 0.91 \times 10^{-4}(d^{1.0240} h_t^{1.7232}) \quad (12)$$

Where, V_{mob} is merchantable overbark volume, V_{tob} is total overbark volume, V_{mub} is merchantable underbark volume, V_{tub} is total underbark volume, d is diameter at breast height, h_m is merchantable height and h_t is total height.

The volume equations selected in this study for a 53-month-old *A. excelsa* stand are limited by the size and age factors, as well as the silvicultural treatments (e.g. fertilization) involved. The extrapolation outside the range of the data, age and/or under other conditions or silvicultural practices should be made with caution, since the prediction accuracy of the equations in other situations is unknown.

CONCLUSIONS

Yield (volume) estimates could provide the basis for a better management of plantations. The Schumacher and Hall's equation, which is commonly used to develop stem volume equations, has been proven to be the best equation for determining *A. excelsa* stem volume in this study. Based on the results obtained, it is suggested that future studies be done for stands which represent a wider range of site quality, age, management practices. More trees per site should also be considered to improve the accuracy of the yield estimation and develop a more comprehensive yield table.

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Biocoversion of Oil Palm Empty Fruit Bunch by *Aspergillus niger* EB4 under Solid-state Fermentation

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ABSTRACT

Oil palm empty fruit bunch (OPEFB) is an abundant lignocellulosic waste material generated from the palm oil industry. In this study, a locally isolated strain *Aspergillus niger* EB4 was cultivated on a pre-treated OPEFB as substrate to produce cellulase in the solid-state fermentation (SSF) process. The cellulase recovered was then subjected to a saccharification process. The strain was grown on the pre-treated OPEFB in a 250 ml Erlenmeyer flask and a 192 L tray cabinet bioreactor, at 80% moisture content and incubated for nine days under a static condition. The activities of the crude cellulase extract in the tray bioreactor were 19.02 ± 0.85, 6.36 ± 0.38 and 4.56 ± 0.26 U/g for β-glucosidase, CMCase and FPase, respectively on day 6 of fermentation. These results were similar to the results obtained from the flask experiment. The results demonstrated the feasibility of solid substrate fermentation of the OPEFB in both flask and tray cabinet bioreactor for the cellulase production. The enzymatic hydrolysis of OPEFB at 5% (w/v) was performed by utilizing the partially purified and crude cellulase incubated at 40°C for seven days. The partially purified cellullases hydrolyzed the pre-treated OPEFB and released 7.7 g/l of reducing sugar which corresponded to a 15% conversion.

Keywords: Cellulase, oil palm empty fruit bunch, *Aspergillus niger*, solid-state fermentation

INTRODUCTION

Cellulase is responsible for the hydrolysis of cellulose and its production is the most important step in the economical production of bio-ethanol, single cell protein and other chemicals. It is a complex mixture of enzyme with different specificities to hydrolyze glycosidic bonds. At present, cellulase is widely used in food, animal feed, beverage, textile and laundry, pulp and paper, as well as agriculture and for research purposes.

Malaysia produces an abundant supply of lignocellulosic OPEFB waste which has not been satisfactorily utilized. Approximately

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2.4 millions tonnes of OPEFB are generated annually. OPEFB consists of mainly cellulose (50.4%), hemicellulose (21.9%), lignin (10%), and ash (17.7%) (Umi Kalsom *et al*., 1997).

The development of a technology with a minimum capital investment is another approach to reduce the cost involved in producing cellulase. This can be accomplished by producing cellulase in solid-state fermentation (SSF), which requires relatively inexpensive equipment as compared to the conventional fermentor in liquid-state fermentation (LSF). The SSF technique has also been shown to reduce energy requirement, improve product recovery, and decrease wastewater output (Hamidi-Esfahani *et al*., 2004). Moreover, these conditions favour the growth of filamentous fungi, which typically grow in nature on solid substrates, such as organic natural materials.

In this study, the production of cellulolytic enzyme by locally isolated *Aspergillus niger* EB4, cultivated on the pre-treated OPEFB in the SSF was investigated. The performance of the locally designed tray cabinet bioreactor for the production of cellulase and the feasibility of saccharification, using crude and partially purified cellulases on the OPEFB were also studied.

MATERIALS AND METHODS

Strain and Inoculum Preparation

The locally isolated fungus *Aspergillus niger* EB4 was obtained from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The culture was maintained on potato dextrose agar (PDA) and incubated at room temperature (21˚C – 26˚C) for seven days. The inoculum was prepared aseptically by adding 10 ml of sterile distilled water, containing 0.1% Tween 80 on the mycelial agar. A spore suspension was measured using a haemocytometer. The inoculum was prepared freshly prior to the experiments. Pre-germination was carried out by inoculating 10% (v/v) of spore suspension to give an initial concentration of 1×10^6 spores per ml of basal medium at pH 5.5. Carboxymethylcellulose (CMC) at 1% (w/v) was added into the basal medium as a substrate. The cultures were pre-germinated on a rotary shaker at 180 rpm at 27 ± 2˚C for 24 h.

Substrate Treatment

The shredded oil palm empty fruit bunch (OPEFB) was obtained from a local palm oil mill in Selangor, Malaysia. The OPEFB was thoroughly washed to remove dust and then sun dried. The shredded OPEFB was reduced to 2 mm length, using a hammer mill. Then, the OPEFB fibres were further treated with 2% (w/v) of sodium hydroxide (NaOH). In this procedure, 50 g of OPEFB was soaked in 500 mL of 2% (w/v) NaOH at 30˚C for 4 h, followed by filtering and rinsing it with distilled water until it was completely free of alkali and then dried at 90˚C for 48 h. The cellulose, hemicellulose and lignin contents in the OPEFB were determined using the gravimetric method as described by Gorring and Van Soest (1970).

Solid-state Fermentation

The solid-state fermentation (SSF) was conducted in a 250 mL Erlenmeyer flask containing 5 g of pre-treated substrate. The flasks were autoclaved at 121˚C, 15 psi for 15 min. Each flask was added with 3 mL of nutrient supplement as follows: 0.5% (v/v) $(NH_4)_2SO_4$, 0.2% (w/v) KH_2PO_4, 0.05% (w/v) $MgSO_4.7H_2O$, 1% (w/v) $CaCO_3$. An inoculum of blended mycelial suspension (10 mL) of *A. niger* EB4 was aseptically inoculated into the flask. At the initial stage of fermentation, the moisture content and pH was set at 80% and 7, respectively. All the flasks were incubated at 30˚C, under a static condition for nine days of fermentation. The control experiment was carried out by excluding the mycelial suspension in the mixture of substrate and fermentation medium. The solid-state fermentation was also performed in a stainless steel tray cabinet bioreactor (width 0.40 m; length 0.60 m; height 0.8 m) with the capacity of 192 L, as shown in *Fig. 1*. The bioreactor consisted of three

perforated trays. Meanwhile, the total weight of the OPEFB for the three perforated trays was 1.05 kg. The OPEFB was placed in a 3 L beaker, sterilized at 121˚C, 15 psi, for 15 min. After cooling, 0.49 L of sterile distilled water, 0.21 L nutrient supplement and 0.7 L pre-germinated fungus were added to the substrate to achieve 80% of the initial moisture content. The well-mixed substrate and fermentation medium were divided into three portions with the same weight and then poured onto each sterile tray to form a thin layer (less than 3 cm) in a sterile condition. Then, the trays were immediately stacked in the cabinet. Meanwhile, the solid state fermentation was carried out at the room temperature (21 – 26˚C) without aeration and static condition. The sampling was carried out aseptically using a sterile sampler. The samples, which were taken from each tray at approximately the same amount, were combined and kept at 4˚C prior to the analysis. The control experiment was conducted by excluding the mycelial suspension on the mixed substrate and fermentation medium.

Extraction Procedures and Analysis

The fermented substrate (5 g) was extracted using 50 mL of 0.01 M phosphate buffer (pH 7) at 4˚C. The extraction was done using a homogenizer at 9700 rpm for 4 min. The culture slurry was then centrifuged at 5000 rpm, at 4˚C for 20 minutes, and the supernatant was stored at -20˚C before the analysis (Shahrim *et al*., 2008; Latifian *et al*., 2007). The CMCase activity was measured according to Dong *et al*. (1992), while the FPase was determined based on Elshafaei *et al*. (1990). After 30 min of incubation at 50˚C, the reducing sugar, liberated in the reaction mixture was measured by the dinitrosalicyclic (DNS) method (Miller, 1959). β-glucosidase was determined based on the procedure suggested by Dong *et al*. (1992). The substrate used for the determination of β-glucosidase activity was p-nitrophenol-β-Glucopyranoside. One unit (U) of the enzyme activities was defined as the amount of enzyme required to liberate 1 µmol of product per min. The enzyme yield was expressed as U/g of dry substrate. The reducing sugars concentration

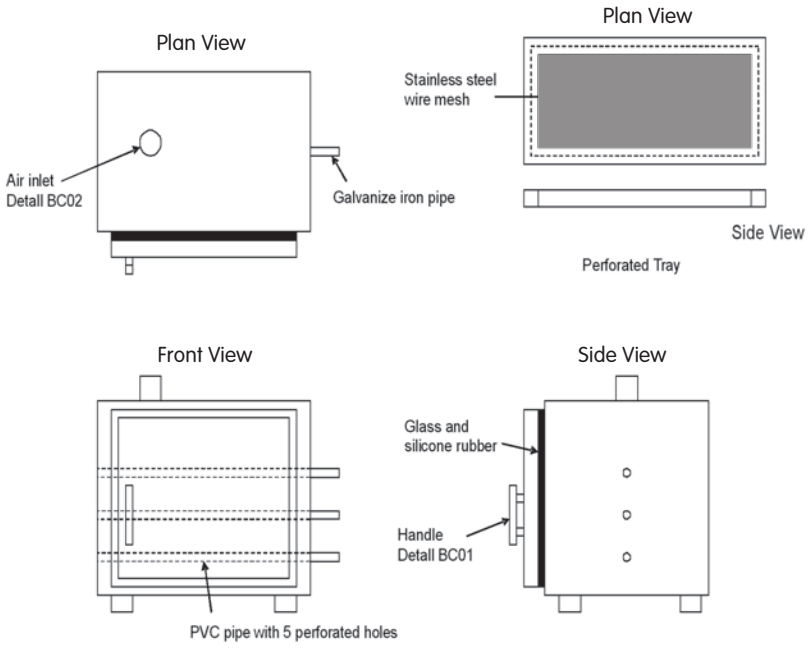

Fig. 1: Schematic diagram of tray bioreactor for SSF

was analyzed according to the DNS method (Miller, 1959). The soluble protein content was determined based on the method proposed by Lowry *et al*. (1951).

Cellulase Recovery and Saccharification of OPEFB

The crude cellulase of *A. niger* EB4 was obtained from the fermentation process in the tray bioreactor. The crude enzyme was assayed before subjecting to the saccharification process and precipitated using 80% ammonium sulphate saturation. The ammonium sulphate precipitation was done according to the method described by Harris (1989). The enzymatic saccharification of OPEFB was carried out in a water bath shaker (Protech Ltd.) at 40˚C and agitated at 100 rpm. For the saccharification experiment, 5 g of the treated OPEFB was placed into a 250 ml Erlenmayer flask, after which 30 ml of crude or partially purified enzyme in 70 ml 0.01 M phosphate buffer (pH 7) was added to obtain 5% (w/v) substrate suspension. Sodium azide (0.02%, w/v) was added to the reaction mixture to prevent bacterial or fungal contamination. Samples (3 ml) were collected every 24 h for the seven days of incubation. The collected sample was centrifuged for 10 min at 5000 rpm. The supernatant was used to determine the reducing sugars. The control experiment was done by excluding the enzyme in the flask and replacing with the buffer solution. The data were the average of three replicates.

RESULTS AND DISCUSSION

Soluble Protein, Reducing Sugars and pH Profile in Flask

The soluble protein of the culture extract during the fermentation is shown in *Fig. 2a*. The soluble protein was highest on the seventh day of fermentation. It should be noted that the increase of the protein content in the culture extract might probably be due to the growth of fungus and the secretion of enzymes such as cellulase. Other extra-cellular enzymes, such as hemicellulases involved in the degradation of the OPEFB, might have also contributed to the soluble protein content. The reducing sugar gradually increased and reached the maximum value on day 6 of fermentation (16.2 mg/g), indicating substrate conversion (*Fig. 2b*). The reducing sugar was found to decrease after day 6 and this correlates with the decrease of the enzyme activities (*Fig. 3*). However, the reducing sugar yield was not a linear function of the concentration of enzyme in the mixture. The pH of the fermented substrate was found to decrease towards the end of the fermentation period. After six days of fermentation, the final

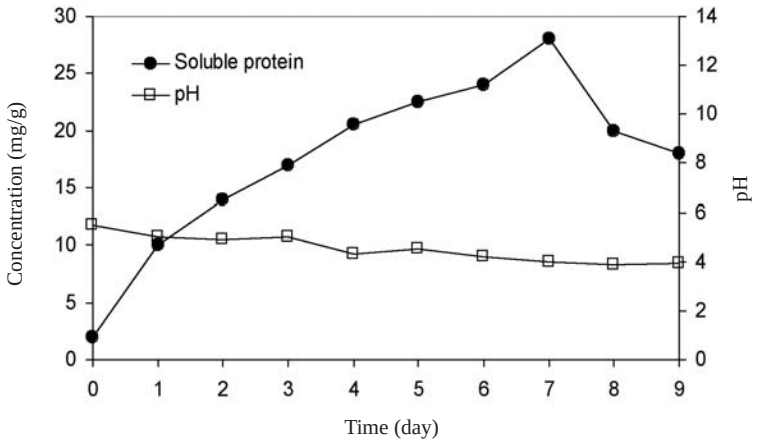

Fig. 2a: Profile of soluble protein, pH during SSF of A. niger *EB4 grown on OPEFB*

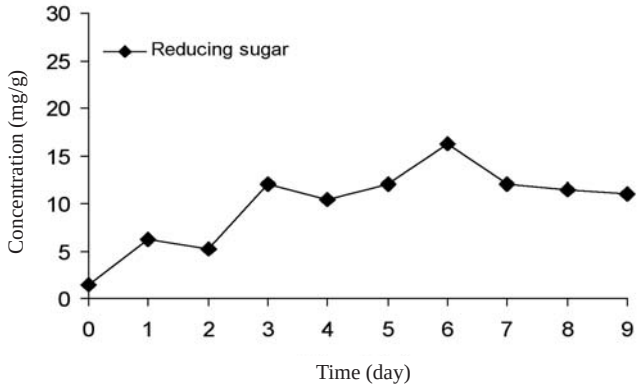

Fig. 2b: Profile of reducing sugar during SSF of A. niger *EB4 grown on OPEFB*

pH detected was in the range between 3.5 and 4.0 (*Fig. 2a*). Most of the filamentous fungi, especially *Aspergillus* and *Trichoderma* strains, were able to grow and metabolize in such a pH range (Jecu, 2000).

Cellulase Production in Solid-State Bioconversion

The lignocellulosic composition of the treated OPEFB used in this study consisted of cellulose (50.7%), followed by hemicellulose (20.4%) and lignin (10%). Based on the composition, the OPEFB could serve as a good substrate for the production of cellulase. Alkali treatment with 2% NaOH was applied on the substrate for delignification. It was reported that 2% NaOH could demolish the structure of cellulose (Yang *et al*., 2004). Filamentous fungi have the ability to penetrate effectively into the intracellular and intercellular spaces of the solid substrate. In the SSF, the pre-treatment of the solid substrate, by either mechanical or chemical means, was employed to improve its amenability to microbial modification by forming smaller permeable molecules, providing sites for easy microbial penetration (Lonsane *et al*., 1992).

The time course study of the cellulase activities of *A.niger* EB4 grown on pre-treated OPEFB in flask is shown in *Fig. 3.* In this study,

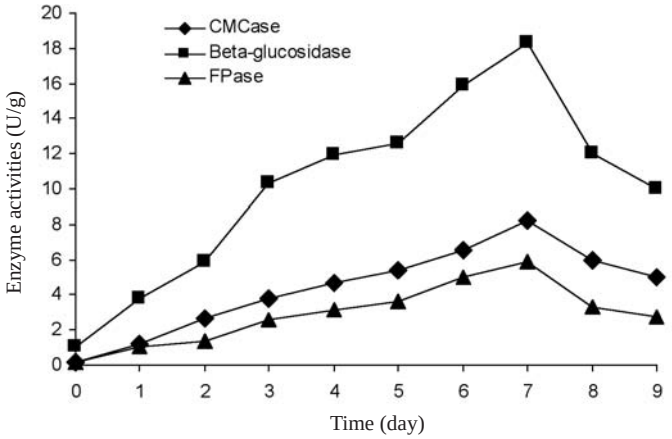

Fig. 3: Time course of the cellulase activities of Aspergillus niger *EB4 grown on OPEFB in SSF at 30˚C*

the cellulase activity was gradually increased and it achieved the maximum value on day six of cultivation and it then gradually declined. The time required to reach the maximum levels of the activity may be affected by several factors, including the presence of the different ratios of amorphous to crystalline cellulose (Gao *et al*., 2008). The highest activity for CMCase, FPase and BGDase were 8.24 U/g, 4.32 U/g and 19.07 U/g, respectively. The decrease in the activity after day 7 might be due to feedback repression by cellobiose. The cellulase activities produced by *A. niger* EB4 and grown on OPEFB in the SSF were comparable with other the cellulase producing fungi (Table 1). The SSF of rice chaff by a microbial consortium showed the highest FPase activity at 5.64 U/g (Yang *et al*., 2004). Alam *et al*. (2005) reported the highest cellulase activity of 0.0433 unit by *Trichoderma harzianum* grown on OPEFB in flask at day 9 in the SSF. The efficiency of enzymatic degradation is also dependent on the chemical composition of the substrate, and the composition of the individual enzymes of the total cellulase (Krishna, 1999; Gao *et al*., 2008).

The experiment of the SSF, using a tray bioreactor, was also conducted in this study. Although the tray bioreactor was conducted at the room temperature, the cellulase activities obtained were comparable to the activities in the flasks. On day 7, the highest FPase, CMCase and β-glucosidase obtained were 4.56, 6.36 and 19.02 U/g, respectively. It should be noted that the cellulase activities for the tray bioreactor were not much affected by uncontrolled temperature (room temperature). The results obtained in this study indicated the effectiveness of the tray bioreactor for the production of cellulase using a simple and economical SSF system.

Enzyme Recovery and Hydrolysis of OPEFB

The ammonium sulphate precipitation, at different saturations for cellulase from *A.niger* EB4, is shown in *Fig. 4*. The results showed that 80% ammonium sulphate saturation gave the highest concentration of the soluble protein and the cellulase activity. The enzyme activities of the crude and partially purified cellulase are shown in Table 2. For enzyme recovery, using 80% ammonium sulphate precipitation, it was shown that 8.1% (2.1 fold purification) was achieved for β-glucosidase, while CMCase showed 7.7% (2 fold purification). As for FPase, only 5.8% recovery with 1.5 fold purification was obtained. The sacharification of the OPEFB

TABLE 1
Comparison of cellulase production from different fungi in SSF

Substrate	Fungal strain	Enyme	Enzyme Yield (U/g substrate)	Reference
Rice bran	*Trichoderma reseei* MGG77	FPase	2.314	Laitifian *et al*. (2007)
Wheat ban	*A. ustus*	FPase CMCase β-glucosidase	5.6 14.0 12.9	Sharmala and Sreekantiah (1987)
Wheat straw	*Thermoascus aurantiacus*	FPase CMCase β-glucosidase	5.5 1709.0 79.0	Kalogeris *et al*. (2003)
Oil empty palm fruit bunch	*A. niger EB4*	FPase CMCase β-glucosidase	4.56 6.36 19.02	This study

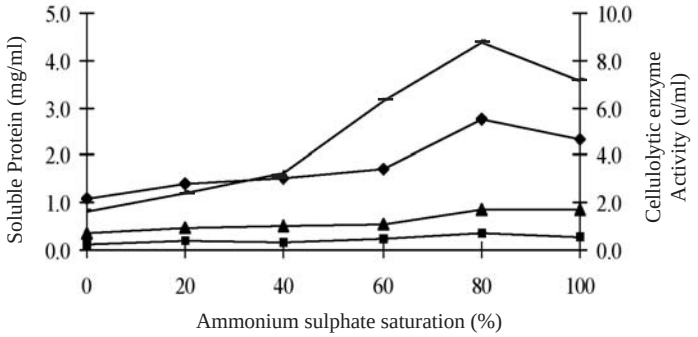

Fig. 4: Ammonium sulphate precipitation for cellulase at various saturations

TABLE 2
Partial purification of cellulase from *A.niger* EB4, using 80% ammonium sulphate saturation

Enzyme	Sample	Activity (U/ml)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
β-glucosidase	Crude filtrate	3.17	0.63	100	1.0
	Partially Purified	5.12	1.35	8.1	2.1
CMCase	Crude filtrate	1.06	0.21	100	1.0
	Partially Purified	1.64	0.43	7.7	2.0
FPase	Crude filtrate	0.53	0.11	100	1.0
	Partially Purified	0.62	0.16	5.8	1.5

was performed using crude and precipitated cellulase. The enzymatic hydrolysis process was monitored by the amount of reducing sugar released.

The profile for the saccharification of the pre-treated OPEFB, using crude and partially purified cellulase from *A. niger* EB4 is shown in *Fig. 5*. The enzymatic hydrolysis of the OPEFB showed a typical upward trend in the rapid release of soluble sugars for four days of fermentation and it then reached a plateau at day 5 onwards. The concentration of reducing sugars was constant after five days of incubation for both the crude and partially purified cellulases. The maximum reducing sugars produced from the OPEFB saccharification, using crude and partially purified cellulase from *A. niger* EB4 are listed in Table 3. The maximum production of the reducing sugars was 7.7 g/l, which corresponded to a yield of 0.15 g/g. The highest glucose concentration produced from the saccharification of the pre-treated OPEFB, obtained for both the crude and partially purified cellulose were 2.62 g/l and 4.87 g/l, respectively; these corresponded with the yield of 0.06 and 0.10 g/g, respectively. The partially purified enzyme gave a better yield of reducing sugars as compared to the crude enzyme in the saccharification experiment. The bioconversion of the pre-treated OPEFB into sugars by the partially purified cellulase was 15%. Baig *et al*. (2004) reported that enzymatic saccharification by precipitated cellulose, containing 0.2 U/ml FPase, 0.41 U/ml CMCase, 0.24 U/ml β-glucosidase from *T. lignorum*, gave banana waste conversion of 15%. The results obtained in this study proved that lignocellulose OPEFB could be used as a potential substrate for enzymatic saccharification.

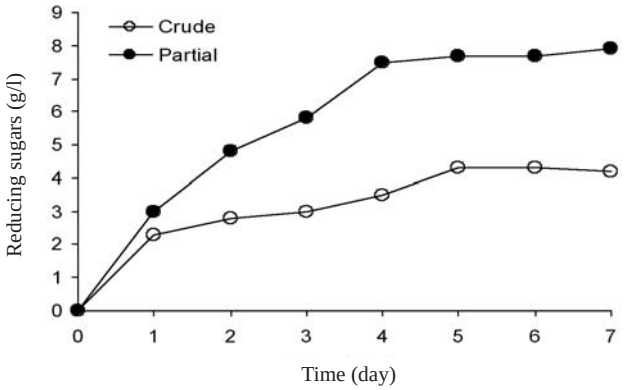

Fig. 5: Time course of the saccharification of the OPEFB, using crude and partially purified cellulase from A. niger *EB4*

TABLE 3
Reducing sugars production in enzymatic saccharification of OPEFB by the crude and partially purified cellulase from *A.niger EB4*

Enzyme	Total reducing sugars		
	Production (g/l)	Yield (g/g substrate)	Bioconversion (%)
Crude	4.37	0.09	9
Partially Purified	7.70	0.15	15

CONCLUSIONS

A. niger EB4 was successfully grown on the pre-treated OPEFB and produced cellulase in both the flask and tray cabinet bioreactors. The activities of the crude cellulase extract in the tray bioreactor were 19.02 ± 0.85, 6.36 ± 0.38 and 4.56 ± 0.26 U/g for β-glucosidase, CMCase and FPase, respectively, and these were almost similar to the results obtained in the flask. The saccharification of the OPEFB, by the partially purified enzyme, gave a higher yield of reducing sugars than the crude enzyme. The maximum production of reducing sugars was 7.7 g/l, which corresponded to 0.15 g/g of the reducing sugars yielded and 15% conversion. The present study indicated that the OPEFB was suitable to be converted into sugars by the cellulase produced from locally isolated fungus.

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Overexpression of Wildtype *Periostin* and *Transforming Growth Factor Beta I* Genes in Colorectal Carcinoma: A Preliminary Study

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ABSTRACT

The majority of deaths from colorectal carcinoma (CRC) occur due to metastasis during the late stage of tumourigenesis. Recently, *periostin*, i.e. a gene encoding a protein which is initially found in osteoblasts, has been reported to be associated with the late-stage tumourigenesis in colon and a variety of human cancers. The researchers investigated the expression of *periostin* mRNA in normal and tumour biopsy specimens using the RT-PCR analysis to elucidate the role of *periostin* in human colorectal carcinoma. The results showed that there was a significantly (P<0.05) higher expression of the *periostin* mRNA in the biopsy specimens obtained from the tumour tissues, as compared to the normal tissues. Nevertheless, sequence analysis revealed no mutation in the full length of the *periostin* gene. As the over-expression of *periostin* in human colorectal carcinoma did not appear to be due to the mutation in the *periostin* gene, the involvement of other collaborative factors was therefore deduced. Consistent with this finding, the researchers focussed on studying the *transforming growth factor (TGF)* β_1 which has been reported to be associated with the increasing in the expression of *periostin*. The analysis (RT-PCR) in this study revealed that *TGF-*β_1 gene was also highly expressed in tumour biopsy specimens (P<0.05). This gene mutation is also absent. These data validated that both *periostin* and *TGF-*β_1 work together to control colorectal organogenesis.

Keywords: Metastasis, colorectal carcinoma, *periostin*, tumourigenesis, RT-PCR, *TGF-*β_1

INTRODUCTION

Colorectal carcinoma (CRC), which is also known as the cancer of the colon and rectum, was recorded as the most commonly diagnosed cancer in men, and the third most common cancer in women (Malaysia National Cancer Registry, 2003). Typically, major fatalities from colorectal cancer are due to the dissemination of the primary tumours, which lead to formation of metastases which are resistant to conventional chemotherapy (Fidler, 1990). This event, known as metastasis, is the hallmark of malignant cancers. Usually, it occurs during the late stage of tumourigenesis to the liver (Galandiuk *et al.*, 1992). For metastasis to take place, the tumour cells must undergo a series of interrelated steps which involve numerous complex molecular interactions (Fidler, 1990; Scanlon and Murthy, 1991).

In the process of identifying genes associated with tumour metastasis, *periostin*, a gene encoding a protein with similarity to the fasciclin family (Takeshita *et al.*, 1993), has been shown to promote tumour metastasis and angiogenesis

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of the colon cancer (Bao *et al.,* 2004). Formally known as the osteoblast-specific factor 2 (OSF-2), *periostin* is found to be over-expressed in several human tumours including ovarian carcinoma (Gillan *et al.,* 2002), colon cancer (Bao *et al.,* 2004; Sim *et al.,* 2006), breast cancer (Shao *et al.,* 2004), nasopharyngeal carcinoma (NPC) (Chang *et al.,* 2005), oral cancer (Siriwardena *et al.,* 2006), head and neck squamous cell carcinoma (HNSCC) (Kudo *et al.,* 2006) and papillary thyroid carcinomas (Puppin *et al.,* 2008). *Periostin* activates the serine/threonine kinase (Akt/PKB) signaling pathway, which is known to increase cellular and endothelial cell survival, by promoting angiogenesis (Bao *et al.,* 2004). An exposure of colorectal cancer cells to anti-*periostin* antibodies activated apoptosis and potentiates the effects of 5-fluorouracil chemotherapy (Tai *et al.,* 2005). Therefore, further studies are suggested to target this protein as a therapeutic option in colorectal cancers.

Although several *periostin* regulating genes have been reported, including bone morphogenetic protein-2 (BMP-2) (Lindner *et al.,* 2005), the bHLH transcription factor (TWIST) (Oshima *et al.,* 2002) and fibroblast growth factor 2 (FGF2) (Li *et al.,* 2004), transforming growth factor beta (*TGF-β*) has been identified as one of the possible candidates for the regulating factor which is responsible for the over-expression of *periostin* in the colorectal cancer in this study, since *periostin* contains similar structure to *β*ig-h3, a molecule induced by *TGF-β*. This gene plays an important role in controlling proliferation, differentiation, and is involved in many important cellular functions (Derynck *et al.,* 2001; Xie *et al.,* 2003). Three highly homologous isoforms of the *TGF-β* (*TGF-β_1, TGF-β_2, and TGF-β_3*) have been reported in mammals (Friedman *et al.,* 1995). Mutations have been reported in transforming growth factor beta receptor two (*TGF-βRII*) gene (Takenoshita *et al.,* 1997).

To date, there has been no report written on the mutation analysis of the *TGF-β_1* gene in colon cancer. The presence of *TGF-β* has been reported to increase the expression of *periostin* in primary osteoblast cell (Horiuchi *et al.,* 1999). In addition, *TGF-β* has also been shown to be involved in tumour progression by modulating angiogenesis in colorectal cancers (Xiong *et al.,* 2002). There has been no report on the expression or mutation analysis of the human *periostin* gene in colorectal cancer. Hence, this study aimed to look at the association between the over-expression with the mutations which might be present within the full length sequences of human *periostin,* as well as in the *TGF-β1* gene.

MATERIALS AND METHODS

Total RNA Specimens

The total RNA from the colorectal biopsy tissues (labelled in numeric number) were provided by the Institute for Medical Research (IMR), Kuala Lumpur (courtesy of Pauline Balraj). The samples from the IMR were initially provided by Hospital Universiti Kebangsaan Malaysia (courtesy of Prof. Dr. A. Rahman A. Jamal) to the IMR, where the total RNA extraction using trizol method was subsequently carried out. The research group was provided with these samples (total RNAs only) as the group is a part of the national team in the multi-institutional research programme approved by the National Biotechnology Directorate (a division within the Ministry of Science, Technology and Environment), and coordinated by the IMR. Two sets of commercially available total RNA, designated as CN and CT, were purchased from BD Biosciences, Inc., USA and Chemicon, Inc., USA. The total RNA was quantitated using a spectrophotometer (Biochrom, England).

Primer Design

The upstream and downstream synthetic oligonucleotide primers were constructed using Primer 3.0 free-ware (*http://frodo.wi.mit.edu/*). The primers for the genes of interest were *periostin* (5'-AATCATCCATGGGAACCA GA-3' and 5'-TATTCACAGGTGCCAGC AAA-3'), *TGF-β_1* (5'-CCCGTCGGTCGCTAG

CTC-3' and 5'- CGTGTACTGGCCGTTACC TT-3') and *GAPDH* (5'-TGCACCACCAA CTGCTTAGC-3' and 5'-GGCATGGACTG TGGTCATGAG-3').

Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Analysis

The first-strand cDNA was prepared using Moloney Murine Leukemia Virus (MMLV) RT enzyme and a range of reagents from Promega, USA. The PCR was performed using PTC-200 Peltier Thermal Cycler, with an initial 2 minutes of denaturation at 95˚C, followed by 35 cycles of 30 seconds of denaturation at 95˚C, 45 seconds of annealing at 58ºC, 1 minute of elongation at 72˚C, and ended with 5 minutes of final elongation at 72˚C. The total 25µl PCR reaction volume contained 1µl of first-strand cDNA template, 1X PCR buffer, 1.5mM $MgCl_2$, 0.2mM each of dNTP, 1µM of upstream and downstream *periostin* or *TGF*β_1 primers and 1 unit of Go*Taq* DNA polymerase (Promega, USA).

Cloning of Partial cDNA Fragments and Sequencing

Purified PCR fragments were cloned into Promega pGEM®-T Easy Vector System II and transformed into chemically competent *E. coli* JM109. Positive colonies with insert of interest were screened using T7 and SP6 promoter primers. The positive clones were selected for the downstream plasmid DNA isolation, using Promega Wizard® *Plus* Minipreps DNA Purification Systems and the procedures were carried out according to the manufacturer's instructions. After that, the isolated plasmids were sent to FirstBase Sdn. Bhd. for sequence acquisition.

Statistical Analysis

The RT-PCR images were captured using the Alpha DigiDoc™ Imaging System and the quantification of the bands intensity (ng) was performed using the AlphaEase®FC Stand Alone software. The statistical values were analysed using the SPSS Paired Sample t-Test to check for significant differences between the normal and tumour samples (in this case, *P*-value < 0.05 is considered as significant).

RESULTS AND DISCUSSION

Expression Analysis of Periostin *and* TGF-β_1 *mRNA*

Quantitative RT-PCR yielded the PCR product with band size of 602 bp from the eighteen samples. Over-expression of *periostin* was observed in all nine tumour samples. In this study, a significant difference ($P<0.05$) was found in the level of mRNA expression of *periostin* between the local or commercial normal and tumour samples. Nevertheless, the *periostin* expression level was not compared among the tumour and metastatic tumour since the samples used in this study were mainly of Dukes'B stage. Overall, although the sample size was rather small, the consistency of the preliminary result with the findings of Bao *et al.* (2004) and Sim *et al.* (2006) further supported the expression behaviours of *periostin* in colorectal carcinoma. For the analysis of *TGF-*β_1, a product band of 869bp was amplified from all the normal and tumour samples. The expression of *TGF-*β_1 gene was found to be significantly higher in all cases of colorectal tumour samples ($P<0.05$). This is consistent with the other findings (Friedman *et al.*, 1995; Xiong *et al.*, 2002), in which *TGF-*β_1 demonstrated high levels of expression in the tumour samples from colorectal carcinoma.

In this study, *GAPDH* was used as the house-keeping gene to show that all the samples tested had equimolar starting concentrations. The values of both genes (*periostin* and *TGF-*β_1) were normalised with the *GAPDH* values before it was continued with the statistical analyses (Table.1). The representative RT-PCR results for *periostin* and *TGF-*β_1 are shown in *Figs. 1* and *2*, whereas *Fig. 3* shows a gel photo of the *GAPDH* expression.

TABLE 1
Distribution of the samples according to their origin and the RT-PCR signal of *periostin* and *TGF-β_1*

No.	Samples		RT-PCR value (ng) after normalisation with GAPDH	
	Local biopsy	Commercially available	*Periostin*	*TGF-β_1*
1		CN1	1.25	5.40
2		CT1	1.55	6.80
3		CN2	0.24	0.05
4		CT2	0.63	3.50
5	61N		0.30	1.66
6	61T		0.54	2.10
7	63N		0.31	0.00
8	63T		0.54	1.57
9	67N		0.45	3.00
10	67T		0.57	3.10
11	69N		0.41	1.92
12	69T		0.57	3.10
13	43N		0.13	0.07
14	43T		0.57	0.10
15	44N		0.07	1.58
16	44T		0.46	1.70
17	53N		0.13	0.02
18	53T		0.46	3.10

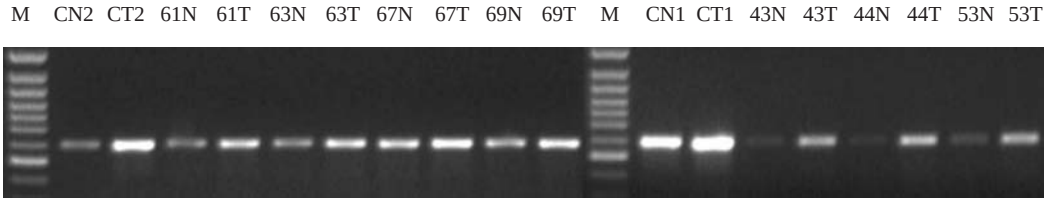

Fig. 1: Agarose gel (1.5% w/v) electrophoresis analysis of the PCR amplified periostin *gene with lane M, 100bp DNA ladder (Fermentas), lane 2-11 and lane 13-20 represent amplicon (602bp) from nine normal (N) and tumour (T) tissues. Note that all samples were paired normal and tumour tissues, except for the CN/CT*

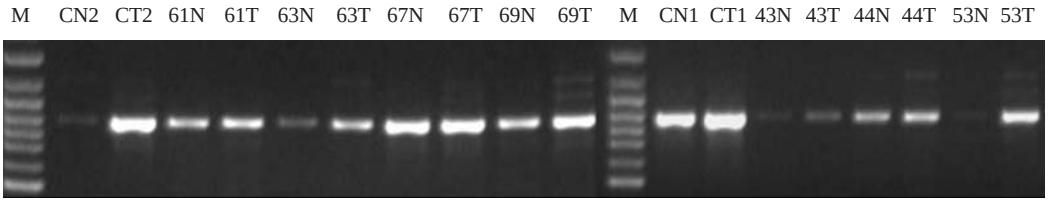

Fig. 2: Agarose gel (1.5% w/v) electrophoresis analysis of the PCR amplified TGF-β_1 *gene with lane M, 100bp DNA ladder (Fermentas), lane 2-11 and lane 13-20 represent amplicon (869bp) from nine normal (N) and tumour (T) tissues. Note that all the samples were paired normal and tumour tissues, except for the CN/CT*

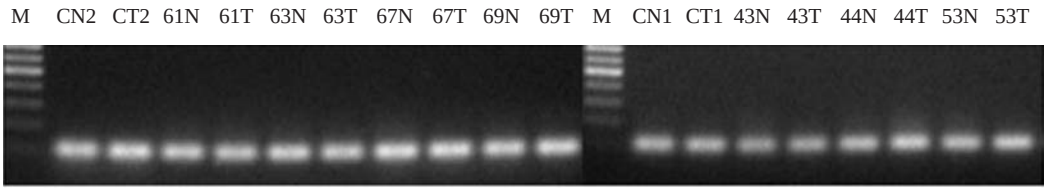

Fig. 3: Agarose gel (1.5% w/v) electrophoresis analysis of the housekeeping gene GAPDH with lane M, 100bp DNA ladder (Fermentas), lane 2-11 and lane 13-20 represent amplicon (87bp) from nine pairs of normal (N) and tumour (T) tissues

Mutational Analysis of Periostin *and* TGF-β_1 *Gene*

The full length of the *periostin* gene, including all nine tumours and five normal samples (CN1, CN2, 44N, 67N, 69N) were sequenced. The sequence analysis of all the nine tumour samples showed no mutation throughout the 2,511 bp open reading frame; however, for samples 53T, 61T and 69T, there is a 16bp nucleotide repeat inserted after the termination codon in the 3' untranslated region (*Fig. 4*). After that, 69T was compared to the corresponding paired 69N and found that the repetition fragment was not present in the normal. Nevertheless, the

TCTCAG*TGA*AAATCCAAAAACCAGAAAAAAATGTTTATACAACCCTAAGTCAATAACCTG
ACCTTAGAAAATTGTGAGAGCCAAGTTGACTTCAGGAACTGAAACATCAGCACAAAGAAG
CAATCATCAAATAATTCTGAACACAAATTTAATATTTTTTTTTCTGAATGAGAAACATGAG
GGAAATTGTGGAGTTAGCCTCCTGTGG**AGTTAGCCTCCTGTGG**TAAAGGAATTGAAG
AAAATATAACACCTTACACCCTTTTTCATCTTGACATTAAAAGTTCTGGCTAACTTTGGAA
TCCATTAGAGAAAAATCCTTGTCACCAGATTCATTACAATTCAAATCGAAGAGTTGTGA
ACTGTTATCCCATTGAAAAGACCGAGCCTTGTATGTATGTTATGGATACATAAAATGCAC
GCAAGCCATTATCTCTCCATGGGAAGCTAAGTTATAAAAATAGGTGCTTGGTGTACAAAA
CTTTTTATATCAAAAGGCTTTGCACATTTCTATATGAGTGGGTTTACTGGTAAATTAT
GTTATTTTTTACAACTAATTTTGTACTCTCAGAATGTTTGTCATATGCTTCTTGCAATGC
ATATTTTTTAATCTCAAACGTTTCAATAAAACCATTTTTCAGATATAAAGAGAATTACTTC
AAATTGAGTAATTCAGAAAAACTCAAGATTTAAGTTAAAAAGTGGTTTGGACTTGGGAA

Fig. 4: Nucleotide 2,514 – 3,213 of human periostin nucleotide sequence. The stop codon, TGA (nucleotide 2,520-2,522), is italicized and highlighted. The putative 16bp sequence is underlined and the repeated sequence is highlighted and denoted in bold

researchers were unable to sequence the other paired normal (53N and 61N), and since the samples provided from the IMR were limited in quantity. Due to the fact that the number of the samples used in this study was rather small, future studies with more samples are required to ascertain the findings of this study. As for the *TGF-β_1*, the sequence analysis of all nine tumours and two normal samples (CN2 and 67N) showed no mutation throughout the gene. Together, these data suggested that the over-expression of both *periostin* and *TGF-β_1* was not due to the mutation of the gene itself.

CONCLUSIONS

The expression of *periostin* and *TGF-β_1* mRNA was detected at a significantly higher level in the tumour biopsy specimens suggesting that both genes might play a role in the carcinogenesis of colorectal cancer. However, no mutation was detected in the coding region of *periostin* and *TGF-β_1* genes, suggesting that their over-expression might be due to the effect of other cis-trans elements. Future studies, consisting of more samples, are therefore required to ascertain the findings of this study.

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Improved Accuracy for Diagnosis of Nasopharyngeal Carcinoma by the Combination of Recombinant EBV Proteins ZEBRA/IgA and LMP2A/IgG ELISA

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is a common cancer in Malaysia and elevated serum antibodies to Epstein-Barr virus (EBV) proteins are useful diagnostic markers of the NPC. Coding sequences of EBV proteins LMP2A, EA-D and ZEBRA were cloned from RNA of B95.8 cell line, an EBV-transformed marmoset cell line, into yeast *Saccharomyces cerevisiae* expression vectors. In this study, ELISA was used to immobilize the recombinant EBV proteins for the detection of serum antibodies in NPC patients. The sensitivities and specificities of serum IgG and IgA against recombinant EBV proteins LMP2A, EA-D and ZEBRA in 124 histopathologically diagnosed NPC and 124 age, gender and ethnic-matched healthy individuals were determined. ZEBRA/IgA was found to be the most sensitive single test, which correctly predicted 90.3% of the NPC cases, followed by LMP2A/IgG (77.4%) and EA-D/IgG (73.4%). For specificity, ZEBRA/IgA, EA-D/IgG and EA-D/IgA were each able to exclude 96.0% of the non-NPC cases. The combination of ZEBRA/IgA and LMP2A/IgG ELISA achieved a sensitivity of 95.2% and a specificity of 99.2%. Among the 124 NPC patients recruited in this study, 100 (80.6%) had elevated VCA/IgA determined by the reference method of indirect immunofluorescence assay (IFA). Thus, a higher sensitivity (95.2%) was achieved by the combination of ZEBRA/IgA and LMP2A/IgG. In addition, the combined ELISA could distinguish the 24 NPC sera which had VCA/IgA titers not detectable by IFA.

Keywords: Nasopharyngeal carcinoma, Epstein-Barr virus, ZEBRA, LMP2A, enzyme-linked immunosorbent assay

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a disease with remarkable geographical and ethnic distribution (Busson *et al*., 2004). It is common in the Chinese populations, particularly in South China and Southeast Asian countries, including Malaysia. NPC is difficult to diagnose in the early stages, and much research has been carried out to identify diagnostic markers of the NPC.

Epstein-Barr virus (EBV) is believed to be an important etiologic agent of the NPC. Elevated serum antibody levels to EBV antigens detected in the NPC patients are useful in the serodiagnosis of this malignancy. The detection of serum antibodies to the EBV viral capsid antigen (VCA) and an early antigen (EA) by indirect immunofluorescence assay (IFA) were among the earliest tests developed (Henle and Henle, 1976; Ho *et al*., 1976).

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IFA remains the gold standard for the EBV serodiagnosis of NPC (Leung *et al*., 2004; Raab-Traub, 2000). However, IFA is rather time-consuming and unsuitable for a large-scale testing or automated handling. Enzyme-linked immunosorbent assay (ELISA) provides an alternative with the potential for automation and mass screening.

The EBV antigens which have been reported as the markers of the NPC, detectable by ELISA, include thymidine kinase (Connolly *et al*., 2001), DNase (Stolzenberg *et al*., 1996), ribonucleotide reductase (Fones-Tan *et al*., 1994), ZEBRA (Chan *et al*., 2003; Dardari *et al*., 2001), and EA (Dardari *et al*., 2001). However, a single EBV antigen may not be sufficient to identify all the individual NPC patients in view of the observed diversity of antibody reactivity among the NPC patients. The combined testing of two EBV antigens yielded more sensitive results in the diagnosis of NPC (Chan *et al*., 2003; Dardari *et al*., 2001).

Three diagnostically relevant recombinant EBV proteins had been produced and evaluated by the researchers in the serodiagnosis of NPC. They are LMP2A from the EBV latent cycle; EA-D from the lytic cycle; and ZEBRA, a transactivator protein which switches on the lytic cycle. LMP2A (expressed from a highly spliced mRNA, containing exons located at both ends of the linear EBV genome), is an integral membrane protein which mimics the B-cell receptor to enable EBV to escape from the host immunity (Kieff and Rickinson, 2001; Caldwell *et al*., 1998). EA-D (encoded by full-length BMRF1) corresponds to a dominant immunogen of the diffuse EA complex (Henle *et al*., 1971). The EBV BZLF1 is an immediate-early gene product, while the ZEBRA is a transactivator protein which is capable of disrupting EBV latency when it is expressed in latently infected cells (Rooney *et al*., 1989).

MATERIALS AND METHODS

Clinical and Biological Samples

Peripheral blood samples were collected from 124 patients with histologically confirmed NPC from the otorhinolaryngology clinic at the University Malaya Medical Centre (UMMC). Serum isolation was performed for each blood sample immediately after the receipt in the laboratory, heat-inactivated at 56˚C for 30 minutes, and stored at -20˚C until use. Sera from age, gender and ethnic-matched healthy individuals served as controls. The collection of clinical samples was carried out with prior ethical clearance from the UMMC Ethics Committee (Ethics Committee Reference No.: 471.2) and an informed consent was also obtained.

B95.8 (ATCC CRL-1612) and P3HR1 (ATCC HTB-62) cell lines were used in this study. These cells were maintained in a RPMI-1640 medium containing 10% of fetal calf serum (v/v), 100 IU/ml penicillin and 100 µg/ml of streptomycin at 37˚C in a humidified 5% CO_2 atmosphere (NuAire water jacketed CO_2 incubator, USA). All reagents used were purchased from Flowlab, Australia.

Cloning and Expression of Recombinant EBV Proteins

Total RNA from B95.8 cells were extracted using the RNeasy Protect Mini Kit (Qiagen, Germany), following the protocol suggested by the manufacturer. The coding sequences of EBV-ZEBRA, EA-D and LMP2A were amplified by the reverse-transcriptase polymerase chain reaction (RT-PCR) from the B95.8 RNA, using gene-specific primers (Table 1) and Superscript III One-Step RT-PCR system with Platinum® *Taq* DNA polymerase (Invitrogen, USA), also following the manufacturer's recommendation. The primers used were designed in the laboratory, based on the known sequences of the EBV genes (Baer *et al*., 1984).

The amplified EBV transcripts were cloned into yeast expression plasmid vectors pYES2.1/V5-His-TOPO® (Invitrogen, USA) and transformed into yeast *Saccharomyces cerevisiae* (INV*Sc*1 strain, Invitrogen, USA). The EBV-ZEBRA, EA-D and LMP2A recombinant proteins were expressed and purified from the yeast *Saccharomyces cerevisiae* transformed by the recombinant pYES2.1/V5-His-TOPO®

TABLE 1
Gene-specific primers used in RT-PCR

Transcript	Primer designation	Genome co-ordinates[a]	Oligonucleotides sequence (5' to 3')	Fragment Size (bp)
ZEBRA	Forward	103155-103136	ATGATGGACCCA AACTCGAC	738
	Reverse	102212-102231	AGAAATTTAAGA GATCCTCG	
EA-D	Forward	79899-79918	ATGGAAACCACT CAGACTCT	1215
	Reverse	81113-81094	TTAAATGAGGGG GTTAAAGG	
LMP2A	Forward	166561-166580	ATGGGGTCCCTA GAAATGGT	1494
	Reverse	1679-1660	TATACAGTGTTGC GATATGG	

[a] Genome co-ordinates are given with reference to the B95.8 genomic sequence (Baer *et al*., 1984).

plasmids, containing the respective EBV genes under the control of the galactose-inducible *GAL1* promoter.

Enzyme-linked Immunosorbent Assay (ELISA)

The serum IgG and IgA against EBV-ZEBRA, EA-D and LMP2A recombinant proteins were determined by ELISA. Individual wells of 96-well microtiter plates (MaxiSorp Nunc, Denmark) were coated with 1µg/ml of purified recombinant EBV proteins. Non-specific binding was blocked with phosphate-buffered saline (PBS) containing 5% milk diluent (KPL Inc., USA) for two hours at room temperature. Plates were incubated with diluted sera (200× dilutions for IgG assays; 100× dilutions for IgA assays) for one hour at the room temperature, followed by five washes with PBS/Tween-20 (0.05%). Bound serum antibodies were incubated with alkaline phosphatase (AP)-conjugated goat anti-human IgG or IgA (5000× dilutions) for two hours at the room temperature, followed by five washes with PBS/Tween-20 (0.05%) and detected by BluePhos® microwell phosphatase substrate reagent (KPL Inc., USA). Negative control wells, comprising of blocking buffer, were included in every test. The cut-off values were calculated as the mean optical density (OD) at 630 nm of the negative samples, plus two standard deviations (SD) for each test. The sensitivity of the test was defined as the percentage of NPC individual detected positive, and specificity was defined as the percentage of the negative scored among healthy individuals.

Indirect Immunofluorescence Assay (IFA)

All sera were titrated for IgA antibodies to VCA by the in-house IFA assay. The expression of the VCA in P3HR1 cell line was induced with 20 ng/ml 12-*O*-tetradecanoyl phorbol 13-acetate (TPA, Sigma) and 3 mM sodium butyrate (NBA, Merck). These induced P3HR1 cells were fixed on the Teflon-coated multi-wells slides in cold acetone-methanol (1:1) and air dried. The fixed cells in each well were then incubated with a serially diluted serum samples and later, incubated with the fluorescein-conjugated goat anti-human IgA which allowed indirect detection of VCA-specific antibody, using a UV microscope. A sample was considered IgA-VCA positive when the titre was ≥ 1:10.

Statistical Analysis

Data analyses were performed using the statistical software SPSS version 11.5 (SPSS Inc., USA). In addition, student's *t*-test was also used to compare the mean OD_{630} values of the anti-EBV recombinant proteins between the NPC patients and the healthy controls. A p value of < 0.05 was considered as statistically significant.

RESULTS

In this study, three EBV antigens from the different phases of the virus infection cycle (i.e. LMP2A in the latent phase, ZEBRA in the immediate early phase of active replication, and EA-D in the lytic phase) were cloned from B95.8 RNA (*Fig. 1*) and transformed into yeast *Saccharomyces cerevisiae* strain INV*Sc*1 for expression. The optimum yields of the recombinant EBV proteins in *S. cerevisiae* strain INV*Sc*1 were obtained 24-hour post induction with 2% galactose. Purified recombinant EBV proteins were coated onto 96-well microtiter plates, at 1µg/ml for the detection of specific serum IgG and IgA.

One hundred and twenty four NPC sera and 124 age, gender and ethnic-matched healthy individuals were also tested. Significant differences (p value ranged from < 0.0001 and 0.005 were shown between the NPC patients and the healthy controls for all the three recombinant EBV proteins in this study (Table 2). All the tests were found to be significant at 0.0001 level, except for the ZEBRA/IgG with p value being 0.005.

ZEBRA/IgA was the most sensitive single test, which correctly predicted 90.3% of the NPC cases, followed by LMP2A/IgG (77.4%) and EA-D/IgG (73.4%). For specificity, the ZEBRA/IgA, EA-D/IgG and EA-D/IgA were able to exclude 96.0% of the non-NPC cases (Table 3).

Higher sensitivities were achieved using the combination of ZEBRA/IgA and EA-D/IgG (93.6%), as well as the combination of ZEBRA/IgA and LMP2A/IgG (95.2%). The combination of EA-D/IgG and LMP2A/IgG showed a sensitivity of 86.3%, which was higher than the sensitivities shown by either of the individual tests involved: EA-D/IgG (73.4%) and LMP2A/IgG (77.4%). The specificity was generally higher by combining two ELISA: 96.8% for EA-D/IgG and LMP2A/IgG; 99.2% for ZEBRA/IgA and EA-D/IgG, as well as for ZEBRA/IgA and LMP2A/IgG, as compared to the best specificity achieved by individual ELISA test, which was 96.0%.

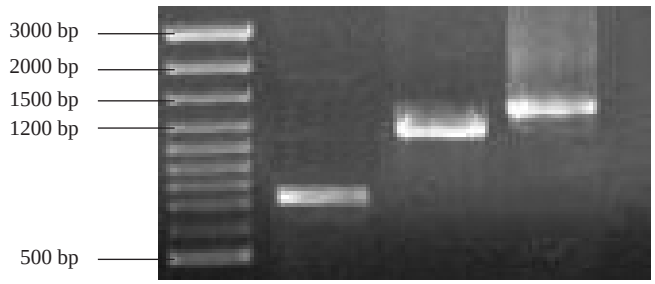

Fig. 1: Three EBV genes amplified by RT-PCR (1% agarose stained with ethidium bromide) that were used to clone into yeast pYES2.1/V5-His-TOPO® vectors

Lane 1: Gene-Ruler™ 100bp DNA Ladder Plus
2: ZEBRA transcript (738 bp)
3: EA-D transcript (1215 bp)
4: LMP2A transcript (1494 bp)

TABLE 2
Comparison of the serum IgG and IgA to the EBV recombinant proteins, between the NPC patients and healthy controls

Reactivity of Recombinant EBV protein	Mean value of OD_{630} ± standard deviation		Student's *t*-test (*p* value)	Significance Level
	NPC Patients	Healthy Controls		
ZEBRA/IgG	1.232±0.587	0.407±0.246	0.00239	< 0.005*
ZEBRA/IgA	0.586±0.376	0.147±0.071	0.00000	< 0.0001*
EA-D/IgG	1.398±0.607	0.392±0.250	0.00000	< 0.0001*
EA-D/IgA	0.524±0.407	0.126±0.080	0.00000	< 0.0001*
LMP2A/IgG	1.186±0.492	0.364±0.202	0.00000	< 0.0001*
LMP2A/IgA	0.465±0.338	0.133±0.086	0.00008	< 0.0001*

* Statistically significant.

TABLE 3
Sensitivities and specificities of the recombinant EBV proteins

Reactivity of Recombinant EBV protein	Sensitivity	Specificity
Single test		
ZEBRA/IgG	66.9	94.4
ZEBRA/IgA	90.3	96.0
EA-D/IgG	73.4	96.0
EA-D/IgA	72.6	96.0
LMP2A/IgG	77.4	95.2
LMP2A/IgA	66.9	93.6
Combination of two tests		
ZEBRA/IgA and EA-D/IgG	93.6	99.2
ZEBRA/IgA and LMP2A/IgG	95.2	99.2
EA-D/IgG and LMP2A/IgG	86.3	96.8

All the sera tested by ELISA were also tested for VCA/IgA by the in-house IFA. Among the 124 NPC sera used in this study, 100 (80.6%) had elevated VCA/IgA determined by the in-house IFA (titers ≥ 1:10), but 24 (19.4%) had undetectable levels of VCA/IgA by IFA (titers < 1:10). All healthy controls (*n* =124) were found to be negative for VCA/IgA by IFA (< 1:10).

Thus, a higher sensitivity (95.2%) was achieved using the combination of ELISA of ZEBRA/IgA and LMP2A/IgG; furthermore, the combination of ELISA ZEBRA/IgA and LMP2A/IgG could distinguish the 24 NPC sera which were not detected to have elevated IgA/VCA by the IFA.

DISCUSSION

In this study, the potential use of three recombinant proteins of EBV antigens, expressed in the different phases of virus infection (i.e. LMP2A in the latent phase, ZEBRA in the immediate early phase of active replication, and EA-D in the lytic phase) to detect the NPC cases, were determined. It was found that the serum IgA to ZEBRA is the best marker in the serodiagnosis of the NPC, with the sensitivity of 90.3% and specificity of 96.0%. Other studies reported by Yoshizaki *et al*. (2000) had also shown that the antibodies to ZEBRA could significantly be increased ($p < 0.05$) in the newly diagnosed NPC patients. Thus, ZEBRA should be included in the panel of the EBV proteins for the serodiagnosis of the NPC.

A much better performance in the serodiagnosis of NPC could be achieved using the combination of two ELISA tests, as shown in Table 3. The best performance was achieved by the combination of ZEBRA/IgA and LMP2A/IgG ELISA, with a sensitivity of 95.2% and a specificity of 99.2%, respectively. The combined ELISA was found to achieve better sensitivity and specificity. The studies from the other groups such as Dardari *et al*. (2001) and Chan *et al*. (2003) also reported that no single test of the EBV antibody was sufficient to detect every patient with the NPC.

The performance of anti-LMP2A was average in the single ELISA test, but it may help to detect the NPC individuals which are not recognized by either anti-ZEBRA or EA-D recombinant EBV proteins. A recent work by Chen *et al*. (2005) found three sera negative for VCA/IgA were positive for LMP2A/IgA in NPC patients, suggesting that the LMP2A specific antibodies might have the potential significance for the diagnosis of the NPC. The above findings demonstrate that the antibody re-activities among the NPC patients are diverse and that the use of combinations of EBV proteins will lead to a wider coverage of immunodominant epitopes recognised by different subset of antibodies.

The detection of serum antibodies to EBV VCA in the sera of NPC patients is traditionally done by IFA. Although the IFA of IgA against VCA has been the mainstay of the EBV serodiagnosis of NPC, there remain 15% to 20% of the NPC patients who have undetectable levels of IgA antibody to VCA (Sam *et al.*, 1989). In the present study, 24 of the 124 NPC patients (19.4%) did not exhibit elevated levels of serum IgA to VCA determined by IFA. The combination of ZEBRA/IgA and LMP2A/IgG ELISA was found to be able to distinguish the 24 NPC patients.

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New Records of Terrestrial Pteridophytes in Genting Highlands, Pahang, Malaysia

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ABSTRACT

Genting Highlands is the common name for a general area including a group of summits around Gunung Ulu Kali and their ridges. Located at the border between the State of Selangor to the west and Pahang to the east, the entire area was a virgin forest until 1967, when the roadwork was begun. To date, there have been fragmented reports in botanical studies in Genting Highlands. The previous works by Ridley, Holttum, Parris, Latiff and Piggott are lacking on studies, specially the ones focusing on pteridophytes in selected study sites. Fieldworks were conducted to assess the species list of terrestrial pteridophyes on April and September of 2005. From these expeditions, a total of 32 species of terrestrial pteridophytes were collected from two sites in Genting Highlands with 21 species being new records for the area. Two species namely, *Diplazium subintegrum* and *Taenitis dimorpha,* were found to be endemic to Peninsular Malaysia.

Keywords: Endemic, fern, fern-allies, Genting Highlands, pteridophytes

INTRODUCTION

Genting Highlands is a common name for the general area, including a group of summits around Gunung Ulu Kali and their ridges (Chua and Saw, 2001). It is located at the southernmost high mountain in the main range in Peninsular Malaysia and is situated only 30 km (in a direct line) from Kuala Lumpur (Stone, 1981). The range is also the border between the State of Selangor to the west and Pahang to the east. The summit of Genting Highlands is formed by Gunung Ulu Kali and it is the site of a hotel complex. Genting Highlands is located in Pahang, with ca. 3,596,400 ha in size, and only 1,523,252.41 ha or 42 percent of the land designated as the total permanent forest reserve (Table 1).

The vegetation and flora of the summit region of Genting Highlands consists of two vegetation types, the upper montane forest and elfin forest, with the transition between them (Stone, 1981). Meanwhile, the mountainous backbone is largely composed of granite (Whitmore and Burnham, 1969), the soil in the upper montane is peaty gley podzols with thin iron pan, and spread with a blanket of peat near the summit (Whitmore and Burnham, 1969), which is always associated with acidic peat with pH 3.3-3.6 (Burgess, 1969).

The scientific study of pteridophytes in Malaysia dated back since the early 1900s. There were many botanists collecting and enumerating pteridophytes in Malaysia including Ridley, Holttum, Stone, Piggot, Bidin and Latiff. Ridley

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TABLE 1
Permanent forest reserves of Pahang, Malaysia

Forest district	Area (ha) in 31st December 2003
Kuantan/ Pekan/ Maran	240,942.68
Rompin	195,381.39
Temerloh/ Bera	154,539.00
Jerantut	387,917.00
Lipis	257,273.15
Bentong	99,112.24
Raub/ Cameron Highlands	188,086.95
Total	1,523,252.41

Source: Pahang Department of Forestry (http://forestry.pahang.gov.my/web/info_status.htm.)

(1926) in his book *Ferns of Malaya* enumerated 420 species of terrestrial pteridophytes in Peninsular Malaysia. Later in 1954, Holttum reported that there were 480 species of ferns, while Turner (1995) listed 616 species of ferns and 63 species of fern-allies in Peninsular Malaysia. The latest and most comprehensive species list of pteridophytes in Malaysia was reported by Parris and Latiff (1997), which was published in *The Malayan Nature Journal*. They brought together the information compiled from herbarium specimens and numerous publications including the references cited above. As a result, a provisional list of 1,165 Malaysian pteridophytes species has been compiled, 647 of which occur in Peninsular Malaysia, 750 in Sabah and 615 in Sarawak.

Considering a substantial gap between 1997 and the present, the composition of pteridophytes might have increased or perhaps decreased in time. Thus, the latest assessment is much needed. To date, there are fragmented reports in botanical studies on biodiversities in Genting Highlands. There are records of species done by Ridley, Holttum, Parris, Latiff and Piggott, but their works covered wide areas and do not specifically focus on pteridophytes. Therefore, there is no species list for pteridophytes in the study sites. With all the above inadequacy on the botanical information and documentation, this study was hence aimed to: (1) study the diversity of terrestrial pteridophytes in the four selected trails in Genting Highlands and (2) prepare a preliminary checklist of the terrestrial pteridophytes for the selected trails.

METHODOLOGY

Collection was done twice; on 24-25 April 2005 and 17 September 2005. The first collection covered two trails along a waterfall and a river near Genting View Resort (cited later as site A). The second trip covered two trails (*Fig. 1*) in an uphill jungle track and Gunung Bunga Buah old road from Goh Tong Jaya (cited later as site B), as shown in *Fig. 1*. Only specimens, which were structurally complete with rhizome, stalk, frond and sori, were collected. The plant specimens were cut using a pair of scateurs, put into plastic bags and secured with plastic rope before transferring them to the laboratory.

The specimens collected were cleaned immediately by draining them in tap water to remove all the dirt and dust. The methodology employed next for preservation, drying, pressing and mounting of specimen was described by Jain and Rao (1977). All the specimens placed on mounting paper were labelled and deposited in the herbarium of Department of Biology, Faculty of Science, Universiti Putra Malaysia (UPM).

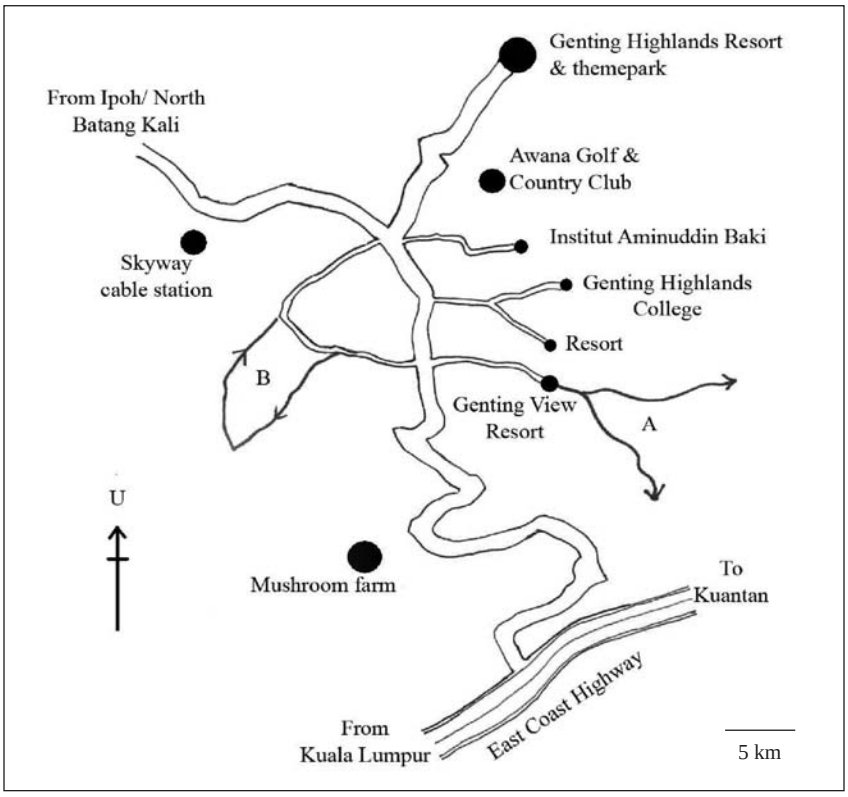

Fig. 1: Map of Genting Highlands, Pahang, Malaysia, showing the study sites A and B

RESULTS AND DISCUSSION

Thirty-two species of terrestrial pteridophytes from twelve families and twenty three genera were collected from two sites in Genting Highlands (Table 2). Out of this number, 21 species (65.6%) were identified as the new records for Genting Highlands. Among the 12 families, nine were true fern families namely *Adiantaceae, Dennstaedtiaceae, Dryopteridaceae, Gleicheniaceae, Marattiaceae, Nephrolepidaceae, Pteridaceae, Thelypteridaceae, Woodsiaceae,* three others were fern-allies namely *Equisetaceae, Lycopodiaceae* and *Selaginellaceae.*

The list of species found in Genting Highlands during this research showed relatively higher differences in the number between true ferns species (26) and fern-allies species (6). The family *Thelypteridaceae* has the most number of genera (5 spp.). Out of 32 species of terrestrial pteridophytes collected, two were endemic species to Peninsular Malaysia, namely *Taenitis dimorpha* and *Diplazium subintegrum.*

Some literature reviewed in this paper was taken from the studies conducted at Gunung Ulu Kali, considering the obvious flora accounts that are substantially the same. The new records of the terrestrial pteridophytess represent a large portion of the findings (65.6%). This new record was found by comparing the species list during this study and the species list by Piggott (1977) in Gunung Ulu Kali. These new species were probably introduced to the study site vegetation by the development during the course of time. Many new infrastructures and facilities were built around the study sites to enhance the structure of the soil around, such as roadside verges, drains, granite walls, and many road cuttings and small

TABLE 2
List of the pteridophytes species collected in trails 1 and 2

Family	Species
Adiantaceae	*Pityrogramma calomelanos* (L.) Link[b]
	Taenitis dimorpha Holtt. *, **, [b]
Dennstaedtiace	*Histiopteris stipulacea* (Hook.) Copel. [b]
	Lindsaya parasitica (Roxb. ex Griff.) Hieron. *, [a]
	Odontosoria chinensis (L.) J. Sm. var. *divaricata* (Christ.) Kramer[a, b]
	Tapeinidium pinnatum (Cav.) C. Chr. var. *pinnatum**, [b]
Dryopteridaceae	*Didymochlaena truncatula* (Sw.) J. Sm. *, [a]
	Pleocnemia irregularis (C. Presl) Holtt. *, [a]
	Tectaria crenata Cav. *, [a]
Equisetaceae	*Equisetum ramosissimum* Desf. ssp. *debile* (Roxb.) Hauke[b]
Gleicheniaceae	*Dicranopteris linearis* (Burm.) var. *linearis*[a]
	Dicranopteris linearis (Burm.) var. *montana*[a, b]
	Sticherus truncatus (Willd.) Nakai var. *truncatus*[a]
Lycopodiaceae	*Lycopodiella cernua* (L) Pic. Serm. [b]
Marattiaceae	*Angiopteris evecta* (G. Forst.) Hoffm. *, [a, b]
Oleandraceae	*Nephrolepis auriculata* (L.) Trimen*, [a]
	Nephrolepis dicksonioides Christ. *, [b]
Pteridaceae	*Pteris longipinulla* Wall ex Agardh[a]
	Pteris tripartita Sw. [a]
Selaginellaceae	*Selaginella plana* (Desv.) Hieron[a]
	Selaginella ornata (Hook. & Grev.) Spring[a]
	Selaginella stipulata (Blume) Spring[a]
	Selaginella wildernowii (Desv.) Bak.[b]
Thelypteridaceae	*Christella parasitica* (L.) Lev. *, [a]
	Mesophlebium motleyanum (Hook.) Holtt. *, [b]
	Pronephrium rubicundum (v.A.v.R) Holtt. *, [a, b]
	Sphaerostephanos penniger (Hook.) Holtt. *, [a, b]
	Trigonospora ciliata (Benth.) Holtt. *, [a]
Wooodsiaceae	*Diplazium accedens* Bl.[a]
	Diplazium cordifolium Bl. *, [a]
	Diplazium subintegrum Holtt.**, [b]
	Diplazium tomentosum Bl.[b]

* New record (in comparison with Piggott, 1977; Piggott, 1981)
** Endemic to Peninsular Malaysia
a = Species collected from site A (Genting View Resort)
b = Species collected from site B (Gunung Bunga Buah)

clearings were made (Piggott, 1981). These new constructions provide a new habitat to certain plant growth. For example *Nephrolepis dicksonioides* was not listed by Piggott (1977), but was found to occupy a small clearing along the road verges in Goh Tong Town.

A comparison between the availability of the pteridophytess species in this study was made to previous accounts by Piggott (1977; 1981) and Turner (1995). According to Piggott (1977; 1981), there were 128 species of pteridophytes in Gunung Ulu Kali. Sixty-three species were

Fig. 2: Several endemic and new records of the pteridophytes species collected from Genting Highlands. Photos by Joannus Unggang and the authors. I.* Didymochlaena truncatula. *II.* Sticherus truncatus. *III.* Diplazium subintegrum* *IV.* Equisetum ramosissimum. *V.* Odontosoria chinensis. *VI.* Taenitis dimorpha*

terrestrial pteridophytes, while 65 species were either epiphytic or litophytic. From 63 species of terrestrial pteridophytes, a total of 12 (19%) species were found during this study, while 51 other species (80.5%) were not found from the study sites. Thirty-one species of the terrestrial pteridophytes were found when compared to the species list prepared by Turner (1995). This might be because the area covered for Turner's list was wider (covering the whole state of Pahang) than the area assessed by Piggott (12 locations in the forest fragments on Gunung Ulu Kali). Certain names of species listed by Turner were new and changed from the old and out-dated names used by Holttum (1968) or Piggott (1988).

CONCLUSION

The notable finding of this study was the comparatively high percentage of the new records collected as compared to the past 25 years (65.6%). Considering the small areas assessed, this number is high, implying a drastic change in both the vegetation and flora of the area. For the record, the study sites only covered two small areas in Genting Highlands, i.e. around ca. 16-18 km^2. Thus, there were possibilities that many more species could be recorded if the area explored was expanded. Therefore, further detailed studies must be carried out to cover the vast and deeper area of the forest so as to provide a more complete and comprehensive species list. From the authors' observations, much of Genting Highlands area has been affected by human activities and no longer retains its full original biodiversity. The result of this study revealed that a big proportion (80.5%) of the pteridophytes recorded previously by Piggott was no longer found. This number implies how certain species may disappear from their habitat in the name of development. Development in rainforests has a global impact through species extinction, habitat destruction, the loss of important ecosystem services and renewable resources, as well as the introduction of alien

species and pathogens. Beyond the responsible development of the rainforests, efforts to rehabilitate and restore degraded forest lands, along with the establishment of protected areas, are key to securing rainforests for the long-term benefits they can provide to mankind.

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Antibacterial Activity of Methanolic Crude Extracts from Selected Plant Against *Bacillus cereus*

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ABSTRACT

Bacillus cereus is a well-known food-poisoning bacterium. In this study, six methanolic crude extracts, from *Azadirachta indica*, *Choromolaena odorata*, *Justicia gendarussa*, *Mangifera odorata*, *Strobilanthes crispus* and *Tinospora crispa*, were investigated for their antibacterial activities against *B. cereus*. For this purpose, different concentrations of the methanol solvent crude extract from selected plants were used (1, 2, 4, 6, 8, 10, 15, and 20 mg/ml) and the diameter of *B. cereus* growth inhibition zone was measured at every 24 hours for 5 days. The antibacterial assay for all the crude extracts showed the inhibition of *B. cereus* growth by concentrations ranging from 2 mg/ml to 20 mg/ml, with a significant correlation between the extract concentrations and degrees of antibacterial activity. Rapid formation of inhibition zones within 24 hours of incubation was obtained, before a slight reduction in the inhibition of the diameter of zone was observed after 120 hours of incubation. The Minimal Inhibition Concentration (MIC) value for *J. gendarussa*, *M. odorata* and *S. crispus* crude extracts were at 2 mg/ml, while *A. indica*, *C. odorata* and *T. crispa* were at 6 mg/ml, 8mg/ml and 10 mg/ml, respectively. However, the Minimal Bactericidal Concentration (MBC) for all the crude extracts were at much higher concentration with the crude extract of *J. gendarussa*, *M. odorata* and *S. crispus* obtained the MBC values at 6mg/ml, whereas *A. indica*, *C. odorata* and *T. crispa* were at 10 mg/ml.

Keywords: Growth inhibition, diffusion assay, methanolic extract, Minimal Inhibition Concentration, Minimal Bactericidal Concentration

ABBREVIATIONS

ANOVA	:	one-way analysis of variance
mg/ml	:	milligram per milliliters
SEM	:	standard error of mean

INTRODUCTION

An interest in the study of medicinal plants, as a source of pharmacologically active compounds, has increased worldwide. Disadvantages of the modern medicines such as its high cost, unavailability in remote areas and the emergence of multidrug-resistant pathogens have become the driving force behind the pursuit of the 'green' medicines (Bandow *et al*., 2003). In addition, multiple resistances in human pathogenic micro-organisms have developed due to the indiscriminate use of commercial antimicrobial

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drugs commonly employed in the treatment of infectious diseases (Zampini *et al*., 2005). The undesirable side-effects of certain antibiotics and the emergence of the previously uncommon infections have forced scientists into looking for new antimicrobial substances from various sources like medicinal plants. Latif (1997) reported that approximately 2000 medicinal plant species from Malaysia have the potential pharmaceutical value and most of them have been widely used as herbal medicines. Generally, the whole plant or its parts such as leaves, roots, flowers or fruits were used to obtain the extract for the preparation of medicinal remedies.

Over the years, medicinal plants have been exploited in traditional medicine for various treatments due to their anti-microbial properties (Kelmanson *et al*., 2000; Srinivasan *et al*., 2000). A number of compounds have been isolated from plants, and their chemical structures were fully elucidated and many of them were tested for possible biological activities (Crombie *et al*., 1990). Edeoga *et al*. (2005) stated that the most important bioactive compounds of plants are alkaloids, flavanoids, tannins and phenolic compounds. Moreover, a study by Saadabi *et al.* (2006) revealed that alkaloid, flavanoids, tannins, sterols as well as triterpenes were present in the plants which exhibited a remarkable antibacterial activity against some pathogenic bacteria.

Among other, *Bacillus cereus* is identified as one of food-borne bacteria causing food-borne diseases with known aetiology (Gasaluk *et al*., 1996; Pan *et al*., 1997). However, the number of cases is likely to be underestimated as a consequence of the short duration and relative mildness of the illness (Granum, 1997). *Bacillus cereus* causes two distinct types of food poisoning, characterized either by diarrhoea and abdominal pain, or by nausea and vomiting (Jensen *et al*., 2003). More importantly, their spores are resistant to many of the heat-treatments used in the food industry such as pasteurisation, and some of the spores are able to germinate and grow at food storage temperatures (Andersson *et al*., 1995). Carlin *et al*. (2000) found that the spore forming and psychrotrophic properties of *Bacillus* species are the main deteriorating factors which would spoil food products and shorten their shelf life.

Even though certain plants have been demonstrated for their effects against pathogenic bacteria, a number of them have not been investigated for their antibacterial activities. In this report, the screening of the antibacterial activities of methanolic crude extracts from six selected plants against common food borne pathogenic bacterium, *Bacillus cereus* is described. Six selected medicinal plants are *Strobilanthes crispus, Azadirachta indica, Justicia gendarussa, Tinospora crispa, Chromolaena odorata* and *Mangifera odorata.* The effectiveness of each plant extract has been determined in the form of the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) values.

MATERIALS AND METHODS

Plant Material and the Preparation of Plant Extracts

The leaves of *A. indica, C. odorata, J. gendarussa, M. odorata, S. crispus* and *T. crispa* were collected from the Agricultural Park, Universiti Putra Malaysia (UPM), in Serdang, Malaysia. All the leaves were picked 30 cm from the aerial part, and wrapped in newspaper to maintain their freshness. The surface sterilization was carried out by washing all the samples with water, followed by 10% (v/v) sodium hypochlorite solution, before they were rinsed with sterile distilled water and air dried at room temperature. Dried samples were then ground into powder using a grinder before they were sieved and fine powder of plant materials was therefore collected.

The preparation of the methanolic crude extract was carried out according to the method proposed by Chandrasekaran and Venkatesalu (2003). Twenty grams of dried samples of each plant were soaked in 50 ml of 80% (w/v) methanol for 24 hours at 30°C, and then filtered using Whatman No.1 filter paper. Finally, the filtrate was evaporated using a rotary evaporator at 60˚C until it was fully dried.

The Preparation of Test Extract Solutions

The preparation of the methanolic crude extract was carried out as suggested by Chandrasekaran and Venkatesalu (2004). A series of different methanolic crude extract concentrations (1mg/ml, 2 mg/ml, 4 mg/ml, 6mg/ml, 8mg/ml, 10mg/ml, 15mg/ml and 20 mg/ml) were prepared by dissolving a known weight of the plant crude extract in 5% (v/v) dimethyl sulphoxide (DMSO), which acted as a solvent.

The Preparation of the Bacteria Culture

Bacillus cereus ATCC 10876 was obtained from the Plant Systematic and Microbial Laboratory, Biology Department, Universiti Putra Malaysia, and maintained on the Nutrient Agar slant. The preparation of the test strain culture was carried out by isolating a single colony of *B. cereus* from the nutrient agar and transferred into 250ml Nutrient Broth, before it was shaken at 180rpm for 24 hours.

Agar Well Diffusion Assay

Nutrient agar was seeded with 1×10^5 cell/ml of *B. cereus* before a series of 0.6cm in diameters wells were made using sterile cork borer. Twenty µL of the methanolic crude extract solutions, in various concentrations, were then transferred into each well and allowed to set. All the plates were incubated at 37°C and the diameters of the clear zone, surrounding each well were measured as the inhibition zone to the nearest millimetre for every 24 hours interval up to 120h. Sets of 5 replicates were used for each type of the extract.

Determination of the Minimum Inhibitory Concentration (MIC)

The same series of the methanolic crude extract, as previously mentioned in the agar well diffusion, were used for the MIC determination. Twenty microlites of methanolic crude extract was mixed into 80µl sterile Nutrient Broth before 1×10^5 cells/ml *B. cereus* culture was added into the mixture. The mixture was then pipetted into the microtiter plate and incubated for 24h at 37˚C. The lowest methanolic crude extract concentration that did not show any growth of *B. cereus* was considered as the MIC value.

The Determination of the Minimum Bactericidal Concentration (MBC)

Ten µL of the test mixture in the microtiter well which did not show any growth of *B. cereus* in MIC determination was sub-cultured onto fresh Nutrient Agar plates before further incubation was carried out at 37˚C for 24 hours. The least concentration, with no *B. cereus* growth, was considered as being the MBC value.

Statistical Analysis

The results obtained were expressed as mean ± SEM. Significant differences among the treatment groups were tested using the one-way analysis of variance (ANOVA), and the comparison of the mean values was made using the Tukey test at 5% significance level. All the statistical analyses were performed using the software program SPSS.

RESULTS AND DISCUSSION

The methanolic crude extracts of the 6 medicinal plants were tested for their antibacterial activities against *Bacillus cereus* using agar well diffusion method. The overall diameter of inhibition for all the methanolic extract concentrations, for all the plant species against *B. cereus*, are shown in Table 1. 80% (v/v) methanol and 5% (v/v) of DMSO were also tested as a control experiment and no activity for these control solutions was observed (data not shown). However, all the methanolic crude extracts from different plant species showed some antibacterial activities towards the growth of *B. cereus*, indicating that the antibacterial activity observed was due to the activity of the bioactive compound present in the plant crude extract and not caused by the solvent used in the extract preparation. The bioactive compound activities in all the methanolic crude extracts seemed to show a similar trend towards the growth of *B. cereus*.

TABLE 1
Antibacterial activity of selected methanolic extracts against *B. cereus*

Extract	Concentration (mg/ml)	Diameter of inhibition zone (cm ± SD)*						MIC**	MBC***
		Incubation Period (h)							
		0	24	48	72	96	120		
Strobilanthes crisaus	Control	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	1	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	2	0.60±0.00^{a}	0.75±0.01^{b}	0.72±0.02^{b}	0.73±0.01^{b}	0.70±0.02^{b}	0.70±0.01^{b}	+	√
	4	0.60±0.00^{a}	0.84±0.01^{c}	0.80±0.01^{c}	0.81±0.01^{c}	0.80±0.02^{c}	0.82±0.01^{c}	+	√
	6	0.60±0.00^{a}	0.90±0.02^{d}	0.89±0.02^{d}	0.90±0.01^{d}	0.88±0.01^{d}	0.90±0.01^{d}	+	X
	8	0.60±0.00^{a}	0.95±0.01^{e}	0.96±0.01^{e}	0.97±0.01^{e}	0.98±0.02^{e}	0.99±0.01^{e}	+	X
	10	0.60±0.00^{a}	0.97±0.01^{e}	1.01±0.01^{f}	1.01±0.01^{f}	1.00±0.01^{ef}	1.04±0.01^{f}	+	X
	15	0.60±0.00^{a}	1.00±0.01^{f}	1.03±0.01^{f}	1.03±0.01^{f}	1.02±0.01^{f}	1.04±0.01^{f}	+	X
	20	0.60±0.00^{a}	1.08±0.02^{g}	1.07±0.02^{g}	1.07±0.02^{g}	1.07±0.02	1.09±0.01^{g}	+	X
Justicica gendarusa	Control	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	1	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	2	0.60±0.00^{a}	0.75±0.01^{b}	0.73±0.01^{b}	0.71±0.01^{b}	0.72±0.01^{b}	0.72±0.02^{b}	+	√
	4	0.60±0.00^{a}	0.80±0.01^{c}	0.79±0.01^{c}	0.77±0.02^{c}	0.76±0.02^{c}	0.76±0.02^{bc}	+	√
	6	0.60±0.00^{a}	0.83±0.02^{cd}	0.82±0.02^{cd}	0.79±0.02^{cd}	0.80±0.01^{d}	0.79±0.01^{cd}	+	X
	8	0.60±0.00^{a}	0.86±0.01^{de}	0.84±0.01^{de}	0.83±0.02^{de}	0.84±0.02^{e}	0.83±0.02^{de}	+	X
	10	0.60±0.00^{a}	0.90±0.02^{ef}	0.88±0.01^{e}	0.86±0.01^{e}	0.87±0.02^{ef}	0.86±0.02^{ef}	+	X
	15	0.60±0.00^{a}	0.92±0.02^{g}	0.93±0.02^{f}	0.91±0.01^{f}	0.89±0.02^{f}	0.89±0.02^{g}	+	X
	20	0.60±0.00^{a}	1.03±0.03^{h}	1.02±0.03^{g}	1.00±0.02^{g}	1.00±0.01^{g}	0.98±0.03^{h}	+	X
Mangifera odorata	Control	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	1	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	2	0.60±0.00^{a}	0.73±0.01^{b}	0.73±0.01^{b}	0.73±0.01^{b}	0.72±0.01^{b}	0.74±0.01^{b}	+	√
	4	0.60±0.00^{a}	0.81±0.01^{c}	0.81±0.01^{c}	0.80±0.01^{c}	0.80±0.01^{c}	0.80±0.01^{c}	+	√
	6	0.60±0.00^{a}	0.84±0.01^{c}	0.85±0.01^{d}	0.83±0.01^{d}	0.82±0.01^{c}	0.83±0.01^{d}	+	X
	8	0.60±0.00^{a}	0.87±0.02^{d}	0.88±0.01^{e}	0.88±0.01^{e}	0.88±0.01^{d}	0.88±0.01^{e}	+	X
	10	0.60±0.00^{a}	0.89±0.01^{d}	0.89±0.01^{ef}	0.88±0.01^{e}	0.88±0.01^{d}	0.88±0.01^{e}	+	X
	15	0.60±0.00^{a}	0.92±0.01^{e}	0.92±0.01^{f}	0.91±0.01^{f}	0.90±0.01^{d}	0.91±0.01^{f}	+	X
	20	0.60±0.00^{a}	0.98±0.01^{f}	0.97±0.10^{g}	0.95±0.01^{g}	0.95±0.01^{e}	0.95±0.01^{g}	+	X

Table 1 (*continued*)

Azadirachta indica	Control	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	1	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	2	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	4	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	6	0.60±0.00^{a}	0.68±0.01^{b}	0.68±0.02^{b}	0.69±0.02^{b}	0.69±0.03^{b}	0.69±0.02^{b}	+	√
	8	0.60±0.00^{a}	0.72±0.01^{bc}	0.71±0.01^{bc}	0.72±0.01^{bc}	0.71±0.01^{bc}	0.72±0.01^{bc}	+	√
	10	0.60±0.00^{a}	0.74±0.03^{c}	0.74±0.02^{c}	0.75±0.02^{c}	0.75±0.02^{c}	0.76±0.03^{c}	+	X
	15	0.60±0.00^{a}	0.80±0.04^{d}	0.81±0.01^{d}	0.81±0.02^{d}	0.81±0.02^{d}	0.81±0.03^{d}	+	X
	20	0.60±0.00^{a}	0.90±0.02^{e}	0.90±0.02^{e}	0.91±0.04^{e}	0.91±0.02^{e}	0.91±0.02^{e}	+	X
Chromalaena odorata	Control	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	1	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	2	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	4	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	6	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	8	0.60±0.00^{a}	0.69±0.02^{b}	0.67±0.02^{b}	0.67±0.02^{b}	0.67±0.02^{b}	0.67±0.02^{b}	+	√
	10	0.60±0.00^{a}	0.72±0.01^{c}	0.72±0.01^{c}	0.72±0.01^{c}	0.07±0.01^{c}	0.72±0.01^{c}	+	X
	15	0.60±0.00^{a}	0.78±0.01^{d}	0.79±0.04^{d}	0.77±0.01^{d}	0.77±0.01^{d}	0.77±0.01^{d}	+	X
	20	0.60±0.00^{a}	0.95±0.01^{e}	0.87±0.01^{e}	0.86±0.01^{e}	0.85±0.01^{e}	0.85±0.01^{e}	+	X
Tinosaora crisaa	Control	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	1	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	2	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	4	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	6	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	8	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	0.60±0.00^{a}	-	√
	10	0.60±0.00^{a}	0.71±0.01^{b}	0.70±0.01^{b}	0.71±0.01^{b}	0.07±0.01^{b}	0.71±0.01^{b}	+	X
	15	0.60±0.00^{a}	0.75±0.02^{c}	0.74±0.01^{c}	0.74±0.01^{c}	0.74±0.01^{c}	0.74±0.01^{c}	+	X
	20	0.60±0.00^{a}	0.85±0.02^{d}	0.80±0.03^{d}	0.81±0.02^{d}	0.81±0.02^{d}	0.82±0.02^{d}	+	X

Means in each column with the same superscript letter are not significantly different amongst themselves when the Tukey tests were used at 5% significance level.

* Diameter of the inhibition zone was included 0.6cm of the well diameter and expressed as the mean ± SD; (N = 5).

** Minimum Inhibition Concentration: (-) no inhibition zone observed; (+) inhibition zone observed.

*** Minimum Bactericidal Concentration: √: Presence of bacteria growth; X: No bacteria growth observed.

After 24 hours of incubation, the growth of *B. cereus* was drastically inhibited by all the crude extracts for all the concentrations. However, after 48 hours, a slight reduction on the inhibition activity against *B. cereus* was observed, before it was steadily maintained until 120 hours. After 24 hours, drastic increases in the diameter of the inhibition zones might directly be caused by immature inocula being less resistant to antibacterial activities. However, after 48 hour of growth, antibacterial activities were diminished; this was probably due to the enzyme denaturation and the bacteria becoming much more dominant (Beukinga *et al.*, 2004).

To evaluate the effectiveness of the concentration of the methanolic crude extracts on the growth of *B. cereus*, the MIC test was conducted using a series of methanolic crude extract concentrations. The MIC values for all the plant crude extracts are shown in Table 1. At a concentration of 2mg/ml, the methanolic crude extracts of *J. gendarussa*, *S. crispus* and *M. odorata* started to show inhibition on the growth of *B. cereus*, with average inhibition rates of 6.18µm/h, 5.83µm/h and 4.63µm/h respectively for *S. crispus, J. gendarussa* and *M. odorata*. All the plant methanolic crude extracts showed the highest mean of bacterial growth inhibition diameter at the concentration of 20mg/ml, with the *S. crispus* methanolic extract as the best inhibitor against *B. cereus* growth, at the rate of 29.51µm/h. In addition, there were strong correlations between the extract concentrations and the diameters of the inhibition zones at this stage, with the correlation coefficients ranging from 0.843 to 0.930 (data not shown). A similar result was also derived by Boer *et al.* (2004) and Sawangjoroen *et al.* (2004). A high antibacterial activity of *S. crispus* leaf extract against the growth of *B. cereus* was expected since several chemical compounds, such as polyphenols, catechins, caffeine, alkaloids, tannins, β-sitosterol and stigmasterol (which could be obtained from the *S. crispus* leaf extract) have a significant antibacterial activity against some pathogenic bacteria (Maznah *et al*., 2000; Endrini, 2003; Abdah *et al*., 2004). *Justicia* species was known as medicinal herbs in the South East Asia region and has a huge potential benefit for diverse biological activities such as lowering the risk of cardiovascular diseases (Lucas *et al.*, 2004) and cancer risk (Marchand, 2002). Although *J. gendarussa* is lignan-free (Lorenza *et al*., 1999), their methanolic crude extracts were still found to be able to inhibit *B. cereus* growth at lower concentrations, due to the presence of other compounds such as β- sitosterol, 2-amino benzyl alcohol and 2-(2'amino-benzylamino) benzyl alcohol (Chakravarty *et al*., 1982).

Azadirachta indica, *C. odorata* and *T. crispa* methanolic extracts were only able to show inhibition on the bacterial growth at higher concentration. The methanolic crude extract of *A. indica* started to inhibit bacterial growth at 6 mg/ml, while this was at 8 mg/ml and 10 mg/ml respectively for *C. odorata* and *T. crispa*. A similar result of the moderate antibacterial activity for these three plant extracts was also reported previously. The alcoholic crude extracts of *A. indica* and *C. odorata* were found to be ineffective as antibacterial; this might be due to the low extractable of phenolic (Apori *et al*., 2000). However, a significant effect was found as larvicidal extract against *Plasmodium falciparum* (Hout *et al*., 2006) and the larvae of *Aedes aegypti* and *Anopheles stephensi* (Kiran *et al*., 2005). On the other hand, the methanolis crude extract of *T. crispa* was not effective in inhibiting the growth of *B. cereus* with only 6.16µm/h, as compared to the control at the concentrations of 10mg/ml. This corresponds with the report by Zakaria *et al.* (2005).

In spite of the MIC results, at 6mg/ml, the values of the MBC were much higher for all the plant crude extracts of *J. gendarussa*, *S. crispus* and *M. odorata* extracts. Meanwhile for *C. odorata*, *T. crispa* and *A. indica* extract, the bactericidal effect can only be observed at 10mg/ml. This means that at the concentrations between the MIC and MBC values, the methanolic crude extracts could only act as bacteriostatic agents rather than as bactericidal for *B. cereus*, because at this concentration, the bioactive compound was unable to eliminate *B. cereus* or sustain the activity for a long period, and thus allowing the

bacteria to grow. Two possible explanations for this bacteriostatic effect are: (i) the bioactive compound in the extract was not adequate to cause a significant mortality to the bacteria (Basri and Fan, 2005); and (ii) the sensitivity of the bioactive compound towards a certain type of solvent might cause or enhance the rate of deactivation or degradation (Mutu and Staden, 2003).

In general, the plant extracts are much more active against Gram-positive bacteria than against Gram-negative bacteria (Lin *et al.*, 1999; Cimanga *et al.*, 2002), and this was demonstrated by the positive effects of several plants extracts on the other Gram-positive bacteria such as *S. epidermidis*, *Bacillus subtilis* (Fatima *et al.*, 2001) and *Pseudomonas aeruginosa* (Nimri *et al.*, 1999). The fact that *B. cereus* was categorized as a Gram-positive bacteria has some contribution towards the effectiveness of the plant methanolic crude extract as a bacteriostatic or bactericidal agent. The density of the lipopolysaccharide layer in the outer surface of bacterial cell wall is much lower in the Gram-positive bacteria as compared to the Gram-negative bacteria (Burn, 1988). Without this layer, certain antibacterial compounds can easily reach the peptidoglycan layer of the bacterial cell wall and penetrate into the cytoplasm to cause the loose of cell's turgor pressure, with a subsequent disorganization of the internal organelle (Clements *et al*., 2002). This might explain the sensitivity of *B. cereus* towards the plant extracts.

In conclusion, this study demonstrated the ability of all the methanolic crude extracts to act as a bactericidal agent. Among the six methanolic crude extracts tested, *Strobilanthes crispus* was found to be the most active, i.e. by showing the largest mean of diameter inhibition zones at the concentration of 20mg/ml. However, this is much dependent on the concentration applied, the types of plant extract and the extraction process. The potential of these plants, particularly the extracts of *S. crispus, J. gendarussa* and *M. odorata*, in pharmacology as an antibacterial agent is enormous. However, further biochemical analysis is still required to prove this.

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Somatic Embryogenesis from Scutellar Embryo of *Oryza sativa* L. var. MR219

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ABSTRACT

Somatic embryogenesis is an efficient plant regeneration system and it is a potentially useful tool for genetic transformation. An experiment was carried out on somatic embryogenesis from scutellar embryo of rice var. MR219. High intensity of callus formation (100%) was initiated through culturing the scutellar embryo on modified MS medium, with the macro nutrients reduced to half-strength and supplemented with different 2,4-D concentrations (1, 2, 4 and 6 mgL^{-1}). Meanwhile, the highest percentage of embryogenic callus formation (80%) was obtained on the modified MS medium containing 4 mgL^{-1} 2, 4-D. The calli produced were yellowish and friable with nodular structures on the surface. Rounded cells with highly dense cytoplasm were also observed under an inverted microscope and their viability was confirmed based on the apple green fluorescence staining in the fluorescein diacetate (FDA) solution. High mean number of somatic embryos was also produced in this treatment, at 85 somatic embryos per explant. Upon transferring the somatic embryos onto the modified MS medium with 2 mgL^{-1} BAP and 0.05 mgL^{-1} NAA for germination, 82.5% of the somatic embryos were germinated into the seedlings.

Keywords: **Rice (*Oryza sativa* L.) var. MR219, 2,4-Dichlorophenoxyacetic acid, α-Naphthalenacetic acid, 6-Benzylaminopurine**

INTRODUCTION

Rice is the most important food crop in developing countries. In fact, it is the basic food for nearly half of the world's population, which is mostly concentrated in Asia. The production of rice has surged over the past 30 years, driven in the beginning by the doubling of yields and the expansion of the cultivated areas. Irrigated rice, which accounts for more than 75% of the global rice production, has been responsible for most of this production increase (IRRI, 1993).

Although rice production has so far kept up with the population growth, new studies suggest that an additional increase of 50 – 70% of the current supply is needed to meet the demand until 2025 (Pinggali *et al*., 1997). As the land resources are shrinking, the present trends

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suggest that tomorrow's rice land will be under even greater pressure (Greenland, 1997).

Tissue culture of rice, which has been developed in the last six decades, was started by Fujiwara and Ojima (1955), and Amemiya *et al.* (1956), by culturing the excised root and immature embryo on nutrient medium. High concentration of strong synthetic auxin, such as 2-4-Dichlorophenoxy acetic acid (2,4-D), has overcome the limitation of rice tissue culture by culturing the undifferentiated and meristematic tissue or the organ tissue explants at defined stage of development in nutrient medium using organogenesis as a pathway for plant regeneration (Ammirato, 1983). Furthermore, rice plant regeneration has been achieved from embryogenic calli derived from immature embryos (Heyser *et al*., 1983), mature seeds (Harke and Lorz, 1989), scutellum (Gupta *et al*., 1989), coleoptiles (Oinam *et al.*, 1995), and microspore (Datta *et al*., 1990). However, according to Bajaj and Rajan (1995), the establishment of the regeneration system of *indica* rice varieties requires a longer period as compared to *japonica* rice.

The MR219 variety is an indica rice hybrid which was produced by the Malaysian Agricultural Research and Development Institute (MARDI). The rice is very good in terms of its quality (shape and taste) and also high in yield. However, this variety is sensitive to environmental changes. In Malaysia, one of the major problems which limit high production of rice is water deficiency. Moreover, several other abiotic stress factors have also been found to affect the cultivation of rice; these include excess soil salinity (Zhu *et al.*, 2000) and temperature stress which affect all the stages of growth and development (Perales *et al.*, 2008).

Conventionally, rice plant can be genetically improved through crossing. However, it takes a long period of at least 2 years for a new variety to be released through such approach. Tissue culture technique, coupled with genetic transformation, can therefore be an alternative approach for improving the rice crop.

This study describes the establishment of somatic embryogenesis, from scutellar embryo of *Oryza sativa* L. var. MR219, which is a potential tool in genetic transformation of rice for high yield and quality improvement.

MATERIALS AND METHODS

Explant Materials and Surface Sterilization Procedure

Explant materials for this research were rice seeds variety MR219, obtained from the Rice and Industrial Research Centre, Malaysia Agriculture Research Development Institute (MARDI) Seberang Perai, Malaysia. Rice caryopses, containing scutellar region of embryo, were isolated by removing lemma and palea from the seeds. The caryopses were sterilized using 70% alcohol for 1 minute, followed by shaking in 40% Clorox containing a drop of Tween-20 on an orbital shaker, at 120 rpm for 40 minutes. Finally, the explants were rinsed with sterile distilled water for 5 times and cultured onto the medium with the different treatments tested in the study.

Basic Media and Treatments

Two basic media used in this study were modified N6 medium supplemented with 500 mgL^{-1} (w/v) L-glutamine and modified MS (Murashige and Skoog, 1962) medium with the macro nutrients reduced to half-strength and supplemented with 500 mgL^{-1} (w/v) of glutamine, 100 mgL^{-1} (w/v) of proline. Both the media were solidified with 0.2% (w/v) phytagel agar. The pH of the media was adjusted to 5.8. Different concentrations of 2,4-D [0, 1, 2, 4, and 6 mgL^{-1} (w/v)] were used as the treatments for embryogenic callus induction. These cultures were then kept at 25 $\pm 2^{\circ}C$ in the growth room (incubation room) in a dark condition for one week and followed by transferring the cultures under 16 hours lighting, provided by fluorescent bulbs with 15.75 $\mu molm^{-2}s^{-1}$ light intensity until the eighth week of culture. Meanwhile, the MS medium containing different concentrations of BAP (0, 0.5, 1, 2 and 4 mgL^{-1}), in combination with different concentrations of NAA (0, 0.01, 0.05 and 0.1 mgL^{-1}) were used as treatments for the

germination of somatic embryos. The cultures were kept at 25 ± 2˚C in the growth culture, with 16 hours of light, provided by fluorescent bulbs and a light intensity of 15.75 $\mu molm^{-2}s^{-1}$ for eight weeks.

Experimental Design and Statistical Analysis

The experiments were arranged in a Completely Randomized Design (CRD) as a single factor experiment, with four replications and each replication per treatment contained ten explants. The gathered data were analyzed using the analysis of variance (ANOVA), while the Duncan New Multiple Range Test (DNMRT), at $\alpha = 5\%$, was employed to carry out a comparison between the treatment means.

Data Recorded

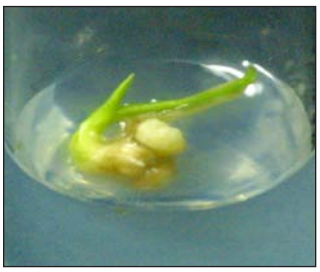
Data recorded in the somatic embryogenesis include the percentage of explant which responded to form callus (%), determination of callus viability based on fluorescein diacetate (FDA) method, the percentage of explant which responded to form embryogenic callus (%), and the mean number of somatic embryos produced per explant. Data were collected every two weeks until the eighth week of culture, while the growth characteristics were observed every week. The parameters on the somatic embryo germination recorded were the percentage of somatic embryo germination (%), the percentage of the normal plant (%) and the percentage of abnormal plant (%), produced after the eighth week of culture.

Assessment of Viability of Callus

The fluorescein diacetate (FDA) method was used to assess the viability of the callus cells. Stock solution of FDA, at a concentration of 0.5%, was prepared in acetone and stored at 0˚C. Firstly, the scutellum derived callus produced was transferred to liquid MS medium containing 1 mgL^{-1} 2,4-D and the FDA solution was added to the cell suspension at a final concentration of 0.01 %. The mixture was incubated for 5 minutes, and the cells were finally illuminated with UV light and visualized under an inverted microscope. The viable cells gave green fluorescence.

RESULTS AND DISCUSSION

Based on the observation, the callus started to grow from scutellar embryo of rice, after three days of culture (Plate 1A). The scutellar embryo derived callus subsequently started to enlarge and some yellowish to greenish nodules grew around the explants after ten days of culture (Plate 1B). After six weeks of culture, calli almost covered the explants surface (Plate 3A). Nodular structure and globular somatic embryo-



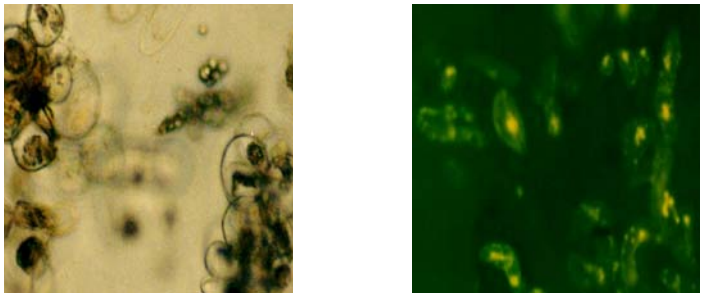
(A)

(B)

Plate 1: The developmental stages of embryogenic callus formation from scutellar embryo of rice (Oryza sativa L.) *var. MR219. Callus formation from the scutellar embryo of rice after three days of culture (A), and the scutellar embryo derived callus started to enlarge and some yellowish to greenish nodules grew at explants, after around ten days of culture (Bar=1cm)*

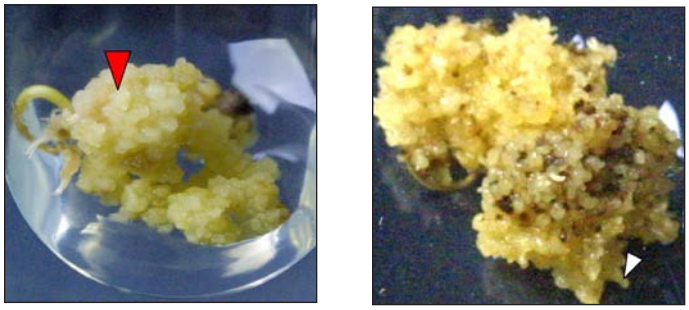
like structures were formed from the calli (Plate 3B). The somatic embryos were then transferred onto the MS medium containing different concentrations of BAP, in combination with different concentrations of NAA for germination (Plate 4A). After three weeks, the somatic embryos were germinated into seedlings (Plate 4B) and grew into a complete plant after sixth week of culture (Plate 4C).

Fig. 1 shows the effect of different media (MS and N6) containing different 2,4-D concentrations on the percentage of explant forming callus after the eighth week of culture. The presence of different 2,4-D concentrations (2, 4, 6, 8 mgL^{-1}) in the media tested gave a significant response for 100% callus formation; meanwhile the absence of 2,4-D (control) inside the media did not produce any callus (*Fig. 1*). The result indicated that 2,4-D was the most suitable to stimulate the formation of callus. However, no significant difference was observed between the 2,4-D treatments. Bonga and Aderkas (1992) stated that in large amount, phenoxy auxin (2,4-D) is a strong promoter of callus formation, while Matsuta and Hirabayashi (1989) stated that suitable concentration of 2,4-D would promote somatic embryogenesis. Although all the 2,4-D concentrations tested produced



(A) (B)

Plate 2: The assessment of callus viability. Rounded cells with highly dense cytoplasm confirmed the embryogenicity of the callus (A) and viable cells with dense cytoplasm fluoresced apple green when assessed using the FDA solution (B)



(A) (B)

Plate 3: The developmental stages of somatic embryo formation, whereby calli with nodule structures (red arrow) almost covered the explants surface after six weeks of culture (A) and nodular structure and globular somatic embryos (white arrow) formed from the callus (Bar= 1cm)

(A) (B) (C)

Plate 4: Stages of somatic embryos germination. Somatic embryos on MS medium containing different concentrations of 2 mgL^{-1} BAP in combination with the different concentrations of 0.05 mgL^{-1} NAA for germination (A) Somatic embryos germinating into seedlings after the third week on germination medium (B), and complete plants regenerated, after the eighth week of culture (C), (Bar=1cm)

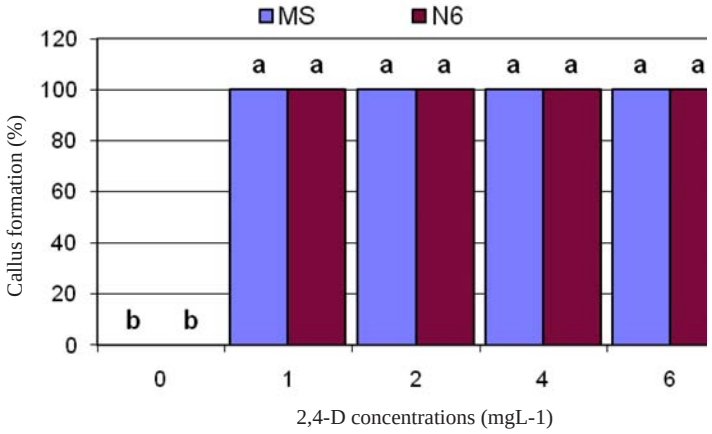


Fig. 1: The effect of the different media (MS and N6) containing different 2,4-D concentrations on the percentage of callus formation after eight weeks of culture

100% formation of callus, the cell viability test performed on the callus showed that not all callus produced were embryogenic. *Fig. 2* illustrates the effect of the different media (MS and N6) containing different concentrations of 2,4-D on the percentage of scutellar embryo of rice explants, producing embryogenic callus after the eighth week of culture. The highest percentage of embryogenic callus formation (80%) from scutellar embryo was observed on the modified MS medium containing 4 mgL^{-1}. Based on the data presented in *Fig. 2*, the growth trend on the percentage of explants produced embryogenic callus showed a quadratic pattern, whereby the addition of 2,4-D up to 4 mgL^{-1} in modified MS and N6 medium showed an increment on the percentage of the scutellar embryo of rice explant producing embryogenic callus. Nevertheless, when the 2,4-D concentration was increased to more than 4 mgL^{-1}, it resulted in a decrement on the percentage of explants forming embryogenic callus. The results indicated that the optimum concentration of 2,4-D was 4 mgL^{-1}. It is important to highlight that the embryogenicity

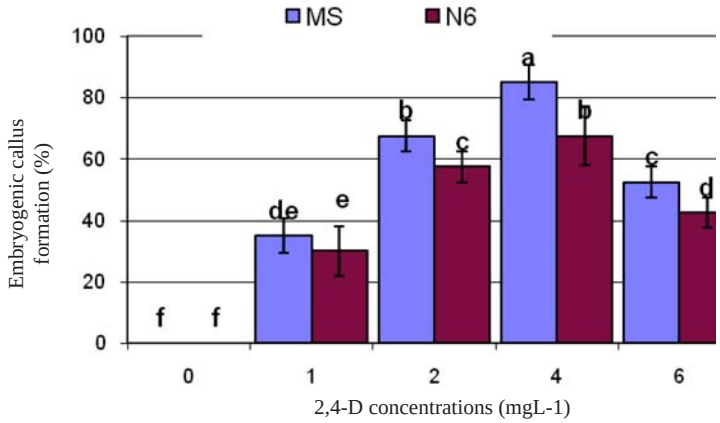


Fig. 2: The effect of the different media (MS and N6) containing different 2,4-D concentrations on the percentage of embryogenic callus formation, after eight weeks of culture

of callus produced is important for plant regeneration. Matsumoto (2003) reported that the selection and differentiation of embyogenic calli is necessary to get efficient regeneration. The cells of the callus were rounded with a highly dense cytoplasm, confirming the embryogenicity of the callus produced (Plate 2A) and furthermore, the cell viability of the callus was confirmed with the fluorescein diacetate (FDA) staining, visualized under an inverted microscope, illuminated with UV luminescence. The viable cells, with a dense cytoplasm and a healthy nucleus fluoresced apple green when assessed using the FDA solution (Plate 2B).

Fig. 3 depicts the effect of the different media (MS and N6) containing different concentrations of 2,4-D on the mean number of somatic embryos formed per explant. The highest mean number of somatic embryos formation (85) was found on the modified MS medium containing 4 mgL^{-1} 2,4-D. Based on *Figs. 2* and *3*, in comparison to N6 medium, the modified MS medium

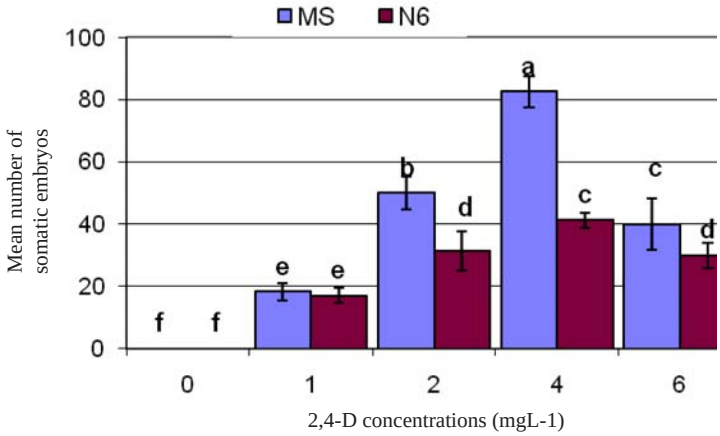


Fig. 3: The effect of the different media (MS and N6) containing different 2,4-D concentrations on the mean number of somatic embryos formed per explant after eight weeks of culture

combined with all the 2,4-D concentrations tested show a higher percentage of embryogenic callus formation from the scutellum embryo of rice explant and the highest mean number of somatic embryos formed per explant. This indicates that the MS medium, which is rich with nutrients is more suitable for the tissue culture of rice, as compared to the N6 medium (Table 2). Minocha (1987) reported that the presence of 2 mgL^{-1} 2,4-D, in the culture medium (MS) of *Pinus radiata* cotyledon explants, caused the formation of callus, while Sane *et al*. (2000) used the MS medium containing 2,4-D at 9.05µM for the induction of somatic embryo on *Acacia tortilis*. Ronald *et al.* (2005) made use of 1mgL^{-1} of 2,4-D for the somatic embryogenesis on slash pine (*Pinus ellotii* Engelm.) and Liu *et al*. (2001) reported that the presence of 4 mgL^{-1} 2,4-D, in the culture medium of rice seeds, caused the formation of embryogenic callus.

Table 1 shows the effect of the different BAP concentrations, in combination with the different concentrations of NAA, on the percentage germination of somatic embryo as well as the percentage of the normal and abnormal plants produced after the eighth week of culture. The normal plant was characterized by the normal growth with bipolar structure, which was indicated by containing shoot and root structures. Meanwhile, the abnormal plant was characterized by the stunted growth. In this study, the highest percentage of somatic embryos

TABLE 1
Effect of different BAP concentrations in combination with different concentrations of NAA on percentage of somatic embryo germination, percentage of normal plant and percentage of abnormal plant produced after the eighth week of culture

Plant Growth Regulators		Somatic embryo Germination (%)	Normal Plant (%)	Abnormal Plant (%)
BAP (mgL^{-1})	NAA (mgL^{-1})			
0	0	20.00 e	18.00 cd	2.00 f
0.5	0	22.50 de	15.50 cd	7.50 ef
1	0	25.00 cde	16.38 cd	8.62 ef
2	0	32.50 c	17.75 cd	14.75 bcd
4	0	23.75 cde	12.63 d	11.12 de
0.5	0.01	25.00 cde	16.75 cd	8.25 ef
1	0.01	27.50 cd	18.75 cd	8.75 ef
2	0.01	60.00 b	39.25 b	20.75 a
4	0.01	32.50 c	20.25 c	12.25 cde
0.5	0.05	21.25 de	12.63 d	8.62 ef
1	0.05	55.00 b	37.00 b	18.00 ab
2	0.05	82.50 a	66.00 a	16.50 abc
4	0.05	30.00 cd	18.50 cd	11.50 cde
0.5	0.1	25.00 cde	13.25 d	11.75 cde
1	0.1	60.00 b	39.00 b	21.00 a
2	0.1	60.00 b	41.75 b	18.25 ab
4	0.1	27.50 cde	13.00 d	14.50 bcd
CV (%)		15.90	9.30	12.30

Means followed by the same letter(s) in the same column are not significantly different using the Duncan New Multiple Range Test (DNMRT) at p=0.05

TABLE 2
The composition of the modified Murashige and Skoog (MS, 1962) and the modified N6 media

Elements	MS (mgL^{-1})	N6 (mgL^{-1})
Macro elements		
Calcium Chloride $CaCl_2$	332.02	166.00
Potassium Dihydrogen Phosphate KH_2PO_4	170.00	400.00
Potassium Nitrate KNO_3	1900.00	2830.00
Magnesium Sulfate $MgSO_4$	180.00	180.00
Ammonium Nitrate NH_4NO_3	1650.00	-
Diammonium sulphate $(NH_4)_2 NO_3$		463.00
Micro elements		
Cobalt Chloride $CoCl_2\ 6H_2O$	0.025	-
Cuprum Sulfate $CuSO_4\ 5H_2O$	0.025	-
Boric Acid H_3BO_3	6.20	1.60
Potassium Iodide KI	0.83	0.80
Manganese Sulfate $MnSO_4\ 4H_2O$	16.90	4.40
Sodium Molybdate $Na_2MoO_4\ 2H_2O$	0.25	-
Zinc Sulfate $ZnSO_4\ 7H_2O$	8.60	1.50
Vitamins		
Glycine $C_2H_5NO_2$	2.00	2.00
Nicotinic Acid $C_6H_5NO_2$	0.50	0.50
Pyridoxine $C_8H_{11}NO_3$	0.50	0.50
Thiamine $C_{12}H_{17}CIN_4O_5$	0.10	1.00
Iron		
Disodium ethylenediaminetetraacetic acid Na_2EDTA	37.25	37.25
Ferrous Sulfate $FeSO_4\ 7H_2O$	27.85	27.85
Others		
Myo-inositol	100	100
Sucrose	30,000	30,000
L-glutamine	500	500
L-proline	-	100
Pytagel agar	2000	2000

germinated into the plant (82.5%) was observed, with 2 mgL^{-1} BAP, in combination with 0.05 mgL^{-1} NAA, and this treatment was also found to produce the highest percentage of the normal plant (66%). Yang *et al*. (1996) stated that the combination of auxin (NAA) and cytokinin (BAP) could enhance the percentage of somatic embryo germination in papaya. On the contrary, Craig *et al.* (1997) obtained somatic embryos germination in *Moricandia arvensis* when the somatic embryos from an auxin medium were transferred into an auxin-free medium.

Based on the data presented in Table 1, the MS medium without both BAP and NAA (MS0) could also result in somatic embryos germinating into seedlings (20%) although with the low percentage of somatic embryos germinating into plant, and 18% of the normal plants were obtained from this treatment. These results indicated that the hormone-free MS medium could be used for the recovery of plants from somatic embryos. Nevertheless, the presence of BAP and NAA was most suitable for somatic embryo germination in rice MR219. Based on the findings of this study, the treatment containing 2 mgL^{-1} BAP combined, with 0.05 NAA, could be chosen as the ideal germination medium for this particular variety.

CONCLUSIONS

Callus was successfully induced on the modified MS and N6 media, containing 2,4-D from scutellar region of rice caryopses embryo var. MR. 219. The study found that eighty percent of embryogenic callus and 85 somatic embryos were produced from the scutellar region on the modified MS medium containing 4 mgL^{-1} 2,4-D. Meanwhile, the MS medium containing 2 mgL^{-1} BAP, in combination with 0.05 mgL^{-1} NAA, resulted in 82.5% of the somatic embryo germination into seedlings. The somatic embryogenesis from the scutellum embryo of rice, developed in this study, could therefore serve as a potential tool with an important application in genetic transformation and production of quality planting materials.

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Influence of Flooding Intensity and Duration on Rice Growth and Yield

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ABSTRACT

An experiment was conducted in the glasshouse of the Faculty of Agriculture, Universiti Putra Malaysia (UPM) in 2005 to evaluate the effect of different flooding treatments on rice growth and yield. Five flooding treatments were used, namely T1 = continuously flooded condition until maturity, T2 = early flooding until 55 DAS (day after sowing) followed by saturated condition until maturity, T3 = early flooding until 30 DAS followed by saturated condition until maturity, T4 = continuous saturated condition until maturity, T5 = continuous field capacity condition throughout the experiment period. The results showed that the response of rice plant to water soil availability varies with its growing stage. At an early stage of rice plant growth (15 and 30 DAS), flooding treatments were found to not affect the growth of rice plant significantly. However, from 45 DAS onwards, the effect was significantly pronounced. All flooding regimes (T1, T2 and T3) significantly favoured rice plant height and the number of tillers as compared to non-flooded regimes (T4 and T5). The positive correlation was observed between the grain yield and yield components. The significant higher number of tillers, high spikelets/ panicle and high 1000-grain weight had contributed to higher grain yield of rice in T1, T2 and T3 as compared to T4 and T5. Shorter duration of flooding (T2 and T3) was found to give a similar performance to continuous flooding, and thus, these methods might save on water use without reducing yields, while over watering might just increase vegetative growth.

Keywords: *Oryza sativa* L., minimal water condition, water regime treatments, glasshouse condition

INTRODUCTION

Rice, *Oryza sativa* L., is one of the most important cereal crops in the world (Wangda *et al.*, 2003). It feeds well in excess of more than 2 billion people in Asia and many in Latin America, providing on average of about 32% of the total calorie uptake (Mclean *et al*., 2002). The lowland rice agriculture is now responsible for 86% of the total world rice crop and the yields are typically in the range of 2.0 – 3.5 t ha^{-1} (Ladha *et al.*, 1997). In Malaysia, rice is the third most important crop, after rubber and oil palm. Rice is mainly grown in the eight granaries in Peninsular Malaysia, covering an area of about 205,548 ha (MOA, 2008).

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However, yield stagnation or even decline has been observed in some rice growing areas of Asia since the early 1980s (Cassman and Pingali, 1994). One of the major factors is the crisis of fresh water. The per capita availability of water resources was found to decline by 40 – 60% in many Asian countries between 1955 and 1990 (Gleick, 1993). Agriculture's share of water will decline at an even faster rate because of the increasing competition from the urban and industrial sectors (Tuong and Bhuiyan, 1994). According to the United Nation's World Food Programme (WFP), the biggest threat to Asia in the future will be the shortage of clean water; this is particularly in Asia as it accounts for 60% of the world's population, but with only 36% of the global freshwater (Sariam, 2004). According to FAO (2000), Malaysia was categorized in Zone 3 in terms of water scarcity in the 20th Century; with the need to increase water management between 25 – 100 % to meet the 2005's water requirement, rice cultivation would be badly affected by this phenomenon. Water is the single most important component for sustainable rice production, especially in the traditional growing areas. Reduction or large withdrawal of water from the field can significantly lower the sustainability of rice production (Belder *et al.*, 2008; Farooq *et al.*, 2006). However, despite the constraints of water scarcity, rice production must rise dramatically to meet the world's food needs. Producing more rice with less water is therefore a formidable challenge for the food, economic, social and water security.

A series of alternate water management in lowland rice have been studied lately, aiming to keep the field not continuously submerged in order to save the water use in rice farming (Farooq *et al.*, 2006). In addition, water inputs can be reduced by introducing periods of non-submerged conditions of several days throughout the growing season unless cracks are formed through the plough sole (Bouman and Toung, 2001). In China, the systems of alternate flooding and drying have been reported to maintain or even increase rice yields and these have widely been adopted by farmers (Belder *et al.*, 2008). Previously, Bhuiyan (1982) reported that the rice plants did not suffer from water stress if the soil was saturated and there was no standing water in the field. Similarly, Tabbal *et al*. (1992) also observed the insignificant difference in the yield between rice grown in flooded condition, alternate flooded conditions and saturated condition. In general, rice plant only uses less than 5% of the water observed through roots from soil (Farooq *et al.*, 2006).

Efforts were made in the past to save water by either reducing the depth of water on the soil surface (Bhuiyan and Palanisami, 1987) or by keeping the root zone saturated without a water head (Ghani and Rana, 1992). Tabbal *et al*. (1992) observed no significant yield difference between rice grown in standing water and those grown under saturated field conditions in the 1988-1989 dry seasons; however, yields under saturated soils were lower in the 1990-1991 dry seasons because of more weed growth, as compared to the previous dry seasons. Therefore, there is a need for a thorough investigation for the changes in rice growth and yield brought out by different water conditions. Thus, a study was undertaken to determine the response of rice plant growth and yield under different water regimes under glasshouse condition.

MATERIALS AND METHODS

The experiment was carried out under a controlled environment in the glasshouse of the Faculty of Agriculture, Universiti Putra Malaysia (UPM), Selangor. The glasshouse had 13:11 h day:night photoperiod and a 21 – 36˚C temperature range, with no artificial lighting. The average day temperature and light intensity inside the glasshouse were recorded at 1-hour intervals (*Fig. 1*).

Ten kilograms of air-dried sandy clay loam soil of Sogomana Series was taken from the Bertam Rice Research Station experimental field. The Sogomana Series is a member of the family of *fine, mixed, isohyperthermic palid Tipik Tuajelkuts* (Paramananthan, 2000). They were developed over sub-recent riverine alluvium, and characterized by light grey to white clays, showing strong to moderate

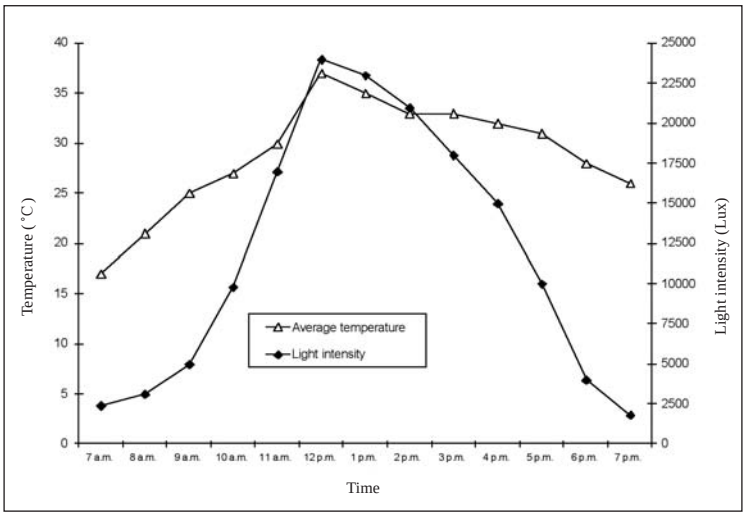

Fig. 1: The average daily temperature and light intensity in the glasshouse

prismatic to coarse angular blocky structures and sticky consistence. The properties of the soil are shown in Table 1. The soils were packed into 20 clay pots. Each pot measured about 32 cm in diameter by 40 cm deep. Pre-germinated rice seeds were sown onto the soil surface at a rate of 500 seeds m^{-2} (approximately 40 seeds per pot). The experiment was begun in May 2005, and completed in September 2005.

The treatments comprised of five flooding treatments, namely T1 = continuously flooded condition (10 cm water level) until maturity, T2 = early flooding (10 cm water level) up to panicle initiation stage (55 DAS – day after

TABLE 1
Physico-chemical properties of Sogomana soil series in MARDI Bertam experimental field

Particle size distribution (%)	
Sand	51.5
Silt	12.0
Clay	36.5
Textural class: Sandy clay loam	
Chemical properties	
pH (1:2.5 in distilled water)	4.49
Cation exchange capacity (CEC), $cmol_c\ kg^{-1}$	7.00
Organic C, %	0.52
Total N, %	0.13
Available P, mg kg^{-1}	10.38
Exchangeable K, $cmol_c\ kg^{-1}$	0.21
Exchangeable Mg, $cmol_c\ kg^{-1}$	0.52
Exchangeable Ca, $cmol_c\ kg^{-1}$	3.60
Available Cu, mg kg^{-1}	0.14
Available Mn, mg kg^{-1}	3.38
Available Fe, mg kg^{-1}	43.30
Available Zn, mg kg^{-1}	1.01

sowing), followed by saturated condition until maturity, T3 = early flooding (10 cm water level) for the first month (30 DAS) followed by saturated condition until maturity, T4 = continuous saturated condition until maturity, T5 = continuous field capacity condition throughout the experiment. However, the soil which was maintained under saturated condition during sowing and flooding treatment were only commenced at 7 DAS. For T4 condition, water was only introduced into the soil until saturated (maintain the soil to muddy condition without standing water) to the maximum of 5 mm standing water condition, depending on the temperature inside the glasshouse at that time. Meanwhile, for T5 condition, a water deficit indicator called Tensiometer was placed inside the pots (each pot contained one Tensiometer). The irrigation of T5 was done when the soil water potential fell between -30 to -50 Centibar, as measured by the Tensiometer.

All crop management practices (i.e. fertigation, pest control, weed control) were done according to MARDI's Cultivation Manual (MARDI 2002). However, all the applications were measured and converted according to glasshouse condition and pot size. Water was drained out from all the flooded and saturated pots at 95 DAS and maintained under the field capacity condition until harvesting. The pots were placed close together and another row of extra pots were placed around the entire perimeter to minimize border effects. A Randomized Complete Block Design (RCBD), with 4 replications, was used in this experiment.

The plant height was measured using a measuring tape from the plant base to the tip of the highest leaf. The average of six readings was taken randomly from each experimental unit. The parameter of the plant height was taken at 15, 30, 45, 60, 75 and 90 DAS. Meanwhile, the number of tillers per plant was recorded as soon as tillering was started (when seedlings have 5 leaves) and ended at the panicle initiation stage when the flag leaf emerged. The number of tillers per plant was taken at 15, 30, 45, 60, 75 and 90 DAS. The days to flowering were recorded when the first flower emerged while the days to grain maturity were recorded when the grain colour turned yellow and the leaves started to senesce. The number of panicles, per square meter, was recorded in each pot of the rice plants and converted into number m^{-2}. The number of spikelet per panicle was recorded for fully-filled grains, half-filled and non-filled spikelets. The rice plants were harvested manually using a sickle at 10 cm above the ground. 1000 filled grains weight, rice straw biomass and rice yield per pot of all treatments were converted into ton ha^{-1} at 14% moisture content. The effects of the different water regime treatments on the rice growth and yields were analyzed using the Analysis of Variance (ANOVA). The statistical analysis was done using the SAS statistical software and the means were tested using the Tukey's studentized range test, at 5% level of probability.

RESULTS AND DISCUSSION

Rice Plant Height

Table 2 shows the height of the rice plant at the different growth stages under different water flooding treatments. At 15 and 30 DAS, there was no significant difference observed. The differences were only recorded at the beginning of 45 DAS. The height of the rice plant increased with time in all the flooding treatments until the time of harvest. Generally, the rice plant which was exposed under T4 (continuous saturated) and T5 (continuous field capacity) conditions were significantly shorter than the rice plant which received continuous flooding (T1), T2 (flooded until 55 DAS followed by saturated) and T3 (flooded until 30 DAS followed by saturated) conditions. Therefore, the height of the rice plant was significantly affected by flooding treatment at all growing stages, except at 15 and 30 DAS. At 45, 60 and 75 DAS, the lowest height of rice plant was observed in T5, where rice grown under the field capacity condition was approximately 10 – 15% shorter as compared to the rice plant under other flooding treatments. At 90 DAS, however, rice plants in both T4 and T5 pots were significantly lower as compared to the other flooding treatments.

TABLE 2
The height of rice plant (cm) under different flooding treatments at various growing stages

Flooding Treatments	Day After Sowing (DAS)					
	15-ET	30-AT	45-MT	60-PI	75-Mk	90-D/M
T1	34.25ab	51.00a	83.63a	92.48a	111.25a	117.50a
T2	37.00a	51.75a	78.50ab	86.00ab	111.50a	115.00ab
T3	35.75a	52.75a	76.00ab	90.25ab	105.00b	111.25bc
T4	32.25a	50.50a	81.25a	88.00ab	101.67bc	107.75c
T5	33.75a	50.75a	74.00b	82.50b	96.00c	100.75d

*In a column, means followed by the same letter are not significantly different at 5% level by Tukey's Test. DAS = Day after sowing: T1 = continuously flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuous saturated condition: T5 = continuous field capacity condition. Growth stages: ET = early tillering; AT = active tillering; MT = maximum tillering; PI = panicle initiation; Mk = milking; D/M = dough/ maturation stage.

In general, the rice plants grown in T1 were taller than the rice plants grown in other flooding treatments, while reduced water regimes of T4 and T5 restricted rice plant growth. Navarez *et al*. (1979), in the glasshouse experiment in Philippines, also found the same results. However, variable flooding regimes (T2 and T3) also resulted in good performance of the rice plant growth in this study. Meanwhile, the effect of the flooding treatments on the height of the rice plant was not obviously significant in all the pots during the vegetative phase (15 and 30 DAS). This might be due to the few and small rice tillers at the early growing stages, which minimized the competition for available water for growth, even under continuous saturated (T4) and continuous field capacity (T5). IRRI (2008) reported that at vegetative stage, water requirement is the least concern affecting rice growth as compared to weed, nutrition and pest management.

As growth advanced, water requirement increased and reduced water conditions such as T4 and T5 significantly restricted plant height, especially at maturity phases. This is because at reproductive stage, water has become the main factor contributing to the growth and production of rice plant (IRRI, 2008). Beyrouty *et al.* (1992) observed the reduction in the height of plant when flood was delayed. In addition, Anbumozhi *et al.* (1998) also found variable and continuous ponding conditions resulted in better performance in plant height compared to shallow ponding condition. The reduced water condition also enhanced weed emergence and significantly reduced the height of the rice plant (Janiya and Moody, 1991).

Number of Tillers

Table 3 shows the effect of different flooding treatments on the number of rice tillers at different growing stages. The results showed that the flooding treatments did not significantly influence the number of rice tiller in the early growing stage (15 and 30 DAS) in both weeded and unweeded pots. A significant effect was recorded starting only at the beginning of the maximum tillering stage, i.e. 45 DAS onwards. The effect of the flooding treatments on rice plant, during the early tillering stages, was not significantly observed because the tillering process was just about to begin at this stage (Sariam, 2004). The number of tillers reached its maximum potential until 75 DAS and at 90 DAS, and tillering process started to slow down in most of the flooding treatments because the rice plants were found to reach their maturity and only a few small tillers were produced.

The production of tiller at 15 and 30 DAS was not significantly affected by the flooding treatments (Table 3). At 45 and 60 DAS, rice plants grown in T3 (flooded until 30 DAS followed by saturated) produced the highest number of tillers (794 tillers m^{-2} and 878 m^{-2} tillers respectively), while the productions of tiller in T5 (continuous field capacity) were significantly the lowest at 625 tillers m^{-2} and 684 tillers m^{-2}, respectively. Meanwhile, at the reproductive stage (75 and 90 DAS), T2 (flooded until 55 DAS followed by saturated afterward) produced the most tillers (1003 tillers m^{-2} and 972 tillers m^{-2}, respectively) as compared to the other flooding treatments, while T5 produced the lowest number of tillers (769 tillers m^{-2} and 772 tillers m^{-2}, respectively). Jahan (2004) and Sariam (2004) also found that the production of tiller was significantly lower under the field capacity than in the flooded and saturated conditions.

Days to Flowering and Grain Maturity

The variability in the flooding treatment did not significantly affect either the day to flowering or the day to grain maturity in all the pots (Table 4). However, T1 (continuous flooded), T2 (flooded until 55 DAS followed by saturated) and T3 (flooded until 30 DAS followed by saturated) enhanced rice plants to flower earlier than T4 (continuous saturated) and T5 (continuous field capacity). This is because at the flowering stage, water demand is very critical, while low or deficit in water availability will delay and lengthen the time of flowering process (Siti Mardina, 2005; IRRI, 2008). According to Williams *et al*. (1990), earlier heading and flowering might have been a stress reaction where rice grown under submerged conditions showed faster heading and flowering than under shallow and saturated conditions.

Meanwhile, the effect of the different flooding treatments on the days for grain maturity showed the opposite result (Table 4). In more specific, rice planted under T5 condition ripened earlier than the rice grown under other flooding treatments in all the pots. It was then followed by T4, T2 and T3. On the contrary, maturity was delayed in T1 with the longest ripening time. This is because less water is needed in the maturity phase (Siti Mardina, 2005; IRRI, 2008) and delay in draining out the water will cause the rice grain to ripen slower.

TABLE 3
The production of rice tillers (number m^{-2}) under different water regime treatments, at various growth stages

Flooding Treatments	Day After Sowing (DAS)					
	15-ET	30-AT	45-MT	60-PI	75-Mk	90-D/M
T1	500a	575a	700bc	759bc	866ab	922a
T2	500a	578a	750ab	831cb	1003a	972a
T3	500a	625a	794a	878a	928ab	966a
T4	500a	581a	684cd	794ab	897ab	891ab
T5	500a	572a	625d	684c	769b	772b

In a column, means followed by the same letter are not significantly different at 5% level by Tukey's Test. DAS = Day after sowing: T1 = continuous flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuously saturated condition: T5 = continuous field capacity condition. Growth stages: ET = early tillering; AT = active tillering; MT = maximum tillering; PI = panicle initiation; Mk = milking; D/M = dough/ maturation stage.

TABLE 4
The effect of the different flooding treatments on the day to flowering and the day to grain maturity of rice plant

Flooding Treatments	Days to flowering ± SE	Days to grain maturity ± SE
T1	59a ±1.15	96a ±1.70
T2	62a ±1.88	92a ±1.88
T3	61a ±1.50	93a ±1.36
T4	63a ±1.94	92a ±1.08
T5	63a ±1.82	92a ±1.44

In a column, means followed by the same letter are not significantly different at 5% level by Tukey's Test.
T1 = continuously flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuous saturated condition: T5 = continuous field capacity condition. SE = Standard Error.

The Number of Rice Panicles

Fig. 2 indicates the effect of the flooding treatments on the number of rice panicles m^{-2}. Generally, the responses of rice panicle number m^{-2} were significantly affected by the flooding treatments. The highest number of rice panicles was produced under continuous flooded condition (T1), which produced 434 panicles m^{-2}, followed by T2 (426 panicles m^{-2}), T3 (425 panicles m^{-2}) and T4 (398 panicles m^{-2}), which were not significantly different among each other. Meanwhile, T5 was found to significantly produce the lowest rice panicle number (320 panicles m^{-2}) as compared to the other flooding treatments.

The result showed that the production of the rice panicles was significantly influenced by the flooding treatments, which were in line with the research done by Jahan (2004) and Sariam (2004). According to Sariam (2004) and Siti Mardina (2005), the production of panicles was significantly reduced when rice was grown under field capacity. From the results, higher number of panicles m^{-2} in all flooded regimes (T1, T2 and T3) is believed to be due to the high number of tillers in the same flooding treatments, as shown in 3.2 (Table 3), indicating the positive interaction between the results in rice growth stages and the results in rice maturity stages.

The Number of Spikelets Per Panicle

The response of spikelets number per panicle to different flooding treatments was found to be significantly different (*Fig. 3*). The number of spikelets per panicle was observed to decrease with the reduction in water availability. Nevertheless, there were no significant differences observed between T1, T2, T3 and T4, as well as between T3 and T4, as compared to T5. A significant difference was only found between T1 and T2 as compared to T5. In more specific, T1 and T2 produced 123 and 122 spikelets/panicles respectively, while T5 produced the lowest number of spikelets (107 spikelets/ panicle). It is believed that a high number of panicles also contributed to a high number of spikelets. The results indicated that the number of spikelets per panicle was much lower under the field capacity condition, as compared to the flooded and saturated conditions. These results are in agreement with the ones by Sariam (2004) who observed that the number of spikelets per panicle under continuous flooded conditions had the highest value, followed by the saturated, while

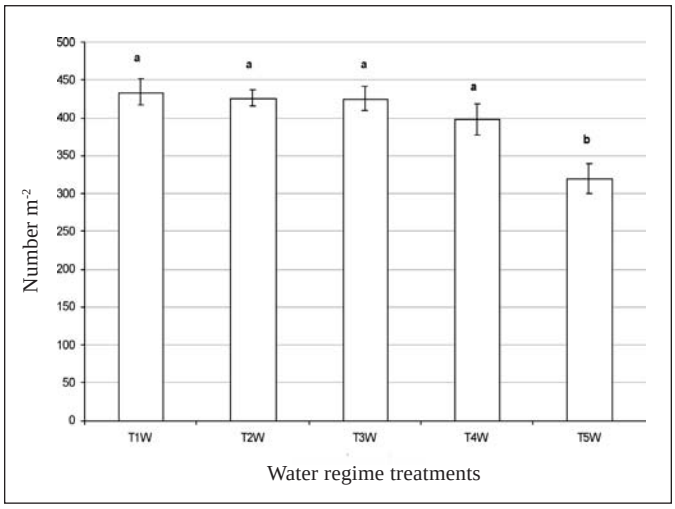

Fig. 2: The effect of different flooding treatments on the number of rice panicles m^{-2}

Means followed by the same letter are not significantly different at 5% level by Tukey's Test.
DAS = Day after sowing: T1 = continuous flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuous saturated condition: T5 = continuous field capacity condition.

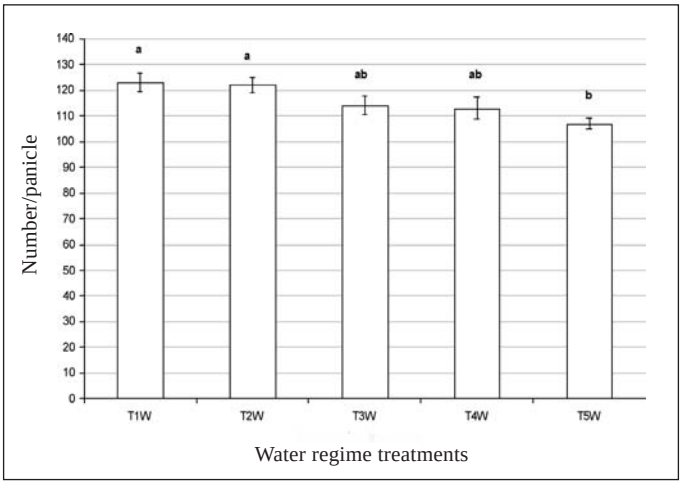

Fig. 3: The effect of different flooding treatments on the number of rice spikelets/panicle

Means followed by the same letter are not significantly different at 5% level by Tukey's Test.
DAS = Day after sowing: T1 = continuously flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuous saturated condition: T5 = continuous field capacity condition.

rice under field capacity condition produced the least spikelets per panicle.

Yield Components and Rice Yield

1000-Grain Weight

One thousand grain weight is a genetic character widely used in yield estimation (Mahfuza, 2006). The 1000-grain weight was affected by flooding treatments, where significant differences were observed in both the weeded and unweeded pots. In the weeded pots, a significant difference was found among almost all the flooding treatments, as shown in *Fig. 4*. Higher 1000-grain weight was obtained under all the flooding regimes (T1, T2 and T3), where T1 (continuous flooded) was indicated to produce the highest grain weight (26.76 g). The weight of 1000-grain under reduced water conditions (T4 and T5) was significantly lower as compared to T1, T2 and T3 with T5 (continuous field capacity) which produced the lowest 1000-grain weight (18.39 g).

Jahan (2004), in his study on rice production under glasshouse condition, indicated similar results where no significant difference of 1000-grain weight was observed under the different flooding regimes. Meanwhile, Sariam (2004) reported that 1000-grain weight varied significantly with water management, where lower grain weight was observed under the field capacity condition as compared to the saturated and flooded conditions. According to Dey and Upadhaya (1996), less biomass and number in grain production under the reduced water regimes could be caused by the lack in water availability at the anthesis (flowering) stage, which restricted rice pollination process and caused the rice to produce infertile and empty rice grain.

Rice Straw Biomass (Rice Straw Yield)

The differences in the flooding treatments had significant effects on the yield of rice straw, as shown in *Fig. 5*. Generally, when water availability declined, the straw biomass gradually decreased in both weeded and unweeded pots. The highest rice straw biomass was obtained in T1 (continuous flooded), which yielded 681.32

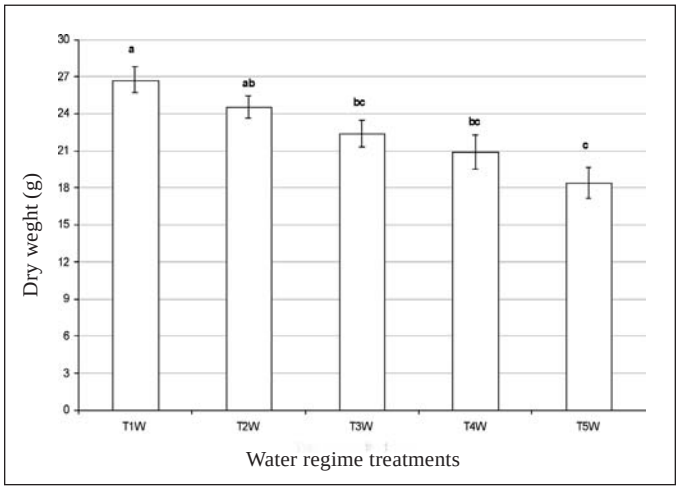

Fig. 4: The effect of different flooding treatments on 1000-grain weight (g)

Means followed by the same letter are not significantly different at 5% level by Tukey's Test.
DAS = Day after sowing: T1 = continuously flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuous saturated condition: T5 = continuous field capacity condition.

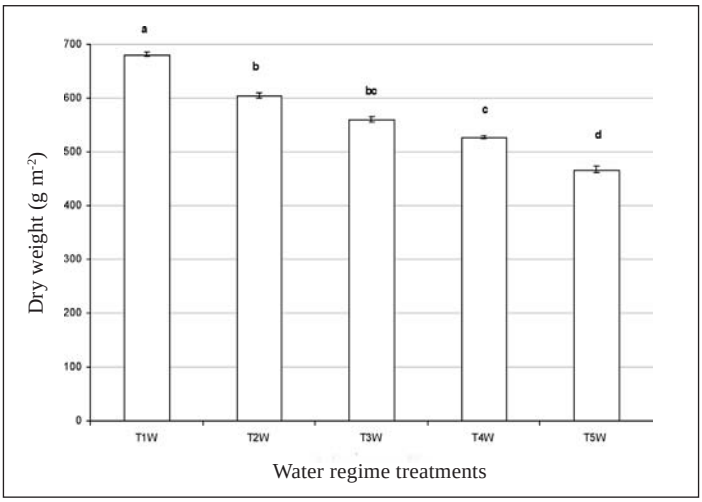

Fig. 5: The effect of different flooding treatments on biomass of rice straw (g m^{-2})

Means followed by the same letter are not significantly different at 5% level by Tukey's Test.
DAS = Day after sowing: T1 = continuously flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuous saturated condition: T5 = continuous field capacity condition.

g m^{-2} of rice straw weight, while T5 (continuous field capacity) produced the lowest straw yield of 467.03 g m^{-2}. From the observation, the amount of rice straw yielded in T5 was in average of 20-30% lesser than the rice straw produced under all flooding regimes (T1, T2 and T3) in both the weeded and unweeded pots. Shorter plants (Table 2) and fewer tillers (Table 3) could have attributed to lower straw yield under the field capacity condition. The results are in agreement with the reported findings (Mishra *et al.*, 1991; Beyrouty *et al.*, 1992; Sariam, 2004). According to Dey and Upadhaya (1996), reducing water availability in soil will adversely affect rice growth especially at vegetative and reproductive stages and this can further decrease the rice straw and grain yield.

Rice Yield

Fig. 6 illustrates the effects of different flooding treatments on the yield of rice grains. From the observation, rice yield was found to decrease significantly with reduced water availability in the soil. Generally, there was no significant difference observed between T1, T2 and T3, but all those flooding regimes significantly produced higher rice yield than T4 and T5. The maximum grain production was obtained from T1 (8534.4 kg ha^{-1}), followed by T2 (7870 kg ha^{-1}) and T3 (6840.8 kg ha^{-1}). Under T4, the rice production was significantly reduced to only 6130 kg ha^{-1}, causing a 23.16% reduction as compared to T1. However, the yield obtained from T4 was only significant when compared to T1 and T2, but not significant when it was compared to T3. Meanwhile, T5 produced the lowest rice grains of 3706.2 kg ha^{-1}, which was 56.57% lower than T1.

The result shows that rice grain yield responded differently under different flooding treatments. Continuous flooding (T1) favours rice growth and produces maximum rice yield. However, the results also suggest that it is not necessary to continuously flood the rice field throughout the rice growing period to obtain high grain yield since maintaining a temporary period of flooding, either until 55 DAS (T2) or 30 DAS (T3) resulted only in a non-significant reduction in rice yield of 1.35% and 14.25%.

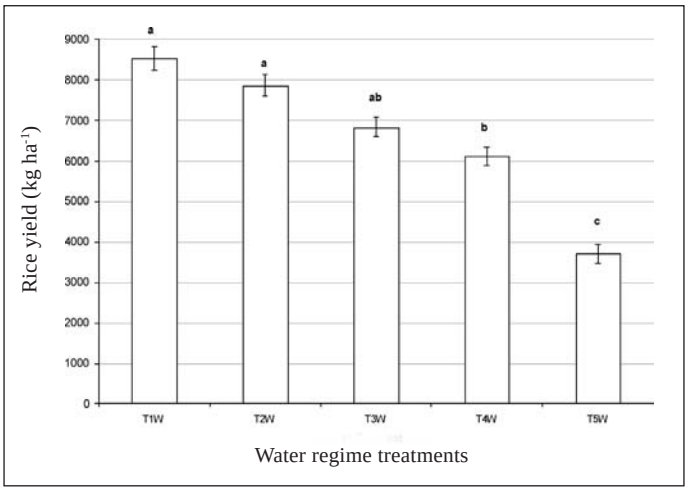

Fig. 6: The effect of different flooding treatments on rice yield (kg ha^{-1})

Means followed by the same letter are not significantly different at 5% level by Tukey's Test.
DAS = Day after sowing: T1 = continuously flooded condition: T2 = early flooding up to panicle initiation stage (55 DAS) followed by saturated: T3 = early flooding for the first month (30 DAS) followed by saturated: T4 = continuous saturated condition: T5 = continuous field capacity condition.

Similar results were also indicated by Mishra *et al.* (1991) and Sariam (2004).

However, grain yield decreased significantly when water was reduced to continuous field capacity (T5). This finding is in line with the results reported by Beyrouty *et al.* (1992), Anbumozhi *et al.* (1998) and Sariam (2004). Meanwhile, under continuous saturated condition (T4), the yield was not significantly different as compared to the moderate flooding period of T3. However, Sariam (2004), in her study, found dissimilar results when the yield of rice under saturated condition was found to be insignificantly different as compared to the rice yield under continuous flooded condition.

From the results, water management is shown as an important tool in rice planting. Water is the single most important component for sustainable rice production, especially in the traditional rice-growing areas (Williams *et al.*, 1990). Water is a major constituent of tissues, a reagent in chemical reaction, a solvent and mode of translocation for metabolites and minerals within plants and is essential for cell enlargement through increasing turgor pressure (Farooq *et al.*, 2006). However, the effect of water deficits on the growth and yield of rice is dependent on the stage of crop growth, at which the water deficits occur (Farooq *et al.*, 2006).

Water stress during vegetative stage reduces plant height, tiller number and leaf area. Immediately after transplanting, adequate land submergence (five to ten centimetres) is necessary to prevent damage to establishing seedlings from high winds and for root development (Farooq *et al.*, 2006). Following the early rooting stage, a shallow depth of land submergence (two to five centimetres) facilitates tiller production and firm root anchorage in the soil. Water deficit during this stage may reduce plant height, tiller number and leaf area, but the yield is least affected if adequate water is provided to permit recovery of the crop before panicle primordial initiation. However, excessive water depth at this stage will hamper rooting and decrease tiller production (Williams *et al.*, 1990). The reduction in grain yield, due to water deficit, during this stage is more related to the degree and duration of water deficits than to the stage of crop growth (Farooq *et al.*, 2006). Meanwhile, milk to grain maturity

stage is the least sensitive to soil moisture stress. After the yellowish ripening stage, there is no necessity for standing water. Water may be drained from the field about 7 – 10 days before harvest so as to facilitate harvesting (Farooq *et al.*, 2006).

Greater yields were observed from flooded rice than rice which was grown under saturated or dry conditions (Castillo *et al.*, 1992). A 10% reduction of rice yield in direct seeded rice flooded at the early reproductive stage was reported afterwards, when compared to the rice grown with a flooding beginning at early tillering (Tanaka *et al.*, 1963). Meanwhile, rice yield was not significantly reduced if water deficit was imposed during vegetative growth, but up to 70% of yield reduction occurred if water deficit was imposed during reproductive period (Lilley and Fukai, 1994).

CONCLUSIONS

The response of rice plant to water soil availability varies with its growing stage and other agronomic practices. At the early stage of 15 and 30 DAS, the flooding treatments did not significantly affect rice growth. However, from 45 DAS onwards, the effect of the different flooding treatments on rice growth was significantly pronounced. All flooding regimes (T1, T2 and T3) significantly favoured the height of rice plant and the production of tillers, while the reduced water regime treatments (T4 and T5) restricted them. Hence, a proper management of water supply, at different growing stages, is important in order to enhance the growth of rice plant to its maximum potential.

The positive correlation between the rice grain yield and rice yield components indicates that the parameters which contribute in producing high grain yield include the number of tillers, number of panicles m^{-2}, number of spikelets/panicle and 1000-grain weight. High rice grain yield, subjected to varying flooding regimes (T1, T2 and T3), was attributed to high number of tillers, high spikelets/ panicle and high 1000-grain weight; whereas, low grain yield under reduced water regimes (T4 and T5) was contributed by the low production of those components. Thus, managing flooding regimes is an important component of the integrated weed management system and to obtain high rice yield.

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Influence of Root Exudate Carbon Compounds of Three Rice Genotypes on Rhizosphere and Endophytic Diazotrophs

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ABSTRACT

Root exudates play an important role in microbial colonization of the rhizosphere. An *in vitro* experiment was conducted to study the root exudate sugars and production of amino acids of three different rice (*Oryza sativa*) genotypes, as well as the influence of these compounds on *Rhizobium* sp. (Sb16) and *Corynebacterium* sp. (Sb26) colonization. Using HPLC, a total of 7 carbohydrate sugars and 16 amino acids were identified from the Mahsuri, Mayang Segumpal and MR219 rice root exudates. A significant ($p<0.05$) relationship was observed between diazotrophic population growth and root exudates sugar and amino acid consumption of the three rice varieties. Higher bacterial population was found in the plant rhizosphere, as compared to the endosphere. *Rhizobium* sp. consumed more sugar and produced higher rhizosphere population as compared to *Corynebacterium* sp. The *Rhizobium* sp. consumed 100% of mannose, xylose, arabinose and sucrose in the root exudates of three rice genotypes. The differences in sugar consumption by *Corynebacterium* sp. were observed between the rice varieties. *Corynebacterium* sp. consumed 100% mannose, xylose and fructose in Mahsuri rice, 100% xylose and arabinose in Mayang Segumpal and 100% arabinose and sucrose in MR219 variety. The identification of the preferred carbon sources by the diazotrophs and the selection of genotypes which produce these compounds may increase the root colonization and subsequently N fixation in the rice plants.

Keywords: Amino acids, carbon sugars, *Corynebacterium* sp., diazotrophic population, *Rhizobium* sp., rice genotypes, root exudates

INTRODUCTION

Diazotrophs are nitrogen fixing bacteria which depend on their environmental carbon sources for their metabolic activity and nitrogen fixation. Besides soil organic matter, plant root exudates are a vital carbon source for microbes. Several plants allocate up to 40% of recently assimilated carbon to the root zone (Degenhardt *et al.*, 2003). Microbes in the vicinity of plant root consumed about 64-86% of the carbon released from the roots (Hutsch *et al*., 2002). The presence of organic compounds, released by the plant roots, stimulates the microbial activity in the rhizosphere (Bacilio-Jiménez *et al.*, 2003). The microbial activity is generally higher in rhizosphere, and the population size of 10 to 100 fold higher was found in this zone as compared to the surrounding bulk soil (Weller and Thomashow, 1994). Sugar, organic acids and amino acids are the major component of the plant root exudates. Soil bacteria perceive and actively move toward niches which are

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optimal for the survival and attached to the plant root surface. This activity plays a key role in the establishment of associative and symbiotic relationships between plants and micro-organisms (Yost and Hynes, 2000).

Root exudate component and concentrations vary with plant genotypes. He *et al*. (2004) observed that the quantity, content and chemical composition of the root exudates of two rice accessions were different. The amount and composition of root exudates entering the soil can be affected by multiple factors such as light intensity, temperature, nutritional status of the plants, activity of retrieval mechanisms, various stress factors, mechanical impedance, sorption characteristics of growth media and microbial activity in the rhizosphere. The differences in the preference of diazotrophs for root exudate carbon compounds are also observed. *Azospirillum brasilense* utilizes fructose and grows poorly on glucose and amino acids as sole carbon and energy sources (Hartmann *et al*., 1988). *Escherichia coli* is attracted by proline, glycerol and succinate and its growth is dependent on the oxidation of these substrates. Root colonization and rhizosphere competence of *Pseudomonas fluorescens* WCS365 is dependent on the synthesis of amino acid. Lectin-specific carbohydrates such as glucose, glucuronic acid, glucosamine, and galactosamine could function as receptors for the lateral surface lectines (Karpunia *et al*., 2003). Different amounts of sugar are consumed by the bacteria when there were more than one preferred sugar present in the root environment.

At present, not much information is available on the types of root exudates and their production by diazotrophs. The search for the natural associative and endophytic diazotroph establishment with the rice is important in increseaing the efficiency of nitrogen fixation. The application of preferred carbon compounds may enhance inoculum growth and provide a conducive environment for nitrogen fixation as well as promotion of direct plant growth. The present study focused on the rhizosphere and endophytic populations of two inoculated diazotrophs strains and their subsequent utilization of sugars and amino acids, released from the root exudates of three different rice genotypes.

MATERIALS AND METHODS

The root exudates of three rice (*Oryza sativa*) varieties, Mahsuri, Mayang Segumpal, and MR219, and the population growth of two diazotrophic strains, *Corynebacterium* and *Rhizobium* spp. (previously isolated from the rice rhizosphere of Tanjong Karang rice irrigation project area, Malaysia) were studied. Mahsuri and MR219 were high yielding rice varieties, while Mayang Segumpal was a local accession. The sugar and amino acid concentrations of the root exudates and the total bacterial population in the growth culture media, rhizosphere and root endophytic were determined at 3, 6, 12, and 18 days after inoculation. The experiment was carried out in a factorial completely randomized design (CRD) with 3 replications. Data were analyzed using the SAS (9.1 version) statistical software.

Seed Surface Sterilization

The seed surface sterilization method was adopted from Amin *et al.* (2004) and Elbeltagy *et al*. (2001). Rice seeds were dehusked and agitated in 70% ethanol for 5 seconds. The ethanol was discarded and the seeds were washed in hypochlorite solution, comprising 3% Chlorox ™ (2.6% NaOCl), with a few drops of Tween20. The seeds were rinsed (three times) with sterile water, followed by 2% sodium thiosulphate solution to neutralize the chloramine residue. The efficacy of the sterilization procedure was checked by germinating the seeds on nutrient agar (NA) plates.

*Preparation of Diazotrophs (*Rhizobium *and* Corynebacterium *spp.) Inoculums*

Rhizobium and *Corynebacterium* spp. (isolated from MR219 rice rhizosphere) were grown in the ATCC broth for 48 hours. The bacterial cells were harvested by centrifugation at 13500

rpm for 10 min in an eppendorf tube and washed with 0.85% sterilized phosphate buffer saline (Bacteriological Analytical Manual, 2001). Optical density (OD_{600}) of the washed cells were checked and adjusted accordingly. The population was confirmed by cell enumeration in drop plate method on NA.

In vitro Growth of Rice Seedlings

The surface sterilized rice seeds were germinated on the sterile petridishes and eight rice seedlings of 5 days old were grown in the growth culture tubes, where a stainless steel sieve was placed above 50 mL of nutrient solution. After that, the seedlings were placed on the sieve in such a way that only the roots were in touch with the nutrient solution. Carbon and nitrogen free plant nutrient solution, modified from Egener *et al.*, (1999), was used for each glass tube. The solution contained (1L): KH_2PO_4, 1.5 g; K_2HPO_4, 0.33 g; K_2SO_4, 0.2g; ferric citrate, 13 mg; $CaCl_2.2H_2O$, 0.4 g; $MgCl_2$., 0.4g; $Na_2MoO_4.2H_2O$, 2 mg; H_3BO_3, 3mg: $MnSO_4.H_2O$, 2 mg; $ZnSO_4.7H_2O$, 0.2 mg; $CuSO_4.5H_2O$, 0.1 mg. Before transplanting, the seedlings were gently washed with sterile distilled water to remove plant metabolites formed during growth on petridishes. Each growth tube was subsequently inoculated with 5 mL of 10^9 cfu / mL of live bacterial cells. The plants were grown for 20 days in a growth chamber with 12 hr. light/ dark cycle at 28 ˚C.

Collection of the Root Exudates

The root exudates of the non-inoculated and inoculated plants were collected from the respective growth tubes at 5, 8, 14 and 20 days after transplanting. The collected plant growth culture solutions of all the glass tubes (50 mL) were filtered through a 0.2 µm Millipore filter and kept in screw-top vials at -20 ˚C for determination of sugar and amino acids.

Determination of Sugars

Sugar concentrations were determined using high performance liquid chromatography (HPLC) with a refractive index (RI) detector. Galactose, arabinose, xylose, fructose, and sucrose were determined on NH_2-carbohydrate column using acetonitrile (75%), as the mobile phase with a flow rate of 1mL min^{-1}. Mannose and glucose were determined by Supel cogel column using phosphoric acid (1%) as the mobile phase at a flow rate of 0.8 mL min^{-1}.

Determination of Amino Acids

The concentrations of amino acid were determined using the HPLC by a modified method proposed by Strydon and Cohen (1994), following the pre-column derivatisation with AQC reagent (6-aminoquinolyl-N-hydroxysuccinimdyl carbamate, Waters, USA). The content of tryptophan was determined using alkaline hydrolyses. Cysteine and methionine were not determined.

Total Sugar and Amino Acids Production

Total productions of sugar and amino acids were estimated as $TP = TC + ER$ where, TP = total sugars and amino acids production by the plant, TC = total sugar and amino acids consumption/ utilization by the diazotrophs over period, ER = Extra sugar and amino acids remaining in the inoculated plant root exudates.

Sugar and Amino Acids Utilization by Diazotrophs

The total sugar consumption/utilization (TC) was determined as $TC = S_t - S_{t1}$ where, S_t is the total sugar production by the control plant over time, and S_{t1} is the total sugar remaining in the culture solution by the inoculated plant over time.

Determination of Diazotroph Population in Growth Culture Solution

At each sampling date, 1.0 mL of plant growth culture solution from each glass tube was diluted 10 folds up to 10^{-11} dilution. Aliquotes of 0.1 mL from each dilution was dropped onto NA plates and the populations were determined following the drop plate count method.

Determination of Rhizosphere Population

At each sampling date, 2 plants were harvested, and roots were gently washed with sterile water and placed in conical flask containing 99 ml distilled water. The contents were then shaken for 15 minutes and a series of 10 fold dilutions were prepared, while the bacterial populations were determined as described previously.

Determination of Root Endophytic Population

The roots (1.0 g) were washed, blotted dry and surface sterilized with 70% ethanol for 5 min and then treated with 3% Clorox for 30 sec. The roots were checked for the efficacy of surface sterilization by rolling them on the NA plates. Using a sterilized mortar and pestle, the roots were macerated (Gyaneshwar *et al.*, 2001). A 10 fold dilution series of was prepared up to 10^{-10} and the diazotroph populations were determined as described previously.

RESULTS

Production and Utilization of Sugar by the Diazotrophs

The Mahsuri rice, inoculated with *Rhizobium* sp., was found to produce and utilize more total sugar (2989 µmol g^{-1} root dry wt) as compared to *Corynebacterium* sp. (2853 µmol g^{-1} root dry wt) during the 20 days of growth (Table 1). *Rhizobium* sp. utilized more arabinose, mannose and xylose, while *Corynebacterium* sp. utilized higher amounts of xylose, mannose and fructose (*Fig. 1a*). Among the sugar, the highest amount of fructose (791 µmol g^{-1} root dry

TABLE 1
Production and utilization of sugar (µmol g^{-1} root dry wt.) in inoculated and non-inoculated Mahsuri rice (*Oryza sativa*) root exudates during 5, 8, 14 and 20 days of growth period in an axenic condition

Sugar	5 days seedling			8 days seedling			14 days seedling			20 days seedling		
	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)
Glu	112.03	--	--	177.03	--	15.2	244.03	--	30.5	265.73	--	30.7
Man	329	--	--	430	--	--	534	--	--	534	--	--
Xyl	290	--	--	585	--	--	591	--	0.9	591	--	--
Arab	204	--	--	346	--	--	346	241	294	346	--	--
Fruc	--	--	--	122	--	233	441	--	--	558	--	--
Galac	--	--	--	--	--	--	--	33	0.61	--	--	--
Suc	--	--	--	300	120	--	300	--	--	300	--	6.6
Total Production (TP)	935	-	-	1960	-	-	2456	-	-	2595	-	-
Total Utilization (TC)	-	935	935	-	1840	1823	-	2302	2363	-	2989	2853

Here, Control = non-inoculated plant, Sb16 = Plant inoculated with *Rhizobium* sp., Sb26 = plant inoculated with *Corynebacterium* sp. (--) = no sugar residue detected. R = residual sugars in the inoculated plant root exudates, TP = total sugar production by the plant, TC = Probable sugars utilization by the diazotrophs ($TC = S_t - S_{t1}$). Where, S_t is the total sugar production by the control plant over time, and S_{t1} is the total sugar remaining in the culture solution by the inoculated plant over time.

wt) was utilized by *Corynebacterium* sp. Both diazotrophs utilized 100 % of released mannose and xylose in the Mahsuri root exudates.

The total sugar production and utilization by the diazotrophs was lower in Mayang Segumpal rice, as compared to the other two varieties. Plants inoculated with *Rhizobium* sp. produced 2686 µmol g^{-1} root dry wt of sugar, while *Corynebacterium* sp. inoculated plants produced half of the sugar during 20 days of growth (Table 2). In Mayang rice root exudates, *Rhizobium* sp. consumed significantly higher amounts of mannose, xylose, arabinose and sucrose, while *Corynebacterium* sp. consumed more xylose and arabinose (*Fig. 1b*). The *Rhizobium* sp. utilized the most released mannose (959 µmol g^{-1} root dry wt) in the Mayang Segumpal. Xylose and arabinose seemed to be completely utilized by both diazotrophs. Nevertheless, glucose was not detected from this rice root.

The MR219 rice plant, inoculated with *Rhizobium* sp., generally exhibited higher sugar exudation (3127 µmol g^{-1} root dry wt), as compared to *Corynebacterium* sp. inoculated

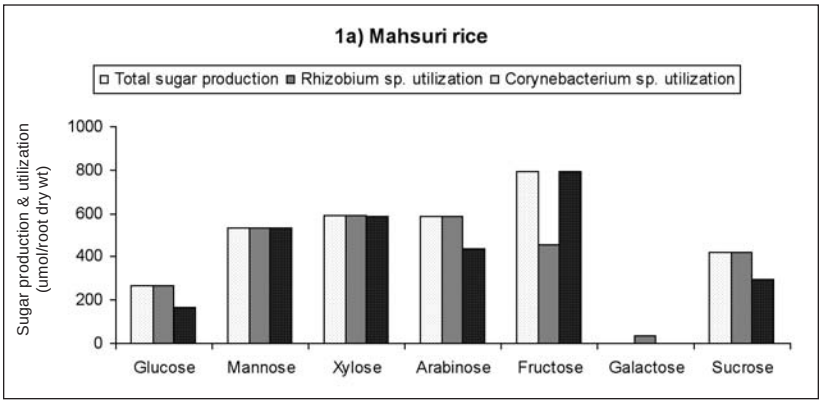

Fig. 1a: The root exudate sugar production and utilization by Rhizobium *and* Corynebacterium *spp. in Mahsuri rice during 18 days of inoculation period (Mean values of 3 replications)*

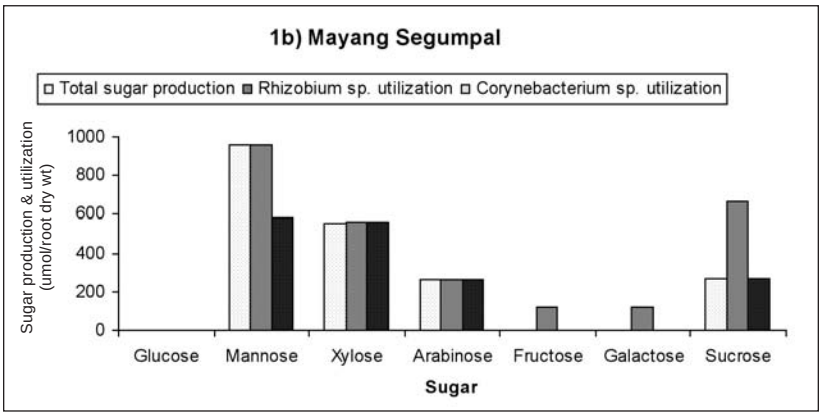

Fig. 1b: The root exudate sugar production and utilization by Rhizobium *and* Corynebacterium *spp. in Mayang Segumpal rice during 18 days of inoculation period (Mean values of 3 replications)*

TABLE 2
Sugar production and utilization (µmol g^{-1} root dry wt.) of inoculated and non-inoculated Mayang Segumpal rice (*Oryza sativa*) root exudates during 5, 8, 14 and 20 days of the growth period in an axenic condition

Sugar	5 days seedling			8 days seedling			14 days seedling			20 days seedling		
	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)
Glu	--	--	--	--	--	--	--	--	--	--	--	--
Man	285	--	155	601	--	216	772	--	221	959	--	376
Xyl	196	10	10	551	--	--	551	--	--	551	--	--
Arab	--	--	--	259	--	--	259	--	--	259	--	--
Fruc	--	119	--	--	--	--	--	466	--	--	466	--
Galac	--	--	--	--	--	--	--	122	--	--	--	--
Suc	--	--	--	--	--	--	266	400	--	266	--	--
Total Production (TP)	481	-	-	1411	-	-	1848	-	-	2035	-	-
Total Utilization (TC)		471	316		1540	1205		1977	1637		2686	1669

Here, Control = non-inoculated plant, Sb16 = Plant inoculated with *Rhizobium* sp., Sb26 = plant inoculated with *Corynebacterium* sp. (--) = no sugar residue detected. R = residual sugars in the inoculated plant root exudates, TP = total sugar production by the plant, TC = Probable sugars utilization by diazotrophs ($TC = S_t - S_{t1}$), where S_t is the total sugar production by the control plant over time, and S_{t1} is the total sugar remaining in the culture solution by the inoculated plant over time.

plant (Table 3). In particular, *Rhizobium* sp. utilized higher amounts of mannose, fructose, sucrose and galactose, whereas *Corynebacterium* sp. utilized higher amounts of mannose, fructose and sucrose (*Fig. 1c*). Mannose was highly utilized (697.5 µmol g^{-1} root dry wt) by *Corynebacterium* sp. Arabinose and sucrose produced by plants was almost completely utilized by both the strains.

Production and Utilization of Amino Acids in the Root Exudates

The production of amino acids was higher in Mahsuri, as compared to the other two varieties. Most of the amino acids produced were utilized by both bacteria. Mahsuri and MR219, inoculated with *Corynebacterium* sp., utilized higher amount of amino acids as compared to *Rhizobium* sp., while *Rhizobium* sp. utilized most of the amino acid from the Mayang rice root exudates.

In Mahsuri rice, *Rhizobium* sp. utilized higher amounts of glycine and isoleucine, while *Corynebacterium* sp. utilized serine, glutamine, glycine, isoleucine and leucine (*Fig. 2a*). In the Mayang Segumpal rice root exudates, *Rhizobium* sp. was found to consume more than 70% of aspartic, glutamine, serine, glycine, threonine and isoleusine, while *Corynebacterium* sp. consumed more than 80% of released leucine and tryptophan (*Fig. 2b*). In MR219 rice root exudates, *Rhizobium* sp. was indicated to utilize higher amounts of glycine, alanine and *Corynebacterium* sp. utilized higher amounts of glycine, alanine, arginine, and isoleucine (*Fig. 2c*).

TABLE 3
Sugar production and utilization (µmol g^{-1} root dry wt.) of inoculated and non-inoculated MR219 rice (*Oryza sativa*) root exudates during 5, 8, 14 and 20 days of the growth period in an axenic condition

Sugar	5 days seedling			8 days seedling			14 days seedling			20 days seedling		
	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)	Control (TP)	Sb16 (R)	Sb26 (R)
Glu	25	--	--	52.3	--	--	77.3	--	--	150	--	24.8
Man	32	--	--	46	--	--	474	--	--	834.9	--	--
Xyl	20	--	--	20	2.9	--	20	--	1.5	20	--	10.6
Arab	186	--	--	186	--	--	198	--	--	198	--	--
Fruc	--	444	--	139	--	--	326	--	--	326	--	14
Galac	48	--	--	48	30	--	258	--	--	591	--	26
Suc	333	--	--	522	9	--	522	--	9	522	--	--
Total Production (TP)	644	-	-	1013	-	-	1875	-	-	2642	-	-
Total Utilization (TC)	-	644	644	-	1415	1013	-	2361	1874	-	3127	2577

Here, Control = non inoculated plant, Sb16 = Plant inoculated with *Rhizobium* sp., Sb26 = plant inoculated with *Corynebacterium* sp. (--) = no sugar residue detected. R = residual sugars in the inoculated plant root exudates, TP = total sugar production by the plant, TC = Probable sugars utilization by the diazotrophs ($TC = S_t - S_{t1}$), where S_t is the total sugar production by the control plant over time, and S_{t1} is the total sugar remaining in the culture solution by the inoculated plant over time.

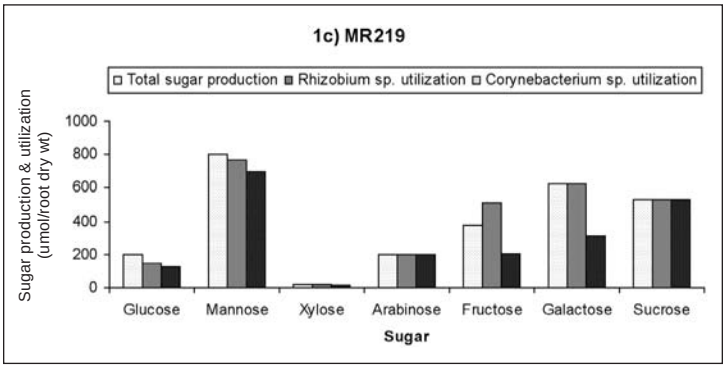

Fig. 1c: The root exudate sugar production and utilization by Rhizobium *and* Corynebacterium *spp. in MR219 rice during 18 days of inoculation period (Mean values of 3 replications).*

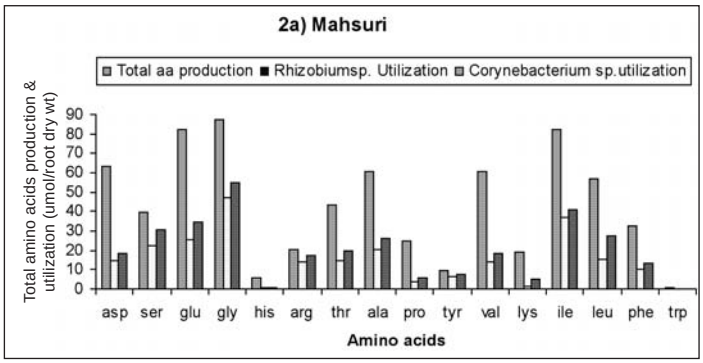

Fig. 2a: The root exudate amino acids production and utilization by Rhizobium *and* Corynebacterium *spp. in Mahsuri rice during 18 days of inoculation period (Mean values of 3 replications)*

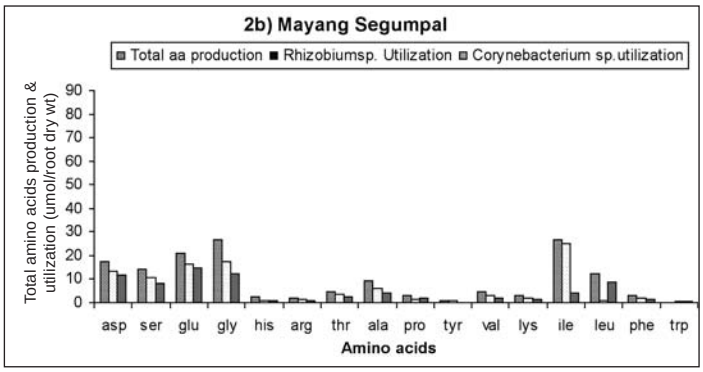

Fig. 2b: The root exudate amino acids production and utilization by Rhizobium *and* Corynebacterium *spp. in Mayang Segumpal rice during 18 days of inoculation period (Mean values of 3 replications)*

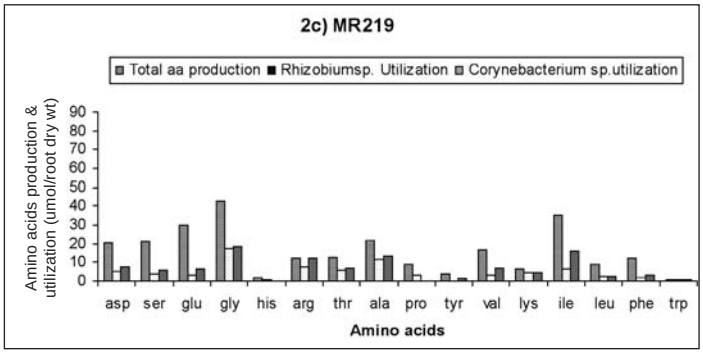

Fig. 2c: The root exudate amino acids production and utilization by Rhizobium *and* Corynebacterium *spp. in MR219 rice during 18 days of inoculation period (Mean values of 3 replications)*

Relationship between Root Exudates Sugar and Amino Acids Utilization and Diazotrophs Colonization

A significant ($P \leq 0.05$) relationship was found between the root exudate sugar and amino acid utilization and diazotroph population growth in the growth culture solution, rhizosphere and root endophytes of the rice varieties (Tables 4 and 5). The population of rhizosphere was significantly higher than in the growth culture solution and root endosphere populations (*Fig. 3*). The population of rhizosphere ranged from 10^8 to 10^{11} cfu g^{-1} root dry weight. As indicated earlier, *Rhizobium* sp. utilized more sugar than *Corynebacterium* sp., and this subsequently produced higher rhizosphere population.

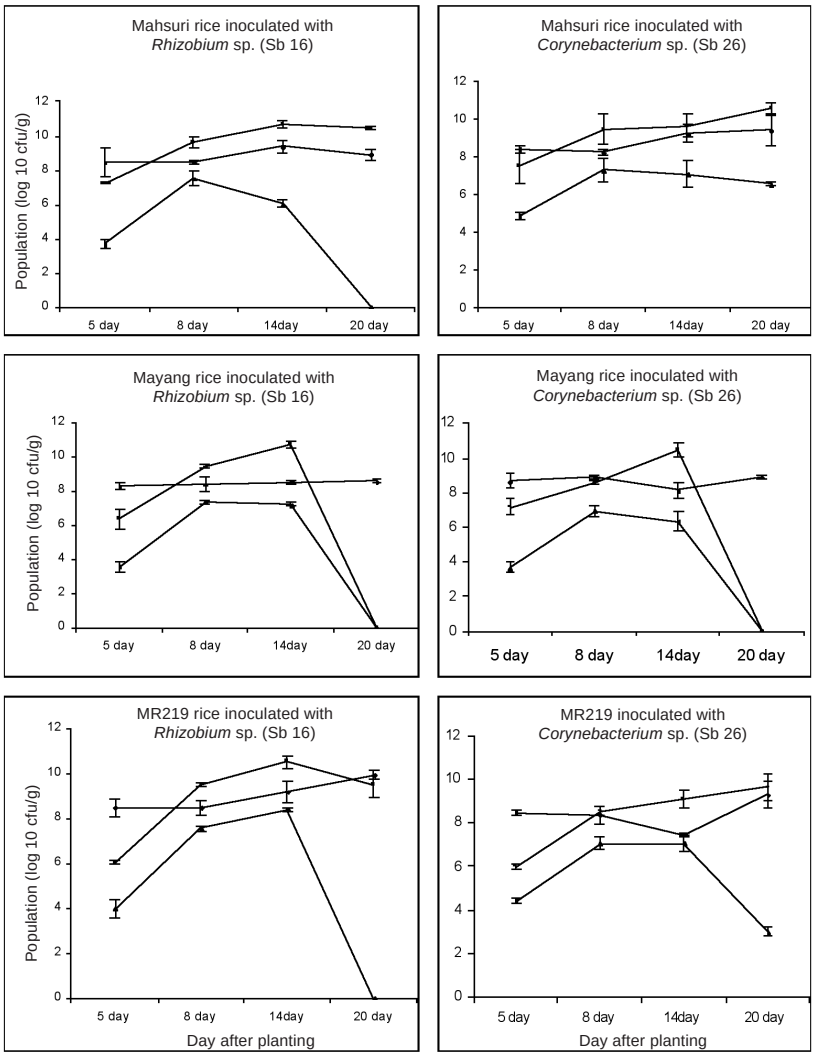

Fig 3: Changes in growth of diazotrophs population in the rice varieties inoculated with Rhizobium *and* Corynebacterium *spp. Populations of* Rhizobium *sp. and* Corynebacterium *sp. in culture solution (◆), rhizosphere (■), root endophytes (▲)of different rice varieties*

TABLE 4

The correlation between the root exudate sugar consumption and the population growth of *Rhizobium* and *Corynebacterium* spp. in Mahsuri, Mayang and MR219 rice varieties. CS = Growth culture solution, Rhi = Rhizosphere, and Root = root endophytes. Negative (-) indicates the absence of sugar. Significance levels are *, 0.05, **, 0.01, ***, 0.001, respectively. NS= not significant from the untreated control

The correlation result of root exudate sugar consumption and population growth in Mahsuri, Mayang and MR219 rice varieties inoculated with *Rhizobium* and *Corynebacterium* spp.

Sugar	Mahsuri						Mayang Segumpal						MR219					
	Rhizobium sp Population			*Corynebacterium sp* population			*Rhizobium sp* population			*Corynebacterium sp* population			*Rhizobium sp* population			*Corynebacterium sp* population		
	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root
Glu	***	NS	NS	NS	***	NS	0	0	0	0	0	0	***	NS	-***	NS	NS	NS
Man	NS	NS	NS	NS	NS	NS	-*	NS	NS	NS	*	**	NS	-***	NS	-***	NS	***
Xyl	-***	NS	*	-***	NS	NS	-*	NS	NS	-*	NS	NS	NS	NS	NS	NS	NS	NS
Arab	***	NS	NS	NS	-***	-*	-*	NS	NS	-*	NS	NS	**	NS	NS	-**	NS	NS
Fruc	NS	NS	*	NS	-***	-*	***	NS	NS	NS	NS	NS	NS	-***	NS	***	-***	-***
Galac	***	NS	*	NS	*	***	NS	*	NS	NS	NS	NS	-**	NS	NS	***	-***	-***
Suc	NS	-***	NS	NS	*	***	*	NS	NS	NS	NS	NS	-*	NS	NS	-***	NS	NS

TABLE 5

The correlation between the root exudate amino acids utilization and the population growth of *Rhizobium* and *Corynebacterium* spp. in Mahsuri, Mayang and MR219 rice varieties. CS = Growth culture solution, Rhi = Rhizosphere and root = root endophytes. Negative (-) indicates the absence of sugar. Significance levels are *, 0.05, **, 0.01, ***, 0.001, respectively. NS = not significant from the untreated control

The correlation result of root exudate amino acid consumption and population growth in Mahsuri, Mayang and MR219 rice varieties inoculated with *Rhizobium* and *Corynebacterium* spp.

Amino acids	Mahsuri						Mayang Segumpal						MR219					
	Rhizobium sp. Population			*Corynebacterium* sp. population			*Rhizobium* sp. population			*Corynebacterium* sp. population			*Rhizobium* sp. population			*Corynebacterium* sp. population		
	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root	CS	Rhi	Root
Asp	NS	-***	NS	NS	-***	NS	NS	**	**	NS	NS	NS	NS	NS	*	-***	NS	*
Ser	NS	-***	NS	NS	-***	NS	NS	**	**	NS	NS	NS	NS	NS	*	-***	NS	*
Glu	NS	-***	NS	NS	-***	NS	NS	***	**	NS	NS	NS	NS	*	NS	-***	NS	NS
Gly	NS	-***	NS	NS	-***	NS	*	*	*	NS	NS	NS	NS	-***	NS	NS	-**	NS
His	NS	NS	*	-*	NS	**	NS	*	NS	NS	NS	NS	NS	-***	NS	NS	-***	NS
Arg	NS	-***	NS	NS	-***	-*	NS	NS	NS	***	-**	NS	**	NS	-***	*	*	NS
Thr	NS	-***	NS	NS	-***		NS	NS	NS	*	NS	NS	NS	-*	NS	-**	-*	NS
Ala	NS	-***	NS	NS	-***	-*	NS	NS	NS	*	NS	NS	NS	-***	NS	-*	NS	NS
Pro	-*	-***	NS	-*	-***	NS	NS	NS	NS	**	NS	NS	NS	-***	NS	NS	NS	NS

Table 5 (continued)

Tyr	NS	-	NS	NS	-	NS	NS	NS	NS	***	-	-	NS	-	NS	-	NS	NS	NS			
		***			***						***	***		***		***						
Val	NS	-	NS	NS	-	NS	NS	NS	NS	**	NS	NS	NS	-	NS	-	NS	NS	NS			
		***			***									***		***						
Lys	NS	-	NS	NS	-	-	NS	NS	NS	**	NS	NS	NS	NS	NS	-	NS	NS	NS			
		***			***	**								***		***						
Ilu	NS	-	NS	NS	-	NS	-	-	-	NS	NS	-	NS	NS	*	-	NS	NS	NS			
		***			***		*	*	*			**		***		***						
Leu	NS	-	NS	-	-	NS	NS	NS	NS	***	-	-	NS	*	NS	-	NS	NS	NS			
		***		*	*						***	***		***		***						
Phe	NS	-	NS	-	-	NS	NS	NS	NS	*	NS	NS	NS	-	NS	-	NS	NS	NS			
		***		*	***									***		***						
Trp	NS	NS	-	NS	NS	**	NS	-	-	***	-	-	NS	-	NS	NS	-	NS	NS			
			***					***	***		***	***		***			***						

Meanwhile, fructose, sucrose, xylose, arabinose, and mannose were significantly correlated with the population of *Rhizobium* sp. On the other hand, sucrose, mannose, glucose, fructose and galactose were significantly correlated with the *Corynebacterium* sp. population.

The population of *Rhizobium* sp. in the Mahsuri rice rhizosphere did not correlate with the concentrations of histidine and tryptophan. In Mayang rice, the concentrations of aspartic, serine, glutamine, glycine and histidine were correlated with the increased *Rhizobium* populations. In MR219 rice, serine, glutamine, isoleusine, arginine and leucine were significantly correlated with the population of *Rhizobium* sp. Meanwhile, the concentrations of histidine, and tryptophan were significantly correlated with the population of *Corynebacterium* sp. in Mahsuri rice. In Mayang rice, the population significantly increased by arginine, threonine, alanine, proline, tyrosine, valine, lysine, leucine, phenylalanine, and tryptophan. In MR219 aspartic, serine and histidine were significantly correlated with the population of *Corynebacterium* sp. The populations of endophytic in all the three rice genotypes were found to decrease after 14 days of inoculation.

DISCUSSION

Inoculation and Stimulation of Sugar and Amino Acids

Root exudates provide a favourable niche for diazotrophic association and roots are prerequisite for the establishment of the symbiotic association between plant and bacteria (Alexandre and Zhulin, 2001). The attraction towards the exudation component is dependent on the concentration and configuration of the compounds (Reinhold *et al*., 1985). In the present study, a significant correlation was found between the utilization of sugar and amino acids in the root exudates and the growth of the diazotrophic population. The populations of rhizosphere were shown to be significantly higher than the populations of non-rhizosphere and root endophytic. The site of exudation may provide suitable environment for colonization on the rhizosphere. The nutrients released in the root exudates were readily consumed by the diazotrophs and consequently, established themselves on the root surface. Conditions provided by the host plants significantly contributed to the initiation of the association process (Reinhold *et al*., 1985).

Root exudates sugar, amino acids production and composition were different between the rice cultivars which might influence the population growth of diazotrophs. Kumar *et al*. (2007) also found differences in the root exudates between cotton cultivars which influence the chemotactic responses in *Azotobacter* spp. The absence of glucose in Mayang Segumpal root exudates, may be due to the re-absorption of plant, as plants were grown in strictly carbon-free condition (Guckert *et al.*, 1991). Another probable cause may be due to the very low amount of glucose present in the root exudates, which could not be detected.

The rice varieties, which were inoculated with *Rhizobium* sp., produced higher rhizosphere population than *Corynebacterium* sp. The higher *Rhizobium* sp. population resulted in more utilization of total sugars from the root exudates of all the three rice varieties. A previous study showed that several compounds, including sugar, and amino acids, attracted *Rhizobium* spp. to the roots (Aguilar *et al*., 1998). The *Corynebacterium* sp. consumed higher amounts of amino acids as compared to *Rhizobium* sp. from Mahsuri and MR219 root exudates. In more specific, amino acids were found to be weak attractants for the bacteria which fixed nitrogen under microaerophilic conditions (Alexandre and Zhulin, 2007). The slightly higher consumption of amino acids observed by *Rhizobium* sp. in Mayang Segumpal may be due to the low sugars in root exudates and compels this strain on amino acids for their physiological activities. Decreased diazotrophs population in the growth culture solution was probably caused by the accumulation of high levels of toxic substances in the vicinity of the root which inhibited growth.

Correlation analyses revealed that the *Rhizobium* sp. significantly consumed almost

all sugar, while *Corynebacterium* sp. consumed glucose, mannose, xylose, arabinose, and sucrose from the root exudates of rice varieties. The concentration of fructose significantly correlated the diazotrophs population in both Mahsuri and Mayang Segumpal rice. Our previous study also showed higher population growth of these diazotrophs in fructose substrate, and this is analogous to the findings of the present study (Naher *et al*., 2008).

Rhizobium sp. seemed to utilize all detected amino acids. Another previous study showed that glutamate, aspartate, alanine, and arginine were significant attractants to *Bradyrhizobium japonicum* (Barbour *et al*., 1991). The attraction to proline was found in many bacteria including *E. coli* and *Sinorhizobium meliloti* (Clancy *et al*., 1981; Götz *et al*., 2000). In the present study, the population of *Corynebacterium* sp. was positively correlated with the concentrations of histidine, tryptophan, arginine, tyrosine, threonine, alanine, proline, valine, lysine, leusine, and phenylanine. In general, it can be concluded that the utilization of sugar and amino acids in root exudates is not only dependent on the concentration of the compounds, but also the presence of the other compounds and the preferences of the diazotrophs to those specific compounds.

In the present study, 7 sugars and 16 amino acids were determined in the root exudates. The utilization of sugar and amino acid by the diazotrophs were assumed to be based on the residual component, which was determined at different dates and deducted from the non-inoculated plants. However, this indirect determination could provide fundamental information on the production of sugar and amino acid, as well as the utilization and growth of diazotrophs in the rice plant system.

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Upland Rice Varieties in Malaysia: Agronomic and Soil Physico-Chemical Characteristics

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ABSTRACT

Rice production is a large industry and there are a lot of opportunities which can be obtained from it. Recently, the demand for specialty and high quality rice has increased remarkably, owing to the affluent and health-conscious consumers in Malaysia. The research on upland rice has been neglected because of its low yield, though it has many good characteristics, including good fragrance and long grains. Furthermore, it has the advantage of being cultivated on dry land without accumulation of water. Therefore, a large track of idle lands in Malaysia can be developed for this purpose. This study involves a documentation of upland rice in natural conditions. Basic information on the varieties of upland rice which produce high grain yields and quality (fragrance, colour) was collected from selected locations in Peninsular Malaysia, Sabah, and Sarawak. For this purpose, both soil and plant materials (at harvest) were collected. The soil and plant materials were analysed for their macro- and micro-nutrient contents. Standard agronomic characteristics, during growing period and at harvesting time, were also measured. The data were analysed using the SAS statistical software and the mean values were then compared using the Duncan's New Multiple Range Test (DMRT) at 0.05 level of significance. Seventeen upland rice fields were identified in several locations during the course of this survey. Thirty-five (35) varieties of upland rice seeds were successfully collected. In particular, upland rice and forest soil (as a control) were acidic, contain low nitrogen content and CEC value at 0 – 20 and at 20 – 40 cm depth. Higher Fe content was also observed, with a major limitation for the growth of upland rice. Ageh, Kendinga, and Strao varieties were selected for further evaluation on nutrient requirements using an idle land soil, owing to its growth cycle, productivity, and seed availability.

Keywords: Upland rice, varieties, agronomic characteristics, physico-chemical characteristics

INTRODUCTION

In Malaysia, rice is normally cultivated either as wet paddy (Peninsular Malaysia, 503,184 ha) or upland rice (Sabah and Sarawak, 165,888 ha) (DOA, 2005). Under wet paddy cultivation, the national average yield is about 3.3 tonnes ha^{-1}, but with a better field management, varieties such as MR 219 and MR 220 can produce yields of about 10 tonnes ha^{-1} at several locations. In 2005, the total national rice production (TNRP) was approximately 2.24 million metric tonnes, which was contributed by eight granary areas; nevertheless, this only catered for 60 – 65% of the domestic requirement. Thus, Malaysia still imports 458,600 metric tonnes of rice to fulfil the requirement of its population (DOA, 2005). In addition to the large import, the rice production areas in Malaysia are decreasing because good rice areas, near development centres are being converted for other uses.

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Upland rice cultivation is practiced mostly by the rural communities living especially in Sabah and Sarawak. It is still an important agricultural activity for home consumption and sometimes the farmers sell their surplus to earn some money. Certain upland rice varieties have desirable characteristics, particularly in terms of their fragrance, colours, sizes, and shapes. These qualities contribute to their popularity among the farmers and health-conscious consumers as an organic food. However, these upland rice varieties have not been commercialized due to their low grain yields. Mariam *et al*. (1991) reported that research on upland rice has been neglected because of the low and unstable grain yields, although it is widely grown in the interior parts of the country. The average yield of the upland rice is lower and this ranges from 0.46 to 1.1 tonnes ha^{-1}. The low grain yields of the upland rice is attributed to the poor management by the farmers during the cultivation period, where fields are left unattended after sowing without any monitoring on plant nutrients and other critical aspects, such as weeds, diseases, and insect-pest attacks. Therefore, with good management practices, the application of adequate plant nutrient and water, together with weeds, diseases, and insect-pests management, the grain yields of upland rice varieties are expected to increase. All these aspects are therefore important in obtaining higher yields. At present, the nutrient supply for upland rice comes from resultant ash and it may not be sufficient to complete its life cycle and produce better yields. Therefore, it is crucial to learn about the nutrient requirements of the upland rice and the fastest way to obtain this information is through analysis of soil and plant.

The results yielded from the analysis on soil and plant would provide information on the nutrient content in soil and plant, respectively. Nutrient partitioning in plant would be useful for further evaluation on the nutrient requirements of selected upland rice varieties. Furthermore, the field operations, such as soil preparation, irrigation, and applications of fertilizers, insecticides, herbicides, and harvesting are possible using machinery and modern technology on flat land, such as idle land, which are comparably difficult in hilly areas. Therefore, a large track of idle lands in Peninsular Malaysia (649,865 ha) can be developed for food production (*http://agrolink.moa.my*). Thus, the evaluation of upland rice varieties should be carried out to identify their potential for future commercialisation on idle lands. Therefore, the objectives of this study were (i) to determine the popular upland rice varieties and their agronomic characteristics based on a survey at several locations in Malaysia, and (ii) to determine the physico-chemical characteristics of the upland rice soils and the nutrient requirements of the plant.

MATERIALS AND METHODS

Selected Upland Rice Cultivation Locations

Several high-yielding upland rice cultivation locations in Sabah, Sarawak, and Peninsular Malaysia were identified with the assistance of the Department of Agriculture (DOA) and Malaysian Palm Oil Board (MPOB). Table 1 shows the list of upland rice fields sampled. The upland rice fields were located in hilly and sloping areas. The classification of upland rice soils is listed in Table 2.

Field Survey of Upland Rice

Basic information, such as the local names of upland rice varieties, grain yield, grain characteristics, and management practices, were recorded using an open-ended questionnaire, supplemented by informal talks with farmers and the observations carried out during planting and at harvesting time. The sampling location coordinates were recorded both in longitude and latitude, using Global Positioning System (GPS) with 15 metres accuracy (Model SILVA).

Soil Sampling

The soils (upland rice field and undisturbed forest areas) were randomly sampled at several points, using an auger at 0 – 20 and 20 – 40 cm depths. The forest soils adjacent to each upland

TABLE 1
The locations of the upland rice fields and their GPS coordinates from the selected locations in Malaysia

Marks[@]	Field locations	GPS coordinate	
		Latitude	Longitude
SSR1	Kg. Kujang Mawang, Tebedu	0°58.301^{N}	110°25.681^{E}
SSR2	Sg. Mujong, Kapit (Rh. Anding)	2°03.305^{N}	113°16.476^{E}
SSR3	Sg. Mujong, Kapit (Rh. Anding)	2°03.463^{N}	113°15.350^{E}
SSR4	Baleh, Kapit (Rh. Milang)	2°01.560^{N}	113°07.458^{E}
SSB1	Kg. Hamad, Tuaran	6°06.389^{N}	116°20.561^{E}
SSB2	Kg. Bonggol, Tuaran	6°06.737^{N}	116°24.454^{E}
SSB3	Kg. Timbang, Kota Belud	6°29.477^{N}	116°32.488^{E}
SSB4	Kg. Timbang, Kota Belud	6°29.522^{N}	116°33.324^{E}
SSB5	Kg. Tangkol, Kota Marudu	6°21.035^{N}	116°44.289^{E}
SSB6	Kg. Kiawayan, Tambunan	5°38.435^{N}	116°18.145^{E}
SSB7	Kg. Kiawayan, Tambunan	5°38.520^{N}	116°18.177^{E}
SSB8	Kg. Baru Jumpa, Tenom	4°56.722^{N}	115°52.966^{E}
SSB9	Kg. Baru Jumpa, Tenom	4°56.763^{N}	115°53.667^{E}
SPH1	RPS Betau, Kuala Lipis	4°16.138^{N}	101°41.019^{E}
SPH2	RPS Buntu, Raub	3°59.349^{N}	101°39.063^{E}
SPH3	Kg. Sg. Mai, Jerantut	3°51.051^{N}	102°20.075^{E}
SPH4	Lembah Kiol, Jerantut	3°52.216^{N}	102°19.118^{E}

[@] SSR1 = Kg. Kujang Mawang, Tebedu; SSR2 = Sg. Mujong, Kapit (Rh. Anding); SSR3 = Sg. Mujong, Kapit (Rh. Anding); SSR4 = Baleh, Kapit (Rh. Milang); SSB1 = Kg. Hamad, Tuaran;SSB2 = Kg. Bonggol, Tuaran; SSB3 = Kg. Timbang, Kota Belud; SSB4 = Kg. Timbang, Kota Belud; SSB5 = Kg. Tangkol, Kota Marudu; SSB6 = Kg. Kiawayan, Tambunan; SSB7 = Kg. Kiawayan, Tambunan; SSB8 = Kg. Baru Jumpa, Tenom; SSB9 = Kg. Baru Jumpa, Tenom; SPH1 = RPS Betau, Kuala Lipis; SPH2 = RPS Buntu, Raub; SPH3 = Kg. Sg. Mai, Jerantut; and SPH4 = Lembah Kiol, Jerantut.

rice field, which were not subjected to burning, were considered as the control for soil physico-chemical characteristics. The soils sampled at each depth were combined to give a composite sample for each area. These soil samples were air-dried, ground, and sieved to pass through a 2.0-mm sieve size. The samples were then kept in labelled plastic containers for further analysis.

Plant Sampling and Agronomic Variables

At harvest, three healthy upland rice hills from each location were randomly sampled. The following variables were measured: the number of tillers per hill, the number of panicles and the weight of roots, straw, panicles (with and without grains), grains (with and without panicles) at 14% moisture, 1000 grain weight and the percentage of unfilled grains. In the laboratory, these plant samples were separated into roots, straw, panicles, and grains. These samples were then oven-dried at 60 ° C for two days, weighed, and ground, to pass through a 2.0- mm sieve size. The samples were kept in the self-adhesive labelled plastic bags for further analysis.

Soil and Plant Analysis

The pH of soil was determined using the pH water (pH_W) and pH KCl (pH_{KCl}) methods at a ratio of 1: 2.5 soil to water; the total N in the soil was determined using the Kjeldahl method (Bremner 1960); the plant availability of P was

TABLE 2
The classification of the upland rice soils in Sarawak, Sabah and Pahang, Malaysia

Marks	Soil series/ Family	Description[@]
SSR1	Merit	Member of the family of fine, mixed, isohyperthermic, yellow Allik Tualemkuts, sedimentary rocks (shale, mudstone, sand stone), well to moderately well drained soils, fine sandy clay texture, argilic horizon, low base saturation, typically occur on rolling, hilly area (6 – 25˚ slopes) at elevations of less than 330 m (1,000 ft).
SSR2 SSR3 SSR4	Kapit Kapit Kapit	Member of the Kapit family, which is a fine-loamy, siliceous, isohyperthermic, Tipik Distroparadanks, weathered sedimentary rocks (West Sarawak granite, diorite, gabbro), within 50 cm of soil surface, moderately well to well drained soils, have a cambic horizon, low base saturation (< 50%), occur at hilly to steep terrain, steep slopes and the shallow soil depth (unsuitable for agriculture).
SSB1 SSB2	Tanjong Lipat Tanjong Lipat	Member of the Tanjong Lipat family which is a fine, loamy, siliceous, Tanjong Lipat isohyperthermic, red-yellow to yellow Allik Tualemkuts, mixed sedimentary rocks dominated by arenaceous material (sandstone/shale), deep well drained profiles with good permeability, fine sandy clay loam textures, brown to yellowish brown and an argilic horizon, occurs on rolling, hilly, and steep terrain (slopes in excess of 12% or 6^{o}) at an elevation of more than 50 m (150 ft), low CEC, low fertility status and erodability.
SSB3 SSB4	Laab Laab	Dystric cambisol, fine loamy, siliceous, isohyperthermic, yellow (sedimentary rocks), mudstone, sandstone and limestone, highly leached and low base saturation, brownish or reddish color of subsoil, well drained, loamy texture.
SSB5	-na	-na
SSB6	Kapilit	Typic Kandiudults, coarse-loamy, siliceous, isohyperthermic, red yellow (sandstone), deep well drained profile, have a kandic horizon, clay distribution pattern that decreased by more than 20% from its maximum within 100 cm of the mineral soil surface.
SSB7	Kumansi	Typic Paleudults, fine, mixed, isohyperthermic, red-yellow (mudstone/shale), deep and well drained profile, do not have a kandic horizon, clay distribution pattern that decreased by more than 20% from its maximum within 100 cm of the mineral soil surface.
SSB8 SSB9	Luasong Luasong	Dystric cambisol, fine, kaolinitic, isohyperthermic, red (sedimentary rocks), coastal and riverine alluvium, highly leached and low base saturation, brownish or reddish color of subsoil, well drained, loamy texture.
SPH1	-na	Schist, phyllite, slate and limestone. Sandstone and volcanic (rock type)
SPH2	-na	Schist, phyllite, slate and limestone. Sandstone and volcanic (rock type)
SPH3 SPH4	Jempol Jempol	Typic Paleudults, fine, kaolinitic, isohyperthermic, brown (tuffaceous shale, acid to intermediate volcanics), deep and well drained profile, do not have a kandic horizon, clay distribution pattern that decreased by more than 20% from its maximum within 100 cm of the mineral soil surface

[@] sources: S. Paramanathan (1998, 2000)
-[na] = information not available due to landform > 25^{o} slope

extracted using the Bray and Kurtz No. 2 method (Bray and Kurtz, 1945) with 2 g soil in 20 mL extractant (1:2; soil/solution ratio); the cation exchange capacity (CEC) and exchangeable bases (K, Ca, and Mg) were determined using the leaching method (Piper, 1950); the aluminium in soil was extracted using 1N KCl and the Al in the solution was measured using the AAS; iron in soil was extracted using the double acid method (0.05 M HCl with 0.0125 M H_2SO_4) at 1:5 soil/solution ratio; the total organic carbon of the soil samples was determined using a Leco® CR-412 T.O.C analyzer; the texture of soil samples was determined using the pipette method (Day, 1965); and the measure of soil moisture tension (pF) on soil dry weight basis was carried out using the pressure plate apparatus based on the soil dry weight basis at pF 0, 1, 2, 2.54, and 4.19. The available water capacity was determined by calculating the difference in the soil moisture content between pF 2.54 and 4.19. Meanwhile, the plant samples (leaves, grains, and roots) were determined using the wet digestion method (Thomas *et al*., 1967). The N, P, and K contents in the solution were measured using an autoanlyser (AA), whereas Ca, Mg, Fe, and Al were measured using the atomic absorption spectrometer (AAS).

Statistical Analysis

The analysis of variance (ANOVA) was carried out using the PROC ANOVA of the Statistical Analysis System (SAS, 2001). The Duncan's New Multiple Range Test (DMRT) was used for the comparison of the mean values when the treatment effects were significant.

RESULTS AND DISCUSSION

The Survey of the Upland Rice Cultivation

The current status

The survey of selected upland rice fields, carried out in October 2003 (first visit) and in February 2004 (second visit), showed that the upland rice cultivation in Malaysia was poor in terms of technology and innovation; therefore, most farmers still practiced shifting cultivation using the slash-and-burn technique.

Field location

Seventeen upland rice fields were selected at various locations in Pahang (4), Sarawak (4), and Sabah (9). The upland rice fields in Sarawak (Kapit) were located in remote areas and can only be accessed using a long boat. The other fields located in Sarawak (Tebedu), Sabah (Tuaran, Kota Belud, Tambunan, Tenom) and Pahang (Raub, Jerantut, Kuala Lipis) were easily accessible with a four-wheel drive vehicle. The topography of these selected upland rice fields varied greatly from the lowland areas to steep hills as well as on the mountain ranges (0 – 500 m). The landscape of most upland rice fields in South and Southeast Asia is level to gently rolling (0 – 80% slope) land (Greenland 1983). According to Mariam *et al*. (1991), the exploitation of forest for timber has probably encouraged cultivators to shift more often and into more remote areas than before, as they take advantage of the partially cleared forest left by the loggers for upland rice cultivation. The observations carried out during the survey indicated that large proportion of the hilly areas was still covered with secondary forest or under cultivation. The GPS coordinates of the selected upland rice fields are listed in Table 1.

Cultural practices

With the exception of Lembah Kiol, Jerantut (SPH4), Pahang, most farmers were found to still practice shifting cultivation (traditional system), in which forest land was cleared using the slash-and-burn technique, cultivated with rice for one or two years, and then abandoned for 3 to 5 years. Fallow is an essential component of the shifting cultivation because it permits a re-growth of the forest species and restores soil fertility. Shorter fallow period, ranging from 3 to 5 years, has been practiced by the farmers at all locations to fulfill the increasing food demands of an expanding population. According to Deegan (1980), the average fallow period of

the upland rice cultivation in Sarawak was 7 years, while 48% of the fields were left fallow for 5 years or less, 34% for 6 to 11 years, and 17% for 12 years or longer. This study showed that all the farmers followed the same basic procedures in the planting of upland rice, such as in land preparation, planting, maintenance, and harvesting. The farmers also intercropped upland rice with short-cycle crops, such as vegetables, cassava, banana, corn, and groundnut at the early growth stages (Table 3). Teng (1991) reported that upland rice farmers in Sarawak also planted maize, tapioca, pumpkin, cucumber, and ginger as the inter-crops in the same fields (Photo 1).

At all locations, seeding was done using a dibbler randomly at planting distances of 30 to 40 cm. Five to six seeds were dropped in each seeding hole which was covered with soil. According to Gupta and Toole (1986), shifting cultivation practices were simple and involved mostly hand tools. Rocks or stumps in the fields did not affect cultivation. This technique was practiced without imposing any disturbance to the ecosystem (Arraudeau, 1983) and able to reduce soil erosion as compared to mechanical clearing (Lal, 1982). The survey also showed that only three of the 17 farmers applied fertilisers by broadcasting methods, and this was found at Kg. Kujang Mawang, Tebedu (SSR1) in Sarawak, and at RPS Betau, Kuala Lipis (SPH1) and at Kg. Sg. Mai, Jerantut (SPH3) in Pahang (Table 3). In Tebedu, Sarawak, the farmers used amorfous (16% N and 48% P_2O_5) fertilisers obtained from the government under the fertiliser subsidy scheme (Table 3). The farmers were unwilling to mix chemical fertilisers with the rice seed because the seeds became wet and sticky, and this caused difficulties during the dibbling time.

The farmers at Kg. Kujang Mawang, Tebedu (SSR1), Kg. Timbang, Kota Belud (SSB3), and Kg. Sg. Mai, Jerantut (SPH3) used herbicides (Paraquat) during land preparation and after germination of weeds, and this was usually done a week after burning (Table 3). During upland rice growing season, weeding was done manually once or twice by the farmers without applying any chemicals. As an input cost-reduction measure, no pesticides and fungicides were applied.

At all locations, upland rice was harvested by hand. During harvesting, all family members and other farmers cooperatively took turn in rotation to expedite the activity (Photo 2). Most farmers used the grains previously harvested for home-consumption and only sold their surplus to earn extra income.

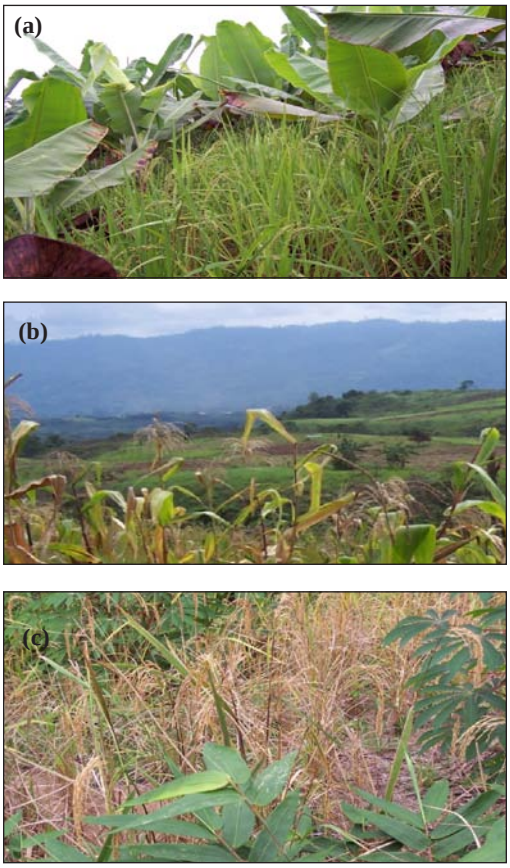

Photo 1: Intercropping of upland rice with other crops: a) Banana, b) Corn, and c) Tapioca

Varieties

The characteristics of upland rice, such as colours, fragrance, and shapes, were the main considerations for the farmers in selecting a

TABLE 3
Cultural practices used in the upland rice fields in several locations in Malaysia

Marks[@]	Cultivation technique	Other crops	Fertilisation	Herbicide
SSR1	Shifting	Corn, Cucumber	Amorfous	Paraquat
SSR2	Shifting	Corn	Nil	Nil
SSR3	Shifting	Corn, Cassava	Nil	Nil
SSR4	Shifting	Nil	Nil	Nil
SSB1	Shifting	Nil	Nil	Nil
SSB2	Shifting	Cassava, Banana	Nil	Nil
SSB3	Shifting	Cassava, Banana	Nil	Paraquat
SSB4	Shifting	Cassava, Banana	Nil	Nil
SSB5	Shifting	Cassava, Banana	Nil	Nil
SSB6	Shifting	Nil	Nil	Nil
SSB7	Shifting	Nil	Nil	Nil
SSB8	Shifting	Corn	Nil	Nil
SSB9	Shifting	Corn	Nil	Nil
SPH1	Shifting	Cassava	Nil	Nil
SPH2	Shifting	Cassava	Nil	Nil
SPH3	Shifting	Corn, Banana, Groundnut	NPK	Paraquat
SPH4	Permanent	Banana	Nil	Nil

[@] SSR1 = Kg. Kujang Mawang, Tebedu; SSR2 = Sg. Mujong, Kapit (Rh. Anding); SSR3 = Sg.Mujong,Kapit (Rh. Anding); SSR4 = Baleh, Kapit (Rh. Milang); SSB1 = Kg. Hamad, Tuaran; SSB2 = Kg. Bonggol, Tuaran; SSB3 = Kg. Timbang, Kota Belud; SSB4 = Kg. Timbang, Kota Belud; SSB5 = Kg.Tangkol, Kota Marudu; SSB6 = Kg. Kiawayan, Tambunan; SSB7 = Kg. Kiawayan, Tambunan; SSB8 = Kg. Baru Jumpa, Tenom; SSB9 = Kg. Baru Jumpa, Tenom; SPH1 = RPS Betau, Kuala Lipis; SPH2 = RPS Buntu, Raub; SPH3 = Kg. Sg. Mai, Jerantut; SPH4 = Lembah Kiol, Jerantut

Photo 2: Cooperation among farmers in all stages of upland rice activity: a) Harvesting of paddy, b) Assembling, and c) Threshing

certain rice variety for planting. In this study, a total of 35 varieties of upland rice seeds were collected from the upland rice farmers at various locations in Malaysia, including Sabah and Sarawak (Table 4). These varieties have been inherited from the previous generation of farmers.

The rice varieties were named by the farmers based on their characteristics, origin, or by maintaining the ancestral name. Therefore, the origin of a variety may be the same but it may have two different names at different locations or districts. Mariam et al. (1991) reported that the varieties with different names might sometimes belong to the same variety when some of these varieties were introduced to places with different ethnic backgrounds.

It is important to highlight the fact that farmers had several upland rice varieties in their collections (Table 4), but they only planted selected varieties (Photo 3). A particular variety of the upland rice was selected for the cultivation based frequently on their preferences, such as fragrance, taste, and texture of the rice (Table 5).

The survey showed that the cultivated upland rice varieties required 4 to 6 months to complete their growth and produce grain yields (Table 5). The growth cycle of the upland rice varieties varied with early maturing rice varieties (90 – 105 days), medium varieties (105 – 130 days), and late-maturing (130 – 150 days) (Jacquot and Courtois, 1987). Teng (1991) observed that almost all upland rice farmers in Sarawak planted late-maturing varieties (150 to 180 days) and these varieties have been selected for planting through generations.

Planting seasons

The upland rice planting season and the duration were found to greatly differ markedly according to the locations and rainfall distributions. Table 6 lists the planting duration of the selected upland rice fields. Basically, the farmers involved in the survey practiced the 'slash-and-burn' activity during the dry season for easier burning process. During the wet season, sowing of the upland rice seeds was continued as there was an adequate supply of soil moisture by the rains to promote seed germination. Rains during the early growth stage usually resulted in better yields. However, heavy rains could result in poor emergence because of seed loss through soil erosion. Lal (1982) reported that shifting cultivation followed a definite pattern, whereby forest was cleared

TABLE 4
The seeds of the upland rice collected during the field survey in Malaysia

Location	Variety
SSR1	Lawi
SSR2 and SSR3	Sarikei, Pulut Sibau, Ukir, Pulut Kawat, Gerung, Seribu, Lentik, Nibong, Sapunak, Kucing, Pakan, Sangking, Ngigit
SSR4	Sebilit, Menalam, Strao, Pulut Besar, Singut, Badang
SSB1 and SSB2	Kendinga, Kungkulob
SSB3 and SSB4	Sarawak, Dorok, But, Ageh
SSB5	Paulok
SSB6 and SSB7	Merah
SSB8 and SSB9	Kendinga, Keninga, Dusun
SPH1	Siam
SPH2	Kurau
SPH3	Liba pasir
SPH4	Siam

Photo 3: Some upland varieties in the fields

TABLE 5
The cultivated upland rice varieties, at the time of survey and the corresponding locations in Malaysia

Location	Variety	Growth cycle (month)
SSR1	Lawi	6
SSR2	Lentik	5
SSR3	Ukir	5
SSR4	Strao	6
SSB1	Kungkulob	5
SSB2	Kendinga	5
SSB3	But	5
SSB4	Dorok	5
SSB5	Paulok	5
SSB6	Merah	5
SSB7	Merah	5
SSB8	Kendinga	4
SSB9	Keninga	4
SPH1	Siam	5
SPH2	Kurau	5
SPH3	Liba pasir	5
SPH4	Siam	5

*SSR1= Kg. Kujang Mawang, Tebedu; SSR2 = Sg. Mujong, Kapit (Rh. Anding); SSR3 = Sg. Mujong, Kapit (Rh. Anding); SSR4 = Baleh, Kapit (Rh. Milang); SSB1 = Kg. Hamad, Tuaran; SSB2 = Kg. Bonggol, Tuaran; SSB3 = Kg. Timbang, Kota Belud; SSB4 = Kg. Timbang, Kota Belud; SSB5 = Kg. Tangkol, Kota Marudu; SSB6 = Kg. Kiawayan, Tambunan; SSB7 = Kg.Kiawayan, Tambunan; SSB8 = Kg. Baru Jumpa, Tenom; SSB9 = Kg. Baru Jumpa, Tenom; SPH1 = RPS Betau, Kuala Lipis; SPH2 = RPS Buntu, Raub; SPH3 = Kg. Sg. Mai, Jerantut; SPH4 = Lembah Kiol, Jerantut

TABLE 6
Planting duration of selected upland rice fields by district in Malaysia

Location	Preparation	Planting	Harvesting
Tebedu (SSR1)	August	September	March
Kapit (SSR2 and SSR3)	July	July/August	January/February
Tuaran (SSB1 and SSB2)	July	August	December/January
Kota Belud (SSB3 and SSB4)	July	August	December/January
Kota Marudu (SSB5)	July	August	January
Tambunan (SSB6 and SSB7)	July	August	January
Tenom (SSB8 and SSB9)	August	September	January
Kuala Lipis (SPH1)	July	August	January
Raub (SPH2)	August	September	February
Jerantut (SPH3 and SPH4)	November	December	April

*SSR1 = Kg. Kujang Mawang, Tebedu; SSR2 = Sg. Mujong, Kapit (Rh. Anding); SSR3 = Sg. Mujong, Kapit (Rh. Anding); SSR4 = Baleh, Kapit (Rh. Milang); SSB1 = Kg. Hamad, Tuaran; SSB2 = Kg. Bonggol, Tuaran; SSB3 = Kg. Timbang, Kota Belud; SSB4 = Kg. Timbang, Kota Belud; SSB5 = Kg. Tangkol, Kota Marudu; SSB6 = Kg. Kiawayan, Tambunan; SSB7 = Kg. Kiawayan, Tambunan; SSB8 = Kg. Baru Jumpa, Tenom; SSB9 = Kg. Baru Jumpa, Tenom; SPH1 = RPS Betau, Kuala Lipis; SPH2 = RPS Buntu, Raub; SPH3 = Kg. Sg. Mai, Jerantut; SPH4 = Lembah Kiol, Jerantut

in the dry season, the cut trees and bushes were left to dry and were burned just before the rainy season. Therefore, the correct timing to complete all major operations, such as land preparation and sowing is critically important, as the farmers will otherwise lose their food supply for the next year.

Rice yield

Upland rice is considered as a home-consumption crop. Therefore, most of the farmers have no record on the performance of their cultivated varieties. The farmers in Sabah and Sarawak used the term "karong" or "tong" to record their harvested yields. According to DOA, a full "karong" and "tong" contain about 50 to 60 kg and 30 to 40 kg grains, respectively. The farmers at each surveyed location stated that the yield of the upland rice was low and unstable. Similar results were also reported by Mariam *et al*. (1991), i.e. low and unstable grain yield were attributed to poor management, cultural practices, and the use of the local non-hybrid varieties. Available record on the surveyed upland rice yield was obtained for the Kendinga variety at Kampung Baru Jumpa (Tenom, Sabah), which was 25 – 30 "karong ha^{-1}" (1.8 tonne ha^{-1}). Benong *et al*. (1989) reported that the yield of 23 early-maturing upland rice varieties was between 1.2 and 3.8 tonne ha^{-1}. Therefore, the results of the survey concluded that the upland rice has the potential to produce higher yields. However, it is totally dependent on the varieties used, the size of fields, cultural practices, and field management, since the interaction of these factors has a great effect on the yield of upland rice.

Major constraints in the cultivation of upland rice

Blast and brown spot diseases and the symptoms of nutrient deficiency were the main problems observed in all locations (Photo 4). In addition, the competition was observed between weed and upland rice for the plant nutrients, water, sunlight, and space (Photo 5). The weed, insect-pest, and disease problems can be solved using the chemical methods for better results. Under

shifting cultivation, upland rice yield is low because the farmers do not apply any chemical fertilisers and are dependent only on the resultant ash from the burning process. Although the varieties have fewer tillers, the number of filled grains can be increased with the application of fertiliser and improved water management. The cultivation of upland rice can be considered as a high-risk activity because it is carried out in hilly and steep sloped areas. Therefore, the cultivation of upland rice in flat land area is less risky and all field management practices are easily applicable. Consequently, the yield and quality of rice grain can be improved.

Potential

This survey suggests that the collected upland rice varieties have not been fully exploited for commercial production. Furthermore, the majority of the upland rice farmers are dependent only on the nutrient in the soil without

Photo 4: The most common symptom of nutrient deficiency, and pest and diseases of upland rice

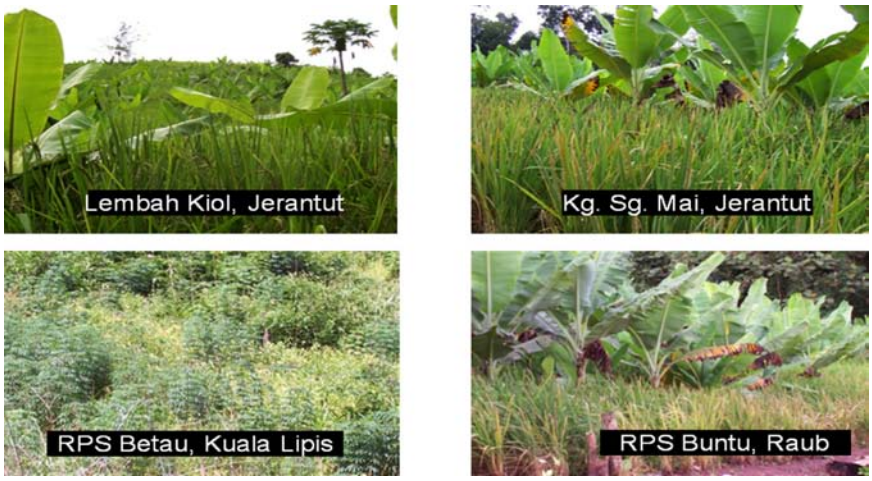

Photo 5: Competition between weeds and upland rice for plant nutrients, water, sunlight, and space in the field

the addition of any chemical fertilisers and their management practices are also not well organized. Therefore, with proper management practices, such as fertilisation, irrigation, weeds and disease management, the performance of upland rice varieties could be improved, particularly in terms of their grain quality and yields.

The Characteristics of Soil

With the exception of clay content, the mean sand and silt contents of upland rice soils were higher in the subsoil (20 – 40 cm depth) as compared to the topsoil (0 – 20 cm depth) (Table 7). The majority of the identified upland rice soils in the top part belong to the sandy clay loam (70%) texture, followed by the sandy loam (24%) and clay loam (6%) textures. Meanwhile, the proportion of the sandy clay loam texture was found to decrease to 18% and the sandy loam texture was increased to 59% for the subsoil. The control soil under virgin forest vegetation showed almost similar particle size distribution values as in the same soil under upland rice cultivation. With higher percentage of the sandy soils type, the upland rice soils are more permeable to air, water, and roots, which are suitable for crop growth, but the limitation includes lower water-holding capacities and poor retention of plant nutrients, due to the small surface areas of its particles. Therefore, adequate water and nutrient supply is crucial to achieve high crop productivity. The texture of the upland rice soils varied widely and this is because of the different parent materials and the degree of soil development. Most of the soils in the surveyed areas were developed from highly weathered sedimentary rocks, low base saturation, CEC, and fertility (Table 2). The soils from basic rocks are mostly clayey, while the soils from intermediate rocks are mainly coarse loamy near the surface and fine loamy to fine clayey in the subsoil (Moormann and Breemen, 1978). With higher distribution of sandy clay loam in the topsoil and sandy loam texture in the subsoil, the upland rice soils tend to have low water-holding capacity in the former than the latter. Moormann and Veldkamp (1978) stated that the abundant, sandy, coarse texture of West Africa upland rice soils limit their production because of the low water-retention capacity. The texture of the upland rice soils may strongly influence the mean percentage water content in the soils. The mean percentage water content was found to decrease with the increasing soil moisture tension, while the pF values were lower in the subsoil as compared to the topsoil. Fine-textured soils hold more water than the coarse-textured soils. Therefore, the higher clay percentage content in the top portion of the upland rice soil influences the mean percentage of the water content and the available water capacity (AWC). However, higher moisture condition, under forest vegetation, was attributed to a slightly higher AWC of these soils, as compared to the upland rice soils (Table 7).

The selected chemical characteristics of the upland and forest soils showed that the values of the topsoil were higher than the subsoil (Table 8). The means soil pH, as measured by pH_w and pH_{KCl} for the upland and forest soils, were similar (with the values of 4.3 and 3.3) for the former and the latter. The means C and N values were also similar for both soils (~3.5% C and 0.17%N). However, for P, K, Ca, and Mg, these were substantially higher in the upland rice than that of the forest soils. The status of the P content for the upland rice soils (28.5 mg P kg^{-1} soil) and the forest soils (24.4 mg P kg^{-1} soil) was higher, based on the routine soil chemical analysis interpretation carried out by Landon (1991). However, there were low, medium, and high for K, Ca, and Mg, respectively (Table 8). On the other hand, Fe and Al were substantially higher in the forest soils as compared to the upland rice soils (Table 8). Nevertheless, the values for Al were low (34.7 and 26.1 mg kg^{-1} soil) and Fe were higher (477.3 and 455.9 mg kg^{-1} soil) than those given by Landon (1991). In all the cases, the CEC of both soils were low at almost similar value (~10.0 $cmol_c$ kg^{-1} soil). Kato *et al*. (1999) reported that greater amount of ash, which was obtained from burning of 10-year-old forest vegetation, increased the soil pH up to 6.5 as compared to the 4-year-old

TABLE 7
Some physico-chemical characteristics of the upland rice and forest soils from various locations in Malaysia

Soil depth		Particle size distribution			Soil pF at		
		Sand	Silt	Clay	2.54	4.19	Available water
cm		%					
a) Upland rice soil							
0 – 20	Mean	59	16	24	28.44	14.47	14
	Max	77	27	40	46.51	28.42	18
	Min	35	8	6	16.07	6.48	10
20 – 40	Mean	62	23	15	28.83	12.24	15
	Max	75	36	26	41.22	21.99	19
	Min	38	13	5	15.24	6.67	9
b) Forest soil							
0 – 20	Mean	59	14	26	30.19	15.38	15
	Max	72	27	38	47.49	28.81	19
	Min	35	8	13	18.21	5.26	13
20 – 40	Mean	59	15	26	29.06	13.15	16
	Max	79	33	44	48.26	23.37	25
	Min	29	5	13	15.04	5.25	10

secondary vegetation (pH 6.0). The total N of the upland rice and forest soils can be considered as low and they showed similar characteristics of the upland tropical soils which are low in their content of nutrient. Therefore, burning of upland rice soil during land clearing did not result in any significant loss of the total N in the topsoil, but a slight increase in N was observed when a greater number of herbaceous leguminous plants capable of symbiotic N_2 fixation or fibrous-rooted plants (i.e. grasses) were burned (Pritchett, 1979). De Bano *et al*. (1998) reported that fire could also organically mineralise bound elements, such as N, P, and base cations, but the availability of these nutrients remained uncertain (Fisher and Binkley, 2000). According to Gupta and Toole (1986), the availability of nutrient in non-fertilised rice soils depends on the parent materials and the degree of weathering or soil formation. Therefore, the results of this study suggested that the slash-and-burn technique practiced by the upland rice farmers could provide certain plant nutrients and increase the pH of soil.

Agronomic Characteristics of the Upland Rice

Number of tillers

There were highly significant differences ($P \leq 0.05$) between the numbers of tillers of the upland rice varieties (Table 9). The number of tillers of selected upland rice varieties ranged from 10 to 18 tillers hill^{-1}. Lentik variety (SSR2) showed the highest number of plant (18 tillers hill^{-1}), whereas the lowest number of tillers was obtained by Strao and Kurau varieties (10 tillers hill^{-1}).

Number of panicles

There were also highly significant differences ($P \leq 0.05$) between the numbers of panicles of

TABLE 8
Selected chemical characteristics of the upland rice and forest soils from various locations in Malaysia

Soil depth		pH_w	pH_{KCl}	C	N	P	K	Ca	Mg	Fe	Al	CEC
cm				— % —		— mg kg^{-1} —						$cmol_c$ kg^{-1}
a) Upland rice soil												
0 – 20	Min	4.29	3.33	1.00	0.10	20.40	92.70	2.40	23.20	80.00	1.60	3.41
	Max	6.48	6.07	6.50	0.26	65.60	240.60	218.90	132.30	1085.00	79.60	15.51
	Mean#	5.21	4.02	3.54	0.17	28.51	152.28	50.71	68.90	455.88	26.05	10.45
20 – 40	Min	4.46	3.34	0.44	0.07	7.90	42.70	2.10	9.40	98.75	6.30	2.29
	Max	6.37	5.00	3.31	0.18	44.40	220.10	153.00	125.70	697.50	115.20	12.92
	Mean#	5.13	3.84	1.89	0.12	15.66	82.16	33.83	52.86	376.69	26.01	9.06
b) Forest soil												
0 – 20	Min	4.32	3.31	0.93	0.13	7.8	66.40	2.00	14.10	47.50	1.60	2.76
	Max	6.91	5.50	6.47	0.24	41.70	305.90	132.10	130.80	1010.00	92.50	16.94
	Mean#	5.10	3.96	3.48	0.17	24.43	111.49	37.64	55.84	477.28	34.72	10.83
20 – 40	Min	4.37	3.29	0.47	0.08	6.70	33.50	1.10	9.20	147.50	1.10	3.22
	Max	6.18	4.47	4.35	0.18	30.10	357.70	113.30	129.40	728.75	69.20	15.83
	Mean#	4.99	3.80	2.22	0.13	13.18	80.29	36.27	48.92	437.79	23.03	10.07

Note: # Mean = mean of 17 locations, = low, = medium, and = high based on routine soil chemical analysis interpretation by Landon (1991).

the upland rice varieties (Table 9). The number of the panicles of upland rice varieties ranged from 7 to 14 panicles hill^{-1} (Table 9). Lawi variety (SSR1) showed the highest number of panicles (14 panicle hill^{-1}), whereas Ukir variety (SSR3) showed the lowest number of panicles (7 panicles hill^{-1}).

Empty grains

There were highly significant differences ($P \leq 0.05$) between the empty grains of the upland rice varieties (Table 9). The empty grains ranged from 10 to 19% and these values correspond to Keninga (SSB9) and Ukir (SSR3) varieties, respectively.

Grain yields

There were highly significant differences ($P \leq 0.05$) between the grain yields of the upland rice varieties (Table 9). In this study, the grain yields of the upland rice varieties ranged from 21 to 50 g hill^{-1}. The highest (50.96 g hill^{-1}) grain yield was observed for Lentik variety (SSR2) and the lowest (21.34 g hill^{-1}) was obtained from Kungkulob variety (SSB1).

Grain yield per panicle

There were highly significant differences ($P \leq 0.05$) between the grain yields per panicle for the upland rice varieties (Table 9). The highest grain yield per panicle was observed for Liba pasir variety (SPH3), which was 5.92 g panicle^{-1}, whereas the lowest (2.25 g panicle^{-1}) yield was obtained from Dorok variety (SSB1).

Dry matter partitioning

The dry matter weight of each plant part of the upland rice varieties was found to remarkably vary (Table 10). Straw constituted the highest proportion of total dry matter, followed by grains, and roots; this suggests that straw is an important dry matter sink, particularly with the larger leaf blades of upland rice, which affect the photosynthesis efficiency and hence the production of dry matter. It was observed that the dry matter partitioning of upland rice varieties ranged between 44 and 61% (straw), 10 and 37% (roots), and 18 and 36% (grains). Meanwhile, the highest straw dry matter weight (121.25 g hill^{-1}) was obtained from Merah variety (SSB6) and the lowest (51.24 g hill^{-1}) from Kungkulob variety (SSB1). The highest dry matter percentage of the roots was obtained by Kungkulob variety (SSB1), which was 27% higher than the Lawi variety (SSR1). The results indicated that Kungkulob rice variety (43.15 g hill^{-1}) had a higher root surface area for nutrient uptake, as compared to Lawi variety (11.02 g hill^{-1}). It was also observed that the upland rice varieties, with a higher proportion of straw and roots dry matter, had a lower grain dry matter weight, indicating that the potential of several upland rice varieties to transfer the photosynthetic products from the panicles into spikelets is highly variable.

Therefore, selecting the upland rice variety, with a higher yield potential, is crucial so as to achieve reasonable grain yields. Lentik (SSR2), Merah (SSB6), and Liba Pasir varieties (SPH3) showed the highest total dry matter weights than other upland rice varieties with the values of 208.71, 198.63 and 169.15 g hill^{-1}, respectively. Among these selected upland rice varieties, Lawi variety (SSR1) had the highest harvest index (0.40), indicating its efficiency to produce grain yields as compared to the other varieties. As expected, a higher total dry matter weight per plant translates into a higher grain yield. This could be achieved by increasing the number of plants per hill or increasing the plant density. The analysis showed that increasing the productivity of the upland rice yield can be realised either by increasing the harvest index or improving the total biomass.

Nutrient partitioning

Highly significant differences ($P \leq 0.05$) were observed for the total nutrients (N, P, K, Ca, Mg, Fe, and Al) storage, between the three plant parts of the upland rice varieties. Specific nutrient partitioning in the dry matter of the upland rice varieties was measured (data did not present).

TABLE 9
Agronomic parameters of the upland rice varieties at harvest from various locations in Malaysia[@]

Location	Variety	No. of Tillers	No. of panicles	Empty grain	Yield^{GW}	Yield$^{A/Pc}$
		—— Tiller or Panicle hill^{-1} ——		— % —	— g hill^{-1} —	—— g panicle^{-1} ——
SSR1	Lawi	17 ab	14 a	16.40 abc	38.35 abc	3.23 cdefg
SSR2	Lentik	18 a	12 ab	12.60 bcd	50.96 a	3.99 bcdef
SSR3	Ukir	14 abc	7 b	19.51 a	24.29 cd	3.14 defg
SSR4	Strao	10 c	9 ab	16.74 ab	22.80 d	2.71 efg
SSB1	Kungkulob	11 c	9 ab	16.81 ab	21.34 d	2.43 fg
SSB2	Kendinga	11 c	9 ab	13.88 bcd	31.60 bcd	3.53 bcdefg
SSB3	But	11 bc	9 ab	14.34 bcd	32.04 bcd	3.50 bcdefg
SSB4	Dorok	12 bc	10 ab	12.57 bcd	22.60 d	2.25 g
SSB5	Paulok	-	-	-	-	-
SSB6	Merah	13 abc	11 ab	11.64 d	45.58 ab	4.18 bcde
SSB7	Merah	11 c	9 ab	12.00 d	45.18 ab	4.85 abc
SSB8	Kendinga	11 c	10 ab	12.24 bcd	32.63 bcd	3.24 cdefg
SSB9	Keninga	13 abc	10 ab	10.49 d	33.81 bcd	3.27 cdefg
SPH1	Siam	12 bc	8 b	10.65 d	40.68 ab	4.78 abcd
SPH2	Kurau	10 c	8 b	13.75 bcd	40.84 ab	5.11 ab
SPH3	Liba pasir	12 bc	8 b	13.06 bcd	47.30 ab	5.92 a
SPH4	Siam	11 c	9 ab	11.65 d	40.58 ab	4.80 abcd

[@] Means in a column with the same letters are not significantly different at 5% level by DMRT.
^{GW}= Gross weight $^{A/Pc}$=Average yield per panicle.

TABLE 10
Dry matter partitioning and harvest index of the upland rice varieties from various locations in Malaysia

Location	Variety	Straw	Root	Grain	Total	Harvest index	Total[@]
		g hill^{-1}					g plant^{-1}
SSR1	Lawi	57.01 (53.59)	11.02 (10.36)	38.35 (36.05)	106.37 (100)	0.40	6.26
SSR2	Lentik	116.34 (55.74)	41.40 (19.84)	50.96 (24.42)	208.71 (100)	0.30	11.59
SSR3	Ukir	77.65 (59.52)	28.51 (21.85)	24.29 (18.62)	130.45 (100)	0.24	9.32
SSR4	Strao	57.90 (55.43)	23.75 (22.74)	22.80 (21.83)	104.45 (100)	0.28	10.45
SSB1	Kungkulob	51.24 (44.28)	43.15 (37.29)	21.34 (18.44)	115.73 (100)	0.29	10.52
SSB2	Kendinga	61.02 (51.16)	26.65 (22.35)	31.60 (26.49)	119.27 (100)	0.34	10.84
SSB3	But	83.86 (58.68)	27.01 (18.90)	32.04 (22.42)	142.91 (100)	0.28	12.99
SSB4	Dorok	56.43 (52.09)	29.29 (27.04)	22.60 (20.86)	108.32 (100)	0.29	9.03
SSB5	Paulok	- -	- -	- -	- -	- -	- -
SSB6	Merah	121.25 (61.04)	31.80 (16.01)	45.58 (22.95)	198.63 (100)	0.27	15.28
SSB7	Merah	81.50 (53.88)	24.59 (16.26)	45.18 (29.86)	151.27 (100)	0.36	13.75
SSB8	Kendinga	53.85 (48.72)	24.05 (21.76)	32.63 (29.52)	110.53 (100)	0.38	10.05
SSB9	Keninga	61.91 (50.94)	25.82 (21.24)	33.81 (27.82)	121.54 (100)	0.35	9.35
SPH1	Siam	76.57 (51.64)	31.03 (20.92)	40.68 (27.43)	148.27 (100)	0.35	12.36
SPH2	Kurau	78.09 (50.93)	34.38 (22.43)	40.84 (26.64)	153.31 (100)	0.34	12.78
SPH3	L.Pasir	84.38 (49.89)	37.47 (22.15)	47.3 (27.96)	169.15 (100)	0.36	16.92
SPH4	Siam	72.21 (50.95)	28.94 (20.42)	40.58 (28.63)	141.73 (100)	0.36	12.88

[@]( ) = % of the total weight
Total = Weight per plant

$$\text{Harvest index: } \frac{\text{Grain dry matter}}{\text{Grain + Straw dry matter}}$$

The highest storage of the total plant nutrients was observed in straw, followed by roots and grains (*Fig. 1*). The results showed that more than 50% of the nutrients accumulated in straw before being transferred and used for grain production.

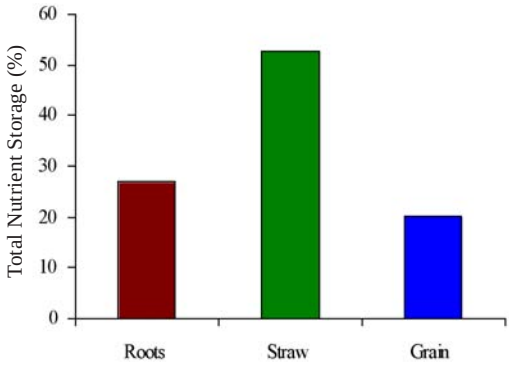

Fig. 1: Total nutrient storage in the dry matter of the upland rice varieties collected at several locations in Malaysia

Highly significant differences ($P \leq 0.05$) were observed in the nutrient partitioning in the total dry matter between the upland rice varieties (Table 11). The observed nutrients in the total dry matter of the upland rice were between 26 and 46% N, 6 and 13% P, 16 and 54% K, 0.30 and 5.6% Ca, 1 and 4% Mg, 2 and 7% Fe, and 3 and 21% Al, suggesting that the upland rice require higher N and K, as compared to the other nutrients to complete their growth cycle and produce grains. It was also observed that the partitioning of N and K in the upland rice varieties was an inverse relationship, since a deficiency in one or both nutrients could cause a yield loss. A balanced N and K fertilisation enhances the growth and improves the uptake of both nutrients, which in turn reduces nitrate losses, during and after the cropping season. The quality of the yield is also dependent on the NK ratio and the fertiliser grades (Marchand and Bourrie, 1998). However, the partitioning of P in the upland rice was less, as compared to N and K. The poor partitioning of P in the upland rice varieties may be due to the lack of soil available P. According to Pande (1994), the availability of P in the upland rice soil was lower than that of the flooded soils; hence, P deficiency may be a limiting factor in the upland soils, particularly in strongly acidic Oxisols.

The highest partitioning of N (46.77%) and Mg (4.07%) was observed in But variety (SSB3), whereas that for P (13.30%), K (54.15%), and Ca (5.60%) was observed in Lawi (SSR1), Kurau (SPH2), and Kendinga (SSB2) varieties, respectively. Dorok variety (SSB4) showed the highest Fe (7.61%) and Al (21.06%). Meanwhile, the lowest partitioning of N (26.42%) and Fe (2.05%) was observed in Kurau variety (SPH2), and that for K (51.67%) was observed in Dorok variety, and P (6.70%) in Merah variety (SSB6), while Ca (0.34%), Mg (1.85%) and Al (3.69%) was observed in Siam (SPH1), Siam (SPH4), and Lawi (SSR1) varieties, respectively (Table 11).

Nutrient uptake

There were highly significant differences ($P \leq 0.05$) in the nutrient uptake between the upland rice varieties (Table 12). The nutrient uptake (in g hill^{-1}) by the upland rice varieties was found to range between 0.7 and 1.9 for N, 0.19 and 0.50 for P, 0.45 and 2.55 for K, 0.01 and 0.17 for Ca, 0.05 and 0.14 for Mg, 0.05 and 0.23 for Fe, and 0.06 and 0.60 for Al. Santos *et al*. (1982) also reported that the uptake of N and K was the highest in the upland rice, followed by Ca, Mg, P, and S; whereas, the highest uptake for the micronutrient was Fe, followed by Mn, Zn, Cu, and B. The nutrient uptake varied with the different growth stages and this increased with the age of plant. Therefore, the timing of plant sampling had an effect on the results of the nutrient uptake. The highest N (1.97 g hill^{-1}), P (0.50 g hill^{-1}), and Fe (0.23 g hill^{-1}) uptakes were observed in Liba pasir (SPH3) variety, whereas the highest Ca (0.17 g hill^{-1}) and Al (0.60 g hill^{-1}) uptakes were observed in Merah (SSB6) variety. The highest uptake of K (2.55 g hill^{-1}) and Mg (0.14 g hill^{-1}) was observed in Kurau (SPH2) and Lentik (SSR2) varieties, respectively. Meanwhile, the lowest N (0.74 g hill^{-1}), P (0.19 g hill^{-1}), and Ca (0.01

TABLE 11
Nutrient partitioning in total dry matter of upland rice varieties at harvest from various locations in Malaysia[@]

Location	Variety	N	P	K	Ca	Mg	Fe	Al
					%[b]			
SSR1	Lawi	43.88 ab	13.30 a	30.77 ef	2.26 bcde	2.77 bc	3.31 def	3.69 g
SSR2	Lentik	34.92 cd	8.40 cde	43.14 b	2.44 bcd	2.93 b	2.93 def	5.21 fg
SSR3	Ukir	33.39 d	8.76 cde	42.20 bc	1.73 cdef	2.13 cde	3.78 cde	8.00 defg
SSR4	Strao	34.72 cd	10.41 bc	38.84 bcd	1.26 cdef	2.40 bcde	4.02 bcde	8.33 defg
SSB1	Kungkulob	45.97 a	8.19 cde	18.13 g	1.51 cdef	2.40 bcde	5.42 bc	18.35 ab
SSB2	Kendinga	31.82 de	9.57 bcd	28.73 f	5.60 a	3.05 b	5.72 b	15.49 bc
SSB3	But	46.77 a	11.46 ab	17.84 g	1.78 cdef	4.07 a	5.64 b	12.42 cd
SSB4	Dorok	43.42 ab	7.87 cde	16.55 g	0.89 def	2.57 bcd	7.61 a	21.06 a
SSB5	Paulok	-	-	-	-	-	-	-
SSB6	Merah	38.44 bcd	6.70 e	32.45 def	3.34 b	2.70 bc	4.25 bcde	12.09 cd
SSB7	Merah	36.75 cd	8.21 cde	42.30 bc	1.77 cdef	2.55 bcd	2.65 ef	5.74 fg
SSB8	Kendinga	35.81 cd	10.41 bc	39.46 bcd	2.70 bc	3.76 a	3.10 def	4.74 fg
SSB9	Keninga	40.97 abc	7.38 de	40.93 bc	1.13 cdef	2.81 b	2.68 ef	4.08 g
SPH1	Siam	36.03cd	8.96 bcde	37.95 bcd	0.34 f	1.98 de	4.14 bcde	10.57 de
SPH2	Kurau	26.42 e	7.78 cde	54.15 a	0.70 ef	1.93 de	2.05 f	6.92 efg
SPH3	Liba pasir	37.39 bcd	9.39 bcde	35.17 cdef	2.29 bcde	2.43 bcde	4.45 bcd	8.86 def
SPH4	Siam	37.95 bcd	8.33 cde	36.66 bcde	0.35 f	1.85 e	4.01 bcde	10.82 de

[@] Means in a column with the same letters are not significantly different at 5% level by DMRT

[b] $\% \text{ Nutrient} = \left[\frac{\text{Nutrient concentration} \times \text{Total dry matter weight}}{\text{Total nutrient in the whole plant}} \times 100\right]$

TABLE 12
Nutrient uptake by the different upland rice varieties at harvest from various locations in Malaysia[@]

Location	Variety	N	P	K	Ca	Mg	Fe	Al	Total[b]
		g hill^{-1}							
SSR1	Lawi	0.76 d	0.23 cd	0.52 fg	0.04 c	0.05 e	0.05 d	0.06 d	1.71 e
SSR2	Lentik	1.72 abc	0.40 ab	2.12 ab	0.12 ab	0.14 a	0.15 abcd	0.27 bcd	4.91 ab
SSR3	Ukir	1.05 d	0.28 bcd	1.37 cde	0.06 bc	0.07 de	0.12 bcd	0.25 abc	3.2 bcde
SSR4	Strao	0.74 d	0.22 cd	0.83 defg	0.03 c	0.05 e	0.08 d	0.18 cd	2.13 de
SSB1	Kungkulob	1.11 cd	0.19 d	0.45 g	0.04 c	0.05 e	0.13 abcd	0.45 abc	2.42 de
SSB2	Kendinga	0.77 d	0.23 cd	0.70 efg	0.14 a	0.07 de	0.14 abcd	0.37 abcd	2.42 de
SSB3	But	1.23 bcd	0.30 bcd	0.47 g	0.05 c	0.10 abcd	0.15 abcd	0.33 abcd	2.63 de
SSB4	Dorok	1.08 cd	0.21 d	0.45 g	0.02 c	0.06 de	0.21 abc	0.57 ab	2.60 de
SSB5	Paulok	-	-	-	-	-	-	-	-
SSB6	Merah	1.83 ab	0.31 bcd	1.47 bcd	0.17 a	0.13 abc	0.21 ab	0.60 a	4.72 ab
SSB7	Merah	1.30 bcd	0.28 bcd	1.56 bcd	0.07 bc	0.09 bcde	0.10 cd	0.21 cd	3.61 abcd
SSB8	Kendinga	0.75 d	0.23 cd	0.84 defg	0.05 c	0.08 cde	0.06 d	0.10 d	2.11 de
SSB9	Keninga	1.21 bcd	0.22 cd	1.21 cdef	0.03 c	0.08 cde	0.08 d	0.12 d	2.95 cde
SPH1	Siam	1.27 bcd	0.32 bcd	1.35 cde	0.01 c	0.07 de	0.15 abcd	0.37 abcd	3.54 abcde
SPH2	Kurau	1.24 bcd	0.37 bc	2.55 a	0.03 c	0.09 bcde	0.09 cd	0.32 abcd	4.69 abc
SPH3	Liba pasir	1.97 a	0.50 a	1.85 bc	0.12 ab	0.13 ab	0.23 a	0.47 abc	5.27 a
SPH4	Siam	1.24 bcd	0.29 bcd	1.36 cde	0.03 c	0.07 de	0.13 abcd	0.35 abcd	3.47 bcde

[@]Means in a column with the same letters are not significantly different at 5% level by DMRT
[b]Total = Total uptake

g hill^{-1}) uptakes were observed in Strao (SSR4), Kungkulob (SSB1), and Siam (SPH1) varieties, respectively. Kungkulob, and Dorok varieties showed the lowest K uptake (0.45 g hill^{-1} each), and the lowest uptake of Mg (0.05 g hill^{-1}) was observed in Lawi (SSR1), Strao (SSR4), and Kungkulob (SSB1) varieties. In summary, the total nutrient uptake of the selected upland rice varieties ranged from 1 to 5 g hill^{-1}, while the highest and lowest total nutrient uptakes were observed in Liba pasir (SPH3) and Lawi (SSR1) varieties, with the mean values of 5.27 g hill^{-1} and 1.71 g hill^{-1}, respectively. These values would be used as a basis for further evaluation in the glasshouse and in the field for fertiliser recommendation practices.

CONCLUSIONS

The cultivation of the upland rice crop is still an important activity for the rural community as it provides them with staple foods. Most upland rice farmers in Sarawak, Sabah, and Peninsular Malaysia are practicing a shifting cultivation using the slash-and-burn technique for land clearing. This is also used as a method to control weeds, insect-pest, and diseases. Three out of the 17 locations surveyed showed that the farmers applied fertiliser, such as amorfous (SSR1) and NPK fertiliser (SPH1 and SPH3), and used paraquat as herbicide (SSR1, SSB3 and SPH3). The number of tillers, panicles, and grain yields of the selected upland rice varieties ranged from 10 to 18 tillers hill^{-1}, 7 to 14 panicles hill^{-1}, 21 to 50 g grain yield hill^{-1}, respectively. Meanwhile, the uptake of N, P, and K in the upland rice varieties ranged from 80 to 211 kg N ha^{-1}, 20 to 53 kg P ha^{-1}, and 20 to 272 kg K ha^{-1}; these quantities would be used as a guide for fertiliser application rates in the glasshouse and field experiments. Ageh, Kendinga, and Strao varieties, which were early, medium, and late-maturing varieties, would respectively be selected for further evaluation on the nutrient requirements, using an idle land soil due to its growth cycle, productivity, and seed availability.

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Seed Germination and Proline Accumulation in Rice (*Oryza sativa* L.) as Affected by Salt Concentrations

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ABSTRACT

Plant cells accumulate praline as an osmoprotectant to conserve osmotic stability and prevent damage. However, the accumulation of proline may not be associated with tolerance to salinity. This study considered the influences of NaCl and Na_2SO_4 compositions, at different concentrations, on seed germination and accumulation of proline in eleven rice genotypes. Rice seeds were grown in petri dishes in the laboratory and treated with distilled water as a control and NaCl and Na_2SO_4 (1:1 molar concentration ratio) at 2.5, 5.0, 7.5 and 10 dS m^{-1} electrical conductivity for 14 days. The mean germination time (MGT) was positively affected by the increase in the concentration of salt. Conversely, there was a negative relationship between germination index (GI) and salt concentration. Shirodi, Fajr, and Shafag can be classified into salt tolerant group, while Tarom-e-Hashemi was identified as salt susceptible, based on MGT and GI. Slight changes were recorded within dry weight and water content of seedlings at different salt levels. The maximum accumulation of proline was observed at 5 dS m^{-1} salt concentration. No relationship was established between the accumulation of proline and the growth parameters.

Keywords: Rice genotypes, seed germination, proline accumulation, salt stress, salt composition

INTRODUCTION

Salinity is an agro-environmental problem limiting plant growth and development in most of the coastal, arid, and semi-arid regions of the world (Ashraf and Khan, 1994). Two major effects have been identified as the probable causes of high salt toxicity in crop plants, i.e. the osmotic and the ionic effects. Germination percentage is not affected by salt stress, but at the same concentration of salinity, significant differences in plant height and dry weight have been reported (Ahmad *et al.*, 2000). The biochemical response of plant cells to osmotic stress is the synthesis of special organic solutes (osmolytes) which accumulate at high cytoplasm concentrations (Serrano and Gaxiola, 1994). The cells of plant accumulate proline as an osmoprotectant to conserve the osmotic stability and prevent damages. Plants culture, under salt stress, shows a high accumulation of proline (Delauney and Verma, 1993; Roosens *et al.*, 1999). The understanding of the role of proline accumulation in salt-tolerant rice, under salt stress, is still unclear (Moons *et al.*, 1995;

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Lutts *et al.,* 1999). Salt treatment significantly increased the proline content of rice, but this also appeared to be a reaction to stress damage and was not associated with salt tolerance, because the contents of proline were higher in the more sensitive cultivars (Hoai *et al*., 2003). There is a general acceptance that under salt stress, many plants tend to accumulate proline as a defense mechanism against osmotic challenge by acting as a compatible solute (Liu and van Staden, 2000; Ghoulam *et al.,* 2002). It seems that rice accumulates proline as a symptom of injury, rather than an indicator of salt tolerance (Gracia *et al.*, 1997).

Rice is rated as an especially salt-sensitive crop (Maas and Hoffman, 1977; Shannon *et al.*, 1998). The response of rice to salinity varies with growth stage. In the most commonly cultivated rice cultivars, young seedlings were very sensitive to salinity (Flowers and Yeo, 1981; Heenan *et al.*, 1988; Lutts *et al.*, 1995). In particular, salinity causes a significant reduction in the growth of seedling, very soon after planting. As the duration of salinity stress increases, there is a significant reduction in the growth of seedling (Zeng and Shannon, 2000).

The major cations in salt-affected soils are Sodium (Na^+), Calcium (Ca^{2+}), Magnesium (Mg^{2+}), while the major anions are Chloride (Cl^-) and Sulphate ($SO4^{2-}$). Many studies carried out on studying salinity have utilized sodium chloride (NaCl) alone, or a combination of NaCl and calcium chloride ($CaCl_2$) salts. Since the soil solution is a mixture of solutes, the rice responses to the mixture of NaCl and Na_2SO_4, at different salt concentrations, would be investigated to approach a saline soil solution. Therefore, the objective of the present study were: (i) to determine the influences of both NaCl and Na_2SO_4 concentrations and the composition on seed germination and proline accumulation in eleven rice genotypes, and (ii) to examine the relationships between the content of proline and the physiological characteristics of the rice seedlings.

MATERIALS AND METHODS

Plant Materials

Rice seeds (*Oryza sativa* L., Japonica cultivar-group), from 11 genotypes, were obtained from Iran. These genotypes include Pouya, Shafag, Neda, Kadous, Tabesh, Tarom-e-hashemi, Sahel, Khazar, Shirodi, Fajr and Nemat, which were selected from the widely cultivated cultivars in Iran.

Growth Condition and Salt Treatments

Twenty rice seeds were placed on filter paper-lined petri dish of 9.0 cm diameter. Salt treatments of NaCl and Na_2SO_4 (1:1 molar concentration) were dissolved in distilled water, at 2.5, 5.0, 7.5 and 10 dS m^{-1} electrical conductivity. Distilled water was applied as a control. Twenty ml of this solution was applied to each petri dish. The experiment was conducted in the laboratory, using a completely randomized design (CRD) in 3 replications at room temperature ($27 \pm 2^{\circ}C$), and under dark condition for 14 days. The number of germinated seeds was counted for 7 days (i.e. from the 3rd to 9th day after soaking). Significant differences between the treatments were determined using the Tukey's studentized procedure.

Germination was observed daily according to the recommendation by International Seed Testing Association (ISTA, 1993). The mean germination time (MGT) was calculated according to the equation proposed by Ellis and Roberts (1981), as follows:

$$MGT = \frac{\sum Dn}{\sum n}$$

Where MGT is the mean germination time, n is the number of seeds, which were germinated on day D; D is the number of days counted from the beginning of germination.

Germination index (GI) was calculated as described in the Association of Official Seed Analysis (1983), using the following formulae:

$$GI = \frac{\text{No. of germinated seeds}}{\text{Days of first count}} + \text{.....} + \frac{\text{No. of germinated seeds}}{\text{Days of final count}}$$

Three seedlings from each replicate were randomly sampled at 14th day after soaking. Root, shoot length, and seedlings fresh weight were measured. Seedlings were dried in a forced-air oven (70˚C) for 72 h and then measured for their dry weights.

Proline was measured as described by Bates *et al*. (1973). Five hundred mg of shoot of seedlings were homogenized in 10 ml of 3% sulphosalicylic acid, and the homogenate was filtered through Whatman No. 2 filter paper. Two ml of the extract was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) for 1 h at 100˚C; the reaction was then terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene. The chromophore-containing toluene was collected and the absorbance was read at 520 nm. The amount of proline was determined from a standard curve and presented in µmol g^{-1} fresh weight.

RESULTS AND DISCUSSION

Seed Germination

In the experiment, the imposition of salt stress was found to significantly ($P \leq 0.01$) affect the growth of seedling. The results showed a significant difference in both rice genotypes, and salt treatments. Considerable effect due to salinity, genotype and their interaction was observed for most of the traits evaluated during the seedling stage (Table 1).

The results showed that salinity stress affected rice phenology but to different extents in different genotypes. The germination of rice seeds was significantly ($P \leq 0.01$) influenced by salinity (Table 1). Meanwhile, the imposition of salt stress showed that the MGT was significantly decreased by increasing the salt levels up to 5 dS m^{-1}, as compared to the control, and an upward tendency above 5 dS m^{-1} was recorded (*Fig. 1*). The lowest and the highest MGT were observed for Shirodi and Tarom-e-Hashemi genotypes, respectively (*Fig. 2*).

Germination index (GI) and final germination percentage (FGP) decreased by increasing the salinity level, while the genotypes were found to respond differently to salinity (*Fig. 3*). There

TABLE 1
Analysis of variance of genotype (G), treatment (T) and their interactions (G×T) for the mean germination time (MGT), germination index (GI), final germination percentage (FGP), root length and shoot height, seedling dry matters, water and proline contents

Dependent variables	Independent variables		
	G	T	G × T
MGT (day)	57.0**	95.1**	4.43**
GI	39.0**	64.2**	2.96**
FGP	3.88**	4.78**	1.31ns
Root length (cm)	24.3**	30.24**	1.8**
Shoot height (cm)	11.8**	52.6**	0.95ns
Dry weight	28.4**	9.35**	1.0ns
Water content (%)	5.56**	4.04**	0.18ns
Proline (µmol g^{-1}FW)	13.1**	243.5**	16.0**

Numbers represent F-values at 1% level; ns: not significant; ** $P \leq 0.01$

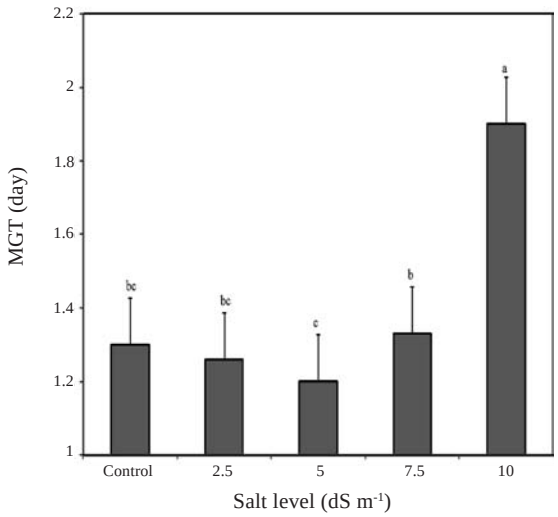

Fig. 1: The mean germination time at five different salt concentrations. Each value is the mean of eleven genotypes, with three replications and vertical bars represents SE

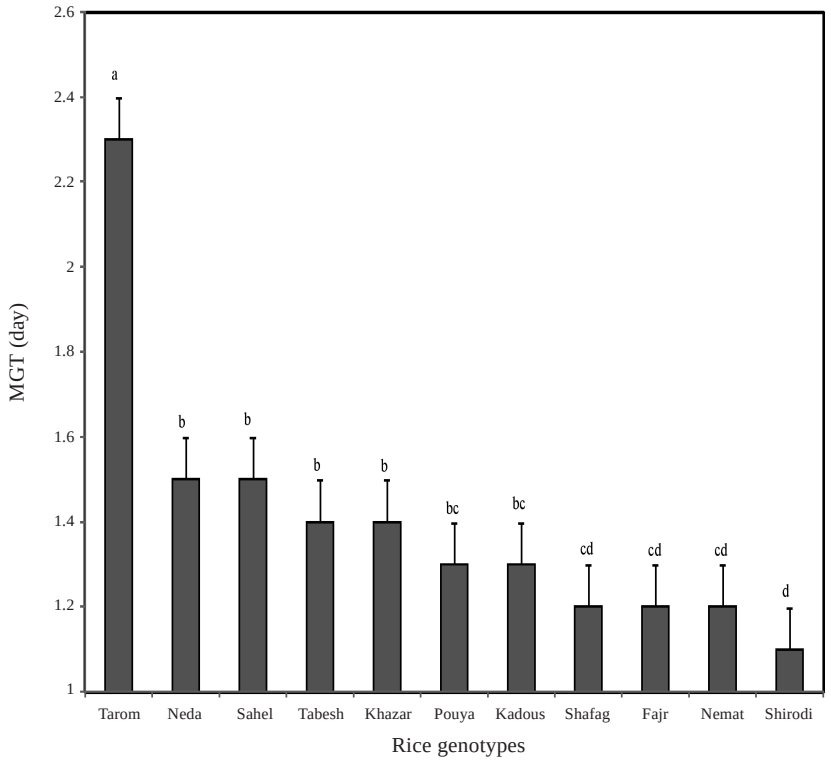

Fig. 2: The effect of salinity on the mean germination time of eleven rice genotypes. Each value is the mean of five salt levels and three replications and vertical bars represents SE

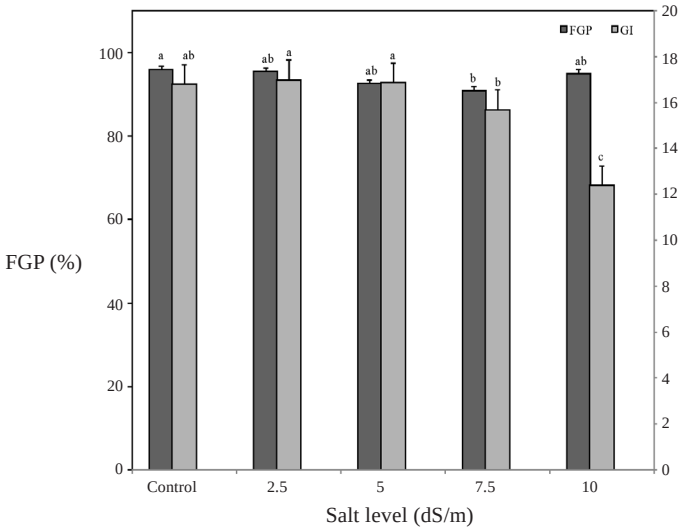

Fig. 3: The effects of salinity on germination index. The value is the mean of eleven genotypes, with three replications and vertical bar represent SE

was no significant interaction between the FGP and the rice genotypes (Table 1). This result was similar to the results obtained by Ahmad *et al*. (2000).

The highest and the lowest GI were recorded for Shirodi and Tarom-e-Hashemi genotypes, respectively. Tarom-e-Hashemi had the lowest FGP, while Shirodi and Fajr genotypes demonstrated the highest FGP (*Fig. 4*).

Salinity affects the germination of seeds by creating an external osmotic potential which prevents water uptake. Mechanism of salt tolerance in plant is divided into two categories, namely avoidance and tolerance against salt. In the present study, significant difference in the salt sensitivity was observed at the germination stage of the eleven genotypes tested (*Figs. 2* and *4*). The salt tolerant genotypes showed a faster growth rate under saline conditions (Walia *et al.*, 2005). Meanwhile, the plant growth can decline steadily when external salinity increases. Consequently, the tolerant plants elevate their germination rate to avoid the salinity stress. Both the MGT and GI can be acceptable parameters to be used in evaluating salt tolerance during germination stage because the genotype, which is salt tolerant, has the lowest MGT and the highest GI. Based on the MGT and GI, 11 genotypes can be assigned into three groups, tolerant (with the lowest MGT and the highest GI), semi-tolerant, and sensitive (with the highest MGT and lowest GI). Shirodi, Fajr and Shafag genotypes showed the lowest MGT and the highest GI; therefore, they can be classified into salt tolerant group. Meanwhile, Tarom genotype (with the highest MGT and lowest GI) is categorized into the salt sensitive group (*Figs. 2* and *4*).

Seedlings Growth

Rice seedling growth were significantly ($p \leq 0.01$) influenced by the levels of salt it contained. The growth of rice seedling was evaluated by root measurements, shoot length, and dry matter. The maximum rice seedling growth occurred at 2.5 dS m^{-1}. The data showed that the length of roots of all genotypes was reduced with the increasing salt concentrations. In general, Fajr and Tabesh genotypes had the highest and lowest root lengths, respectively (*Fig. 6*). The results revealed that the length of shoot of the rice seedlings increased at 2.5 dS m^{-1}, and above this concentration, no significant differences were

TABLE 2
The correlation between phenological characteristics and proline content at early seedling stage

	Root length	Shoot height	Dry weight	Water content	Proline content
Root length	0	0.55 **	-0.46 **	0.23 **	-0.13 ns
Shoot height		0	-0.38 **	0.44 **	-0.03 ns
Dry weight			0	-0.25 **	-0.01 ns
Water content				0	-0.04 ns
Proline content					0

Results are r values of eleven rice genotypes, five salt concentrations with three replications
** and *: significant different at 1%, 5% level; ns: not significant

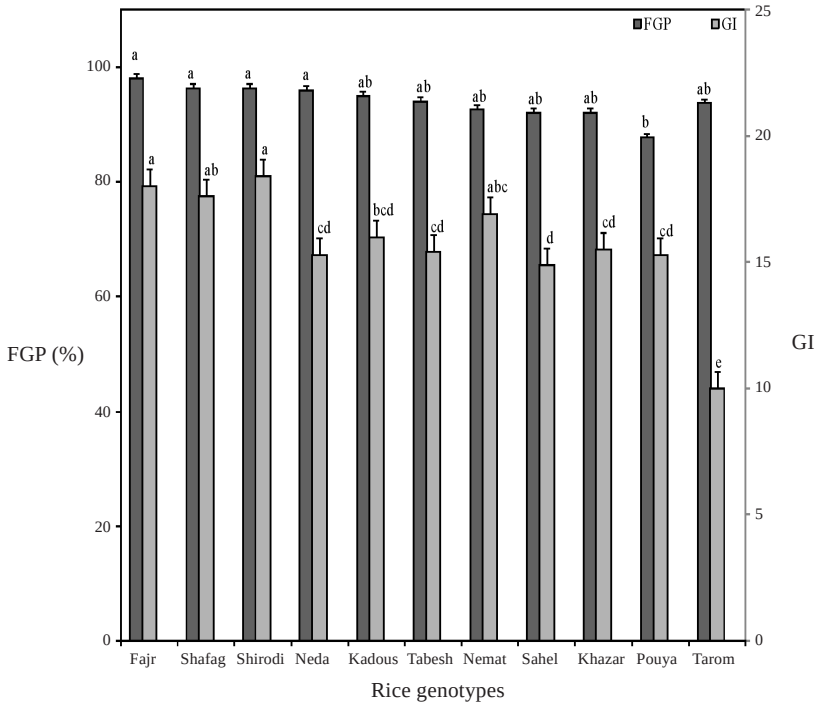

Fig. 4: The effects of salt stress on the germination index of eleven rice genotypes. Each value is the mean of five salt treatments, with three replications and vertical bar represents SE

observed (*Fig. 5*). Eight out of the 11 genotypes showed the lowest shoot length, while Fajr genotype was found to have the highest shoot length (*Fig. 6*).

Moreover, the dry weight and percentage of water content in rice seedling genotypes were significantly affected by salinity. There was a slight increase in the dry weight when the

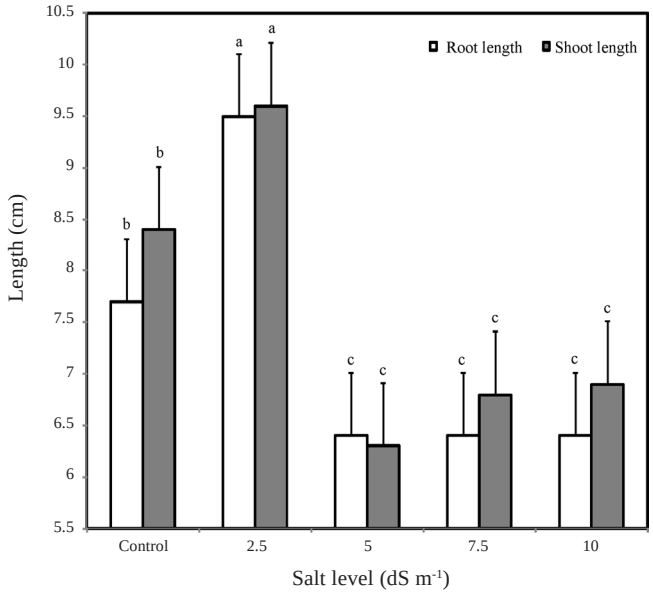

Fig. 5: The lengths of root and shoot at different salt concentrations. Each value is the mean of three replications and vertical bar represents SE

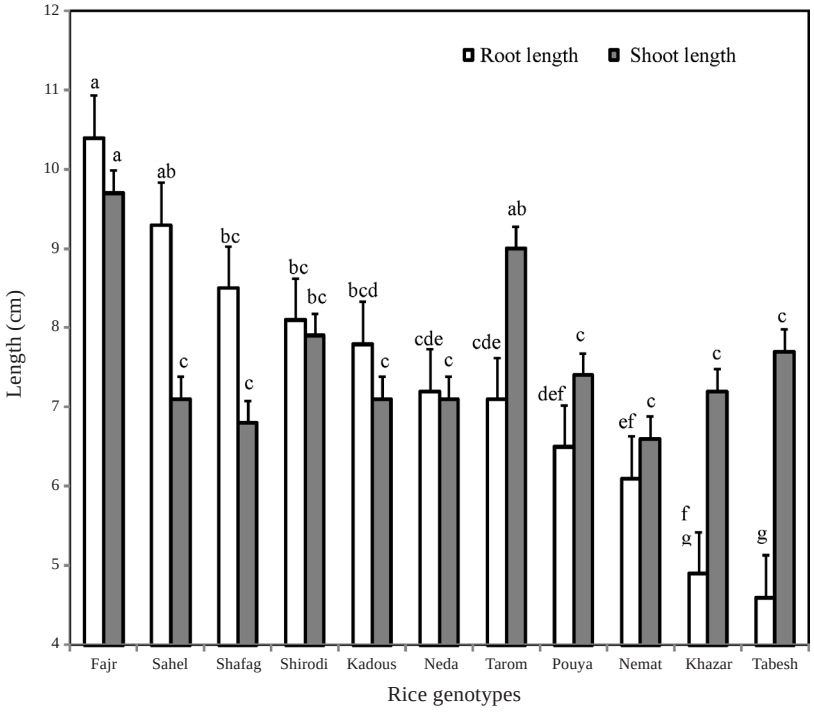

Fig. 6: The root length and shoot height of eleven rice genotypes. Each value is the mean of five salt concentrations, with three replications and vertical bar represents SE

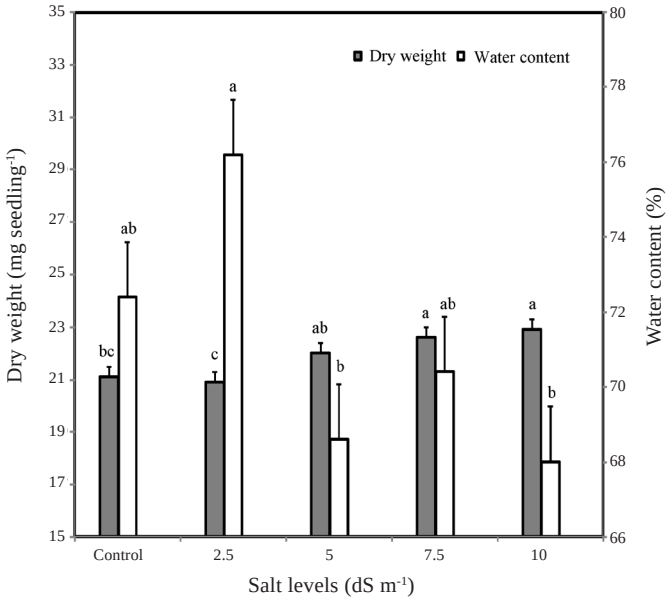

Fig. 7: The percentage of the dry weight and water content at different salt concentrations

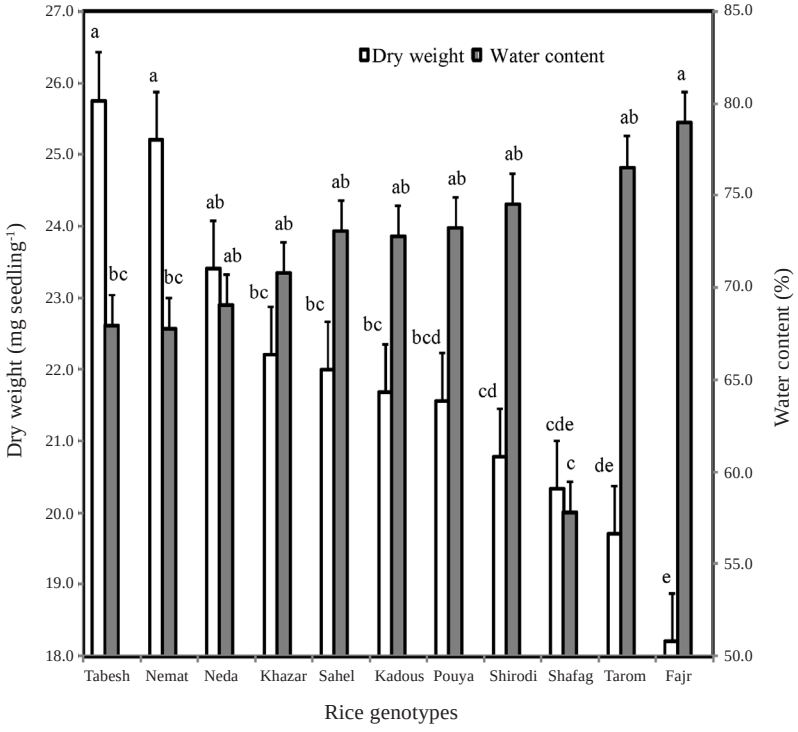

Fig. 8: Dry weight and water content of eleven rice genotypes at seedling stage

salinity stress increased. As the concentration of salinity increased, a significant increase in the water content occurred at lower salt level, thereafter, a decrease was observed (*Fig. 7*). Tabesh and Fajr genotypes demonstrated the highest and the lowest dry weights, respectively. In addition, they also showed the lowest and the highest water contents, respectively (*Fig. 8*). The decrease in the dry weight is accompanied by the increase in the water content in seedling after the salt treatment. The results proved that there was a negative relation between the dry weight and the water content under salt stress (Table 3).

The comparison of the data derived from germination and seedling stages revealed that Fajr genotype, which was found with the longest root, the highest shoot and water content, can be classified into the salt tolerance group at its seedling stage. It seems that Fajr genotype is salt tolerant, not only at germination stage but also at the seedling stage and it could adapt well to salinity condition. However, Tarom-e-Hashemi is salt sensitive at germination stage and it could tolerate at seedling stage.

Proline Content

The content of proline in rice seedlings was differently altered with the increase in salt concentration. There was only a slight increase in the content of proline at 2.5 dS m^{-1} as compared to the control. Generally, rice seedlings tend to accumulate proline at 5 dS m^{-1} and a downward trend beyond 5 dS m^{-1} was observed (*Fig. 9*). The considerable decrease in the content of proline at 10 dS m^{-1} might be due to the inhibition of the seedling growth by salt stress (Table 3). The highest and the lowest amounts of proline were found in Shafag and Fajr genotypes, respectively (*Fig. 10*).

In addition, proline also plays a role as an osmoprotectant to adjust osmotic stability; it was expected that the accumulation of proline was associated with the percentage of water content, as compared to the control especially at 10 dS m^{-1}. However, no significant correlation among the physiological parameters and proline content was found. It seemed that salt stress provoked rice genotypes to different extents. The accumulation of proline in Shafag genotype

TABLE 3
The relationship between proline content and shoot length

Rice Genotype	Proline (µmol g^{-1}FW)			Water Content (%)		
	Control	EC=10	Change%	Control	EC=10	Change%
Fajr	#0.58a	0.23b	-62.1	79.3a	75.3a	-5.0
Kadous	0.59a	0.02b	-96.6	73.6a	67.9a	-7.7
Khazar	0.75a	0.16b	-78.7	75.6a	68.1b	-9.9
Neda	0.53a	0.14b	-73.6	70.6a	65.3a	-7.5
Nemat	0.45a	0.01b	-98.7	69.8a	62.9a	-9.9
Pouya	0.67a	0.02b	-97.0	74.3a	68.8b	-7.4
Sahel	0.45a	0.01b	-98.7	73.4a	72.1a	-1.8
Shafag	0.69a	0.05b	-92.7	57.7a	57.7a	0
Shirodi	0.63a	0.01b	-98.4	75.0a	72.0a	-4.0
Tabesh	0.65a	0.02b	-96.9	70.5a	66.7a	-5.4
Tarom	0.60a	0.09b	-85.0	77.0a	71.7a	-6.9

Results are the mean values of three replications. The values with the same letter in a row are not significantly different

$$\text{Change\%} = \left[\left(EC_{10} - \text{Control}\right)/\left(\text{Control}\right)\right] \times 100$$

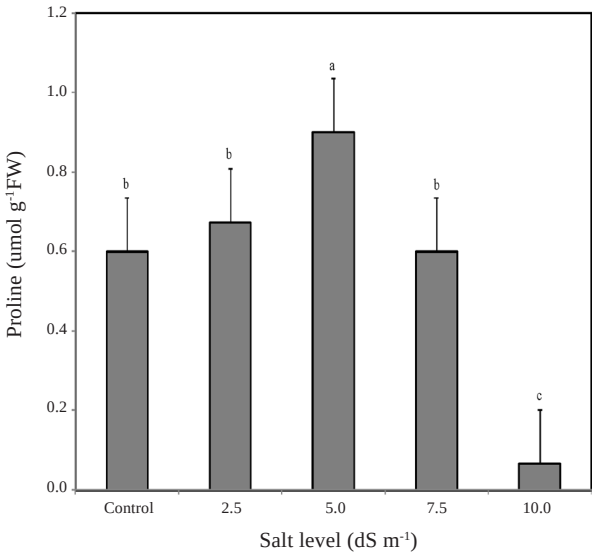

Fig. 9: The comparison of the proline content of rice seedlings at different salt concentrations

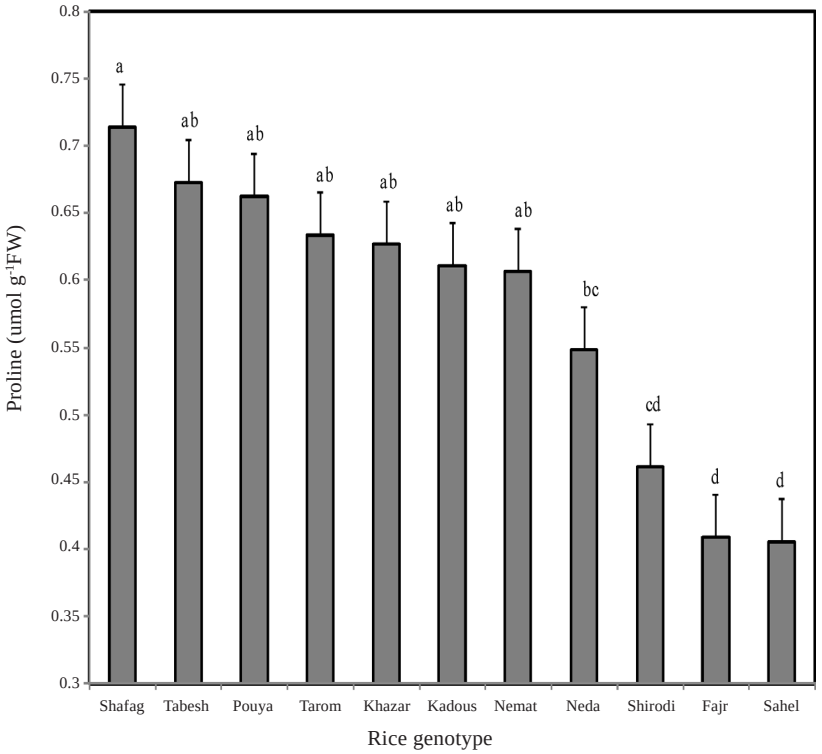

Fig. 10: The accumulation of proline in eleven rice genotypes. Each value is the mean of five salt concentrations, with three replications and vertical bar represents SE

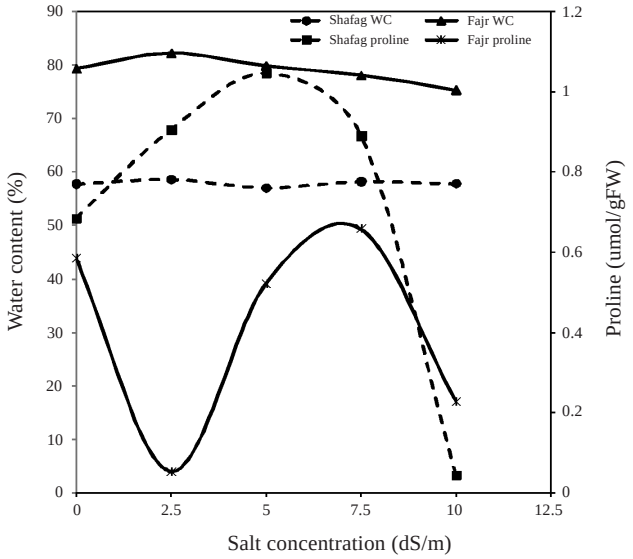

Fig. 11: The effect of salt levels on the contents of water and proline in Shafag genotype as salt susceptible and Fajr genotype as salt tolerant at the early seedling stage

as a salt susceptible followed a regular pattern, as compared to Fajr genotype as a salt tolerant. Fajr genotype also tended to accumulate proline less than Shafag genotype (*Fig. 11*). This result was similar to the ones obtained by Luttus *et al.* (1996). Therefore, the content of proline did not play a functional role to enhance water uptake by rice seedlings, at least in these rice genotypes (Tables 2 and 3). This conclusion is in agreement with the previous report by Khedr *et al.* (2003).

Salt stress can affect the synthesis of organic compatible solutes such as proline, betaine and soluble sugars (Phap *et al.*, 2006). The accumulation of proline has been reported in many plants under salt stress. However, there are reports which indicate that salt tolerant cultivated rice accumulates less free-proline than salt sensitive ones (Lutts *et al.*, 1996). Jain *et al.* (2001) suggested that salt stressed plants accumulate increased amount of low-molecular weight water-soluble metabolites, like proline and betaines, in their cells, possibly for osmotic adjustment. However, it is not clear whether the accumulated proline is involved in ameliorating salt tolerance because the accumulation was the highest in more sensitive genotypes (*Fig. 10*). The result suggested that the increase in proline content might not be associated with salinity tolerance, but rather with the extent of damage encountered by salt stress, as shown by the greater increase in the content of proline in the susceptible genotypes. This conclusion is confirmed by Hoai *et al.* (2003).

CONCLUSIONS

Increasing salt levels had detrimental effects on germination percentage, root and shoot length, water content and seed germination index of the rice genotypes tested. In particular, Fajr and Shirodi genotypes as salt tolerant group and Tarom-e-Hashemi genotype as salt susceptible were classified based on their MGT and GI. However, the relationship between the growth parameters and MGT and GI was not found. The salt tolerant and salt sensitive groups showed that there was no significant relationship between proline and salt tolerant, at least in these rice genotypes, because proline content also increased in salt tolerant.

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Upland Rice Root Characteristics and Their Relationship to Nitrogen Uptake

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ABSTRACT

Nitrogen and phosphorus efficiencies are the main constraints to food production in the sub-humid and humid tropic soils. A laboratory and glasshouse study was initiated to examine the differences in the root architecture of Malaysian upland rice landraces and relate them to efficiency of the nitrogen fertilizer uptake. Six upland rice landraces, obtained locally, were soaked in water and allowed to germinate using the cigar role method. The seedlings were fertilized with a complete nutrient solution daily and the roots which were allowed to develop after 14 days were measured using the WINRHIZO. The same landraces were planted in the glasshouse in polybags containing 25 kg soil in four replications. N-15 labelled urea was applied at 170 kg N/ha and N use efficiency was measured at harvest. Significant differences in root length, surface area, root volume, average root diameter, and number of forks, between the 6 landraces were studied. Nitrogen in the plant (derived from fertilizer applied) was found to range from 6.22 – 27.6%. Nevertheless, a poor correlation was obtained between the length of root and the dry matter yield and the total N uptake. Five of the landraces tested showed a good potential in taking up the fertilizer N applied.

Keywords: Upland rice, root architecture, nitrogen fertilizer, nitrogen use efficiency, nitrogen derived from fertilizer

INTRODUCTION

Upland rice is grown by subsistence farmers on approximately 20 million hectares in the sub-humid and humid tropics, generally on infertile, strongly weathered soils. The main constraint to food production in these soils is the deficiency of N and P. The low yield of upland rice obtained is largely a consequence of its production being limited to infertile or drought-prone upland soils and to a low harvest index (HI) of traditional cultivars (George *et al*., 2001). IRRI (2000) reported the importance of N input in determining yield. It has been shown that root architecture, the spatial configuration of a root system in the soil, varies between and among species, and plays an important role in below-ground resource acquisition (Lynch, 1995).

Various strategies have been developed to synchronize plant N demand and N supply from soil and fertilizers. These include proper timing, rate, placement, and the use of modified form of fertilizer (IAEA, 2006). Modifying the soil-plant environment with fertilizers and amendments may not be the most practical or economical solution to address all the mineral nutrient problems in plants and soils. On the

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contrary, genetic selection and plant breeding techniques have been used to develop rice varieties which are resistant to diseases and adverse environmental conditions, such as drought, nutrient deficiencies, toxicities, and salinity. However, genetic selection to improve the efficiency of N use in the upland rice crop has not been carried out. In addition, little information is available on the upland rice cultivar differences in response to N fertilization in Asia (Saito *et al*., 2006).

Our study was aimed at identifying the genotypic differences in soil and fertilizer N-use efficiency and the relationship between the seedling root characteristics to yield.

MATERIALS AND METHODS

Six upland rice landraces obtained locally (AN 1334A, AN 753, AN 582, and AN 1084 obtained from the Department of Agriculture, Sabah, and Bertih and Satang obtained from Jerantut, Pahang) were soaked in water and allowed to germinate between two sheets of moistened filter paper, placed on a water-proof brown paper, rolled up like a cigar (cigar role method) and placed in a standing position in a plastic container. The seeds were sprayed with complete Hoagland nutrient solution twice daily (morning and evening) and allowed to grow for 14 days. The roots which developed after 14 days were scanned using the WINRHIZO. The root parameters, such as their length, diameter, volume, and number of root tips were recorded. The same landraces were planted in the glasshouse in polybags containing 25 kg soil in four replications. The soil used was Munchong series (Typic hapludox) with the following properties: pH of 4.38, Bray-1P of 4.16 mg/kg and total N of 0.16%. An equivalent rate of 100 kg P/ha as triple super phosphate (20% P) was mixed into the top 20 cm of the soil prior to seed planting. Nitrogen and potassium fertilizers were applied at an equivalent rate of 170kg N, and 150 kg K/ha applied in three split applications at 10, 45 and 60 days after planting. The source of nitrogen used was ammonium sulphate (21% N), which was enriched with 5% N-15 atom excess. Potassium was applied as muriate of potash (50% K). The seeds were planted on 20th November 2007 and harvested on 10 April 2008 (i.e. 140 days after planting).

The straw and grains were weighed and sub-sampled. The sub-samples were dried in an air-forced oven at 70˚C, until constant weights were achieved. They were ground to pass through a 1 mm sieve size and stored in plastic containers. Total nitrogen was determined using the Kjeldahl method (Bremner and Mulvaney, 1982) and the N-15 enrichment of the samples was determined using the emission spectrometer at the Malaysian Nuclear Agency. The analysis of variance for the data obtained was carried out using the SAS version 9.0 package and the comparison of the means was made using the studentised Tukey method.

RESULTS AND DISCUSSION

Root Characteristics

The two landraces obtained from Pahang showed significantly longer roots than those obtained from Sabah (Table 1). Significantly lower root surface area and the root volume were observed for AN 1334. Nevertheless, there were no significant differences between the landraces for the number of root tips produced.

It has been known that plant root systems are highly plastic in their development and can adapt their architecture in response to prevailing environmental conditions (Zhang and Forde, 1998 and Zhang *et al*., 1999). In *Arabidopsis*, it has been shown that uniformly high nitrate (10 mM) suppresses lateral root development, while for plants grown at low levels of nitrate (10 μM), and when a section of the primary root was exposed to a high nitrate level, lateral root production was stimulated specifically in that area. The main effect of nitrate appears to be on the rate of the lateral root elongation rather than on the lateral root initiation. The elongation rate of the primary root is identical at 10 μM and 10 mM nitrate and the metabolism of nitrate is not required for the architectural changes (Zhang and Forde, 1998). Since metabolism of nitrate

TABLE 1
Root characteristics of the upland landraces, 14 days after germination

Upland rice landraces	Root length	Root Surface Area	Root Volume	No. of root tips
	cm	cm^2	cm^3	
AN1334A	72.36 c	6.96 b	0.053 b	818.3 a
AN753	97.37 b	10.79 a	0.096 a	1026.7 a
AN582	91.55 b	11.56 a	0.117 a	951.7 a
AN1084	93.09 b	12.34 a	0.131 a	1017.3 a
SATANG	114.53 a	13.28 a	0.125 a	791.3 a
BERTIH	102.60 ab	12.74 a	0.129 a	1090.3 a

is not required for the root architectural changes, the differences observed in the root length of the upland rice landraces shown must be due to their genetic differences.

Agronomic Characteristics

The agronomic parameters which showed significant differences among the upland rice landraces were only observed for plant height and panicle length (Table 2). AN 1334A, Satang, and Bertih showed no significant differences in their height but they provided the tallest plant. AN 1084 was about 10 cm shorter, while AN 753 and AN 583 were about 20 cm significantly shorter than the tallest plant. The plant height obtained was much taller than those grown in the field, as described by the Department of Agriculture, Sabah. This was due to the lower light intensity available in the greenhouse, where the experiment was carried out. The date to maturity was at 140 days while under field conditions, these landraces upland rice could be harvested at 110 to 150 days.

The straw yield obtained from Satang was the highest amongst the 6 landraces tested, while the grain yield was lowest. All the other landraces showed a similar grain yield (Table 3). The total N yield was also highest in Satang, due to the high straw yield produced. The weight/1000 seeds (Table 2) showed similar values, except for Bertih.

The N contents in grain were similar for all the landraces used, while for the N content

TABLE 2
Agronomic parameters obtained from the harvested upland rice

Upland rice landraces	Plant height	No of tillers/pot	No of panicles/pot	Panicle Length	No of Spikelet/ panicle	Weight/1000 grains
	cm			cm		g
AN1334A	151.8 a	6.75 a	10.25 a	26.49 ab	254 a	23.15 a
AN753	133.4 b	9.25 a	13.00 a	25.21 ab	252 a	21.61 a
AN582	134.9 b	7.75 a	10.75 a	27.88 ab	136 a	24.96 a
AN1084	142.3 ab	4.25 a	6.25 a	29.17 a	361 a	29.03 a
SATANG	151.0 a	8.25 a	12.50 a	20.50 b	124 a	25.45 a
BERTIH	151.7 a	8.25 a	12.50 a	22.45 ab	104 a	16.20 b

TABLE 3
Straw, grain and nitrogen yield and harvest index of the upland rice landraces

Upland rice landraces	Straw Yield	Grain Yield	Total Yield	Straw N Yield	Grain N Yield	Total N Yield	N in Grain	Straw: GrainYield	Straw: Grain N
	g pot^{-1}			mg pot^{-1}			(g kg^{-1})		
AN1334	38.88 d	11.27 a	50.15 c	1268.7 cd	408.8 a	1677.6 ca	24.37 a	5.08 b	4.61 b
AN753	51.33 c	15.96 a	67.29 b	1532.6 c	637.0 a	2169.6 c	29.36 a	3.96 b	2.92 b
AN582	35.02 d	11.68 a	46.70 c	1208.8 cd	457.5 a	1666.2 cd	27.46 a	3.96 b	4.15 b
AN1084	29.82 d	12.26 a	42.08 c	971.1 d	475.7 a	1446.8 d	32.88 a	3.27 b	2.89 b
SATANG	111.96 a	7.04 b	119.00 a	3702.1 a	263.4 a	3965.5 a	6.64 c	21.39 a	19.04 a
BERTIH	88.27 b	14.41 a	103.36 a	2506.1 b	602.2 a	3136.6 b	19.20 b	9.46 b	6.49 b

in straw, only AN 753 and Bertih showed significantly lower values than the others (Table 3). Similar values were also seen in the upland rice varieties in Brazil (Arf *et al*., 2003).

The harvest index (HI) (grain yield/total yield) was similar to the traditional upland rice variety from Northern Laos used by Saito *et al*. (2006). This low HI has been contributed to the low yield obtained from the upland rice (Table 3). The percentage of N in the grain was the lowest in Satang (6.64%), due to the low grain yield obtained. All the AN lines showed between 24 to 33% of the total N in grain. Thus, showing a clear difference in the ability to take up and mobilize the N in the plants.

Fertilizer nitrogen contributed 20 to 27% of the total N taken up by the AN lines, 16% in Bertih, and only 6% in Satang; they were equally distributed in the straw N and the grain N (Table 4). Nitrogen use efficiency in the range of 23 – 30 % has been recently reported for the upland rice when 300 kg N ha^{-1} was applied (Wang *et al*., 2008).

Significant relationships between root length and dry matter weight, root length and total N uptake were obtained (*Figs. 1* and *2*), indicating the suitability of using root characteristics and agronomic parameters. It has also been mentioned that rooting depth is one of the root characteristics which determines the ability of a crop to intercept N, particularly NO_3^- during the periods of leaching (Gastal and Lemaire, 2002). Since upland rice landraces are grown under aerobic conditions, most of the N will be in the

TABLE 4
Nitrogen derived from fertilizer by straw and grain, and the percentage of N derived from fertilizer N added

Upland rice landraces	Straw N from Fertilizer	Grain N from Fertilizer	Total N from Fertilizer	N derived from Fertilizer
	mg pot^{-1}			%
AN1334A	172.82 b	183.34 a	356.2 b	19.62 b
AN753	298.04 a	232.25 a	473.8 ab	26.69 a
AN582	180.42 b	196.65 a	377.1 b	21.35 ab
AN1084	209.64 ab	212.38 a	422.0 ab	27.61 a
SATANG	126.72 c	118.62 b	245.3 c	6.22 c
BERTIH	242.06 a	278.37 a	555.9 a	16.32 b

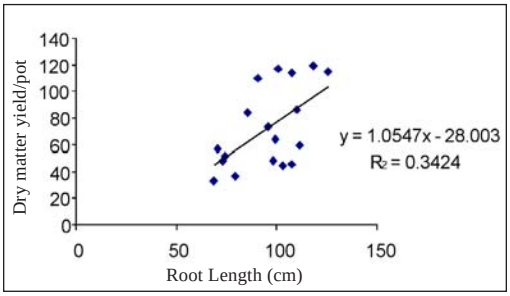

Fig. 1: Relationship between root length and dry matter weight of upland rice

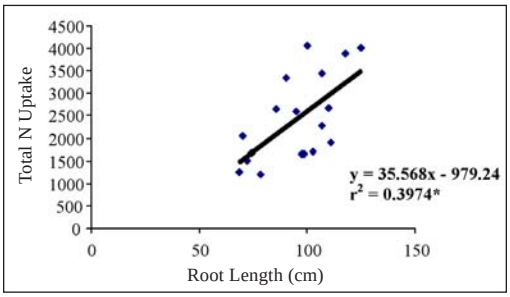

Fig. 2: Relationship between root length and total N uptake

form of NO_3^-, and is liable to be leached under heavy rainfall conditions of the tropics. Thus, varieties capable of producing long seminal roots will be potentially capable of producing deep roots. Other parameters, such as root surface area, root diameter and number of root tips did not correlate well with the agronomic parameters.

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Deterministic Model Approaches in Identifying and Quantifying Technological Challenges in Rice Production and Research and in Predicting Population, Rice Production and Consumption in Malaysia

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ABSTRACT

In general, rice production and sufficiency is the main concern to all Asian countries in currently facing the ever growing population and climatic uncertainties. The consumption in Malaysia relies largely on the locally produced (70%) and imported (30%) rice for years. The price hike of this staple food, which can be categorized as a security food crop with an annual production of 1.6 million tons (beras) yielded from about 650,000 ha of the harvested paddy irrigated- and non-irrigated growing areas nationwide, could possibly be expensive to the lower-income consumers. With "no further reduction" in the modelled per capita rice consumption (82.3 kg/person/ year) versus the increasing population, various efforts must be made in term of research and technological advancement, increased cropping hectarage, as well as active extension program to increase the production of rice for consumption, self-sufficiency and more importantly, for having strong rice stock-file accumulation. Based on the data gathered from the past 27-years (1980 – 2007), the deterministic mathematical models of the Malaysian population, rice per capita consumption and five rice yield models versus years (1980 – 2007 and 2008 – 2030) were developed and predicted. The proposed model was based on the national average yields over the years and the model could be used to predict the yield 'close' to the nation's rice production in the years ahead. The data on the crop cutting test or survey were used for comparison purposes. With the derivatives of the yield models, the quantitative technological advancement indexes were used in identifying the research objective, scope and areas, as well as in quantifying the contribution of crops and their management-related technologies in the past, present and predicted technological performances in rice production. To reach sufficient rice production at a relatively faster rate, the scope of the research's objective should be based on the high yield model, in which the averaged yield could reach 13.4 t/ha in the year 2030. The priority order of the research areas would be irrigation/water > crop establishment-related management > sustainability of the existing management technology > large plot production-related adaptive studies (technological uniformity studies) > continual varietal improvement. The local released varieties are ecologically suited to the Malaysian rice growing areas, where varietal development and improvement are generally time consuming. With the current planted hectareage, coupled with the inclusion of the planned additional 100,000 ha (assumed to be staggered), as planned by the Ministry of Agriculture and with the conversion of the non-fully to fully irrigated areas by 2012, the Malaysian rice self-sufficiency is predicted to be observed/achieved in 2012. The 'modified higher-order polynomial' yield model which was conditioned

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with the scope of the above research objective and the area priorities predicts the rice production of 2.0, 4.4 and 9.1 million t/ha in 2010, 2020 and 2030, respectively. With the modelled minimum per capita consumption (82.3 g/person/year) and the predicted population of 29.3 (2010), 36.7 (2020) and 45.7 million (2030), the respective consumption, surplus and self-sufficiency would be 2.4, 3.0 and 3.8 million tons, -0.4, 1.3 and 5.3 million tons and 83, 144 and 241%, respectively. The surplus could then be used for the stock-pile accumulation and export.

Keywords: Population model, rice production and technological advancement indexes and models, rice consumption, self-sufficiencies, irrigation and management related technologies, rice import and surplus

INTRODUCTION

The production of rice (*Oryza sativa*) in most Asian countries should increase in tandem with the continent's population growth. In 1997, the total production of rice in these countries was estimated at 574.2 million tones (Workman, 2008) with the estimated population of 3.73 billions in 2006 (UNESCAP, 2008). When narrowing down to the South East Asian countries, almost 100% of the population are taking rice in their daily diet. Literally, we could 'equate' the population growth with the increase in the production of rice for this particular region, or rice production is positively associated with its population. The population and rice production for this sub-continent are estimated at 564 million and 102 million tons, respectively in 2008 (USDA, 2008). The per capita rice consumption in the Southeast Asian countries (not including Malaysia) is currently ranging between 101 – 217 kg/person/year (NationMaster.com, 2008). The figure for Malaysia, calculated by the exponential decay curve model (i.e. based on the data from 1980 – 2007), has its lower plateau of 82.3 kg/person/year.

Recently, Malaysia is also facing the issue of shortages in the locally produced rice. The consumption in Malaysia relies heavily on the locally produced and imported rice (i.e. on the average of 70% and 30%, respectively) for years (DOA, 2007). Even though the self-sufficiency figure was initially set by the Government (National Agriculture Policy) in the earlier years, with the recent food global crisis, it is believed that Malaysia should have the 100% rice sufficiency because rice is a nationally and politically related crop. The later is probably evidenced by the Government's plan in opening an additional new rice growing land of 100,000 ha within two years (Bernama, 2008). The price hike of this staple food (categorized as a security food crop), with the annual production of 1.6 million tons (beras), obtained from about 650,000 ha of the harvested paddy irrigated- and non-irrigated growing areas nationwide (Ministry of Agriculture, 2007), is rather expensive to the lower-income consumers. With a model which indicates "no further reduction" in the per capita rice consumption (82.3 kg/person/year) versus the increasing population, the rice consumption will then be population dependent.

In general, this study attempted to identify, qualitatively and quantitatively, the direction, trend and areas of rice technological research and advancement in rice production so as to meet the Malaysian rice consumption at her highest self-sufficiency level. For this, a specific objective was then developed for the deterministic mathematical models and functions to be used in identifying and quantifying technological challenges in rice production and research, as well as in predicting population growth, rice production and consumption in Malaysia up to the year 2030. The rice self-sufficiency, in relation to the net import was also computed.

The data, which were mainly obtained from the Departments of Agriculture and Statistics and Malaysian Agricultural Research and Development Institute for the period between 1980 and 2007, were used for the development of the above model. In specific, the deterministic mathematical models of the population, the growth rate of the population, yield versus year,

total and crop and management technology indexes, as well as per capita consumption, were also developed. The models were used in assessing and evaluating the past, present and future (prediction) of rice production, research and technology advancement, import and self-sufficiency. The yield and technology analyses were largely based on the national average data. The sensitivities of the models to: [i] planted hectareages [mean, additional new land, as planned by the Ministry of Agriculture (Bernama, 2008)] and the conversion of the existing paddy fields into fully irrigated areas) and [ii] five proposed technology index-related yield trends ('logistic', linear, exponential, polynomial-logistic and modified polynomial) were also computed.

Therefore, the models were developed based on mainly the national average rice yield data versus years to have a practical or 'real' implication. The data of the crop cutting tests (CCT) or surveys (CCS) were only used for comparative purposes.

POPULATION AND RICE CONSUMPTION

Population and Its Growth

As clocked on 21 May 2008, the Malaysian population stood at 27,486,789. Based on the data presented by United Nation Organization (UNEP, 2008), the world's population is predicted to grow with a logistic exponential growth function from 1750 to 2100. The population will reach the figure of more than 10 billion people in 2100. At present, the world is experiencing its liner phase of population growth and clocked at 6,674,551,067 on 16 June, 2008 (*U.S. Census Bureau, 2008).* Therefore, discussions on food distribution and shortages in certain developing and underdeveloped countries are the main concerns, especially in facing the issues of rapid population increase and food production against the global climatic and weather uncertainties (global warming) ahead.

Based on population from 1980 to 1996 (Anon, 2008), the deterministic Malaysian population model was developed. The model (*Fig. 1*) which contains the features of linear and exponential functions, which incorporate both the natural and planned (or forced) population growth rates (*Fig. 2*) were validated by the population figures for the years between 1997 and 2007, clocked on 21 May 2008 and projected for 2010 (Malaxi.com, 2008). Mathematically, the model could be written as follows:

$$y = (e^{-45.95182}e^{0.02801248x} + 476.8922x - 930968.8431)/2.013$$

$$\text{IF} \quad 1980 < x \leq 2034$$

and

$$y_t = y_{t-1}(1 + e^{3.424772105 - 0.048925316(x - 1980)}/100)$$

where t = year 2035, 2036, ..., 2050

$$\text{IF} \quad 2034 < x < 2050 \qquad (1)$$

Where, y and x are population and year, respectively. The annual population growth rate was averaged at 2.6% during the period of 1980 – 1997 (Department of Statistic, 2001). Since Malaysia will become one of the developed nations at and after 2020, it is therefore assumed that the growth rate will decline from 2.2% in 2034 to merely 1.0% in 2050. The decline would possibly match with the rates, as planned by the Government under the family planning program. Conservatively, the developed and validated model could predict the population of Malaysia with a 'high accuracy' up to the year 2050. The projected Malaysian populations are 29.3, 36.7, 45.7 and 63.1 millions in 2010, 2020, 2030 and 2050, respectively. This trend is possibly in line with the government's target of achieving 70 million people when the country reaches the status of a fully developed nation.

Rice Consumption, Sufficiency and Stock-pile

The total amount of the nation's rice consumption is simply the product of population and the consumption per capita in a particular year, while self-sufficiency is the ratio of the net domestic rice production to the consumption. The data extracted from Padi Beras Nasional (BERNAS) and the Ministry of Agriculture

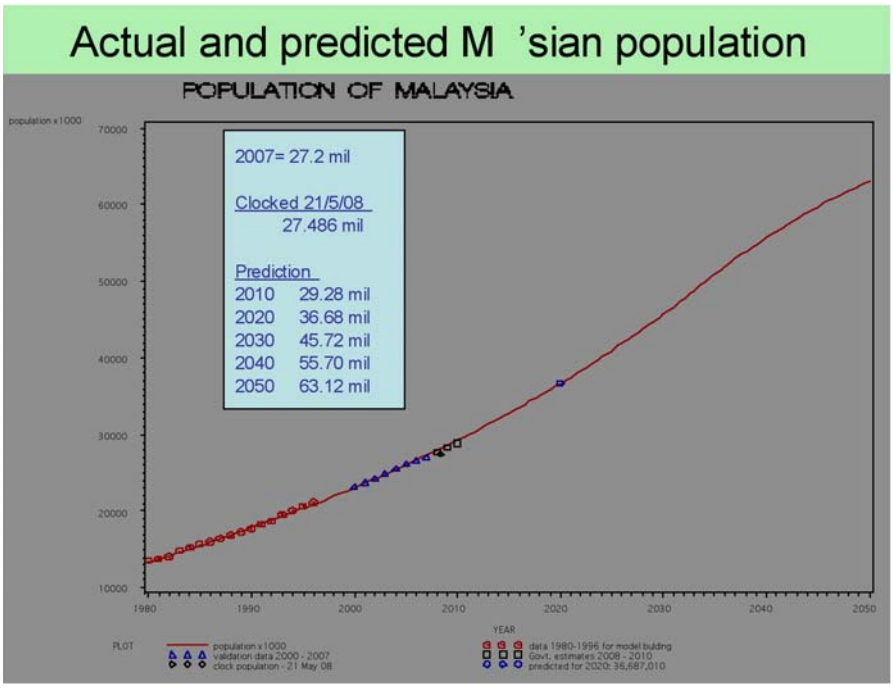

Fig. 1: Predicted Malaysian population until year 2050

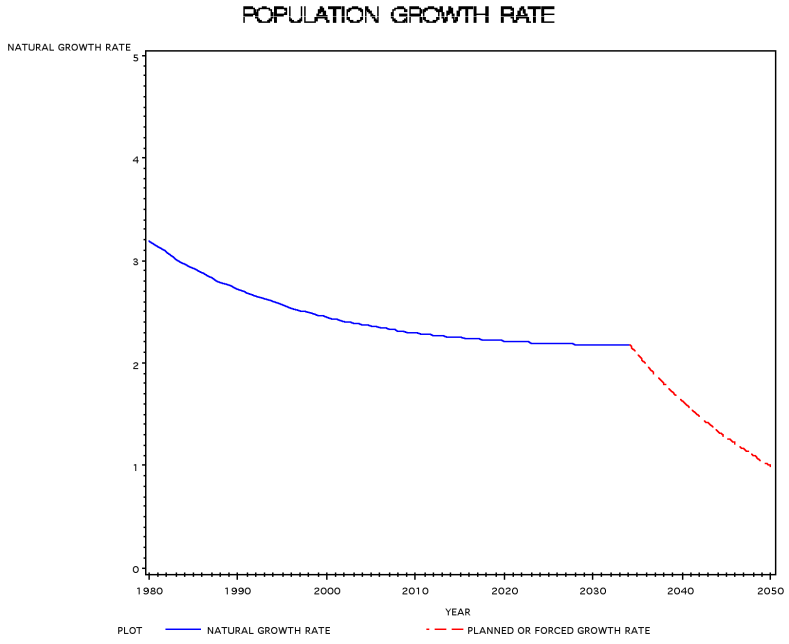

Fig. 2: Calculated malaysian population growth rate (natural and planned growth rate)

(2008), the rice production, rice consumption, import and self-sufficiency during the period of 1980 – 2007 are shown in *Fig. 3*. Based on the data, the rice import (167 – 843 thousand tons/ year) was needed to top up the locally produced rice (1,010 – 1,600 thousand tons/ year) in meeting the yearly consumption of 1,290– 2,230 thousand tons/ha. This led to the figures of 69 – 88 % rice self-sufficiency for the period. The highest sufficiency was recorded in 1980 and subsequently the figure was decreased with increasing population in the years approaching 2007. The figures did not reflect any active stock-pile in the country during the period mentioned. The recent government's announcement of the 3 month's rice stock is probably a short-term temporary measure in ensuring rice sufficiency locally. If in a situation, where the neighbouring rice-producing countries were taking a stringent action in exporting their rice, it was then presumed that the country would have a difficult situation of an accumulated rice shortage nationwide.

The above prediction would strongly suggest that programs and activities, i.e. research and development in generating advanced and sustainable technologies, improving the existing non-irrigated paddy fields and new land opening for rice cultivation, should mandatorily be accelerated. These will certainly increase the production of rice in Malaysia and it is also hoped that the rice self-sufficiency of >100% and the possibility of having 1-year stock-pile of the locally produced rice, be possibly achieved in the near future. This is crucial as rice has high significant implications in the social, political and economic stability in this country.

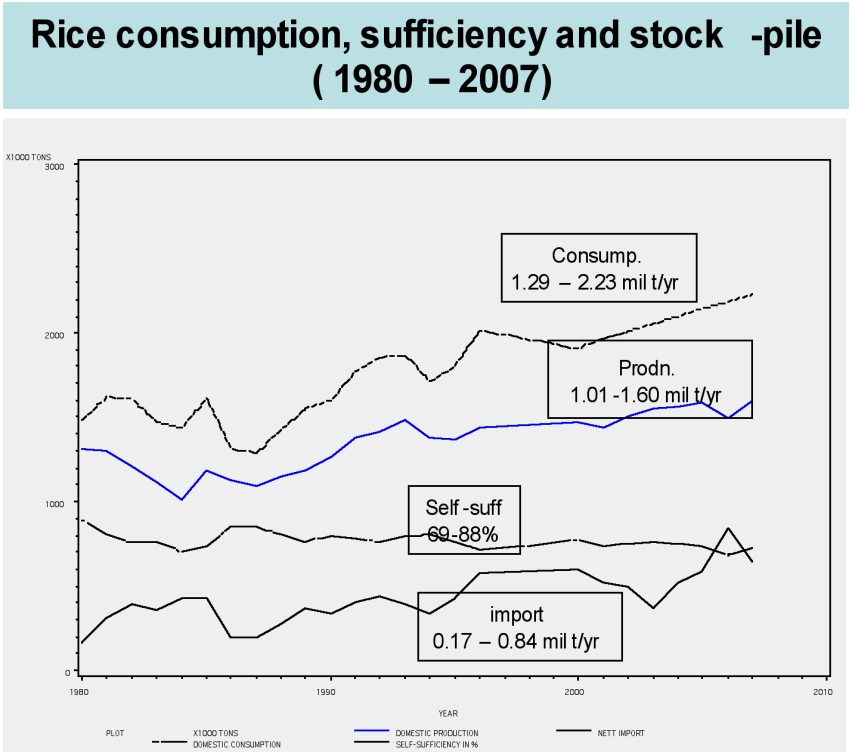

Fig. 3: Rice production, consumption, net import and self-sufficiency from years 1980 – 2007

RESEARCH AND DEVELOPMENT AREAS AND THEIR CHALLENGES

The parameter of yield per unit area (tons/ha) is the focal point of the technological advancement objective quantitatively set in any research and development (R&D) program. In this paper, rice (padi) yield in tons/ha was used in identifying and quantifying the effectiveness of the technologies generated by the past R&D programs and activities. In general, it was also used for identifying the future research areas which should be given a priority in achieving the national's good rice self-sufficiency and stock-pile at a relatively faster rate, to sustain the nation's exponential population growth.

The padi yields in tons/ha, from 1980 – 2007, were used in computing the technology advancement index (TAI) development in rice production. In brief, the TAI is defined as a change in tons/ ha/ year or the tangent at any point of the year when tons/ha is regressed over years. In other words, the instantaneous TAI is the kg/ha-vs.-year model dependence. The higher the TAI, the higher the technological advancement in affecting yield will increase over the time. Therefore, TAI is the total technological combination of crop (varietal development and improvement) and management components (irrigation, fertilization, crop establishment and distribution, pest and diseases control, etc.) in the production of rice.

Yield Models

Based on the average of the national yields (tons/ ha) versus years (1980 – 2007), five models developed in this study are shown in *Fig. 4*. The deterministic models were mathematically written as:

$$\text{Model 1: } y = 19060.4/(1 + 7.76e^{-0.0252\,(x\,-1980)}) \quad (2)$$

$$\text{Model 2: } y = 2105.13 + 62.80(x\text{-}1980) \quad (3)$$

$$\text{Model 3: } y = 2184.38e^{0.02123(x\,-1980)} \quad (4)$$

$$\text{Model 4: } y = (4102731.93 + 4514.33 (x - 1980)^{2.48})/(1758.3957 + (x - 1980)^{2.48}) \quad (5)$$

$$\text{Model 5: } y = 2217.75 - 0.717(x\text{-}1980)^{0.7} + 7.96(x\text{-}1980)^{2} - 0.394 (x-1980)^{3} + 0.00697(x\text{-}1980)^{3.98} \quad (6)$$

where, y and x are tons/ ha and years starting from 1980, respectively. Models 1, 2, 3, 4 and 5 could be termed as the logistic growth, simple linear, exponential, 'logistic polynomial' and 'modified higher-order polynomial' functions, respectively. Based on these models, the increases of yield per ha over the years between 1982 – 2007 (which were due to technological advancement based on models 1, 2, 3, 4 and 5) were 94.6, 94.7, 94.8 , 94.5 and 93.6%, respectively. On the other hand, the respective figures (due to climatic fluctuations) were only 5.4, 5.3, 5.2, 5.5 and 6.4%. These figures suggest that the technological advancement was more dominant in the increase of yield per ha over the years, as compared to the climatic variability during the period. Thus, it would give an ample room for research and development program and activities to generate advanced technology in the effort to increase rice yield in the future!

The predicted yields in kg/ha, computed by the above models (Equations 2, 3, 4, 5, 6 and *Fig. 4*), are shown in Table 1. It is suggested that the yield should increase with Model 3 up to 2013, which subsequently then be switched to Model 5 to obtain a steady increase of higher yields over the years. Model 5 indicated that the overall yield could be increased to 13.2 tons/ha in 2030. The crop cutting test or survey also showed a potential yield of 14.0 tons/ha (Department of Agriculture, 2008). If research and extension activities were not geared to their highest capacities, we are in the opinion that the yield increase would only progress with Model 4, with the overall yield of about 4.0 – 4.3 tons/ ha. Even if the yield increased linearly (Model 2), the predicted yields in 2020 to 2030 would only be in the range of 4.6 – 5.2 tons/ha.

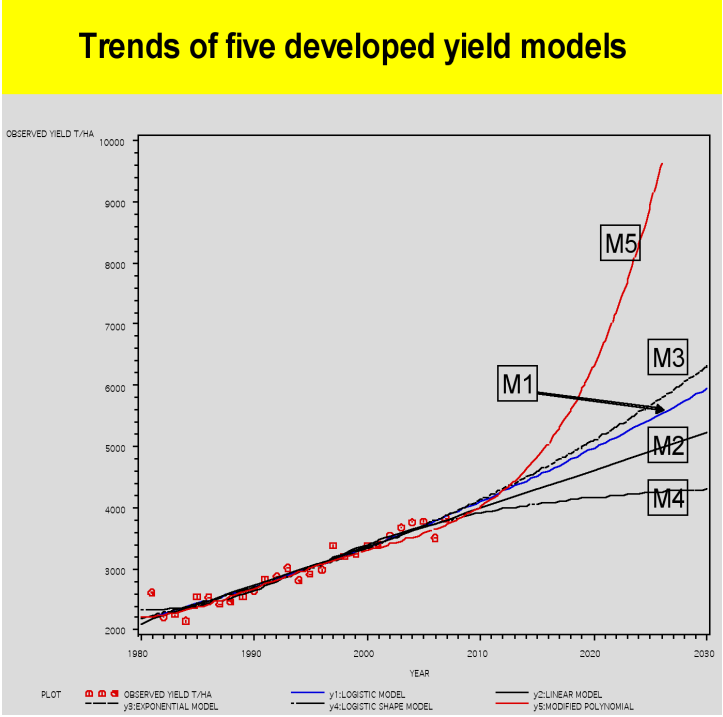

Fig. 4: Five developed yield models versus years (1980 – 2030) in Malaysian rice growing areas

TABLE 1
Predicted padi yields and technological advancement indexes (TAI) in 2010 – 2030, based on Yield models 1, 2, 3, 4 and 5

Models	Years									
	2010		2015		2020		2025		2030	
	Yield Kg/ha	TAI kg/ha/yr	Yield Kg/ha	TAI kg/ha/yr	Yield Kg/ha	TAI kg/ha/yr	Yield Kg/ha	TAI kg/ha/yr	Yield Kg/ha	TAI kg/ha/yr
1: logistic growth	4102	81	4523	86	4971	92	5351	98	5951	103
2: simple linear	3989	62	4303	62	4617	62	4868	62	5245	62
3: exponential	4129	87	4592	97	5106	108	5559	120	6314	134
4: 'logistic polynomial'	3910	36	4062	25	4169	17	4232	13	4301	9
5: 'modified higher-order polynomial'	4016	114	4819	217	6320	396	8300	667	13179	1050

Technology Advancement Index (TAI) and Its Partitioning to Crop and Management Technologies

By taking the derivative of each model, the trends of the TAIs from 2008 to 2030 are shown in *Fig. 5*, while the predicted TAIs for 2010, 2015, 2020, 2025 and 2030 are indicated in Table 1. Model 5 has higher predicted TAIs (114.1 – 1050.1 kg/ha/year) as compared to the other models. This suggests that the technology usage, refinement and development will be significantly reflected in the yield improvement. On the contrary, Model 4 shows that no further technological improvement would occur. This suggests that the scientists should use Model 5 as their research moving target versus years in quantifying the achievements of their research.

Based on limited available data, the technologies are broadly categorized into four groups, viz., crop (varieties), irrigation (with soil physical manipulation), crop establishment and other components (pooling effects of fertilizers and soil fertility, pest, disease and weed control and other practices on rice yield). Due to the limitation, the assumption on time duration required for technological advancement was made, particularly for irrigation and varietal improvement.

TAI Due to Varietal Improvement

Breeding research programs are generally aimed at producing and improving rice varieties in terms of yield quantity (productivity) and quality and/or crop type which is resistant to certain diseases or responsive to certain environmental factors. In this paper, the quantity (tons/ha) is emphasized since rice production and sufficiency are the pressing issues at present. Based on the yield data (national average) of MR84, MR185, MR211, MR219 and MR220 released

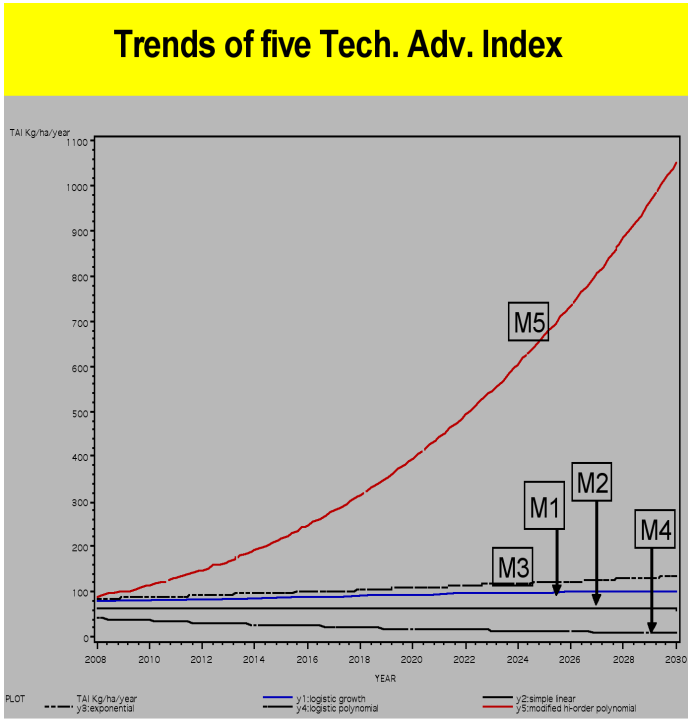

Fig. 5: Technological advancement indexes over years (2008 – 2030) as generated from five yield models

in 1977, 1999, 2001 and 2003, respectively, the yield improvement per year due to the newly released varieties is 41.5 kg/ha/year. The linear increase was assumed in this paper. If this improvement is consistent over the years (up to 2030), then the percentages of the contribution of varietal component in the TAI over the years (2010, 2015, 2020, 2025 and 2030) of five yield models are as indicated in Table 2.

TAI Due to Irrigation Technology and Improvement

Based on the data obtained in the off-seasons of 2005 and 2006 and the main-season of 2006/2007, the differences in the yield per ha between the areas, within the irrigation scheme (3.9 – 4.2 tons/ha) and outside scheme (3.2 – 3.3 tons/ha) were calculated. The average difference was 0.74 tons/ha. Thus, it could be implied that the yield difference was due to the irrigation/ water related technology improvement. It is also assumed that if the areas outside the scheme were converted to the fully irrigated paddy fields, they would then achieve the yield level close to the areas full of irrigation. If the conversion would take in 2, 4, 6, 8 and 10 years' duration, the yield would increase the rates due to irrigation improvement which would then become 0.37, 0.18, 0.12, 0.09 and 0.07 tons/ ha/ year, respectively.

TAI Due to Other Management Technologies and Improvement

After considering the two technologies above, the yield improvement could be contributed by the other component improvements, namely weed, pest and disease control, as well as fertilizer, crop establishment techniques and other soil and agronomic manipulations. The quantum of the technological improvements of these pooled components is based on the targeted yield increased, as based on the above five yield models. It was recorded that the crop cutting test had achieved the highest potential yield of 14.0 tons/ha, i.e., by the conversion from a smaller plot to per ha basis. This indicates that if the crop growth and performance is uniform throughout a large field, a higher average yield per ha should therefore be achieved. This suggests that crop establishment technology (for example, high density planting) and field planting environment should be researched further aggressively in obtaining uniform crop performance over larger growing areas.

Integrated Contribution of Technological Components to the Total TAI

Table 2 shows that the yearly varietal improvement will contribute to about 63% of the total TAI for each year, based on the simple linear yield model (Model 2). The contributions

TABLE 2
Contribution index of varietal improvement against total technology Index (TAI) over years 2010 – 2030 based on five yield models (1 = logistic growth, 2=simple linear, 3= exponential, 4='logistic polynomial', 5=' high order modified- polynomial) of rice production in Malaysia growing environment. Value greater than 100 indicates contribution index saturation of a particular model

Year	Model 1	Model 2	Model 3	Model 4	Model 5
– – – – – – – – – – – – – – contribution index to total TAI (%) – – – – – – – – – – – – – –					
2010	51.2169	66.1715	47.3965	115.150	36.4160
2015	47.8017	66.1715	42.6232	163.767	19.0686
2020	44.8712	66.1715	38.3306	231.068	10.4909
2025	42.3790	66.1715	34.4703	320.805	6.2272
2030	40.2855	66.1715	30.9988	437.064	3.9570

would be reduced from 51% in 2010 to 40% in 2030, when the logistic growth model (Model 1) was used. Model 3 (exponential) predicts the reduction from 36% to 10% for the same period. The high yield model (Model 5: 'modified higher-order polynomial') indicates a small respective varietal contribution to the total TAI (36% to 4%). These suggest that the higher the model, the higher the contribution of the other components will be (other than padi varieties) in the predicted rice yield in the Malaysian rice growing areas. As reported by Longping (Star, 2004), Othman (rice breeder) of MARDI, the varieties were not the factors causing lower yield production in Malaysia, but it was rather due to the poor farm management as compared to China (17 tons/ha). This was due to the different climates and better farm management in China. High temperatures have been indicated to cause higher percentage of spikelet sterility and hence producing lower yield (Battisti and Hinckley, 2008). Othman's statement could be confirmed by Model 4 ('logistic polynomial') which is predicted to be asymptotic over years, which likely to be dominated by the varietal performance. The current performance of rice in Thailand is mostly determined by the climate and management practices (Thailand Extension Authority, 2008).

With the existing improved varieties, rice production in Asian countries is ecologically and mainly associated with flooded paddy growing condition. In the case of water or irrigation requirements, 75% of the world rice is produced from irrigated growing areas (Science Daily, 2007). This particularly indicates the importance of water and irrigation in rice production. About 23% of the rice harvested hectareage in Malaysia is not from the fully irrigated areas. It is then suggested that these areas should be converted into fully irrigated growing areas. The conversion might involve the infrastructural improvement or in situ irrigation technological advancement techniques. If the required durations for the conversion are 2, 4, 6, 8 and 10 years, the calculated contributions of the irrigation and its related technologies to the total TAI are shown in Table 3. These calculations were made by assuming that both areas (inside or outside the scheme) had received equal crop and other-management technologies for rice growing. Even if the conversion would only take two years, Model 5 (high yield trend function) would still show that the increase yield rate would not saturate the yield level over time. A similar situation will also occur when the varietal and irrigation components are combined in causing the rice yield to increase, based on the high yield models, particularly with the extended years of the projection's period (Table 4).

This implies that there will be a room for yield improvement by contributions of other technological components, viz., crop establishment and weed, pest and disease control and soil and agronomic manipulation. For example, if the area gradually requires 10 year's period for the conversion, conditioned by the linear varietal improvement (41.5 kg/ha/yr), about 47 – 89% contribution will then be estimated (years 2015 – 2030) to the total TAI, based on the high yield trend (Model 5) which should be coming from other management technologies. In the IADA granary of Barat Laut Selangor, the average rice yield was 6.5 tons/ha versus about 4.0 tons/ha or less for the other areas in 2007 (IADA Barat Laut Selangor, 2008). It is important to note that the farmers in this area have been practicing good management of rice cultivation (especially with a high plant density planting and higher fertilizer rate application). Mansor (2008) indicated that a high paddy planting density (600 tillers that yield 500 panicles per sq. m) produced high yield obtained from the local verification trial plots (8-10 t/ha). Rice varieties, MRQ50, MR219 and MR220 released in 1999, 2001 and 2003 had local verification trial yields of 4000-5000, 6380 – 7980 and 5000 – 9600 kg/ha, respectively (MARDI, 2008).

This suggests that the yield gaps, among the management practices and the yield range within the same rice variety, indicated that the rice yields under the Malaysian growing condition could be further increased, i.e. above the level as dictated by the variety and water/irrigation components for the other factors. Since the

TABLE 3
Contribution index of irrigation technology improvement against total technology index (TAI) over years 2010 – 2030 based on five yield models (1= logistic growth, 2=simple linear, 3= exponential, 4='logistic polynomial', 5=' high order modified-polynomial) at five assumed durations of irrigation scheme completion period. Value greater than 100 indicates contribution index saturation of a particular model

Year	Irrigation scheme completion period (years)				
	2	4	6	8	10
contribution index to total TAI (%)					
Model 1					
2010	456.233	228.111	152.078	114.055	91.2417
2015	425.811	212.900	141.937	106.450	85.1575
2020	399.707	199.848	133.236	99.924	79.9370
2025	377.506	188.748	125.835	94.374	75.4972
2030	358.857	179.424	119.619	89.712	71.7676
Model 2					
2010	589.447	294.715	196.482	147.358	117.883
2015	589.447	294.715	196.482	147.358	117.883
2020	589.447	294.715	196.482	147.358	117.883
2025	589.447	294.715	196.482	147.358	117.883
2030	589.447	294.715	196.482	147.358	117.883
Model 3					
2010	422.201	211.095	140.734	105.548	84.4357
2015	379.681	189.836	126.560	94.918	75.9322
2020	341.444	170.717	113.815	85.359	68.2850
2025	307.057	153.524	102.352	76.762	61.4080
2030	276.133	138.063	92.044	69.031	55.2236
Model 4					
2010	1025.74	512.86	341.91	256.428	205.137
2015	1458.82	729.39	486.27	364.694	291.747
2020	2058.32	1029.13	686.11	514.566	411.642
2025	2857.68	1428.80	952.56	714.402	571.506
2030	3893.31	1946.60	1297.77	973.300	778.619
Model 5					
2010	324.388	162.190	108.129	81.0949	64.8742
2015	169.860	84.928	56.620	42.4640	33.9703
2020	93.451	46.724	31.150	23.3622	18.6893
2025	55.471	27.735	18.490	13.8674	11.0936
2030	35.248	17.624	11.749	8.8119	7.0493

yield per ha is directly and mathematically a function of planting density and fertilizer rate, the crop establishment and fertilization are therefore other important technologies to be further improved in obtaining high rice yield in relatively short time, in addition to the existing management practices (weed, pest and diseases control and other cultural practices).

Research Priority and Challenges

Based on the above five yield models and the related brief discussion, the main tasks and challenges faced by researchers and scientists are objectively in making the high yield model over time as their core research's target. By using the models, the research progress, output and success could be identified and quantified for the

TABLE 4
Contribution index of combined irrigation & variety technology improvement against total technology index (TAI) over years 2010 – 2030 based on five yield models (1= logistic growth, 2=simple linear, 3= exponential, 4='logistic polynomial', 5=' high order modified-polynomial) at five assumed durations of irrigation scheme completion period. Value greater than 100 indicates contribution index saturation of a particular model

Year	Irrigation scheme completion period (years)				
	2	4	6	8	10
contribution index to total TAI (%)					
Model 1					
2010	507.450	279.327	203.295	165.272	142.459
2015	473.612	260.701	189.739	154.251	132.959
2020	444.578	244.719	178.107	144.795	124.808
2025	419.885	231.127	168.215	136.753	117.876
2030	399.143	219.709	159.905	129.997	112.053
Model 2					
2010	655.618	360.887	262.654	213.529	184.055
2015	655.618	360.887	262.654	213.529	184.055
2020	655.618	360.887	262.654	213.529	184.055
2025	655.618	360.887	262.654	213.529	184.055
2030	655.618	360.887	262.654	213.529	184.055
Model 3					
2010	469.598	258.492	188.130	152.944	131.832
2015	422.305	232.459	169.184	137.541	118.555
2020	379.774	209.048	152.145	123.689	106.616
2025	341.527	187.995	136.823	111.232	95.878
2030	307.132	169.062	123.043	100.030	86.222
Model 4					
2010	1140.89	628.01	457.06	371.58	320.29
2015	1622.58	893.16	650.04	528.46	455.51
2020	2289.39	1260.20	917.18	745.63	642.71
2025	3178.49	1749.61	1273.37	1035.21	892.31
2030	4330.37	2383.67	1734.83	1410.36	1215.68
Model 5					
2010	360.804	198.606	144.545	117.511	101.290
2015	188.929	103.997	75.689	61.533	53.039
2020	103.942	57.215	41.641	33.853	29.180
2025	61.698	33.962	24.717	20.095	17.321
2030	39.205	21.581	15.706	12.769	11.006

assessment of the technological advancement. This is crucial since the arable land is becoming lesser in the future. For example, the global per capita arable land had decreased from 0.42 to 0.23 ha/person (1961 – 2002) [FAO, 2008] and this figure could become worsened when the global population would be estimated above 10 billions in 2100.

In a medium term (up to 2030), the technologies in the Malaysian rice production should be improved further at a reasonably rapid rate. In signifying the positive and additive effects of the technologies, research and management in sustaining the performances of existing technologies are equally important. In more specific, the scope of the medium terms

and the priority of the Malaysian rice research are as followings:

(1) Use Model 3 (exponential) until 2013 and then switch to Model 5 ('modified higher-order polynomial') from 2013 onwards to form year-dependence moving quantitative objective or the target in all research programs and activities leading to the improvement or generation of relevant and applicable technologies. Avoid any research which quantitatively yield technologies that reflect the yield trend, as indicated in Model 4 ('logistic polynomial'). Based on the yield statistics of 1980 – 2007 (Dept. of Agric., 2008), Model 4 would likely be the trend to obtain the national average rice yield versus years until 2030. If this was true, i.e., without any additional hectareage of rice areas, Malaysia would face a high rice importation for the increased consumption, due to the growth in its population.

(2) Priority of research areas (*in the order of time-related importance*)

The suggested order of the priority of the research area is irrigation/water > crop establishment-related management > sustainability of existing management technology > large plot production-related adaptive studies > continual varietal improvement. A brief discussion is given below:

a. Research on the infrastructural and in-situ irrigation-related technological development, refinement and improvement. Infrastructural-related technology is mostly suited for upgrading and conversion of the rain-fed or not-fully irrigated areas into fully irrigated scheme, or for the opening of new rice growing areas. The in-situ research is probably suited to the existing fully-irrigated fields, especially in controlling water loss and seepage, controlling water level and technique used for reducing water evaporation loss and water quality in the paddy fields, as related to rice growth and development. The above research will definitely take into account the physical structure and type of soil and the climatic fluctuation of the locality.

b. Research on crop establishment, especially on the relationship of high planting density versus fertilizer rate in rice growth and development.

c. Research on sustainability of the existing management and cultural related technologies. Climatic uncertainties will, to certain extent, affect the performance and efficiencies of certain management technologies. The world is now facing the global warming trend in the years ahead.

d. Local verification and adaptive studies with increasing trial plot size (technological uniformity studies) should be carried out nationwide over all rice growing areas. These studies will determine the stability and modification of certain technologies over Malaysian rice growing areas. This is an effort to obtain a quite "uniform" rice yield performance over all rice growing areas and thus increasing the national average yield with high degree of confidence.

e. Apart from improving the quality of grains, varietal improvement works should be carried out continuously in obtaining an average nationwide yield increase rate of 42 kg/ha/year or higher. With a quite disadvantaged climatic condition relative to those in China and Japan for rice growing, the above yield increase rate could possibly be achieved annually. Most locally developed rice varieties are adapted well to the Malaysian rice growing areas.

PREDICTION OF RICE PRODUCTION, CONSUMPTION, SELF-SUFFICIENCY AND STOCK-PILE ACCUMULATION (2008 – 2030)

The mean, standard deviation and coefficient of variation of rice planted hectareage for the duration of between years of 1998 and 2007 were 674,548 ha, 12,144 ha and 1.8%, respectively, as shown in *Fig. 6* (Ministry of Agriculture, 2007). The respective values for the harvested areas were 656,805 ha, 26,763 ha and 4.1%. About 23% of the areas did not receive a full-irrigation system and it is also classified as the rain-fed rice growing areas. With the current local and global rice sufficiency, the Ministry of Agriculture has decided to open an additional area of 100,000 ha for rice cultivation (BERNAMA, 2008).

The developed per capita consumption model (*Fig. 7*), coupled with the developed population model (*Fig. 1*) was employed in the calculation of rice consumption per year. The per capita consumption had been decreased with an exponential decay function which yielded an averaged 'non-reducible' level at 82.3 kg/person/year after 2003 onwards.

Based on the above discussion and possibilities, the rice (beras) production, domestic consumption, net import and self-sufficiency were predicted for years 2008 – 2030, as shown in Tables 5, 6, 7, 8 and 9. The predictions were subjected to five predicted yield models (logistic growth, simple linear, exponential, 'logistic polynomial' and 'modified higher-order polynomial'), as well as the options of the planted hectareages for rice cultivation. The hectareage options are: [1] based on the planted hectareage mean for years 1998 – 2007, [2] as in (1) but with the inclusion of conversion of partially irrigated (or rain-fed) areas into fully-irrigated scheme by 2012, [3] as in (1) but with additional new rice growing land and [4] as in (3) but with full irrigation conversion. For a practical consideration, the additional new land for rice growing would be implemented in stages, i.e. 40,000, 40,000 and 20,000 ha in 2010, 2011 and 2012, respectively. According to Chang (2002), crop yield is a function of climate, technology, management and land.

Table 5 shows that the total nation's rice consumption was predicted to increase exponentially from 2.4 million tons in 2010 to 3.0 and 3.8 million tons in 2020 and 2030, respectively. This trend is in tandem with the population increase, whereby the predicted figures for 2010, 2020 and 2030 were 29.3, 36.7 and 45.7 million people, respectively. If the rice production was purely based on the current planted hectareage, the self-sufficiency would be

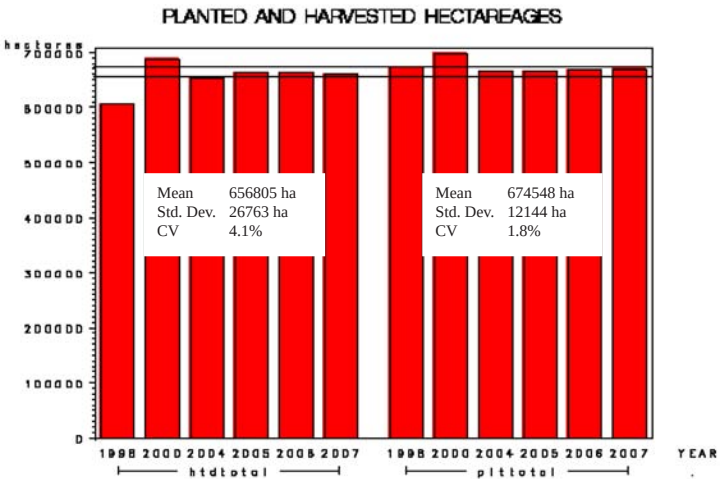

Fig. 6: Harvested and planted hectareages of rice growing in Malaysia from 1998 – 2007

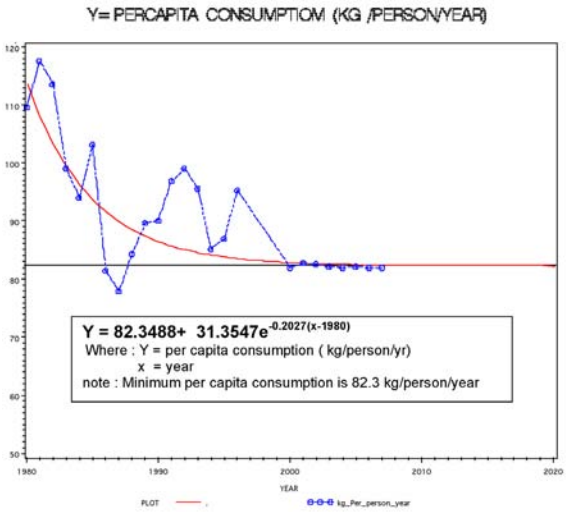

Fig. 7: Per capita rice consumption model. The model was developed based on the data gathered from 1980- 2007, from Department of Agriculture, Malaysia

TABLE 5
Predicted population, per capita rice and total consumption and mean planted rice hectareage

Year	Population (×1000)	Per capita consumption (kg/person/year)	Consumption (ton)	Planted (ha)
2008	27958.95	82.4563	2305391.38	674548
2009	28614.30	82.4366	2358864.89	674548
2010	29281.54	82.4205	2413398.24	674548
2011	29961.00	82.4073	2469006.04	674548
2012	30653.04	82.3966	2525705.82	674548
2013	31358.01	82.3878	2583517.73	674548
2014	32076.27	82.3807	2642464.27	674548
2015	32808.21	82.3748	2702570.04	674548
2016	33554.21	82.3700	2763861.61	674548
2017	34314.67	82.3661	2826367.32	674548
2018	35090.01	82.3630	2890117.22	674548
2019	35880.64	82.3604	2955142.90	674548
2020	36687.01	82.3582	3021477.51	674548
2021	37509.55	82.3565	3089155.60	674548
2022	38348.73	82.3551	3158213.17	674548
2023	39205.02	82.3539	3228687.59	674548
2024	40078.90	82.3530	3300617.58	674548
2025	40970.88	82.3522	3374043.22	674548
2026	41881.47	82.3516	3449005.93	674548
2027	42811.20	82.3511	3525548.50	674548
2028	43760.61	82.3507	3603715.06	674548
2029	44730.26	82.3503	3683551.13	674548
2030	45720.72	82.3500	3765103.62	674548

obtained in 2021, i.e. when Model 5 was used in estimating rice production (Table 6) and the rice surplus in 2030 would be 2.5 million tons (165% self-sufficiency). The level would not be achieved when the other four models were used in the yield prediction. In a situation where Hectareage option 2 was used (100% fully-irrigated rice growing areas), the self-sufficiency could be observed in 2012 for the yield Models 1, 2, 3 and 5 (Table 7). Due to the strength of the positive population growth, self-sufficiencies were short-lived in Models 1 and 2. In spite of Model 3 which was continually yielding surplus rice production, its figures were much lower than those of Model 5. With Hectareage option 3 (mean planted hectareage with new additional rice growing areas), self-sufficiency would be seen in 2017 when Model 5 was used. The predicted self-sufficiencies would be 109 and 190% in 2020 and 2030, respectively. This result was not shown in other yield models (Table 8). It reveals that Option 3 has yielded figures which lie between those of Options 1 and 2.

As hypothesized and predicted, Option 4 of rice hectareage (mean planted hectareage plus new additional rice growing areas and with 100% fully-irrigated areas) has resulted in promising figures (Table 10). The self-sufficiency was predicted for 2012. However, due to the relatively strong exponential population growth, the sufficiency would be ended in 2025 and 2017 for Models 2 and 4, respectively. The predicted surplus or negative net rice import for Model 5 in 2020 and 2030 were 1.3 and 5.3 million tons, respectively. The respective figures for Models 1 and 3 were less than a million ton (0.3 – 0.6 million tons). The above information suggests that the rice Hacterage for Option 4 had significantly provided a conducive environment for Model 5 in producing relatively high rice production, surplus and self-sufficiency in Malaysia. Based on the consumption figures of 2.4 – 3.7 million tons per year for 2010 – 2030, there would generally be (on the average) 2.0 and 1.0 million tons of the annual rice stock-pile and export, respectively, during the stated period.

TABLE 6
Predicted rice production, net import and self-sufficiency based on five yield models (1= logistic growth, 2 = simple linear, 3 = exponential, 4 = 'logistic polynomial', 5 = 'high order modified-polynomial) and mean planted hectareage

Year	Model 1	Model 2	Model 3	Model 4	Model 5
			production (tons)		
2008	1866826.42	1829241.85	1874042.65	1814774.68	1806216.80
2009	1904414.43	1858975.26	1914253.91	1833751.10	1851082.01
2010	1942553.86	1888708.67	1955327.98	1851450.35	1901833.74
2011	1981244.57	1918442.08	1997283.37	1867949.89	1959490.70
2012	2020486.03	1948175.49	2040139.00	1883326.51	2025142.55
2013	2060277.38	1977908.90	2083914.18	1897655.10	2099949.94
2014	2100617.34	2007642.30	2128628.64	1911007.79	2185144.37
2015	2141504.26	2037375.71	2174302.54	1923453.36	2282028.21
2016	2182936.12	2067109.12	2220956.47	1935056.93	2391974.63
2017	2224910.44	2096842.53	2268611.44	1945879.76	2516427.59
2018	2267424.38	2126575.94	2317288.94	1955979.19	2656901.74
2019	2310474.63	2156309.35	2367010.92	1965408.71	2814982.44
2020	2354057.50	2186042.76	2417799.78	1974217.99	2992325.71
2021	2398168.82	2215776.17	2469678.41	1982453.15	3190658.15
2022	2442804.02	2245509.57	2522670.20	1990156.81	3411776.96
2023	2487958.05	2275242.98	2576799.03	1997368.38	3657549.90

Table 6 (*continued*)

2024	2533625.42	2304976.39	2632089.31	2004124.22	3929915.19
2025	2579800.21	2334709.80	2688565.94	2010457.85	4230881.57
2026	2626476.00	2364443.21	2746254.39	2016400.15	4562528.19
2027	2673645.93	2394176.62	2805180.66	2021979.59	4927004.64
2028	2721302.69	2423910.03	2865371.31	2027222.34	5326530.87
2029	2769438.48	2453643.44	2926853.47	2032152.54	5763397.19
2030	2818045.05	2483376.85	2989654.84	2036792.40	6239964.22
			net import (tons)		
2008	438564.96	476149.53	431348.73	490616.71	499174.59
2009	454450.46	499889.63	444610.98	525113.79	507782.89
2010	470844.38	524689.57	458070.26	561947.90	511564.50
2011	487761.47	550563.96	471722.67	601056.15	509515.34
2012	505219.79	577530.33	485566.82	642379.31	500563.26
2013	523240.35	605608.83	499603.55	685862.63	483567.79
2014	541846.93	634821.96	513835.62	731456.48	457319.90
2015	561065.77	665194.33	528267.49	779116.68	420541.83
2016	580925.49	696752.48	542905.14	828804.68	371886.97
2017	601456.88	729524.79	557755.88	880487.56	309939.73
2018	622692.84	763541.28	572828.27	934138.02	233215.48
2019	644668.27	798833.56	588131.98	989734.20	140160.46
2020	667420.01	835434.75	603677.73	1047259.51	29151.80
2021	690986.78	873379.43	619477.19	1106702.45	-101502.55
2022	715409.16	912703.60	635542.97	1168056.36	-253563.79
2023	740729.54	953444.60	651888.56	1231319.21	-428862.31
2024	766992.15	995641.18	668528.27	1296493.36	-629297.61
2025	794243.01	1039333.41	685477.27	1363585.37	-856838.35
2026	822529.93	1084562.72	702751.54	1432605.78	-1113522.26
2027	851902.56	1131371.88	720367.84	1503568.91	-1401456.15
2028	882412.37	1179805.03	738343.75	1576492.72	-1722815.81
2029	914112.65	1229907.70	756697.67	1651398.59	-2079846.06
2030	947058.58	1281726.78	775448.78	1728311.23	-2474860.59
			self-sufficiency (%)		
2008	80.9766	79.3463	81.2896	78.7187	78.348
2009	80.7344	78.8080	81.1515	77.7387	78.473
2010	80.4904	78.2593	81.0197	76.7155	78.803
2011	80.2446	77.7010	80.8942	75.6559	79.364
2012	79.9969	77.1339	80.7750	74.5663	80.181
2013	79.7470	76.5588	80.6619	73.4524	81.283
2014	79.4946	75.9761	80.5547	72.3192	82.693
2015	79.2395	75.3866	80.4531	71.1713	84.439
2016	78.9814	74.7906	80.3570	70.0128	86.545
2017	78.7198	74.1886	80.2660	68.8474	89.034
2018	78.4544	73.5810	80.1798	67.6782	91.931
2019	78.1849	72.9680	80.0980	66.5081	95.257
2020	77.9108	72.3501	80.0204	65.3395	99.035
2021	77.6319	71.7276	79.9467	64.1746	103.286
2022	77.3477	71.1006	79.8765	63.0153	108.029
2023	77.0579	70.4696	79.8095	61.8632	113.283
2024	76.7622	69.8347	79.7454	60.7197	119.066
2025	76.4602	69.1962	79.6838	59.5860	125.395

Table 6 (*continued*)

2026	76.1517	68.5543	79.6245	58.4632	132.285
2027	75.8363	67.9093	79.5672	57.3522	139.751
2028	75.5138	67.2614	79.5116	56.2537	147.807
2029	75.1839	66.6108	79.4574	55.1683	156.463
2030	74.8464	65.9577	79.4043	54.0966	165.732

-ve indicate surplus after consumption

TABLE 7
Predicted rice production, net import and self-sufficiency, based on five yield models (1 = logistic growth, 2 = simple linear, 3 = exponential, 4 = 'logistic polynomial', 5 =' high order modified-polynomial) and mean planted hectareage with the non-irrigated conversion into fully-irrigated areas

Year	ha	Model 1	Model 2	Model 3	Model 4	Model 5
			production (tons)			
2008	674548.00	1866826.42	1829241.85	1874042.65	1814774.68	1806216.80
2009	674548.00	1904414.43	1858975.26	1914253.91	1833751.10	1851082.01
2010	674548.00	1942553.86	1888708.67	1955327.98	1851450.35	1901833.74
2011	674548.00	1981244.57	1918442.08	1997283.37	1867949.89	1959490.70
2012	882564.91	2643562.92	2548953.27	2669276.46	2464106.18	2649655.42
2013	882564.91	2695625.10	2587855.86	2726551.02	2482853.42	2747531.88
2014	882564.91	2748405.09	2626758.44	2785054.52	2500323.80	2858998.54
2015	882564.91	2801900.72	2665661.03	2844813.32	2516607.33	2985759.39
2016	882564.91	2856109.31	2704563.62	2905854.37	2531789.21	3129611.06
2017	882564.91	2911027.67	2743466.21	2968205.17	2545949.59	3292442.78
2018	882564.91	2966652.04	2782368.80	3031893.82	2559163.48	3476236.31
2019	882564.91	3022978.12	2821271.39	3096949.05	2571500.86	3683065.90
2020	882564.91	3080001.05	2860173.98	3163400.16	2583026.76	3915098.22
2021	882564.91	3137715.41	2899076.57	3231277.11	2593801.46	4174592.36
2022	882564.91	3196115.20	2937979.15	3300610.49	2603880.78	4463899.74
2023	882564.91	3255193.81	2976881.74	3371431.56	2613316.25	4785464.05
2024	882564.91	3314944.08	3015784.33	3443772.23	2622155.45	5141821.27
2025	882564.91	3375358.23	3054686.92	3517665.11	2630442.24	5535599.57
2026	882564.91	3436427.89	3093589.51	3593143.51	2638217.04	5969519.29
2027	882564.91	3498144.08	3132492.10	3670241.44	2645517.05	6446392.88
2028	882564.91	3560497.21	3171394.69	3748993.67	2652376.57	6969124.89
2029	882564.91	3623477.10	3210297.27	3829435.67	2658827.14	7540711.91
2030	882564.91	3687072.94	3249199.86	3911603.72	2664897.84	8164242.54
			net import (tons)			
2008	674548.00	438564.96	476149.53	431348.73	490616.71	499174.59
2009	674548.00	454450.46	499889.63	444610.98	525113.79	507782.89
2010	674548.00	470844.38	524689.57	458070.26	561947.90	511564.50
2011	674548.00	487761.47	550563.96	471722.67	601056.15	509515.34
2012	882564.91	-117857.10	-23247.45	-143570.65	61599.64	-123949.60
2013	882564.91	-112107.37	-4338.13	-143033.29	100664.31	-164014.15
2014	882564.91	-105940.82	15705.82	-142590.25	142140.46	-216534.28
2015	882564.91	-99330.68	36909.01	-142243.28	185962.70	-283189.35

Table 7 (*continued*)

2016	882564.91	-92247.71	59297.98	-141992.76	232072.39	-365749.46
2017	882564.91	-84660.34	82901.11	-141837.84	280417.74	-466075.46
2018	882564.91	-76534.82	107748.42	-141776.61	330953.74	-586119.09
2019	882564.91	-67835.21	133871.52	-141806.14	383642.04	-727922.99
2020	882564.91	-58523.55	161303.53	-141922.65	438450.75	-893620.72
2021	882564.91	-48559.81	190079.03	-142121.51	495354.14	-1085436.76
2022	882564.91	-37902.02	220234.02	-142397.32	554332.39	-1305686.57
2023	882564.91	-26506.22	251805.85	-142743.97	615371.34	-1556776.46
2024	882564.91	-14326.51	284833.25	-143154.65	678462.12	-1841203.70
2025	882564.91	-1315.02	319356.30	-143621.90	743600.97	-2161556.36
2026	882564.91	12578.04	355416.42	-144137.58	810788.89	-2520513.36
2027	882564.91	27404.42	393056.40	-144692.95	880031.44	-2920844.38
2028	882564.91	43217.85	432320.38	-145278.61	951338.49	-3365409.83
2029	882564.91	60074.03	473253.86	-145884.54	1024723.99	-3857160.78
2030	882564.91	78030.68	515903.76	-146500.10	1100205.79	-4399138.91
			self-sufficiency (%)			
2008	674548.00	80.977	79.346	81.290	78.7187	78.348
2009	674548.00	80.734	78.808	81.151	77.7387	78.473
2010	674548.00	80.490	78.259	81.020	76.7155	78.803
2011	674548.00	80.245	77.701	80.894	75.6559	79.364
2012	882564.91	104.666	100.920	105.684	97.5611	104.908
2013	882564.91	104.339	100.168	105.536	96.1036	106.348
2014	882564.91	104.009	99.406	105.396	94.6209	108.194
2015	882564.91	103.675	98.634	105.263	93.1190	110.479
2016	882564.91	103.338	97.855	105.137	91.6033	113.233
2017	882564.91	102.995	97.067	105.018	90.0785	116.490
2018	882564.91	102.648	96.272	104.906	88.5488	120.280
2019	882564.91	102.295	95.470	104.799	87.0178	124.632
2020	882564.91	101.937	94.661	104.697	85.4889	129.576
2021	882564.91	101.572	93.847	104.601	83.9647	135.137
2022	882564.91	101.200	93.027	104.509	82.4479	141.343
2023	882564.91	100.821	92.201	104.421	80.9405	148.217
2024	882564.91	100.434	91.370	104.337	79.4444	155.784
2025	882564.91	100.039	90.535	104.257	77.9611	164.064
2026	882564.91	99.635	89.695	104.179	76.4921	173.079
2027	882564.91	99.223	88.851	104.104	75.0385	182.848
2028	882564.91	98.801	88.003	104.031	73.6012	193.387
2029	882564.91	98.369	87.152	103.960	72.1811	204.713
2030	882564.91	97.928	86.298	103.891	70.7789	216.840

-ve indicate surplus after consumption

TABLE 8
Predicted rice production, net import and self-sufficiency based on five yield models (1= logistic growth, 2 = simple linear, 3 = exponential, 4 ='logistic polynomial', 5 =' high order modified-polynomial) and mean planted hectareage with new additional rice growing areas

Year	ha	Model 1	Model 2	Model 3	Model 4	Model 5
			production (tons)			
2008	674548	1866826.42	1829241.85	1874042.65	1814774.68	1806216.80
2009	674548	1904414.43	1858975.26	1914253.91	1833751.10	1851082.01
2010	714548	2057745.30	2000707.14	2071276.91	1961239.44	2014610.52
2011	754548	2216216.08	2145965.35	2234157.06	2089484.89	2191882.25
2012	774548	2320017.87	2236987.47	2342584.34	2162524.80	2325364.71
2013	774548	2365708.18	2271128.78	2392849.08	2178977.57	2411262.10
2014	774548	2412028.43	2305270.09	2444192.35	2194309.76	2509086.38
2015	774548	2458976.74	2339411.40	2496637.28	2208600.35	2620333.00
2016	774548	2506550.76	2373552.71	2550207.53	2221924.12	2746578.70
2017	774548	2554747.67	2407694.02	2604927.23	2234351.41	2889481.48
2018	774548	2603564.19	2441835.33	2660821.05	2245948.06	3050780.56
2019	774548	2652996.53	2475976.64	2717914.18	2256775.47	3232296.32
2020	774548	2703040.44	2510117.95	2776232.35	2266890.72	3435930.27
2021	774548	2753691.16	2544259.26	2835801.86	2276346.71	3663664.98
2022	774548	2804943.41	2578400.57	2896649.55	2285192.42	3917564.09
2023	774548	2856791.40	2612541.88	2958802.84	2293473.09	4199772.23
2024	774548	2909228.85	2646683.19	3022289.75	2301230.46	4512514.97
2025	774548	2962248.93	2680824.50	3087138.90	2308503.03	4858098.84
2026	774548	3015844.29	2714965.81	3153379.52	2315326.27	5238911.22
2027	774548	3070007.04	2749107.12	3221041.45	2321732.84	5657420.37
2028	774548	3124728.79	2783248.43	3290155.21	2327752.82	6116175.33
2029	774548	3180000.59	2817389.74	3360751.94	2333413.91	6617805.95
2030	774548	3235812.95	2851531.05	3432863.46	2338741.61	7165022.80
			net import (tons)			
2008	674548	438564.96	476149.53	431348.73	490616.71	499174.59
2009	674548	454450.46	499889.63	444610.98	525113.79	507782.89
2010	714548	355652.94	412691.10	342121.33	452158.80	398787.72
2011	754548	252789.96	323040.69	234848.98	379521.15	277123.79
2012	774548	205687.95	288718.34	183121.47	363181.01	200341.11
2013	774548	217809.55	312388.95	190668.65	404540.16	172255.63
2014	774548	230435.83	337194.17	198271.92	448154.51	133377.88
2015	774548	243593.29	363158.63	205932.75	493969.69	82237.04
2016	774548	257310.84	390308.89	213654.08	541937.48	17282.91
2017	774548	271619.65	418673.30	221440.09	592015.91	-63114.16
2018	774548	286553.03	448281.88	229296.17	644169.15	-160663.35
2019	774548	302146.37	479166.26	237228.72	698367.43	-277153.42
2020	774548	318437.06	511359.55	245245.15	754586.79	-414452.76
2021	774548	335464.44	544896.34	253353.74	812808.89	-574509.38
2022	774548	353269.77	579812.60	261563.63	873020.75	-759350.92
2023	774548	371896.18	616145.70	269884.75	935214.50	-971084.64
2024	774548	391388.72	653934.38	278327.82	999387.11	-1211897.40
2025	774548	411794.28	693218.71	286904.31	1065540.18	-1484055.62
2026	774548	433161.64	734040.12	295626.41	1133679.66	-1789905.29

Table 8 (*continued*)

2027	774548	455541.45	776441.38	304507.04	1203815.66	-2131871.87
2028	774548	478986.27	820466.63	313559.85	1275962.24	-2512460.27
2029	774548	503550.55	866161.39	322799.19	1350137.22	-2934254.82
2030	774548	529290.67	913572.57	332240.17	1426362.01	-3399919.18
			self-sufficiency (%)			
2008	674548	80.9766	79.3463	81.2896	78.7187	78.348
2009	674548	80.7344	78.8080	81.1515	77.7387	78.473
2010	714548	85.2634	82.9000	85.8241	81.2646	83.476
2011	754548	89.7615	86.9162	90.4881	84.6286	88.776
2012	774548	91.8562	88.5688	92.7497	85.6206	92.068
2013	774548	91.5693	87.9084	92.6198	84.3415	93.333
2014	774548	91.2795	87.2394	92.4967	83.0403	94.953
2015	774548	90.9866	86.5625	92.3801	81.7222	96.957
2016	774548	90.6902	85.8781	92.2697	80.3920	99.375
2017	774548	90.3898	85.1869	92.1652	79.0538	102.233
2018	774548	90.0851	84.4891	92.0662	77.7113	105.559
2019	774548	89.7756	83.7853	91.9723	76.3677	109.379
2020	774548	89.4609	83.0758	91.8833	75.0259	113.717
2021	774548	89.1406	82.3610	91.7986	73.6883	118.598
2022	774548	88.8143	81.6411	91.7180	72.3571	124.044
2023	774548	88.4815	80.9165	91.6410	71.0342	130.077
2024	774548	88.1420	80.1875	91.5674	69.7212	136.717
2025	774548	87.7952	79.4544	91.4967	68.4195	143.984
2026	774548	87.4410	78.7173	91.4286	67.1302	151.896
2027	774548	87.0788	77.9767	91.3628	65.8545	160.469
2028	774548	86.7085	77.2328	91.2990	64.5931	169.719
2029	774548	86.3298	76.4857	91.2367	63.3469	179.658
2030	774548	85.9422	75.7358	91.1758	62.1163	190.301

-ve indicate surplus after consumption

TABLE 9
Predicted rice production, net import and self-sufficiency based on five yield models (1 = logistic growth, 2 = simple linear, 3 = exponential, 4 = 'logistic polynomial', 5 =' high order modified-polynomial) and mean planted hectareage with new additional rice growing areas and with the non-irrigated conversion into fully-irrigated areas

Year	ha	Model 1	Model 2	Model 3	Model 4	Model 5
			production ($\times$1000 tons)			
2008	674548.00	1866826.42	1829241.85	1874042.65	1814774.68	1806216.80
2009	674548.00	1904414.43	1858975.26	1914253.91	1833751.10	1851082.01
2010	714548.00	2057745.30	2000707.14	2071276.91	1961239.44	2014610.52
2011	754548.00	2216216.08	2145965.35	2234157.06	2089484.89	2191882.25
2012	982564.91	2943094.76	2837765.25	2971721.81	2743304.48	2949877.57
2013	982564.91	3001055.91	2881075.74	3035485.92	2764175.89	3058844.04
2014	982564.91	3059816.19	2924386.23	3100618.22	2783625.77	3182940.56
2015	982564.91	3119373.20	2967696.72	3167148.06	2801754.33	3324064.18
2016	982564.91	3179723.96	3011007.21	3235105.43	2818656.41	3484215.13

Table 9 (*continued*)

2017	982564.91	3240864.90	3054317.70	3304520.96	2834421.24	3665496.68
2018	982564.91	3302791.85	3097628.19	3375425.93	2849132.35	3870115.14
2019	982564.91	3365500.02	3140938.68	3447852.31	2862867.63	4100379.78
2020	982564.91	3428984.00	3184249.17	3521832.74	2875699.48	4358702.79
2021	982564.91	3493237.75	3227559.66	3597400.56	2887695.03	4647599.20
2022	982564.91	3558254.59	3270870.15	3674589.84	2898916.39	4969686.87
2023	982564.91	3624027.17	3314180.64	3753435.36	2909420.96	5327686.38
2024	982564.91	3690547.51	3357491.13	3833972.67	2919261.70	5724421.06
2025	982564.91	3757806.96	3400801.62	3916238.07	2928487.43	6162816.85
2026	982564.91	3825796.18	3444112.11	4000268.63	2937143.16	6645902.32
2027	982564.91	3894505.19	3487422.60	4086102.24	2945270.31	7176808.60
2028	982564.91	3963923.31	3530733.09	4173777.57	2952907.05	7758769.35
2029	982564.91	4034039.21	3574043.58	4263334.15	2960088.51	8395120.67
2030	982564.91	4104840.85	3617354.07	4354812.33	2966847.05	9089301.13
			net import (tons)			
2008	674548.00	438564.96	476149.53	431348.73	490616.71	499174.59
2009	674548.00	454450.46	499889.63	444610.98	525113.79	507782.89
2010	714548.00	355652.94	412691.10	342121.33	452158.80	398787.72
2011	754548.00	252789.96	323040.69	234848.98	379521.15	277123.79
2012	982564.91	-417388.94	-312059.44	-446015.99	-217598.66	-424171.76
2013	982564.91	-417538.18	-297558.01	-451968.19	-180658.16	-475326.31
2014	982564.91	-417351.92	-281921.97	-458153.96	-141161.51	-540476.29
2015	982564.91	-416803.16	-265126.69	-464578.02	-99184.29	-621494.15
2016	982564.91	-415862.35	-247145.61	-471243.82	-54794.80	-720353.52
2017	982564.91	-414497.58	-227950.38	-478153.63	-8053.92	-839129.36
2018	982564.91	-412674.63	-207510.98	-485308.71	40984.87	-979997.92
2019	982564.91	-410357.11	-185795.78	-492709.40	92275.27	-1145236.87
2020	982564.91	-407506.49	-162771.67	-500355.23	145778.03	-1337225.28
2021	982564.91	-404082.15	-138404.06	-508244.96	201460.57	-1558443.60
2022	982564.91	-400041.42	-112656.98	-516376.67	259296.78	-1811473.69
2023	982564.91	-395339.58	-85493.05	-524747.78	319266.63	-2098998.80
2024	982564.91	-389929.94	-56873.56	-533355.10	381355.88	-2423803.48
2025	982564.91	-383763.74	-26758.41	-542194.86	445555.78	-2788773.63
2026	982564.91	-376790.25	4893.82	-551262.70	511862.77	-3196896.39
2027	982564.91	-368956.69	38125.90	-560553.74	580278.19	-3651260.10
2028	982564.91	-360208.25	72981.97	-570062.51	650808.01	-4155054.29
2029	982564.91	-350488.07	109507.55	-579783.01	723462.62	-4711569.54
2030	982564.91	-339737.22	147749.55	-589708.71	798256.57	-5324197.50
			self-sufficiency (%)			
2008	674548.00	80.977	79.346	81.290	78.719	78.348
2009	674548.00	80.734	78.808	81.151	77.739	78.473
2010	714548.00	85.263	82.900	85.824	81.265	83.476
2011	754548.00	89.761	86.916	90.488	84.629	88.776
2012	982564.91	116.526	112.355	117.659	108.615	116.794
2013	982564.91	116.162	111.518	117.494	106.993	118.398
2014	982564.91	115.794	110.669	117.338	105.342	120.453
2015	982564.91	115.422	109.810	117.190	103.670	122.996
2016	982564.91	115.046	108.942	117.050	101.983	126.063
2017	982564.91	114.665	108.065	116.918	100.285	129.689
2018	982564.91	114.279	107.180	116.792	98.582	133.909

Table 9 (*continued*)

2019	982564.91	113.886	106.287	116.673	96.877	138.754
2020	982564.91	113.487	105.387	116.560	95.175	144.257
2021	982564.91	113.081	104.480	116.453	93.478	150.449
2022	982564.91	112.667	103.567	116.350	91.790	157.358
2023	982564.91	112.245	102.648	116.253	90.112	165.011
2024	982564.91	111.814	101.723	116.159	88.446	173.435
2025	982564.91	111.374	100.793	116.070	86.795	182.654
2026	982564.91	110.925	99.858	115.983	85.159	192.690
2027	982564.91	110.465	98.919	115.900	83.541	203.566
2028	982564.91	109.995	97.975	115.819	81.941	215.299
2029	982564.91	109.515	97.027	115.740	80.360	227.908
2030	982564.91	109.023	96.076	115.662	78.799	241.409

-ve indicate surplus after consumption

CONCLUSIONS

The predicted Malaysian population of 36.7 and 45.7 million people in 2020 and 2030 are 'certainly' to occur and thus the staple food (rice) consumption would then be in tandem with the population trend. The decay rice per capita consumption model had plateau values in the vicinity of 82.0 kg/person/year after 2003. With years ahead, the per capita arable land will decrease and based on the mean rice planted hectareage, the Malaysian per capita rice area in 2007 was 0.025 ha/person. It is envisaged therefore, the role of scientists in research and development is crucial in producing applicable technologies for higher rice production with limited land suitable for agriculture.

Based on the national (average) data for 1980 – 2007, the developed deterministic mathematical models in predicting population increase, rice production and consumption, rice-related (crop and management) technological advancement are then unlikely arbitrary in nature. Model applications which are subjected to several logical options and sensitivities will yield predicted figures with a high accuracy level!

The recoded paddy yields by the Department of Agriculture for 1998 – 2007 were less than 4.0 tons/ha (2.9 – 3.5 tons/ha). This trend is likely to reflect the lowest developed yield model (Model 4: 'logistic polynomial') in this paper. Even for 2030, the average predicted national average paddy yield is only 4.3 tons/ ha. If this continued to happen, the country would always be facing with high import for rice and thus rice stock-pile would be built from imported supplies (and not from the local). This is supported by the data which indicated that we were on the average of 70% self rice sufficiency for years. Stock-pile is important since rice is Malaysian security and staple food and it has the social and political implications for this country. Since rice is now one form of crisis globally, what will happen if the rice producing countries stop exporting their rice?

Model 5 yields a function ('modified higher-order polynomial') which provides quantitative moving target (over years) which should be used as a focal objective in research activities for generating advanced and applicable technologies for rice production. This would be used in research success and areas, respectively. Yield movement over time is basically driven by technology, whereby only about 5% is affected by the climatic fluctuation. Model 5 had predicted the national average yield of 13.4 tons/ha in 2030. This is comparable to the crop cutting test yield of 14.0 tons/ha.

Based on the average rice hectareage (674, 548 ha) which conditioned with the completed conversion of the non-fully to fully irrigated paddy fields (by 2012), the high yield model predicted the self-sufficiency would be firstly attained in 2012. The yearly rice production,

surplus and self-sufficiency would be 2.6 – 8.2 million tons, 0.12 – 4.4 million tons and 104 – 216%, respectively from 2012 to 2030. With the inclusion of an additional of 100,000 ha new rice areas (40,000, 40,000 and 20,000 ha which would be implemented in 2010, 2011 and 2012, respectively), the respective figures would be 2.9 – 9.1 million tons, 0.42 – 5.3 million tons and 117 – 241 %, respectively.

In summary, the above prediction could be 'realized' if Model 5 ('modified higher-order polynomial') was used as the quantitative moving objective versus the years of the R&D programs and the activities in the Malaysian rice production. The order of priority of research areas would be irrigation/water related methods and technique > crop establishment related technology (especially high planting density with proper fertilization rate) > sustainability of existing management practices > large adaptive technology testing (technological uniformity studies) > continual varietal improvement and development. In the medium term, the existing released varieties are locally and generally well suited and adaptable in all the Malaysian rice growing environments.

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Pollen and Seed Yield Components of Water-stressed Cultivated and Weedy Rice

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ABSTRACT

Water stress occurring during the early phase of the reproductive growth stage may influence plant reproduction success. The objectives of this study were to evaluate the responses of pollen and seed yield components to water stress during anthesis in cultivated rice (*Oryza sativa* L.) varieties and weedy rice strains. Studies were conducted in 2007 using three cultivated rice varieties; MR 84, MR 219 and MR 232. In 2008 three weedy rice strains were obtained from Seberang Perak, Kuala Pilah and Tanjung Karang areas. Studies were conducted in the field where plants were grown in polybags and submerged in polyethylene tanks. Prior to anthesis, plants were subjected to water stress by taking the plants out of the polyethylene tanks for five days. Flag leaf relative water content (RWC) and relative injury (RI) were measured daily during the stressed period. Pollen production and pollen viability were measured upon re-watering. Seed yield components measured were grain number per panicle, 100-grain weight, percentage filled grain and percentage spikelet sterility at harvest. Water stress caused a 13 – 34% decline in the number of pollen grains per anther in cultivated varieties but increased pollen production in weedy strains. Percentage pollen viability declined by 40 – 45% in MR 219 and MR 232, but increased by 15% in MR 84 when plants were water-stressed. Percent pollen viability in weedy strains never exceeded 52% and significantly declined with water stress. For cultivated varieties, water stress increased total number of grain per panicle by 31%. In weedy strains, only Seberang Perak increased in the number of grain per panicle due to water stress. Spikelet sterility was relatively higher in cultivated varieties (16 – 50%) compared with weedy strains (10 – 23%). The 100-grain weight was not affected by water stress in both cultivated varieties and weedy strains. This study indicated that weedy rice strains would gain a competitive advantage by producing more filled grains when water deficit occurs during anthesis.

Keywords: Water stress, weedy rice, pollen, seed yield components

INTRODUCTION

For successful seed set in cereal crops, viable pollen, receptive stigma and well developed ovule are required. Unfavourable environments occurring during the early phase of the reproductive growth stage will interrupt normal pollen grain and ovule developments and subsequently will lead to reduced or complete failure in seed set. Water availability during early reproductive growth stage especially during anthesis is one of the major environmental limitations and has been implicated in inconsistent yield potential and reduced seed yields in several grain crops (Boyer and Westgate, 2004; Barnabas *et al*. 2008).

The degree of damage caused by water deficit in several grain crops is strongly dependent on the genotype, severity of water shortage and plant growth stage at which it occurs (Jongdee

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et al., 2002; Lafitte, 2002; Liu *et al*. 2006; Vanuprasad *et al*. 2007). During early growth in plant reproduction, different phases show different susceptibility to water deficits. The most sensitive growth stage to water stress in cereals falls between booting stage until anthesis. Water deficits within this short span of time, particularly during meiosis, anthesis or early zygote formation, may subsequently cause the failure of seed set in grain crops. Sheoran and Saini (1996) reported that the most damaging effect of water stress occurred during meiosis in pollen mother cells This clearly indicates that the early reproductive growth stage is the most critical time for reproduction success. Thus, avoiding water deficit through good water management system during this critical stage will likely improve yield potential.

It appears that water availability during pollen grain formation will directly disrupt the reproductive process. The incidence of pollen grains sterility had been reported in barley, maize, wheat and rice when low water potential occurred during microsporogenesis and microgametogenesis (Saini, 1997; Saini and Westgate, 2000). In cultivated rice varieties, reduction in seed yield due to drought or water stress during anthesis had been frequently associated with increased percentage of spikelet sterility (Fukai *et al.*, 1999; Jongdee *et al*., 2002; Liu *et al*., 2006) and spikelet number per panicle (Boonjung and Fukai, 1996). Prolonged stress occurring during anthesis causes changes in panicles and flag leaf transpiration which contributed to severe spikelet desiccation and white heads (Ekanayake *et al.*, 1993).

The degree of seed yield reduction due to water deficit is highly dependent on the timing of stress (Garrity and O'Toole, 1994). Water deficit during meiotic stage had been reported to reduce seed set in some cultivated rice varieties (Sheron and Saini, 1996) and maize (Boyer and Westgate, 2004). In hybrid rice, water deficit during the grain filling stage enhanced grain yield due to remobilization of carbon reserves (Yang *et al*., 2003). Therefore, water deficit prior to or during anthesis in several cultivated varieties is well documented and as accepted evidence which reduces seed set in majority of cereal crops. However, little information are available on whether water deficit during early reproductive growth stage will affect pollen production and viability in weedy rice genotypes in comparison with cultivated rice varieties. The objectives of this study were to compare the differences in pollen and seed yield components response to water stress during anthesis in cultivated rice varieties and weedy rice strains.

MATERIALS AND METHODS

Plant Materials and Rice Culture

This study was conducted at the Faculty of Agriculture, Universiti Putra Malaysia in 2007 and 2008. In 2007 season, three cultivated rice varieties (the old popular MR 84 variety and the new widely planted MR 219 and MR 232 varieties) were used. In 2008, three wild strains of weedy rice seeds collected in November 2007 from three different rice growing areas in Malaysia were used.

Seeds of the cultivated varieties and wild strains of weedy rice were sown in black perforated plastic polybags of 30×30 cm size containing approximately 10 kg of soil obtained from a rice growing area. Several seeds were sown in each polybag, but only two emerging seedlings were allowed to grow to avoid overcrowding. The polybags were submerged in water in polyethylene tanks of 90×90 cm size. A total of 10 polybags were housed in each polyethylene tank. The polyethylene tanks containing seedlings in polyethylene bags were placed in an open field and were arranged in a completely randomized design with two replications and with several samplings per experimental unit. Standard procedures of rice growing culture were followed throughout the studies. Seedlings growth and development were monitored daily.

Water Stress Treatment

When a panicle was protruded through the flag leaf from any tiller within a polyethylene tank, all the 10 polyethylene bags were taken out to

impose water stress treatment for five days. Re-watering of stressed plants by putting back the plants into the polyethylene tank was done on day 6. The control plants were left submerged in water in the polyethylene tank throughout the study. Within this imposition of water-stress period, the polybags were placed under the shade in the glasshouse to avoid additional water from rain. A tensiometer (Irrometer, Model SR, Riverside, CA, USA) was inserted into each polybag of the stressed plants at a depth of 15 cm to monitor soil moisture potential (ψ_w) during the water stress period (Table 1). The first reading of ψ_w was recorded one hour after imposition of water stress in the morning (9.00 – 10.00 h). The final ψ_w reading (day 6) was taken within one hour after the stressed plants were re-watered in the polyethylene tanks.

Measurement of Leaf Water Relations Parameters

Fully extended flag leaves were randomly cut from the stressed plants beginning from 1 day until 5 days stressed period to determine RWC. A total of three flag leaves from different plants were sampled daily at mid-morning (10.00 – 11.00 h). The last flag leaf samples (day 6) were taken within one hour after the stressed plants were re-watered in the polyethylene tanks. Flag leaf samples of the control plant were collected at the same time when flag leaf samples from stressed plants were collected. The cut pieces of 5 cm section flag leaves were put in vials and immediately brought to the laboratory to determine fresh weight (FW). Distilled water was added to the vials containing the flag leaf sections and were kept for 24 h at room temperature (25˚C) in the dark. The flag leaf sections were weighed to determine turgid weight (TW). Dry weight (DW) of the leaf sections were obtained after oven-drying at 80˚C for 48 h. The RWC was calculated using the formula:

$$RWC = [(FW - DW)/(TW-DW)] \times 100$$

Determination of cell membrane thermal stability (CMTS) of flag leaf for control and water stressed plants was done according to the procedures outlined by Prasad *et al.* (2006). The flag leaves collected for RWC determination were also used to determine CMTS. The CMTS was estimated by using the following formula:

$$\% CMTS = [1- (T_1/T_2)]/[1- (C_1/C_2] \times 100$$

where T and C refer to electrolytic leakage (conductivity) in the control and heat-treated samples and subscripts 1 and 2 refer to

TABLE 1
Soil water potential (ψ_w) at different days in polybags during stressed period for cultivated varieties (2007) and weedy rice strains (2008)

Day	Soil Water Potential (ψ_w)	
	Cultivated varieties	Weedy strains
	---------- kPa ----------	
1	5.0 ± 0.01 ‡	5.0 ± 0.02
2	13.13 ± 1.38	10.00 ± 0.57
3	43.75 ± 4.75	34.63 ± 4.5
4	78.13 ± 4.88	61.0 ± 6.01
5	83.0 ± 0.01	87.00 ± 5.00
6 (re-watered)	2.5 ± 0.50	2.0 ± 0.01

‡ ± values indicate standard error of the means.

conductivity before and after autoclaving. Relative Injury (RI) was then derived from the following formula:

$$\% \text{ RI} = 100 - \text{CMTS}$$

Determination of Pollen Quality

Pollen quality determined in this study was referred to as pollen number per anther and pollen viability. During the water stress period, stressed and control plants were monitored daily for anthesis. Prior to anthesis, a total of 6 matured anthers from 20 spikelets from different panicles of stressed plants were randomly selected within 1 h following re-watering (day 6). Spikelets of the control plants were collected on the same day as spikelets from stressed plants were collected.

Pollen number was estimated by placing an anther on a glass slide with grids and squashed with a needle to disperse pollen grains on the slide. Estimation of pollen grain number was done under a light microscope (Hirox Hi-Scope, KH-2700, Japan).

The remaining anthers after determination of pollen number were used for a pollen viability test. A total of six matured anthers were randomly selected from stressed and control plants. Tetrazolium chloride (tetrazolium 2,3,5-triphenyl chloride) test was used to test pollen viability for cultivated varieties. Pollen grains were dusted onto glass Petri dishes. Five to six drops of 0.5% Tetrazolium chloride solution were added. The Petri dishes containing pollen grains were than placed in an oven at 38˚C for two hours. At the end of two hours, a uniform red stain on pollen grains observed under a light microscope (Hirox Hi-Scope, KH-2700, Japan) were considered as viable. Percentage of pollen viability was estimated based on 300 pollen grains. For weedy rice strains, 1% IKI was used to estimate pollen viability (Prasad *et al.* 2006). IKI was used to avoid the damage to pollen grains at high temperature exposure (Huang *et al.*, 2006). A similar procedure was followed as used in cultivated varieties except that pollen grains were not exposed to high temperature, instead the pollen grains were left at room temperature, at approximately 25˚C, while staining in IKI solution.

Determination of Seed Yield Components

Seed yield components were determined when the seed reached full maturity (when seeds changed from green to yellow). The process of seed ripening took 25 – 35 days. A total of twelve randomly selected panicles were harvested from each experimental unit. Panicles were air-dried at room temperature for 24 h before seed yield components were recorded. The grains were separated from panicles to determine the number of grain and grain weight (filled and unfilled spikelets) per panicle. The grains from twelve panicles were then bulked. Percentage sterility and filled grain were calculated based on 15 g of bulked grains. Filled grains were separated from empty spikelets using a General Seed Blower (Hearson, MO., USA). Sub-samples of four random 100-grain samples were counted to determine 100-grains weight.

The data collected were analyzed for analysis of variances by means of SAS statistical analysis package (SAS, 1995). Data from each study date were analyzed separately. Multiple mean comparison analysis for treatment combinations of variety and stress treatment was performed by using least significant different at $\alpha = 0.05$ level when F-test was significant.

RESULTS

Soil Moisture Potential

Soil moisture potential in polyethylene bags of stressed plants increased from 5 kPa at the beginning of stress period (day 1) to 83 kPa and 87 kPa for cultivated and weedy rice, respectively (Table 1). The stressed plants started to show some curled leaves beginning at day 3. By day 4, all leaves on stressed plants were curled, but none of the plants died during the stressed period. The curled leaves of stressed plants recovered their normal shape and structure 3 – 4 h after re-watering.

Flag Leaf Relative Water Content

The RWC of flag leaf in cultivated rice varieties for water-stressed and control plants ranged between 89 – 96% and 82 – 99% at the beginning of stressed and unstressed (control) period, respectively (*Fig. 1*). The RWC for both stressed and control plants were maintained above 90% at the end of day 4. This indicates that stressed period for four days was not severe enough to cause differences in RWC.

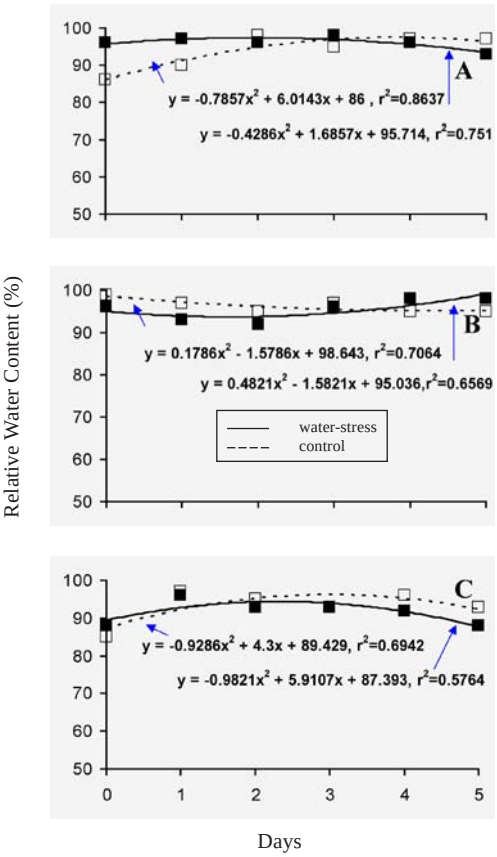

Fig. 1: Relative water content of MR 232 (A), MR 219 (B) and MR 84 (C) varieties for control (□) and water stress (■) treatments

The flag leaf RWC for Seberang Perak strain was between 55 – 60% at the beginning of the stress period (day 1) (*Fig. 2A*). RWC was >91% at day 1 for Kuala Pilah and Tanjung Karang strains (*Fig. 2B* and *2C*). Similar to cultivated rice varieties, RWC for both stressed and control weedy strains was maintained above 90% at the end of day 4.

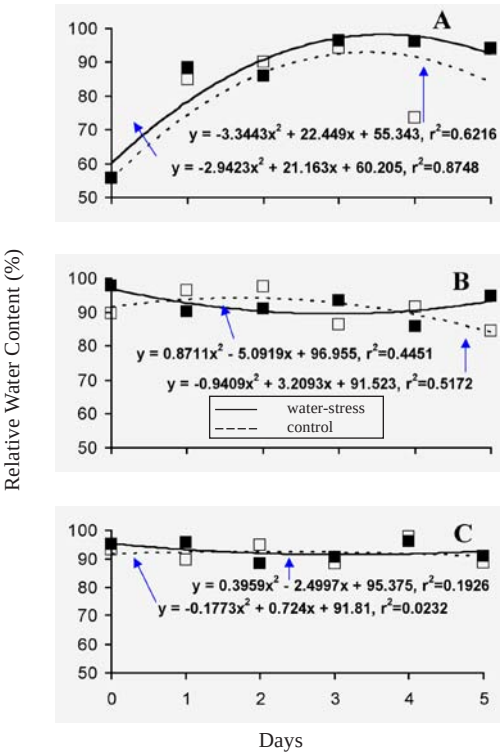

Fig. 2: Relative water content of Seberang Perak (A), Kuala Pilah (B) and Tanjung Karang (C) strains for control (□) and water stress (■) treatments

Flag Leaf Relative Injury

There were slight differences between cultivated varieties in %RI for water stressed and control plants. The stressed plants showed a declining pattern of RI with increased number of stressed period (*Fig. 3*). The RI due to water stress from day 1 to day 5 in MR 219 variety was in the range of 16 – 32%, and 37 – 47% for MR 84 variety. In MR 219 control plants, RI never exceeded 25% when recorded within same period of time. In MR 84 control plants, percent RI increased from 24% at day 1 to 55% at day 5.

RI for weedy rice strains never exceeded 15% during 5 days of stress (*Fig. 4*). Two

strains, Seberang Perak and Kuala Pilah, showed increasing trend of % RI with increased days of stress period. Similarly, the control strains had <15% RI. Tanjung Karang strain showed the lowest % RI after 5 days of water stress compared with other two strains (*Fig. 4C*).

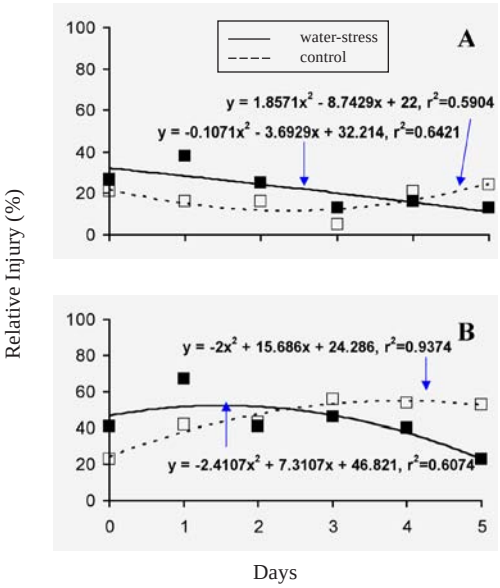

Fig. 3: Relative injury of MR 219 (A) and MR 84 (B) varieties for control (□) and for water stress (■) treatments

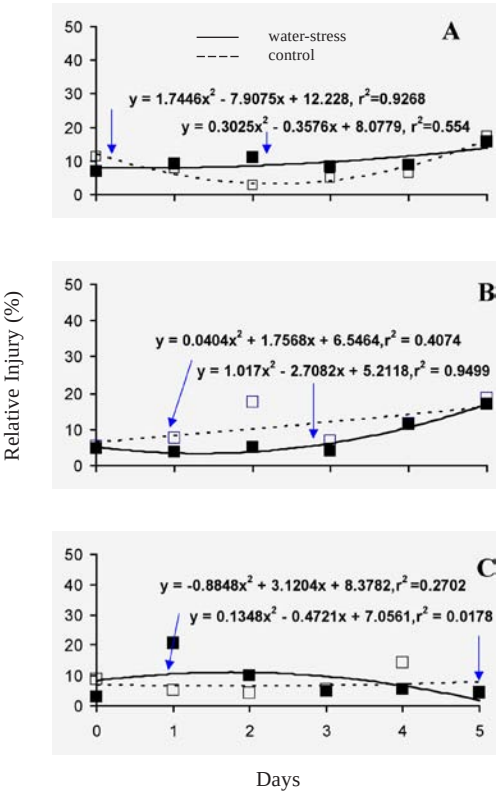

Fig. 4: Relative injury of Seberang Perak (A), Kuala Pilah (B) and Tanjung Karang (C) strains for control (□) and water stress (■) treatments

Pollen Quality

Water-stressed plants of cultivated varieties showed a decline in pollen number per anther by an average of 20% over the control. The number of pollen per anther for cultivated varieties ranged between 448 and 635 for the control plants and declined to 382 – 553 when the plants were water-stressed (Table 2). Only the MR 219 variety showed significant decline in pollen number per anther by 35% when plant were stressed for 5 days. The decline in pollen number per anther due to water stress for MR 232 and MR 84 was on the average of 14%.

Pollen viability as measured by TTC staining significantly declined in MR232 and MR219 (Table 2). The decline in pollen viability in MR232 variety was 45% and 40% for MR219.

Total pollen production in weedy rice strains was much higher than in cultivated varieties for both the control and water stressed plants. The pollen number per anther recorded in control plants ranged between 1276 and 1806 and 1529 – 1888 in water-stressed plants (Table 3). Only Seberang Perak strain significant increased (by 29%) pollen production when the plants were water-stressed during anthesis. The increase in pollen production when subjected to water stress for Kuala Pilah and Tanjung Karang strains was <5%. An average increase of pollen number per anther for water stressed plants was 10% over the control. This indicates that when weedy rice strains experience water stress during anthesis, it will trigger plants to produce more pollen.

Pollen viability was low (21 – 52%) for all weedy rice strains regardless of water stress

TABLE 2
Number of pollen and pollen viability of cultivated varieties of unstress (control) and water stress treatments

Variety	Treatment	No. pollen anther^{-1}	Viability (%)
MR232	Control	635	90
	Water stress	553	49
MR219	Control	511	71
	Water stress	336	42
MR84	Control	448	58
	Water stress	382	67
LSD		152	22
ANOVA		F-value (P- value)	
Stress		8.94 (0.024)	8.45 (0.027)
Variety		10.61 (0.011)	1.16 (0.037)
Stress x variety		0.90 (0.456)	4.64 (0.061)

TABLE 3
Number of pollen and pollen viability of weedy rice strains of unstress (control) and water stress treatments

Strain	Treatment	No. pollen anther^{-1}	Viability (%)
Seberang Perak	Control	1276	21
	Water stress	1650	16
Kuala Pilah	Control	1806	52
	Water stress	1888	37
Tanjung Karang	Control	1519	52
	Water stress	1529	49
LSD		116	4
ANOVA		F-value (P-value)	
Stress		15.33 (0.008)	37.44 (<0.001)
Strain		38.44 (0.004)	236.66 (<0.001)
Stress x strain		8.34 (0.019)	8.15 (0.019)

treatments. Seberang Perak strain had only 21% pollen viability and significantly declined to 16% viability when plants were stressed. Similarly, Kuala Pilah strain showed a significant decline in pollen viability from 52% to 37% when plants were subjected to stress. Tanjung Karang strain did not decline in pollen viability when the plants were stressed for 5 days during anthesis.

Seed Yield Components

Water stress during anthesis for four days increased total number of grain per panicle for all the three cultivated varieties. In MR 84 variety, short period of water stress caused the plant to produce less pollen grains but without a significant reduction in percentage filled grain although high spikelet sterility. The significant

increase in total grain number was only observed in MR 84 variety which was increased by 58% (Table 4). Control plants of MR 232 variety had the highest number of grains per panicle and the lowest was recorded in MR 219. The 100-seed grain weight was not affected when plants were subjected to water stress for 4 days during anthesis. The 100-grain weight were on an average of 2.14 g for MR 219 and MR 232 and 1.72 g for MR 84. Percentage filled grain showed no significant difference between control and water stressed plants for all three varieties. The average percentage filled grain was highest in MR 232 which was 84% compared with 55% in MR84. Percentage sterility (empty spikelets) per panicle was not severely affected when cultivated varieties were stressed for 5 days during anthesis. MR 84 variety had 46% spikelet sterility average across stress treatment. MR 219 showed insignificant increased in percentage sterility from 37% for control to 50% when water-stressed. This indicates that MR 219 was the most sensitive to water stress among the three varieties tested.

Water stress for 5 days during anthesis showed inconsistent and no significant effect on total number of grain per panicle for all three weedy rice strains (Table 5). The total number of grain per panicle was increased in Seberang Perak strain but was decreased in Kuala Pilah strain when plants were water stressed. The 100-seed grain weight for all three weedy rice strains on the average was much higher than the cultivated rice varieties. Only Seberang Perak strain showed a significant increase in 100-grain weight following stress treatment. Percentage filled grain was not affected by water stress in Kuala Pilah and Tanjung Karang strains which was on the average of 89%. Similarly, percentage sterility was not affected by water stress in Kuala Pilah and Tanjung Karang strains. Increase in percentage filled grain from 77% in control plants to 83% was observed in Seberang Perak strain. Concomitant decline in percentage sterility from 23% to 17% was also observed in Seberang Perak strain.

DISCUSSION

Leaf relative water content (RWC) is one of the commonly use indicators of leaf water status when plants are subjected to water stress. Measurement of leaf relative water content is easy to perform; however, inconsistent and

TABLE 4
Seed yield components of cultivated rice varieties of unstress (control) and water stress treatments

Variety	Treatment	No. of grain panicle^{-1}	100-grain wt. (g)	Filled grain (%)	Sterility (%)
MR232	Control	125	2.11	83	18
	Water stress	146	2.20	85	16
MR219	Control	90	2.10	63	37
	Water stress	105	2.17	50	50
MR84	Control	105	1.70	56	45
	Water stress	166	1.74	53	47
LSD		43	0.22	18	15
ANOVA		F-value (P-value)			
Stress		4.81 (0.071)	0.23 (0.648)	0.06 (0.813)	6.21 (0.048)
Variety		4.43 (0.066)	26.39 (0.001)	2.44 (0.168)	13.32 (0.006)
Stress x variety		0.12 (0.889)	0.70 (0.532)	0.61 (0.573)	1.53 (0.290)

TABLE 5
Seed yield components of weedy rice strains subjected to unstress (control) and water stress treatments

Strain	Treatment	No. grains panicle^{-1}	100-grain wt. (g)	Filled grain (%)	Sterility (%)
Seberang Perak	Control	172	2.86	77	23
	Water stress	198	3.37	83	17
Kuala Pilah	Control	150	2.63	90	10
	Water stress	131	2.80	89	11
Tanjung Karang	Control	160	2.81	90	10
	Water stress	159	2.68	87	13
LSD		27	0.60	4	4
ANOVA		F-value (P-value)			
Stress		0.12 (0.744)	0.81 (0.487)	0.08 (0.0790)	0.17 (0.690)
Strain		16.49 (0.003)	1.63 (0.271)	6.09 (0.035)	9.97 (0.012)
Stress x strain		4.23 (0.072)	0.84 (0.487)	1.04 (0.408)	7.75 (0.255)

varying results in relation to seed yields was reported in rice (Jongdee *et al*., 2002; Lafitte, 2002). Similarly, in this study measurement of flag leaf RWC during the stressed period was inconsistently correlated to most of the parameters measured. These results suggest that other factors are as important as RWC in determining response to water stress during flowering. Therefore, RWC of flag leaf recorded during stressed period appeared to be an unreliable plant water stress indicator. The concept of leaf membrane thermal stability to measure plant relative injury to water stress was based on the leaf cell membrane integrity by measuring electrolytes leakage following hot and cold exposure of leaf cells (Agarie *et al*., 1995). The flag leaf RI in MR 219 observed in this study was lower than that in MR 84, but the low percentage value of RI in MR 84 did not contribute to an increase in grain production per panicle. In an earlier study (Prasad *et at*., 2006), rice varieties grown at higher temperature and had lower RI also did not contribute to higher percent spikelet fertility and grain yields. This observation suggests that RI may not be a reliable selection criteria for plant stress in rice. Furthermore, the versatility of leaf membrane thermal stability which measures plant responses to water deficits has yet to be tested on several crop species.

Withholding water from rice plants for five days appeared not to be severe enough to have any critical effect on pollen production in cultivated varieties, instead the weedy strains tended to produce more pollen. In general, pollen production in weedy rice strains was increased by an average of 10% when water-stressed; however, in cultivated varieties it was reduced by an average of 20%. Only the MR 219 variety decreased in pollen production following water stress during anthesis. For MR 219, not only pollen production was reduced but pollen viability was also significantly lowered. Reduction in pollen viability in this particular variety led to reduced proportion of filled grain on panicle in response to higher percentage of spikelet sterility. It indicates that this variety was sensitive to even a short period of water stress, especially during anthesis compared with other two cultivated varieties. Wilcox and Neiland (2002) reviewed several stages which lead to pollination failure and one of them was due to insufficient pollen quantity. Liu *et al*. (2006) implicated increased spikelet

sterility in rice after six days of water stress at anthesis to reduced anther dehiscence and low stigmata-pollen density rather than due to reduced pollen viability. For MR 232 variety, pollen viability was affected but the proportion of filled grain on the panicle was not affected because of low percentage of spikelet sterility. The ability of MR 232 to produce high number of pollen, >550 pollen per anther, under water deficit environment appears to compensate for the reduction in pollen viability and subsequently resulted in high percentage of filled grain. This suggests that high amount of pollen in an anther may give a high chance of successful fertilization. A minimum of 20 pollen grains per stigma are required for a successful fertilization and seed set in rice (Matsui and Kagata, 2003). In this study, it was found that seed size as measured by 100-grain weight was not affected by water stress in both cultivated varieties and weedy strains. This indicates that accumulation of dry weight by seed which determines the final seed size is not affected if water stress occurred within five days during early part of reproductive growth stage in rice.

In general, percentage spikelet sterility in weedy strains was relatively lower (10 – 23%) compared with cultivated varieties (16 – 50%). Pollen viability in weedy strains was relatively low compared with cultivated varieties. However, number of grain and the proportion of filled grain formed in weedy strains were higher than in cultivated varieties. In one of the strains, Seberang Perak, a short period of water stress significantly increased percentage of filled grain. A possible explanation for the increase in the proportion of filled grain in this particular strain was higher pollen number being produced which subsequently caused a greater chance of successful fertilization. Another possibility is that the nature of those weedy strains which can tolerate water stress without affecting the yield components. In hybrid rice, mild water stress prior to anthesis facilitates remobilization of C reserves, increase grain-filling rate and increase grain yield (Yang *et al*., 2003).

CONCLUSIONS

Tolerance to water stress based on RWC and flag leaf percentage relative injury seem to be unreliable indices of plant stress on which to select new rice variety. The data suggest that water stress for less than five days will enhance weedy rice strains to produce higher amount of seeds. Water stress for less than five days during early reproductive growth stage will contribute to a competitive advantage for weedy rice. This study also suggests that longer duration of water stress (>5 days) should be conducted to confirm the differences in the effect of water stress on pollen and seed yield in cultivated and weedy rice.

High amount of pollen per anther and high pollen viability appeared to be important genotypic factors and should be included as a selection criterion for a new rice variety. The new cultivated varieties with high number of pollen per anther can withstand short water stresses, especially during the early reproductive growth stage without negative effect on seed set and seed yields.

The presence of weedy rice in the field and a short period of water stress occurring prior to or during anthesis may contribute to higher incidence of weedy rice infestation in the following growing season.

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Critical Period of Weed Competition in Direct Seeded Rice Under Saturated and Flooded Conditions

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ABSTRACT

Field experiment was conducted at the Malaysian Agriculture Research and Development Institute (MARDI) station, Seberang Perai, Penang in off-season 2005 and main season 2005/2006, to determine the critical period of weed competition in saturated and flooded conditions. The experiment consisted of different seasons, namely weed free and no weeding periods. Sum Dominance Ratio showed that the weed compositions were different in the saturated condition, as compared to the flooded condition for both seasons. The dominance ranking of weed groups in the off- season in 2005 in saturated condition was sedges, followed by grasses and broadleaved, while during the main season of 2005/2006, grassy weeds were the most dominant, followed by sedges and broadleaved weeds. In the flooded condition, the dominance rankings of weed groups (such as broadleaved>grass>sedges) were the same in both seasons. The number of tillers, along with rice grains yield, was significantly affected by the weed competition in both saturated and flooded conditions. Yield loss due to weed competition was higher in the saturated condition (54.5%) than in the flooded condition (35.2%). Based on the 5% level of yield loss, the critical period in the off-season of 2005 was between 2 – 71 days, after sowing (DAS) in saturated condition, and 15 – 73 DAS in flooded condition. Meanwhile in the main season of 2005/2006, the critical period was between 0 – 72 DAS in the saturated condition and 2 – 98 DAS in the flooded condition.

Keywords: Rice-weed competition, weed control, minimal water condition

INTRODUCTION

Global climatic change, either directly or indirectly, induces changes in land use (Kathiresan, 2005). Scarcity and growing competition for fresh water resources also reduce its availability for irrigation and rice cultivation will be affected by this phenomenon. Weed has always been a "perennial problem" in rice fields in Malaysia. Weed emergence in relation to crop emergence is an important factor in weed-crop competition. Weeds which emerge along with crop plants have an adverse effect

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on the crop yields. A crop loss due to weed competition varies with the duration of weed infestation of the crop. The crop is likely to experience yield reduction, unless weeds are kept free during a part of its growing period (Azmi *et al*., 2007). Weed interference and yield losses in direct seeded rice was 55% (Ampong Nyarko and De Datta, 1991) and uncontrolled *Fimbristylis miliacea* alone reduced grain yields by 42% (Begum, 2006). The optimum time, at which crop must be free of the adverse effect of weeds, is referred to as the critical period of weed competition. Almost all the annual crops are susceptible to weed competition during the early stage of development, particularly within the first one-third to one-half of the crop life cycle (Mercado, 1979). The critical period of weed competition represents the time interval (overlap) between the two separate components: (i) the length of time crop must be free of weed after planting so that later-emerging weeds do not reduce yield, and (ii) the length of time weeds which emerge with the crop can remain before they begin to interfere with crop growth (Ghosheh *et al*., 1996; Hall *et al.*, 1992). Thus, weed control during this period is necessary to avoid considerable reduction in crop yield (Weaver, 1984). This may be accomplished by removing weed at the beginning of the critical period or keeping the crop weed-free until the end of the critical period (Woolley *et al*., 1993). Begum *et al*. (2008) observed that based on the predicted logistic and gompertz curves, the critical period for controlling of rice weed *F. miliacea* in direct seeded rice was between 14 – 28 DAS. Ghosheh *et al*. (1996) pointed out that long critical periods are indicative of more competitive weeds or less competitive crops. In general, the critical period is one of the important alternative weed management strategies in order to minimize the labour requirement for weeding operations, enhance the efficiency of herbicide use and maximize economic returns. However, determining when the critical period occurs is difficult due to many interacting factors involved, including crop cultivar, weed community, crop management practices and environment (Hartzler, 2008). The critical period of weed interference in direct-seeded rice, under saturated and flooded conditions, are very meager. Thus, this study was carried out to determine the critical period of weed competition in direct-seeded rice under saturated and flooded conditions.

MATERIALS AND METHODS

Two experiments were carried out in two cropping seasons, off season 2005 and main season 2005/2006, at the experimental field in MARDI Seberang Prai Research Station, in Penang. The soil was Sogomana soil series with an average pH 5.0, 1.1% organic matter content and 8.4 Cation Exchange Capacity (CEC). The local climate is tropical with the annual average rainfall ranges between 156 – 208mm. Meanwhile, the minimum and maximum annual temperatures were 25 and 35˚C, respectively. Land preparation was done according to the MARDI Rice Cultivation Manual 2002. Healthy rice seeds of the MR220 variety (150 kg ha^{-1}) were used and drum-seeded to facilitate and avoid crop damage, due to manual hand weeding, and water was applied according to the treatment throughout the growing period. The total required fertilizer was applied at 170 kg N/ha, 80 kg P_2O_5/ha and 150 kg K_2O/ha as urea, Triple super phosphate (TSP) and Muriate of potash (MOP) respectively. The fertilizers were applied at 15, 35, 55 and 75 DAS, in accordance with the split of N as 15%, 35%, 25% and 25%; P_2O_5 70%, 20% and 10%; K_2O 15%, 45%, 30% and 10%. The experiment was established in the split plot design, with four replications. Two water regimes (flooded and saturated) were the main plot, and the times of weed removal were subplots. The weed removal treatments were divided into two components, the weed-free period and weed competition periods (*Fig. 1*). These plots were kept free of weeds by hand weeding for 0, 15, 30, 45, 60 and 75 days, after sowing (DAS) and subsequently weeds were allowed to grow until harvest in weed-free treatment; the weeds were allowed to compete for 0, 15, 30, 45, 60 and 75 DAS, after which

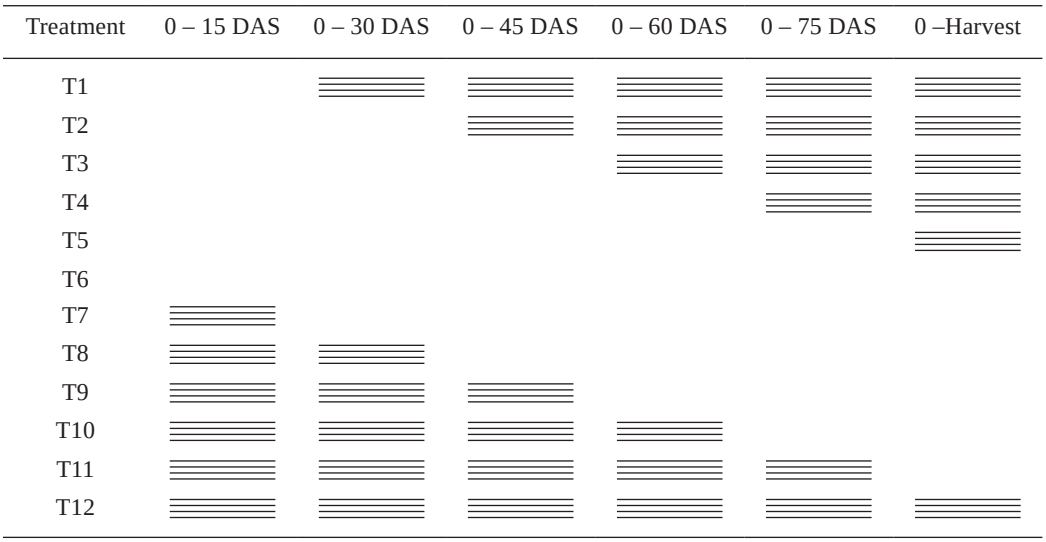

Fig. 1: Time of weed removal treatments in saturated and flooded conditions

Weed competition

Weed free (Manual weeding)

the plots were free from the weeds until harvest for the weed competition treatment.

The twelve weed removal treatments (T1 – T12) for both water regimes were as follows:

- T1: Weed-free until 15 DAS
- T2: Weed-free until 30 DAS
- T3: Weed-free until 45 DAS
- T4: Weed-free until 60 DAS
- T5: Weed-free until 75 DAS
- T6: Weed-free from sowing to maturity
- T7: Weedy until 15 DAS
- T8: Weedy until 30 DAS
- T9: Weedy until 45 DAS
- T10: Weedy until 60 DAS
- T11: Weedy until 75 DAS
- T12: Weedy from sowing to maturity

A total of 96 plots were prepared and each plot size was 8m $\times$ 7m. These plots were separated by 25 cm width and 25 cm height of levee constructed before seeding. Overflow canals were also constructed to ensure that the saturated condition was maintained throughout the experiment. Water was introduced at 7 DAS, and maintained thereafter in both treatments, less than 2 cm water depth for the saturated condition and 5 – 10 cm water depth in the flooded condition. At 60 DAS, weeds were recorded using 0.5m $\times$ 0.5m quadrat according to Kim and Moody (1983) at 30, 60 and 90 DAS. The weeds were separated into different species and dried in an oven for 48 hours at 65˚C and dry matter was determined. The tillers of rice were recorded at the day of sampling 30, 60 and 90 DAS, using the same size of quadrat. The yields of rice grain were obtained from the centre of the 5 m $\times$ 5 m area of each plot at harvesting 115 DAS and converted to t/ha at 14% moisture.

The summed dominance ratio (SDR) of the weed species was computed using the following equation (Janiya and Moody, 1989):

$$SDR = \frac{\text{Relative density (RD)} + \text{Relative dry weight (RDW)}}{2}$$

Where,

$$RD = \frac{\text{Density of a given species}}{\text{Total density}} \times 100$$

$$RDW = \frac{\text{Dry weight of a given species}}{\text{Total dry weight}} \times 100$$

The number of tiller and grain yields were subjected to the Analysis of Variance using the Statistical Analysis System (SAS). The means separation was done using the Duncan New Multiple Range Test (DNMRT). The grain yield was analyzed using the non-linear models. The critical weed-free period and the critical time of weed removal were calculated by substituting rice yields, expressed as per cent of control, into the Gompertz and logistic equations, respectively. The yield loss levels of 5 and 10% were chosen arbitrarily (Kiani and Faravani, 2003; Martin *et al.*, 2001; Hall *et al.*, 1992). The equation with the highest co-efficient of determination (r^2) value was judged as the most appropriate (Papamichail *et al.*, 2008).

RESULTS AND DISCUSSION

Weed Composition and Summed Dominance Ratio (SDR)

In saturated condition, the number of weed species was higher (10 species) in the off-season than the main season (*Figs. 2a* and *2b*). Sedges were dominant weeds (6 – 27 % SDR), followed by grasses (8 – 12 % SDR) and broadleaved weeds (2 – 6 % SDR) in the off-season, while in the main season, grasses were among the dominant (4 – 35% SDR), followed by broadleaved weeds (8 – 13 % SDR) and sedges (6% SDR) (*Fig. 2b*). According to Bhagat *et al*. (1996), the composition of rice weed communities is strongly influenced by water management practices. The saturated condition usually favoured germination of sedges and grassy weeds. Tanaka (1976) found that sedges and grasses accounted for more than 90 % of the total dry weight in saturated condition. In addition, Bhagat *et al*. (1999) also reported that the dominance of *Echinochloa* species and *Lepthocloa chinensis* was favoured by the saturated condition. The six most dominant weeds in the saturated condition in the off-season were *Cyperus iria, F. miliacea, Cyperus digitatus, Echinochloa crus-galli, Echinochloa colona* and *L*. chinensis. Weed succession from

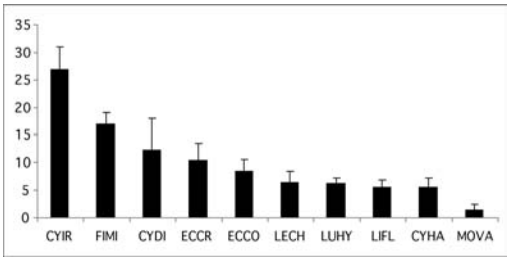

Fig. 2a: Weed dominance ranking (SDR) in off-season 2005 under saturated condition

CYIR = *Cyperus iria*, FIMI = *Fimbristylis miliacea*, CYDI = *Cyperus difformis*, ECCR = *Echinochloa crus-galli*, ECCO = *Echinochloa colona*, LECH = *Leptochloa chinensis*, LUHY = *Ludwigia hysopifolia*, LIFL = *Limnocharis flava,* CYHA = *Cyperus haspan*, MOVA = *Monochoria vaginalis*

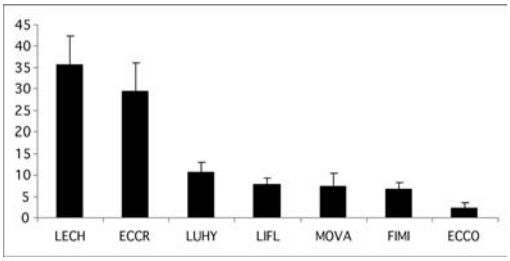

Fig. 2b: Weed dominance ranking (SDR) in main season 2005/06 under saturated condition

LECH = *Leptochloa chinensis*, ECCR = *Echinochloa crus-galli*, LUHY = *Ludwigia hysopifolia*, LIFL = *Limnocharis flava,* MOVA = *Monochoria vaginalis*, FIMI = *Fimbristylis miliacea*, ECCO = *Echinochloa colona*

sedges in the off-season to more competitive grassy weed were observed in the main season (*Fig. 2b*), followed by broadleaved weeds and sedges. *Lepthocloa chinensis* and *E. crus-galli* were at the higher rank of dominant in the main season. This succession was due to ability of the weeds to produce more seeds which contribute to additional soil seed bank in the off-season and emerge in the main season. Early raining in the main season had also changed the environment which encouraged weed seeds to germinate better. On the other hand, the composition of the weed flora might differ, depending on the water supply (Bhan, 1983), cultural practices such as tillage, crop establishment technique, irrigation and fertilizer used at various times during the year (Mabbayad *et al.*, 1983).

In flooded condition, broadleaved weeds *Monochoria vaginalis* and *Limnocharis flava* were recorded dominant in both seasons (*Figs. 2c* and *2d*). Tanaka (1976) reported that broadleaved weeds were dominant over grassy and sedges in the flooded condition. At IRRI, De Datta (1981) reported that flooding to a depth of 15 cm from 4 days after seeding to the late dough-ripening stage allowed more broadleaved weeds and suppressed grass and sedge emergence. Kent and Johnson (2001) also observed that the increase in flooding depth and flooding duration encouraged most of the broadleaved weeds.

Number of Tillers and Rice Grain Yield

In the off-season, the numbers of tillers at 30, 60 and 90 DAS were significantly affected by the weeding interval treatments, both in saturated and flooded conditions (Tables 1 and 2). The significant highest number of tillers, at 30 DAS, were recorded from T6 (weed free throughout) in the flooded condition (598 tillers) and saturated condition (549 tillers), respectively. The numbers of tillers were significantly reduced as the weed free periods reduced or as the weed competition periods increased in both the flooded and saturated conditions. The trend is almost the same in the main season, but the numbers of tillers were generally much lesser as compared to the off-season (Tables 3 and 4).

The yield of rice grains was significantly affected by the weeding interval treatments and water regime treatments in both seasons (Tables 1, 2, 3 and 4). Significant higher rice grain yields of 5.4 and 5.2 ton/ha were recorded in the flooded, weed free treatment throughout sowing to maturity (T6) in the off-season and main season, respectively (Tables 1 and 3). This rice grain yield was not significantly different as compared to the rice grain yield of weed free until 30 DAS to 75 DAS, and the weed competition until 15 DAS in the off-season (Table 1) and the weed free until 60 DAS to 75 DAS and the weed competition until

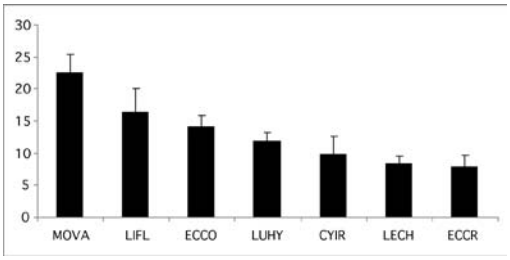

Fig. 2c: Weed dominance ranking (SDR) in off-season 2005 under flooded condition

MOVA = *Monochoria vaginalis*, LIFL = *Limnocharis flava,* ECCO = *Echinochloa colona*, LUHY = *Ludwigia hysopifolia*, CYIR = *Cyperus iria*, LECH = *Leptochloa chinensis*, ECCR = *Echinochloa crus-galli*

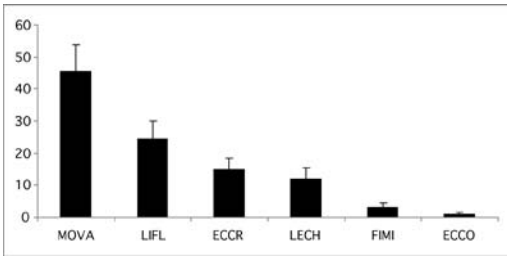

Fig. 2d: Weed dominance ranking (SDR) in main season 2005/06 under flooded condition

MOVA = *Monochoria vaginalis*, LIFL = *Limnocharis flava,* ECCR = *Echinochloa crus-galli*, LECH = *Leptochloa chinensis*, FIMI = *Fimbristylis miliacea*, ECCO = *Echinochloa colona*

TABLE 1
Effect of weeding regime on tiller and grain yield in flooded condition (off-season 2005)

Weed Removal Treatments	Number of tiller/m^2			Grain Yield (t/ha)
	30DAS	60DAS	90DAS	
Weed-free until 15 DAS (T1)	488de	395bc	364bc	4.48bc
Weed-free until 30 DAS (T2)	440fg	407bc	394bc	4.66abc
Weed-free until 45 DAS (T3)	488cde	415abc	385bc	4.76abc
Weed-free until 60 DAS (T4)	511c	433ab	387bc	4.80abc
Weed-free until 75 DAS (T5)	559b	435ab	425b	5.18ab
Weed-free from sowing to maturity (T6)	598a	473a	489a	5.40a
Weedy until 15 DAS (T7)	493cd	394bc	399bc	5.34a
Weedy until 30 DAS (T8)	462ef	432ab	397bc	4.36c
Weedy until 45 DAS (T9)	488cde	400bc	419b	4.36c
Weedy until 60 DAS (T10)	430g	369c	370bc	4.18cd
Weedy until 75 DAS (T11)	471de	397bc	371bc	4.08cd
Weedy from sowing to maturity (T12)	399h	364c	356c	3.50d
R^2	0.934	0.510	0.578	0.649
C.V	3.439	8.893	9.454	10.274
Significant values	0.0001	0.0111	0.0018	0.0001

Means within columns with the same alphabets are not significantly different at P> 0.05

TABLE 2
Effect of weed competition period on the number of tiller and grain yield in saturated condition (off-season 2005)

Weed Removal Treatments	Number of tiller/m^2			Grain Yield (t/ha)
	30DAS	60DAS	90DAS	
Weed-free until 15 DAS (T1)	509bc	345d	343de	2.88d
Weed-free until 30 DAS (T2)	536ab	364d	383bc	3.14bcd
Weed-free until 45 DAS (T3)	485cd	381d	384bc	3.20bcd
Weed-free until 60 DAS (T4)	460de	414bc	373cd	3.32bcd
Weed-free until 75 DAS (T5)	405f	423b	408b	3.52bc
Weed-free from sowing to maturity (T6)	549a	501a	500a	4.40a
Weedy until 15 DAS (T7)	510bc	400bc	374cd	3.56b
Weedy until 30 DAS (T8)	530ab	361d	361cde	3.34bcd
Weedy until 45 DAS (T9)	440e	354d	346de	3.20bcd
Weedy until 60 DAS (T10)	360f	348d	344de	2.92cd
Weedy until 75 DAS (T11)	391f	299e	332ef	2.90cd
Weedy from sowing to maturity (T12)	309g	288e	307f	2.00e
R^2	0.958	0.892	0.889	0.748
C.V	3.848	6.218	5.375	11.832
Significant values	0.0001	0.001	0.0001	0.0001

Means within columns with the same alphabets are not significantly different at P> 0.05

TABLE 3
The effect of weed competition period on the number of tiller and grain yield in flooded condition (main season 2005/2006)

Weed Removal Treatments	Number of tiller/m^2			Rice Grain Yield (t/ha)
	30DAS	60DAS	90DAS	
Weed-free until 15 DAS (T1)	280bc	283bc	297b	1.24e
Weed-free until 30 DAS (T2)	280bc	326abc	327ab	2.68bcde
Weed-free until 45 DAS (T3)	355ab	335abc	311b	3.15bdc
Weed-free until 60 DAS (T4)	361ab	339abc	331ab	3.68abc
Weed-free until 75 DAS (T5)	393a	352ab	364a	4.31ab
Weed-free from sowing to maturity (T6)	403a	363a	368a	5.24a
Weedy until 15 DAS (T7)	370ab	352ab	345ab	3.89abc
Weedy until 30 DAS (T8)	381ab	276bc	302b	2.13cde
Weedy until 45 DAS (T9)	319ab	275bc	330ab	1.65de
Weedy until 60 DAS (T10)	290bc	282bc	309b	1.62de
Weedy until 75 DAS (T11)	285bc	280bc	297b	1.50de
Weedy from sowing to maturity (T12)	205c	268c	295b	1.11e
R^2	0.565	0.474	0.469	0.677
C.V	18.587	14.813	9.924	41.793
Significant values	0.0013	0.0205	0.0187	0.0001

Means within columns with the same alphabets are not significantly different at P> 0.05

TABLE 4
Effect of weed competition period on the number of tiller and grain yield in saturated condition (main season 2005/2006)

Weed Removal Treatments	Number of tiller/m^2			Grain Yield (t/ha)
	30DAS	60DAS	90DAS	
Weed-free until 15 DAS (T1)	376cd	363bcd	238de	2.18de
Weed-free until 30 DAS (T2)	387bc	363bcd	237de	2.40cd
Weed-free until 45 DAS (T3)	364cd	416ab	278abcd	2.82bc
Weed-free until 60 DAS (T4)	357cd	374bcd	301abc	3.06ab
Weed-free until 75 DAS (T5)	448ab	354bcd	320ab	3.29ab
Weed-free from sowing to maturity (T6)	461a	490a	332a	3.38.3a
Weedy until 15 DAS (T7)	410abc	399abc	311ab	2.37cd
Weedy until 30 DAS (T8)	385c	427ab	303abc	1.74ef
Weedy until 45 DAS (T9)	364cd	297cd	274bdc	1.62fg
Weedy until 60 DAS (T10)	397abc	367bcd	266bcde	1.62fg
Weedy until 75 DAS (T11)	390bc	376bcd	251cde	1.20gf
Weedy from sowing to maturity (T12)	310d	293d	213e	1.15g
R^2	0.572	0.513	0.603	0.871
C.V	10.800	16.705	12.711	15.666
Significant	0.0017	0.0084	0.0004	0.0001

Means within columns with the same alphabets are not significantly different at P> 0.05

15 DAS in main season (Table 3). Thus, weed control during this period is necessary to avoid considerable reduction in the crop yield. The yield of rice grains in the saturated condition was lower as compared to the flooded condition in both seasons. The highest rice grain yields in the saturated condition were only 4.4 and 3.4 ton/ha in the weed-free treatment throughout sowing to maturity (T6) in the off-season and main season, respectively. Mohankumar and Alexander (1989) observed the highest grain yield from the flooding condition as compared to the continuous saturated condition. The results also showed that the weed competition period from sowing to maturity (T12) produced the lowest rice grain yield in the main season (1.1 ton/ha) and the off-season (2.0 ton/ha) at saturated condition. Meanwhile, Becker and Johnson (1999) also found that rice yield was drastically reduced as a consequence of increased weed infestations in the saturated condition.

Critical Period of Weed Competition

The Gompertz and Logistic equation was fitted to determine the critical period of the weed competition. Based on 5% and 10% levels of yield loss, the critical period can be predicted from the weed-free and weed interference duration curves (Norsworthy and Oliveira, 2004). In the off-season, the critical periods of the weed competition in the saturated condition, at 5% and 10% yield losses, were between 2 to 71 DAS and 5 to 52 DAS, respectively (*Fig. 3a*). Meanwhile in the flooded condition, the critical period was predicted between 15 to 73 DAS and 25 to 51 DAS at 5% and 10% yield loss, respectively (*Fig. 3c*). In the main season 2005, the critical periods of the weed competition in the saturated condition, at 5% and 10% yield loss, were between 0 to 72 DAS and 2 to 55 DAS (*Fig. 3b*). In the flooded condition, the critical period was predicted between 2 to 98 DAS and 4 to 84 DAS (*Fig. 3d*). Johnson *et al*. (2004) found that the critical periods of the weed control

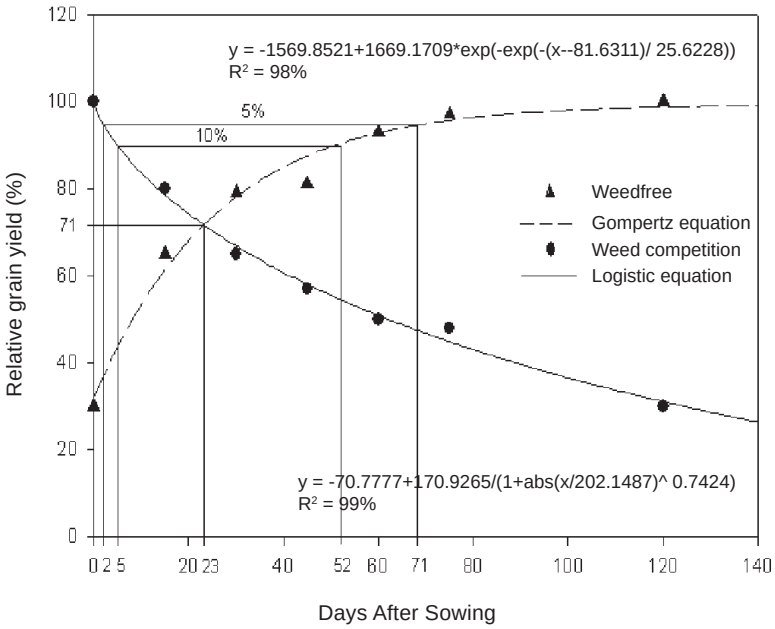

Fig. 3a: Critical period of the weed competition under saturated condition in off-season 2005

to obtain 95% of weed-free yield were estimated to be between 29 to 32 DAS in wet-seeded rice and 4 to 83 DAS in dry-seeded rice.

The result also showed that a critical period of weed competition in the off-season started early and a longer weed-free period was needed in the saturated condition compared to flooded condition, due to the higher weed infestation. Smith and Fox (1973) reported that few or no weed seedlings emerged when the soil was flooded, but at the field capacity, all the weed species emerged readily. The critical period was also observed to be early in the main season (*Fig. 3b* and *Fig. 3d*) as compared to the off-season. To maintain a 95% of rice yield in the main season in the saturated condition, weed control has to be done as early as 0 DAS and needs to be maintained until 72 DAS (Table 5), and the infestation of weeds above certain density at this time will cause a significant yield reduction. In the off-season, weed control must be done on the second day after sowing

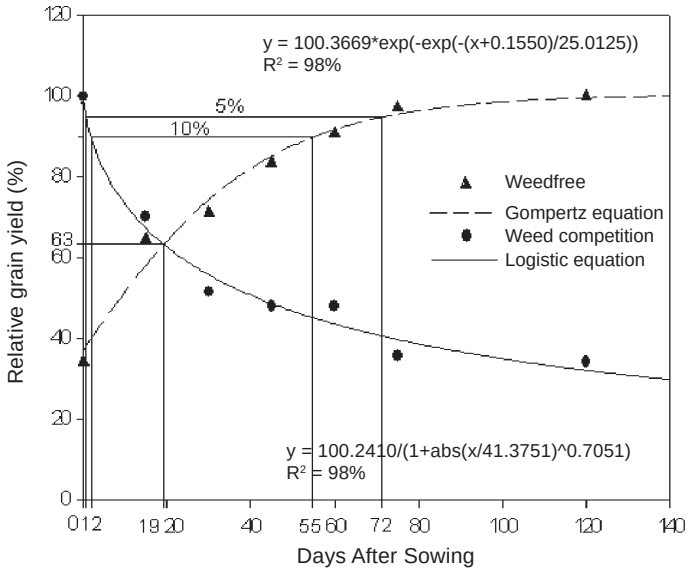

Fig. 3b: Critical period of the weed competition under saturated condition in main season 2005/2006

TABLE 5
The estimated critical periods of weed competition for 5 and 10 % yield losses

Seasons	Water Regimes	Maximum weed infestation period		Minimum weed free period	
		5%	10%	5%	10%
Off-season 2005	Saturated (DAS)	2	5	71	52
	Flooded (DAS)	15	25	73	51
Main season 2005/2006	Saturated (DAS)	0	2	72	55
	Flooded (DAS)	2	4	98	91

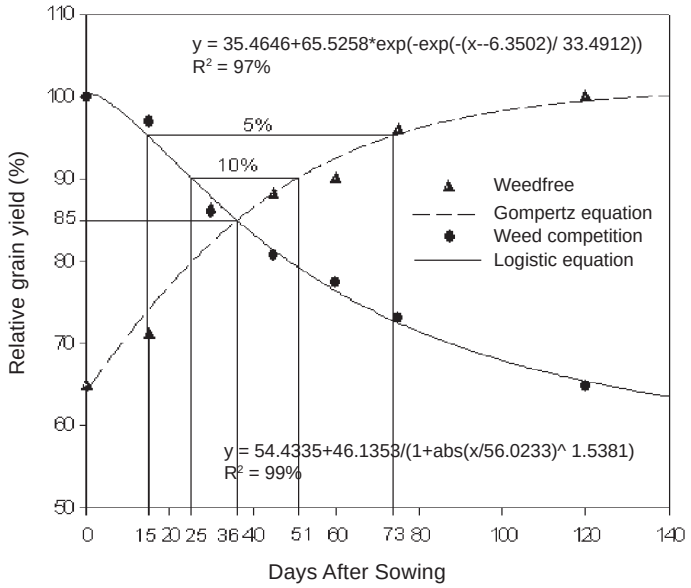

Fig. 3c: Critical period of the weed competition under flooded condition in off-season 2005

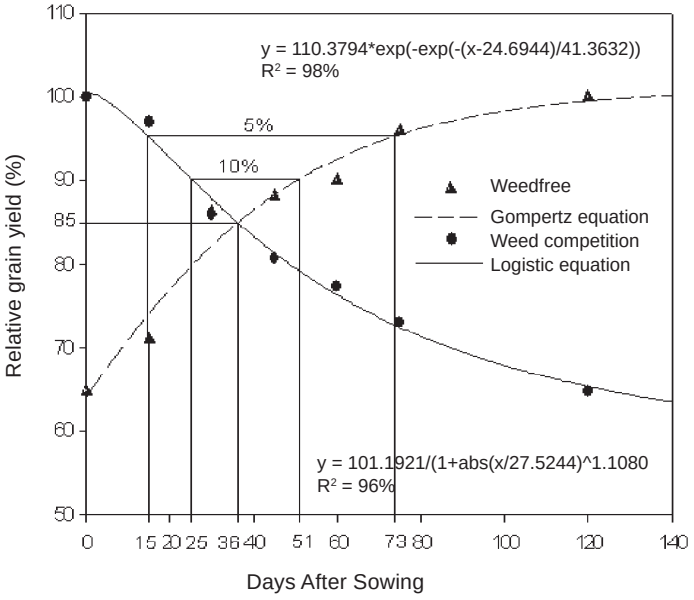

Fig. 3d: Critical period of the weed competition under flooded condition in main season 2005/200

to prevent 5% yield loss and this needs to be maintained until 71 DAS. The success of the weed control operations is dependent on the time of weed seedling emergence, weed species and stage of crop growth. Timely applications of effective herbicide are able to reduce losses when there is an occurrence of targeted weeds (Azmi and Supaad, 1990), optimize herbicides efficacy against weeds and also minimize production cost or protect crops against injury (Baki and Azmi, 1992).

CONCLUSIONS

Weed composition and critical period of weed competition were obviously influenced by water regime treatments in both seasons. In the off-season, the hierarchical dominance of weed group in saturated condition was sedges>grasses>broadleaved weeds, while in the main season, the hierarchical ranking was grasses>sedges>broadleaved weed. In the flooded condition, the dominance ranking was reversed than the saturated condition in both seasons. The reduction in the grain yield, caused by increasing the duration of weed competition, was found to be higher in the saturated condition than in the flooded condition in both seasons. Based on the results gathered in the present study, to prevent 5% yield loss the critical periods in the off-season were 2 – 71 and 15 – 73 DAS in the saturated and flooded condition, respectively; whereas, in the main season, the critical periods were 0 – 72 and 2 – 98 DAS in the saturated and flooded conditions, respectively.

ACKNOWLEDGMENTS

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Critical Time of Nitrogen Application During Panicle Initiation on the Yield of Two Malaysian Rice Cultivars (*Oryza sativa* L.)

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ABSTRACT

Nitrogen is the most limiting nutrient in rice production. N fertilizer is susceptible to losses when the time of application does not match with period of crop demand. A glasshouse experiment was conducted to determine the critical time of nitrogen fertilizer application at panicle initiation on grain yield of two Malaysian rice cultivars (MR219 and MR232). The experiment consisted of five N treatments applied each at 60 kg ha^{-1} at 45 (N1), 50 (N2), 55 (N3), 60 (N4) and 65 (N5) days after seeding (DAS) with five replications. Prior to this stage, a total of 75 kg N ha^{-1} was applied during vegetative growth stage (at 15 and 35 DAS). Plant physiological parameters such as height, SPAD value and LAI showed statistical difference among some treatments. Application of N also resulted in an increase in plant biomass. The results demonstrate that the split appication of fertilizer N at PI stage (55 DAS) significantly increased percentage of filled grains, 1000-grain weight and total grain yield. Incorrect timing of N application (65 DAS) at PI stage, drastically reduced rice yield to approximately 39% for MR219 and 17% for MR232. Farmers should be advised to apply N between 50 DAS and 55 DAS, even though application of N at 55 DAS was far better than 50 DAS in terms of yield parameters.

Keywords: *Oryza sativa*, urea, days after seeding (DAS), glasshouse, grain yield, timing, panicle initiation, critical growth stages

INTRODUCTION

Rice (*Oryza sativa* L.) is a unique crop of great antiquity and akin to progress in human civilization (Smith *et al.*, 2003). It is estimated that about 40% of the world's population consume rice as their major source of food. The importance of rice for food security and socioeconomic stability is self-evident. Rice production has been described as the world's single most important economic activity. The increase in production is possible if soil, water, nutrients and other production inputs are used efficiently. Sustainable rice production is a key to improving global livelihood of both small scale farmers in developing countries and rice producing countries worldwide. Thus meeting the challenges for sustainable increase in rice production and production efficiency is vital for alleviation of poverty and attainment of food security worldwide.

Nitrogen is the most important and yield-limiting nutrient in rice production worldwide (Lin *et al.*, 2006). Nitrogen promotes rapid growth of rice, increases leaf area, spikelet number per panicle, percentage of filled grains and grain protein content (Dobermann and

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Fairhurst, 2000). Rice production consumes approximately 20% of the total N fertilizer used for agriculture in the world (Mew *et al.,* 2003).

In rice plants, leaf N is remobilized to the grains during the grain filling period along with actively produced photosynthates. That is, there is a compromise between the supply of N and photoasimilates from leaves to the grains during grain filling such that enough N must remain in leaves to allow photosynthesis to continue, yet enough N must be transported to the grains to allow normal grain development and storage of adequate reserves (Shiratsuchi *et al*., 2005).

This N fertilizer is often not effectively used by irrigated rice because of improper timing and rates of application. It is typically required in greater quantities than any other nutrient if rice farmers are to reap high yields and profits. Inappropriate N management has detrimental effects on crop yield and the environment and aggravates disease and pest incidence. Nitrogen fertilization is a key input in increasing rice production. The introduction of high-yielding varieties has greatly increased the prospect of increasing yields but this goal will not be reached without great increases in the use and efficiency of N on rice.

Since fertilizer is an expensive input, an economical and appropriate method of application needs to be determined to enhance productivity and profit of the growers under given situation (Manzoor *et al.,* 2006). Dobermann and Cassman (2002) noted that average farm yield levels of 70 – 80% of the attainable yield potential are necessary to meet expected food demand in the next 30 years; research must seek to develop nutrient management approaches that optimize profit, preserve soil quality, and protect natural resources in systems that consistently produce at these high yield levels. Achieving these goals will require novel strategies for more precise plant nutrient management tailored to the technologies, dynamics and spatial scales relevant to each system.

Efficient use of N applied to rice has been a course of concern in rice production especially in flooded rice. Irrigated rice (*Oryza sativa* L.) yield increases in Asia have slowed down in recent years (Dobermann *et al.,* 2003). Further, yield increases are likely to occur in smaller increments through fine-tuning of crop management. In field experiments, flooded rice generally recovers only 20 – 40% of applied N, whereas upland rice normally recovers about 40-60% (De Datta, 1981). The split application of fertilizer N remains an essential component of recommendation, however the time of application especially at critical stages varies depending on the type of rice cultivar. Application of N during critical stages may optimize leaf N distribution, thereby maintaining high canopy photosynthesis, especially during grain filling stage (Qi Jing *et al.,* 2007). Inappropriate N management also has detrimental effects on crop yield and the environment and aggravates disease and pest incidence. Varying the application of fertilizer N to match the specific needs of rice can increase yield, while also reducing N loss and maximizing recovery of fertilizer N. Therefore, the objective of this study was to investigate the correct time of N application, specifically at the panicle initiation stage for two indica rice cultivars (MR219 and MR232) taken from Malaysian Agricultural Research and Development institute (MARDI).

MATERIALS AND METHODS

A greenhouse experiment was conducted at field 10, Universiti Putra Malaysia (02˚N 59.476' 101˚E 42.867', 51 m altitude) between December 2007 and March 2008. Two indica rice cultivars (MR219 and MR232) from Malaysian Agricultural Research and Development Institute (MARDI) were planted for the experiment. The average yield of MR219 and MR232 is estimated at 8 – 10 t ha^{-1} (MARDI, 2006).

Pots of 40 cm height and 34 cm diameter size filled with 15 kg of uniformly mixed soil were used. The experimental soil was Bakau series obtained from Tanjong Karang, a major rice growing area located in Kuala Selangor, Peninsular Malaysia. Characteristically, the soil is loamy clay with pH 5.1, CEC 16 cmol kg^{-1} soil, 16.3 g organic C kg^{-1} , 1.46 g total N kg^{-1}, 5.3 mg available P kg^{-1} and 72.6 mg K kg^{-1} .

Weather records were taken between the months of December 2007 and February 2008. Average minimum and maximum temperatures were recorded with a minimum and maximum thermometer at ten days interval (*Fig. 1*).

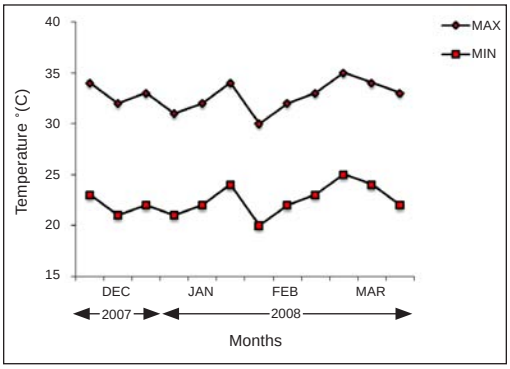

Fig. 1: Minimum and maximum temperature distribution of the glasshouse

The experiment consisted of five N treatments and two varieties (MR219 and MR232) with five replications arranged in a completely randomized design. The varieties were separately arranged within the same glasshouse. The five N treatments comprised of different timings of urea N-fertilizer (60 N kg ha^{-1}) applied at five days interval during the panicle initiation stage (45 (N1), 50 (N2), 55 (N3), 60 (N4) and 65 (N5) days after seeding (Table 1)). During early crop establishment (15 DAS) and midtillering stage (35 DAS), 50% of N fertilizer (75 kg ha^{-1}) was top dressed in all treatments. At 75 DAS, 10% of the N (15 kg ha^{-1}) was also applied to all treatments. A total amount of 150 kg N/ha equivalent was applied in all treatments.

Phosphorus and potassium fertilizers were applied based on the standard recommended rate in all treatments. Phosphorus (90 kg P_2O_5 ha^{-1}) as rock phosphate and potassium (150 kg K_2O ha^{-1}) as muriate of potash were applied basally at early crop establishment stage (14 DAS). Vita-Grow ® (90 mL/18 L) was sprayed as a micronutrient foliar fertilizer at the midtillering stage (30 DAS) in all treatments.

The rice seeds were pre-germinated in ZAPPA® solution for 24 hours and then broadcast into the soil moistened at saturation. There were ten seedlings sown per pot. After seedling establishment (14 DAS), about 10 cm water depths were maintained throughout the growing period until two weeks before harvesting, in order to allow ripening and drying of the grains. Adequate pest and disease control measures were taken throughout the plants' growth.

Sampling was conducted to determine the yield and yield components of rice plants. Ten flag leaves were sampled from each pot at grain filling stage (85 DAS) to measure the chlorophyll content (SPAD value) and leaf area index (LAI). Plant height was measured on 10 plants per pot at 80 DAS. At maturity (110 DAS), 10 panicles were sampled from each pot to determine the yield and yield components of the rice plants. The panicles were separated into filled and unfilled spikelets. The samples were then oven-dried at 70˚C for 48 hours to constant weight. Data were subjected to analysis of variance and means comparison (DMRT at 5% P level) of different measured parameters were performed using SAS system.

RESULTS AND DISCUSSION

Application of N fertilizer yielded significant differences among some treatments for both cultivars in terms of plant height, leaf chlorophyll content (SPAD reading) and LAI. Treatment N3 produced highest plant height for cultivar MR219 (92.2 cm) and was significantly different from N1 (85.6 cm) and N5 (84.2 cm) (*Fig. 2*). In MR232, statistical difference was noted in the plant height of treatments N2 (96.0 cm) and N5 (85.4 cm). Difference observed in plant height could be attributed to increase in panicle length due to application of N at critical stage of PI.

SPAD value for MR219 was significantly higher in N2 (39) and N3 (38) compared to N4 (33), where as in MR232 cultivar, treatments N3 and N4 recorded statistical difference with N5 in terms of SPAD value (Table 2). Comparatively, cultivar MR219 recorded higher average SPAD reading than MR232. LAI measurement was

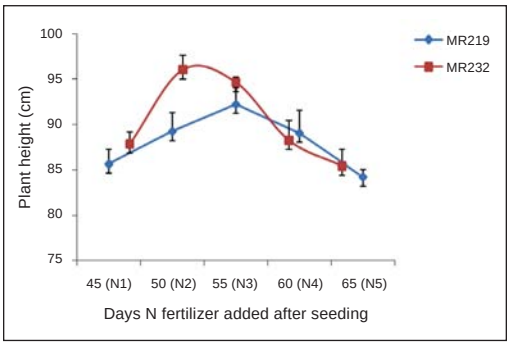

Fig. 2: Plant height patterns of the rice cultivars at 80 DAS. Error bars represent standard error of the means

TABLE 1
Amount of N applied on rice cultivars (MR219 & MR232) at different time during the panicle initiation stage

Treatments	Days After Seeding				
	45	50	55	60	65
	kg N ha^{-1}				
N1	60	-	-	-	-
N2	-	60	-	-	-
N3	-	-	60	-	-
N4	-	-	-	60	-
N5	-	-	-	-	60

significantly higher in treatment N3 for both cultivars MR219 and MR232 (8.4 and 7.9) respectively. The high occurrence of LAI in treatment N4 indicates better plant canopy structure.

Application of N resulted in an increase in plant biomass (*Fig. 3*). The highest plant biomass was observed in N3 (400.2 g pot^{-1}) for MR219, where as in MR232, N2 recorded the highest (367.2 g pot^{-1}). The fact that N2 and N3 yielded the highest plant dry weight might be attributed to application of N at critical growth stages such as 50 and 55 DAS. This experiment confirms the observation made by Dobermann *et al*. (2000) that N uptake at midtillering and panicle initiation stage tends to increase the biomass of plants' leaves, stems and panicles.

Spikelets number per panicle are presented in *Fig. 4*. Treatment N3 for both cultivar MR219 and MR232 accounted for the highest grain number per panicle, (135) and (131) respectively. However, lowest grain number per panicle was recorded in N5 for both cultivars. It was observed that application of N before or after 55 DAS, steeply decreased grain number. Application of N during these periods, may lead to wastage because the N might not be efficiently utilized by the plant. In both MR219 and MR232, grain number per panicle was considerably higher due to application of N at 55 DAS (*Fig. 4*). This indicated that the applied N was efficiently utilized by the plant, thus making it possible for

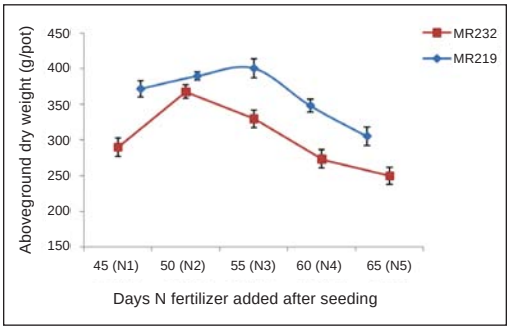

Fig. 3: Effects of partitioning of N on aboveground dry weight of the rice cultivars. Error bars represent standard error of the means

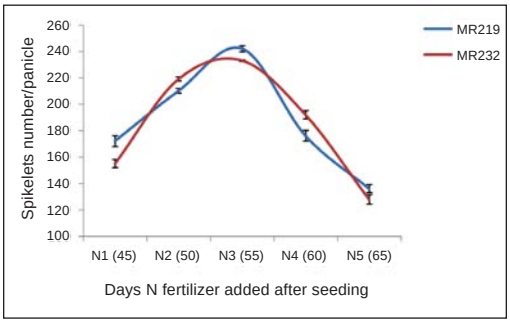

Fig. 4: Spikelet number per panicle as affected by N application at PI stage. Error bars represent standard error of the means

the plant to translocate the carbohydrates into the organs.

Results shown in *Figs. 5, 6* and *7* demonstrated that appication of N fertilizer at 55 DAS during the PI stage (N3) increased the percentage of filled grains, 1000-grain weight and total grain yield. The increase in yield could be due to efficient N uptake by the plants, that led to better photosynthetic rate as shown by the SPAD value (Table 2). Treatment N3 accounted for the maximum paddy yield in both MR219 (225.75 g pot^{-1}) and MR232 (214.61 g pot^{-1}), while N5 produced the lowest yield for both MR219 (137.58 g pot^{-1}) and MR232 (177.63 g pot^{-1}). The results suggest that delaying application of N at PI stage may drastically reduce paddy yield up to approximately 39% for MR219 and 17% for MR232.

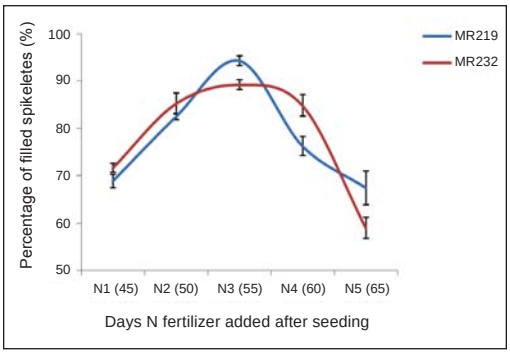

Fig. 5: Effects of N applied on percentage of filled spikelets. Error bars represent standard error of the means

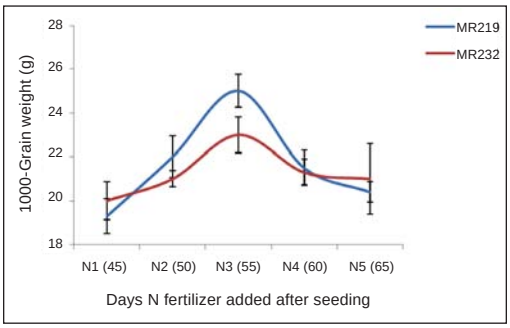

Fig. 6: Effects of N partitioning at PI stages on 1000-grain weight. Error bars represent standard error of the means

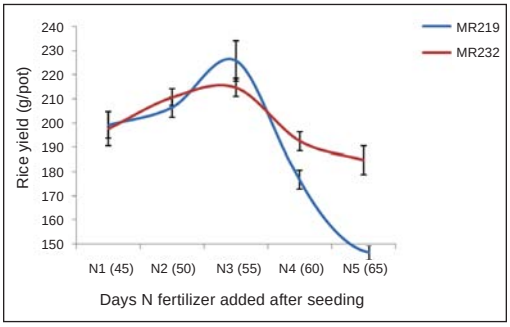

Fig.7: Rice yield as affected by application of N at different days of PI stage. Error bars represent standard error of the means

TABLE 2
Effects of N splitting on chlorophyll content (SPAD value) and LAI

Treatments	SPAD value		Leaf Area Index	
	Cultivar			
	MR219	MR232	MR219	MR232
N1	37ab (1.10)	34b (0.74)	6.6cd (0.16)	6.5c (0.09)
N2	39a (1.34)	35ab (0.59)	6.7d (0.15)	6.6c (0.06)
N3	38a (1.02)	36a (1.33)	8.4a (0.11)	7.9a (1.12)
N4	33b (2.00)	37a (1.16)	7.8b (0.14)	7.0b (0.33)
N5	35ab (1.30)	31c (0.50)	6.0e (0.08)	5.7d (0.06)

In each column, means followed by the same letter(s) are not significantly different at 5% level by DMRT. Numbers in parenthesis are standard error of the mean.

CONCLUSIONS

The results showed that application of N fertilizer at 55 DAS (N3) increased the number of spikelets/panicle, % of filled spikelets, 1000-g grain weight, and rice yield probably due to efficient N uptake. Application of N fertilizer before 50 DAS or after 55 DAS reduced all these parameters. Generally, late application of N after 65 DAS may reduce rice yield to about 39% for MR 219 and 17% for MR 232. The results suggested that practically, farmers should be advised to apply N between 50 DAS and 55 DAS, even though application of N at 55 DAS was far better than 50 DAS in terms of yield parameters. This was to avoid late application of N by the farmers as this may result substantial yield losses.

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Growth, Physiological and Biochemical Responses of Malaysia Rice Cultivars to Water Stress

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ABSTRACT

The response of water deficit on rice plants varies substantially according to cultivars. Drought tolerant cultivars possess better morphological, physiological and biochemical adaptation to reduce water availability. An experiment involving water stress on rice varieties was carried out under rain shelter to examine the morphological changes (leaf rolling, root depth), stomatal responses and biochemical processes (proline and peroxidase accumulation) of five different local rice varieties. These varieties were selected based on their drought tolerant potential from an earlier varietal screening trial. The varieties were taken from both traditional (Muda, Jawi Lanjut and newly breed commercial varieties, MR 84, MR219 and MR 220) obtained from Genebank, MARDI Research Station, Seberang Prai, Kepala Batas, Pulau Pinang. These varieties were exposed to two different water regimes; water stress by withholding water and well watered condition (control). The experiment was carried out in a Complete Randomized Design (CRD) with 4 replicates. Water stress plants exhibited lower growth rate with obvious variation among rice varieties on the sensitivity to water stress. Meanwhile, the overall sensitivity of the varieties to water stress was ranked in the order; MR220>Muda>MR84>MR219>Jawi Lanjut. Water deficit decreased stomatal conductance, relative water content and root depth while peroxidase activities and proline accumulation were increased in rice grown under water stress treatment.

Keywords: Leaf rolling, relative water content, root growth, stomatal conductance, water stress and rice cultivars

INTRODUCTION

Water scarcity is a severe environmental limitation to plant productivity. Drought-induced loss in crop yield may exceeds loses from all other causes, since both the severity and duration of the stress are critical (Farooq *et al*., 2008). A slow pace in revealing drought tolerance mechanisms has hampered both traditional breeding efforts and the use of modern genetics approaches in the improvement of drought tolerance of crop plants. Fukai and Cooper (1995) listed the traits of rice under four categories; drought escape, drought avoidance, drought tolerance and drought recovery. The physiological avoidance can be achieved by cultivars which were able to reduce water loss, such as by leaf rolling and decreased stomatal conductance. In retrospect, these features may

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also affect the net photosynthesis that will in turn cause a detrimental effect to yield. The optimization of these processes will lead to a significant achievement in sustaining growth under limited water conditions.

Plants have evolved a wide range of enzymatic and non-enzymatic mechanisms to scavenge the generation of reactive oxygen species (ROS), as a result of environmental stresses. Peroxidase is thought to increase during abiotic stress as it is an important antioxidant enzyme in scavenging or utilizing hydrogen peroxide (Okuda *et al*., 1991).

Rice is the most important cereal crop in the world and it is the primary source of food and calories for about half of mankind (Khush, 2005). Rice provides as much as 80% of the dietary calories in some Asian countries. The predominantly rice-growing areas in Asia (130 million hectares, more than 85% of the total world rice production) are often threatened by severe abiotic stresses, and the most common being the drought. Climate change has rendered several areas unsuitable for rice cultivation, especially the 'rice bowl' area in Kedah and this has caused losses in millions of Ringgit. The Malaysian Agricultural Research and Development Institute (MARDI is trying to develop new varieties of rice which are resistant to flood, drought, and high temperatures. The development of rice tolerance to limited water conditions particularly for granary areas, has not been successful in general, although there are cultivars reputedly tolerant to water deficit. The present study was undertaken to determine the physiological and biochemical changes in rice cultivars, when exposed to limited water conditions.

MATERIALS AND METHODS

Origin of Plant Material

Seeds of five rice genotypes (*Oryza sativa* cv. MR84, MR219, MR220, MUDA and Jawi Lanjut) were obtained from Genebank, MARDI Research Station, Seberang Prai, Kepala Batas, Pulau Pinang.

Growth Condition and Plant Culture

Seeds were sown on a plastic tray for raising seedlings and placed in the dark until seedlings emerged. At the 1.5-leaf stage, seedlings of uniform size were transplanted into plastic pots with clay loam soil from BERTAM. The experiment was conducted at a rain shelter house at Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia.

Water Stress Treatment

Water stress was imposed by withholding irrigation until leaf rolling symptoms were observed as a stress indicator while equal amount of water (1 liter per pot per day) was given in well watered treatment. The experiment was carried out in a Complete Randomized Design (CRD) with 4 replicates.

Measurements

Leaf rolling

Leaf rolling was assessed visually in each pot, in all the treatments. Several plants were assessed and the pots were given a mean leaf rolling score, ranging from 1 to 5, with 1 being flat and 5 a tightly rolled leaf (O'Toole and Moya, 1978). These ratings were made during midday, i.e. about twice per week during the period of water deficit of all the treatments.

Stomatal conductance

Stomatal conductance of leaves was determined using a portable porometer (Delta-T AP4, Delta-T Devices, Cambridge, UK). The measurements were taken on the abaxial surface of the leaf once a week between 11.00 h and 14.00 h. The readings were accomplished during one-hour to avoid the diurnal pattern of variation of the leaves. The terminal part of the main leaf lobe was placed into the cup, i.e. on the head unit which was positioned normal to the sun. The measurements were conducted during cloudless periods on the exposed leaves between 10.00 h and 14.00 h.

Relative water content

The leaves were cut, and the relative water content (*RWC*) was determined according to the procedures by Ghannoum *et al*. (2002). The relative water content of leaf was determined as follows:

$$RWC = (\textit{fresh weight} - \textit{dried weight}) / (\textit{fully turgid weight} - \textit{dried weight}) \times 100$$

To determine the fully turgid weight, the leaves were kept in distilled water in the darkness at 4˚C to minimize respiration losses until they reached a constant weight (full turgor, typically after 12 h). The leaf dry weight was obtained after 48 h at 70˚C in an oven. Five to six replicates were obtained per treatment.

Proline measurement: Fresh flag leaf tissue (0·5 g) was ground in liquid nitrogen and then extracted in 20 mL of hot water for 30 min with a moderate shaking. The homogenate was centrifuged at 5000 g for 10 min. The proline concentration was quantified using the ninhydrin acid reagent method described by Bates et al. (1973) using the L-proline as a standard.

Protein expression: For protein expression, leaves were collected using liquid nitrogen and protein was extracted in a buffer 62.5mM Tris-HCL pH 6.8; 10% glycerol 2% SDS and 1.4M 2-mecapthanol (2ul/mg of tissue) and incubated the mixture at 70˚C for 10 minutes and centrifuged at 15000rpm for 10minutes at 40˚C. The supernatants (protein) were taken for the treatment with the cracking buffer before electrophoresis was conducted in the SDS-PAGE.

Peroxidase activity**:** Peroxidase activity was assayed as an increase in optical density due to the oxidation of guaiacol to tetra-guaiacol. The 3 ml reaction mixture contained 16 mM guaiacol, 2 mM H_2O_2, 50 mM phosphate buffer (pH 6.1) and 0.1 ml enzyme extract. Enzyme extract was prepared as in case of SOD. The reaction mixture consisted of 50 mM, pH 6.1, phosphate buffer, 16 mM guaiacol, 2 mM H_2O_2, 0.1 ml enzyme, 0.4 ml water, to make final volume of 3.0 ml. The absorbance due to the formation of tetra-guaiacol was recorded at 470 nm and the enzyme activity was calculated as per extinction co-efficient of its oxidation product, tetra-guaiacol 2 ¼ 26.6 mM71 cm71. The enzyme activity was expressed as mmol tetra-guaiacol formed per min per g fr. wt. or per mg protein.

Root shoot ratio: The shoot samples were harvested by cutting about 2 cm above the soil surface at maturity stage and were separated into grain and straw. The shoots were washed and oven dried for 70 to 72 hours and weighed.

Statistical analysis

All data were analyzed using SAs software (SAS Institute Inc. 1997). Each treatment was analyzed in four replications. When ANOVA showed significant treatment effects, the Duncans multiple range test was applied to compare the means at P‹ 0.05.

RESULTS

Growth Parameters

Root depth

The root depth of rice was reduced when the soil subjected to drought condition (water stress) for all the rice varieties, as shown in *Fig. 1*. Under well watered treatment, root depth was higher than under water stress condition for all varieties. Under the well watered treatment, the root depth of MR220 and MR84 were comparable with a minor difference and were higher than the other varieties. The Jawi Lanjut variety indicated lower root depth under well watered treatment than the other varieties. However, under the water stress treatment, the MR220 indicated lower root depth than the other varieties. From the observation, the Jawi Lanjut variety indicated a higher root depth than the other varieties under the water stress treatment.

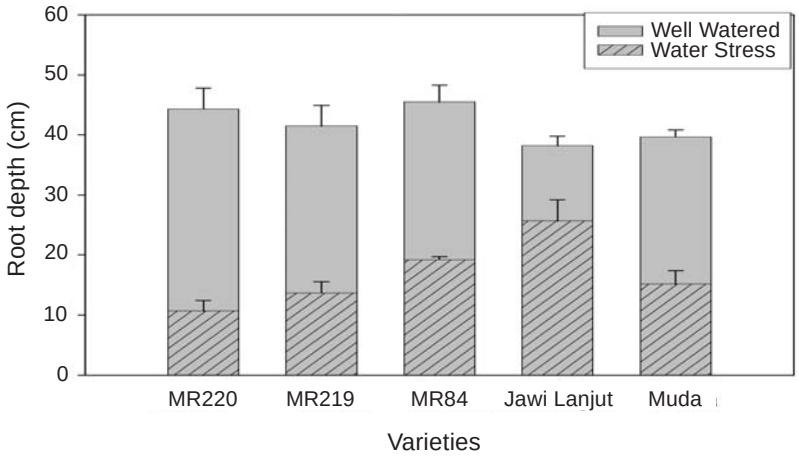

Fig. 1: Root depth of five cultivars exposed to well-watered and drought conditions. Vertical bars indicate ± standard errors, n = 4

Leaf rolling

In the present study, leaf rolling was observed first in MR220, which also showed the higher leaf rolling score at subsequent measurements, indicating that this cultivar was the most sensitive to water deficit (*Fig. 2*). Leaf rolling occurred later in Jawi Lanjut and it was lower than the other cultivars, showing a maximum score of 4. Similarly, the extent of leaf rolling was found to be gradual in MR84 and MUDA. Five days after the stress treatment, the leaves of all cultivars were partially rolled at midday, starting with MR220, and this was followed by MR219, MUDA, MR84 and JAWI LANJUT which showed a higher mean score in leaf rolling.

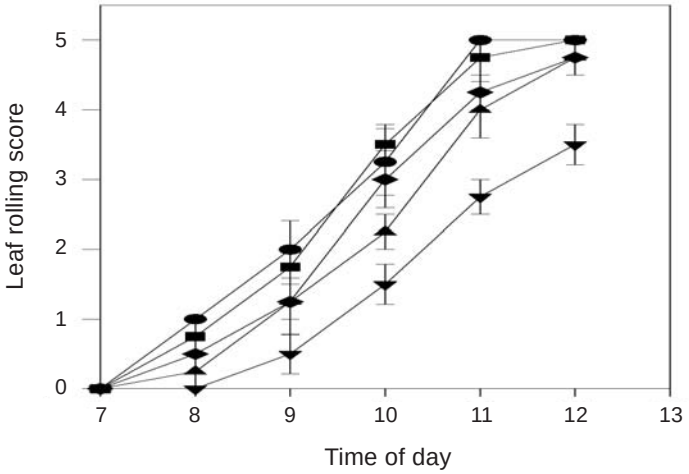

Fig. 2: Diurnal changes in leaf-rolling score of five cultivars (● MR220, ■ MR219, ▲ MR84, ▼ Jawi Lanjut and ◆ MUDA). Vertical bars indicate ± standard errors, n = 4

Physiological Parameters

Relative water contents (RWC)

The relative water contents of all the varieties were similar under the well-watered condition on all the measurement occasions. However, it declined progressively in stressed plots with the development of severe water deficit (*Fig. 3*). The decline in the RWC was more rapid in MR220 than in the other varieties. The Jawi Lanjut had relatively higher water content than the other varieties, even after 10 days of exposure to soil drying. However, all the varieties had similar and lowest values of the RWC at the end of the soil drying cycle.

Stomatal conductance

Stomatal conductance decreased in all the varieties of rice as the intensity of water deficit increased with the time of soil drying (*Fig. 4*). The decline in stomatal conductance was faster after 6 days of stress development than under well watered condition. Stomatal conductance of MR220 and MUDA declined more rapidly than in other varieties; however, after 10 days of soil drying, all varieties (except for Jawi Lanjut) showed a considerable decrease in stomata conductance. Jawi Lanjut exhibited a higher stomatal conductance under stress than the other varieties under stress although it also had consistently lower values after 6 days of stress treatment.

Root-shoot ratio

The root shoot ratio was reduced when the soil was subjected to drought condition (water stress) for all the rice varieties, as shown in *Fig. 5*. Under the well watered treatment, the root shoot ratio was higher than under water stress treatment for all the varieties. Under the well watered treatment, MR220 and MR219 did not show much difference in the root shoot ratio, and were higher than the other varieties. However, under water stress treatment, MR220 showed a lower root shoot ratio than the other varieties. Based on the observation, the Jawi Lanjut varieties showed high root shoot ratio than the other varieties under the water stress treatment.

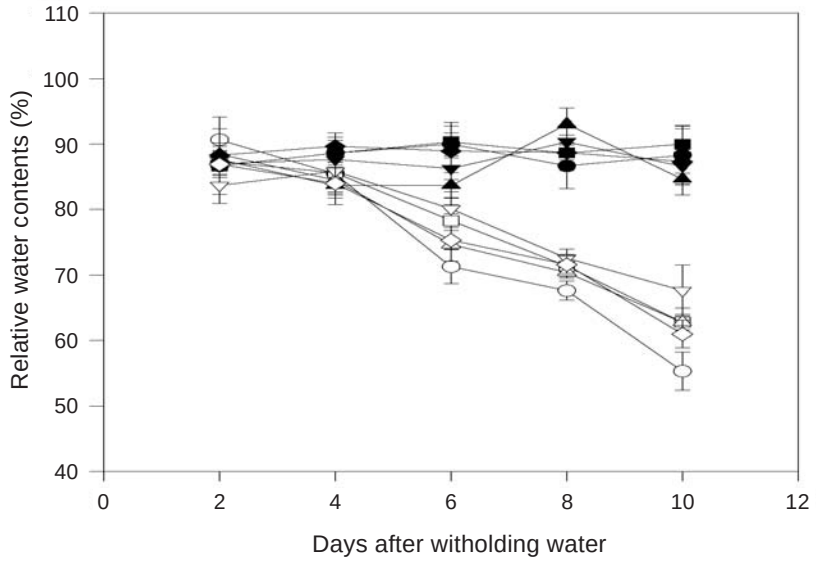

Fig. 3: Relative leaf water content of five cultivars exposed to well-watered (closed symbol) and drought (open symbol) conditions (○ MR220, MR219, △MR84, ▽ Jawi Lanjut and ◊ MUDA). Vertical bars indicate ± standard errors, n=4

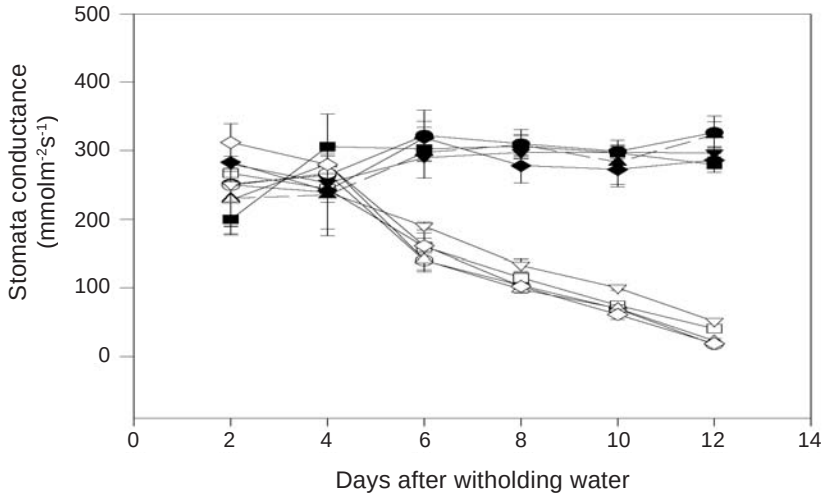

Fig. 4: Stomatal conductance of five cultivars exposed to well-watered (closed symbol) and drought (open symbol) conditions (○ MR220, MR219, △MR84, ▽ Jawi Lanjut and ◊ MUDA). Vertical bars indicate ± standard errors, n=4

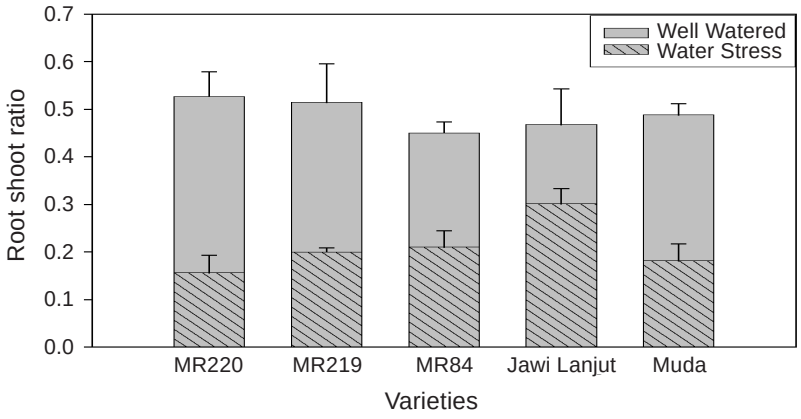

Fig. 5: Root-shoot ratio of five cultivars exposed to well-watered and drought conditions. Vertical bars indicate ± standard errors, n=4

Biochemical Parameters

Proline accumulation

The accumulation of proline was affected by water regimes and rice varieties, as depicted in *Fig. 6*. The accumulation of proline was higher under water stress treatment than the well watered treatment for all the rice varieties. The level of increase in the proline concentration in response to water stress varied between the rice varieties. In particular, Jawi Lanjut was observed to have a high proline accumulation under water stress treatment than the other varieties, while MR220 had a lower proline accumulation than the other rice varieties.

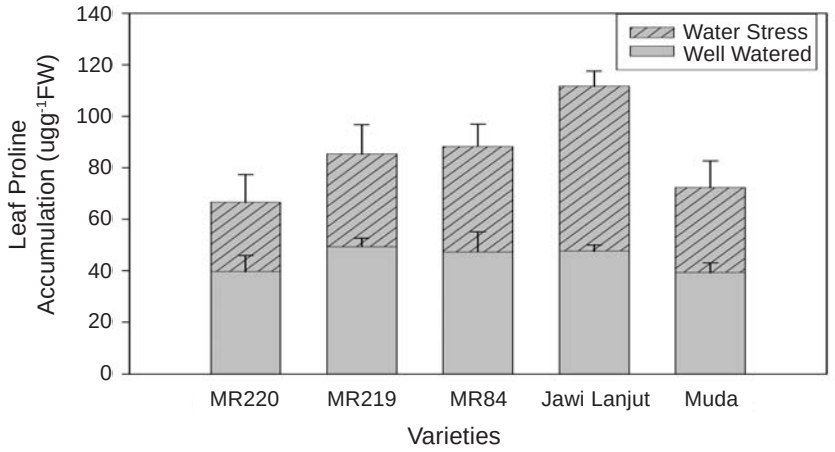

Fig.6: Leaf proline accumulation ratio of five cultivars exposed to well-watered and drought. Vertical bars indicate ± standard errors, n=4

Peroxidase activity

The peroxidase activity was also affected by the water regimes and rice varieties, as shown in *Fig. 7*. The peroxidase activity was high under the water stress treatment than under the well watered treatment for all the rice varieties except for MUDA. Jawi Lanjut was observed to have a high peroxidase activity under the water stress but lower peroxidase activity under well watered treatment than the other varieties. However, MR219 showed a high peroxidase activity for both the treatments (water stress and well watered), and MR220 showed a lower peroxidase activity than the other varieties under water stress treatment.

Protein expression

The protein expression of the rice varieties, under different water regime, is shown in *Fig* 8. The MR219 and Jawi Lanjut varieties showed more protein when exposed to the soil drying

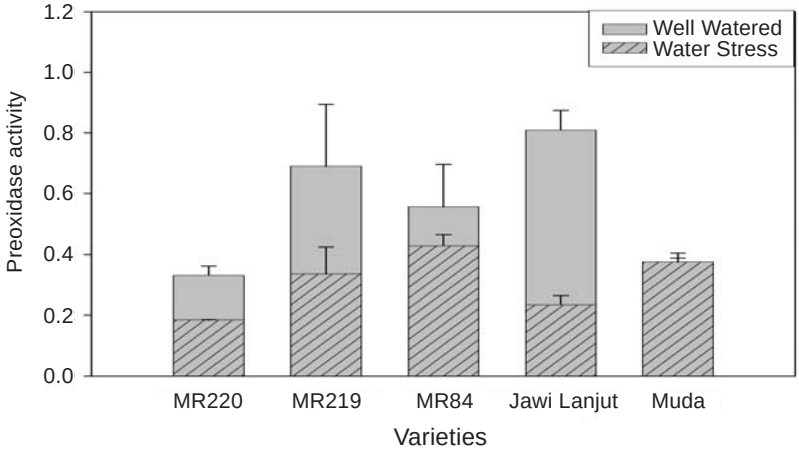

Fig. 7: Peroxidase contents of five cultivars exposed to well-watered and drought conditions. Vertical bars indicate ± standard errors, n=4

(water stress treatment). However, it is different between the MR220, MR84 and MUDA varieties because they did not show much difference in terms of protein expression. However, under the well watered treatment, all the varieties were shown to be comparable and did not differ much in protein expression.

DISCUSSION

As observed in the first experiment, a rice variety was varied by its sensitivity to soil drying. In general, leaf rolling score indicated that the sensitivity of the varieties follows the order MR220>Muda>MR219>MR84>Jawi Lanjut (*Fig. 2*). Therefore, leaf rolling is commonly used as an important criterion during screening of genotypes for drought tolerance (Cutler *et al*., 1980; Sloane *et al*., 1990; Rosario *et al*., 1992; Lilley and Fukai, 1994). Accordingly, several varieties such as MR220, Muda and MR84 were found to be more sensitive than MR219 and MR84, while other variety like Jawi Lanjut was relatively tolerant to the water stress treatment imposed. Leaf rolling and leaflet closure during periods of soil moisture depletion have also been observed in rice (Lilley and Fukai, 1994). These leaf movements, such as the adjustment of leaf angle or modification of leaf orientation to reduce the interception of solar radiation and, thus, decrease leaf temperature and water loss by transpiration, are regarded as one of the drought avoidance mechanisms evolved in plants (Pugnaire *et al*., 1999; Carr, 2001). On the other hand, lower rate of stress development (leaf rolling) as a result of maintenance of turgor has been used as an important criterion during screening genotypes, such as mungbean (*Vigna radiate*) accessions (Rosario *et al*., 1992), soybean plant introductions (Sloane *et al*., 1990; Carter and Rufty, 1992) and rice cultivars (Cutler *et al*., 1980; Price *et al*., 1992; Lilley and Fukai, 1994) for tolerance towards drought. It has been suggested that greater leaf rolling may be an important attribute linked to drought tolerance and may have a positive impact on crop yield under water stress conditions (Joshi, 1999; Lima *et al*., 2002).

Relative water content (RWC) substantially declined with prolonged period of soil drying, but the rate of decline was faster at the later stages of the stress (*Fig. 3*). Relative water content of all the varieties were similar under well-watered condition on all the measurement occasions. However, it declined progressively in stressed plots with the development of severe water deficit. The decline in the RWC was more rapid in MR220 than in the other varieties. In particular, Jawi Lanjut had relatively higher relative water content than the other varieties, even after 10 days of exposure to soil drying. All the varieties had similar and lowest values of RWC at the end of the soil drying cycle. On the other hand, the leaf RWC of MR220 was found to be similar to that of the control plants, but it decreased sharply in water stressed plants of Jawi Lanjut, two days after withholding water. The differences among the rice varieties in terms of the rate of decline in the leaf RWC could also be associated with the variations in other physiological responses to water stress, such as reduction in stomatal conductance (g_s).

Stomatal conductance (g_s) considerably decreased as the intensity of water stress increased with the time of soil drying (Figure 4). The stomatal conductance decreased in all the rice varieties, as the intensity of water deficit stress increased with the time of soil drying. The decline in the stomatal conductance was faster after 6 days of stress development. Stomatal conductance of MR220 and MUDA declined more rapidly than the other varieties. However, after 10 days of soil drying, all the varieties (except for Jawi Lanjut) showed a considerable decrease in the stomata conductance. Instead, Jawi Lanjut exhibited a higher stomatal conductance under stress than the other varieties, under stress although it also had consistently lower values, after 6 days of the stress treatment. Such a reduction in g_s appeared to be the primary response and a common phenomenon during water stress, which is believed to be one of the most important desiccation-avoidance mechanisms evolved in plants (Pugnaire *et al*., 1999; Carr, 2001).

The accumulation of proline was high under water stress treatment than the well-watered treatment for all the rice varieties (*Fig. 6*). Similarly, that the leaves of the unstressed plants have been reported to be free from proline contents, which are very small and its accumulation increases 100-folds when the plants are subjected to drought stress (Widyasari and Sugiyarta, 1997). Thus, proline is known to play an important role as an osmoprotectant in plants subjected to hyperosmotic stresses such as drought and soil salinity.

Water regimes treatment and interaction between the varieties have been shown to have effects on the peroxidase activity (*Fig. 7*). During soil drying, the different mechanisms of protection appear to act at the different stages of water loss. The survival strategy during early dehydration is to avoid protein unfolding and restrict membrane disturbance by preferential hydration. Upon further removal of water from the hydration shell, sugar molecules have to replace water at hydrogen bonding sites to preserve the native protein structure and spacing between phospholipids (Folkert *et al*., 2001). These results suggested that plants were capable of surviving surface soil drying. This capability could be related to increases in antioxidant activities, particularly the SOD and catalase (CAT). However, full drying suppressed antioxidant activities and induced lipid peroxidation (Fu and Huang, 2001). The protein expression of rice varieties exposed to different water regime are shown in *Fig. 8.* The MR219 and Jawi Lanjut varieties showed that more protein was expressed when they were exposed to soil drying (water stress treatment). Based on the observation, the well-watered treatment for all the varieties showed a low level of protein express, as compared to water stress treatment, except for in Jawi Lanjut and MR219. The findings therefore positively agree with Gulent and Eris (2004), who studied on the effect of heat stress on the total protein content in strawberry plants.

CONCLUSIONS

In general, the differences between rice varieties were observed in their sensitivity to water stress. Based on the mean values of visual stress score, leaf rolling during the drought period, overall sensitivity of the varieties was ranked in the order of MR220>Muda>MR84>MR219>Jawi Lanjut. Water deficit decreased stomata conductance, relative leaf water content and root depth. However, peroxidase activity and proline accumulation were increased in rice grown under stress treatment.

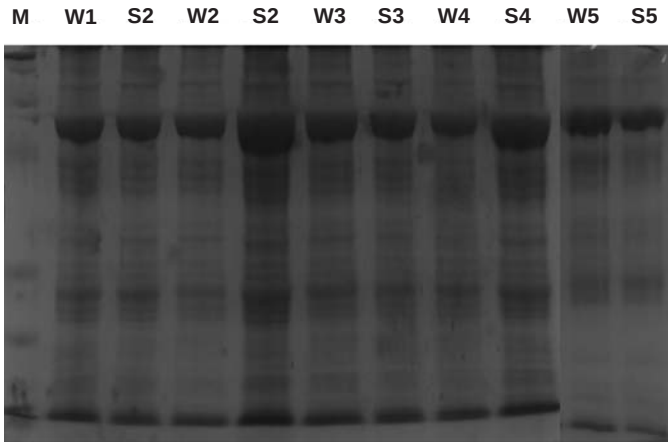

Fig. 8: Protein expression in rice leaves exposed to well-watered and drought conditions

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Biochemical Diversity of Bacterial Isolates from Paddy Soils of Peninsular Malaysia

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ABSTRACT

In this study, the diversity of bacterial isolates from paddy soil located in several rice growing areas of Peninsular Malaysia was evaluated. Phenotypic and physiological characteristics of the isolates were recorded to categorize and identify the bacteria. Ten strains of bacteria were recovered from six different locations (Alor Bakat, Kg. Seligi, Bachok, Kelantan; Sekinchan, Selangor; Kobah, Kedah; and Sg. Batu Pahat, Perlis). The spread-plate technique on nutrient agar at pH 7.0 was used to isolate and purify all the strains. The characteristic of the bacteria strains were determined using the Gram staining, motility test, as well as the shape and size of the single colony on solid media. From the various tests conducted, nine isolates were identified as Gram-positive rods, and only one was Gram-negative cocci. Further biochemical tests were carried out to determine the ability of these bacteria strains to hydrolyze starch, casein and gelatine, ferment carbohydrate (glucose, lactose and sucrose), enzyme production (catalase and oxidase), MR-VP tests and growth under anaerobic condition; the elevated NaCl was also examined. On the basis of these tests and the biochemical characteristics, nine of the strains belonged to the same genus, *Bacillus*, with three potentially different species. Meanwhile, only one strain showed the characteristics related to *Proteus mirabilis*. This study also showed that the dominant bacteria genera are generally limited, despite the contrasting geographical location and soil characteristics in which the strains were isolated.

Keywords: Bacteria diversity, biochemical tests, morphology, paddy soils

INTRODUCTION

Rice is the only major grain crop grown almost exclusively as human food. Indeed, rice constitutes half of the diet of 1.6 billion people and another million rely on it for more than one fourth of their diet (De Datta and Brady, 1987). It is expected that in the year 2020, an additional 300 million tonnes will be needed to feed the rapidly expanding human population. To meet this demand, its production must increase by 65% within the 30 years and must be achieved with only minimal expansion of the cultivated area (Saito and Watanabe, 1978).

In low input traditional rice cultivation, plant N originated from the soil, and replenished from the atmosphere by spontaneous biological nitrogen fixation (BNF). Research on rice nutrition has shown that even when high amounts of inorganic N fertilizer are applied, rice plant obtain 60 – 70% of their N from the soil (Reddy

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et al., 2002). Therefore, crop intensification may affect rice soil fertility if proper N input does not replenish the N taken up from the soil and replenishment can be achieved by increasing chemical fertilizers and biological N sources, such as green manure crops, enhancing N_2 fixation by indigenous BNF agents (free living bacteria) and decreasing N loss by proper N application and water management (O'Hara *et al*., 2002).

N_2-fixing agents in soil and water can be considered as natural "fertilizer factories". Promoting their growth and N_2-fixing activity is an important strategy in sustaining rice production. Biological nitrogen fixation technologies are especially important for long-term maintenance of soil fertility. The technologies are environmentally safe and reduced environmental pollution is achieved. Fertilizer savings, improved soil properties, reduced pests and diseases, as well as often being economically justifiable are the other advantages related to the BNF (Ladha and Reddy, 2003).

The determination of the composition of microbial communities in soil is not necessary for a better quantification of nutrient transformation. However, the biodiversity of the soil micro-organism is important in relation to the maintenance of soil ecosystem function. The presence of free-living bacteria, within the rice rhizophere, is also an indication of the importance of rhizobacteria in contributing to the nitrogen requirements of the paddy plant. In Malaysia, however, very limited study has been done on the ecological and diversity of rhizobacteria from wetland paddy soil. The purpose of this study was to determine the biochemical biodiversity of rhizobacteria from wetland paddy soil and investigate the distribution of dominant bacterial groups from different geographical locations in Peninsular Malaysia.

MATERIALS AND METHODS

Bacteria Sources

Ten strains were obtained from several sources of six main geographical locations: Alor Bakat, Kg. Seligi, Bachok, Kelantan; Sekinchan, Selangor; Kobah, Kedah, and Sg. Batu Pahat, Perlis. The strains used in this study are listed in Table 1, while the location of each site is indicated in *Fig. 1*. The description and characteristics of the paddy soils are given in Tables 2 and 3, respectively.

Isolates P1, P13, S1, 22, 23, 40, 44 and 47 were recovered from the rhizosphere area (within 3 mm of the root structure) in paddy soil. Isolate P2 was recovered from the paddy soil around the rhizosphere area, while isolate P3 was recovered from the paddy soil between the rhizosphere areas.

TABLE 1
The source of strains

Strain	Location/source
P1	Alor Bakat, Bachok, Kelantan (rhizosphere)
P2	Alor Bakat, Bachok, Kelantan (around rhizosphere)
P3	Alor Bakat, Bachok, Kelantan (between rhizosphere)
P13	Alor Bakat, Bachok, Kelantan (rhizosphere)
S1	Kg. Seligi, Bachok, Kelantan (rhizosphere)
22	Kg. Seligi, Bachok, Kelantan (rhizosphere)
23	Pasir Puteh, Kelantan (rhizosphere)
40	Sekinchan, Selangor (rhizosphere)
44	Kobah, Kedah (rhizosphere)
47	Sg. Batu Pahat, Perlis (rhizosphere)

Fig. 1: The location of sampling sites in Perlis, Kedah, Selangor and Kelantan, which represent the major rice growing areas in Peninsular Malaysia

Preliminary Tests

Colony morphology was determined after 2 to 4 days of growth on the nutrient agar plates incubated at 35 – 37˚C. Each isolate was submitted to Gram staining and was examined for cellular morphology and arrangement. One loop of distilled water was put on a clean slide. After that, one loop of the broth culture was added onto it after 16 hours of incubation, and this was spread until it was well mixed. The slide was subsequently heated to get a dry smear. The crystal-violet solution was then poured on the entire smear for 1 minute. The slide was washed gently with running water, and iodine solution was then added for 1 minute. Then, some drops of 95% alcohol were put on the particular smear to decolourize the blue stain. Safranin gram solution was then added to stain the negative bacteria in red colour for 30 seconds. The dried and coloured smear was examined under oil immersion microscope (Blazevic and Grace, 1975).

The isolates were also examined for endospore formation using the endospore staining technique. A heat smear from 24 hours Schaeffer's sporulation agar of culture was prepared. The smear was covered with 5% aqueous solution of malachite green and steamed over boiling water for 5 minutes. After the slide has cooled sufficiently, it was rinsed with distilled water and counterstained with 0.5% safranin solution for about 20 seconds. The slide was then briefly rinsed with distilled water and blotted dry with tissue paper, before it was examined under oil immersion (Blazevic and Grace, 1975).

Biochemical Tests

The following tests were carried out for all the strains: starch, casein and gelatin hydrolysis; oxidase, catalase, indole and hydrogen sulphite production; Citrate utilization; tyrosine degradation; acid production from carbohydrates (glucose, lactose and sucrose); reduction of Nitrate to Nitrite; MR – VP test; and phenylalanine deamination (Blazevic and Grace, 1975).

Growth on NaCl and Oxygen Requirement

Each isolated colony was streaked on 7% and 10% sodium chloride (NaCl) agar and incubated at 37˚C for 7 days, so as to examine the requirement for NaCl. The isolates were also examined for requirement of oxygen. The culture was streaked on a nutrient agar and placed into an anaerobic jar. Oxygen was evacuated from the jar using the gas-generating kits. The 'gas-kit' (Oxoid) was disposible H_2 and CO_2 generator envelopes. The introduction of water, along with the generator envolope in the lid, induces the generation of hydrogen and carbon dioxide gases. The hydrogen combined with the oxygen in the jar to produce water.

RESULTS AND DISCUSSION

The isolated strains were examined for their cell morphology and Gram reaction. Table 4 shows that nine out of 10 strains were Gram positive bacteria. Therefore, the isolation of bacteria from the rice rhizosphere of different geographical locations in Peninsular Malaysia showed that 90% of the strains are Gram positive rods bacteria and 10% are Gram negative cocci bacteria. No other morphological characteristics were obtained and this indicated a generally low diversity of bacteria in paddy soil. This study indicates that bacteria associated with rice rhizosphere in the Peninsular are widespread, but limited to a couple of genera especially to the *Bacillus* species. This result concurs with the report by Wartiainen *et al*. (2008), indicating

TABLE 2
Description of paddy soil where bacteria were isolated

Soil Series	Location	Taxonomic Class	Parent Material
Kangar	Sg. Batu Pahat, Perlis	Typic Endoaquert, very fine clayey, mixed, Isohyperthermic	Riverine alluvium
Kundor	Kobah, Kedah	Typic Tropaquept, very fine clayey, mixed, acid, Isohyperthermic	Marine alluvium
Briah	Sekinchan, Selangor	Typic Endoaquepts, fine, mixed, isohyperthermic	Mixed riverine and estuarine deposits
Jabil	Bachok, Kelantan	Typic Paleeaqults, fine to very fine, kaolinitic, Isohyperthermic	Recent riverine alluvium
Lubok	Pasir Puteh	Typic Fluvaquent, fine, mixed, isohyperthermic	Recent riverine alluvium

TABLE 3
Selected physical and chemical characteristics of paddy soil series

Properties	Kangar	Kundor	Briah	Jabil	Lubok Itik
Texture class	Silty Loam	Silty Loam	Clayey	Clayey	Clayey
Total (%)					
Clay	16	21	55	83	94
Silt	62	66	34	13	2
Sand	22	23	11	1	1
pH(H_2O)	6.80	4.68	4.60	4.70	4.70
CEC (cmol kg^{-1})	8.85	14.85	15.66	12.66	15.02
Total N (%)	0.05	0.13	Nd*	0.25	0.28
Exchangeable					
K (cmol kg^{-1})	0.06	0.22	0.30	0.14	0.22
Mg (cmol kg^{-1})	0.30	0.86	4.11	0.40	1.65
Ca (cmol kg^{-1})	7.11	6.82	5.42	0.27	2.10
Organic matter	1.25	5.26	Nd*	3.31	5.17

*Nd = Not determined

that the diazotrophic community from a paddy field in China centred around the *Azoarcus* genera. Furthermore, Gram-positive bacteria and actinomycetes were the dominant microbes in rice soils, as reported by Kimura and Asakawa (2006).

TABLE 4
Results of the morphology test

Strain	Gram staining	Cells morphology
P1	Purple, Gram +	Rods
P2	Purple, Gram +	Rods
P3	Purple, Gram +	Rods
S1	Purple, Gram +	Rods
22	Purple, Gram +	Rods
23	Purple, Gram +	Rods
40	Purple, Gram +	Rods
44	Purple, Gram +	Rods
47	Purple, Gram +	Rods
P13	Red, Gram -	Coccus

The presumptive test for the Gram positive bacteria showed that these strains were possibly *Bacillus* species, due to the presence of endospore. This was done using the Schaeffer–Fulton method. The malachite will stain endospores green, whereas the vegetative cells will only be stained by the safranin. Endospore is different from vegetative cells because they have a greater resistance to heat and other destructive agents. All the Gram positive isolates have the ability to degrade hydrogen peroxide by producing the enzyme catalase. Bubbles of free oxygen were released in the presence of catalase (Blazevic and Grace, 1975). Thus, catalase test is important to distinguish the *Bacillus* species from genus *Clostridium*, which is also an endospore-forming, but is catalase negative (*Bergey's Manual of Systematic Bacteriology*, 2005).

P13, the only strain of Gram negative bacteria, exhibited a swarming motility on the solid media, which might be classified in *Proteus* species. The important characteristic which distinguishes these genera from other *Enterobacteria* is their ability to deaminate phenylalanine by changing the media to dark green colour after several days of incubation (*Bergey's Manual of Systematic Bacteriology, 2005*). The production of deaminase phenylalanase by strain P13 enables it to deaminize the amino acid phenylalanine into phenylpyruvic acid.

The ability of each strain to produce enzyme for the hydrolysis of starch, casein and gelatin is given in Table 5. All the isolates have the ability to hydrolyze starch by producing the amylase enzyme. This indicated that the entire Gram positive and Gram negative strains can utilize starch as a carbon source. In the presence of amylase, these macromolecules will be hydrolyzed into dextrins, maltose and glucose molecules. All the Gram positive strains also showed the ability to utilize casein, as compared to the Gram negative strain, P13, which is incapable of hydrolyzing casein as a nitrogen source. This is because these bacteria produce more soluble and transparent derivatives as their nitrogen source. For gelatine liquefaction test, strain S1, 40 and 47 were unable to hydrolyze gelatine into amino acid because they lack gelatinase enzyme. They might be in a same group due to their similar reactions in starch, casein and gelatin hydrolisis test.

Microbes ferment many organic compounds including carbohydrates to obtain their energy source. The fermentation of carbohydrate by the isolates were 100% for glucose, 50% for lactose and 50% for sucrose. Table 6 shows the results derived from the fermentation of carbohydrate for each strain. All the isolates showed their ability to generate energy through the bio-oxidation of simple sugars (glucose), by producing acetic acid, lactic acid, formic acid, ethanol and carbon dioxide as common products. The production of these acids cause the medium to change colour, i.e. red to yellow. However, the only Gram-negative bacteria, P13 and other 4 strains of Gram-positive bacteria (P1, P2, P3 and 23) demonstrated a weak fermentation capability because they were unable to ferment a more complex structure of carbohydrate, such as lactose and sucrose, which are a combination of two monosaccharide molecules. Therefore, these strains might be of the same species (*Bergey's Manual of Systematic Bacteriology*, 2005).

Table 7 shows the results gathered from the other biochemical tests. All the strains

TABLE 5
Hydrolisis tests for starch, casein and gelatine

Test	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
Starch	+	+	+	+	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	+	-
Gelatin	+	+	+	-	+	+	-	+	-	+

+ positive reaction
- negative reaction

TABLE 6
Results of carbohydrate fermentation

Fermentation	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
Glucose	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	+	+	-	+	+	+	-
Sucrose	-	-	-	+	+	-	+	+	+	-

+ positive reaction
- negative reaction

were capable of utilizing sodium citrate when incorporated in a mineral medium as the only carbon source. Oxaloacetate and acetate were the primary products which were subsequently converted to pyruvate and carbon dioxide by an oxaloacetate decarboxylase (O'Brien and Geisler, 1974).

A considerable number of bacteria can be differentiated on the basis of the end products produced, when they ferment glucose in MR-VP medium. Some of the bacteria, such as genus *Proteus* and *Aeromonas* ferment glucose, were used to produce large amounts of acetic, lactic and formic acids, together with carbon dioxide, hydrogen and ethanol. The accumulation of these acids will lower the pH of the medium to five or less, and the indicator will turn red when methyl red is added (Baker and Silverton, 1986). This indicates that the organism is a mixed acid fermenter (e.g *Proteus*, *Citrobacter*). As for butanediol fermenter (e.g *B. cereus, B. polymyxa*), they produced little or none of these acids, as compared to acid fermenter which produced a large amount of polar butylene glycol. Acetoin, which is an intermediate in the production of butylene glycol, is responsible for the development of red colour in the VP test instead of acids. The results indicated that strain P13 is a mixed acid fermenter, while strains 22 and 44 are butanediol fermenters. As for the remaining strains, they cannot be differentiated using the MR-VP test because it was found to give positive results for both tests.

Many facultative bacteria are able to use oxygen in nitrate as hydrogen acceptor. Nitrate (NO_3) will be converted into nitrite (NO_2) when it is utilized by facultative bacteria (Doelle, 1975). This enzymatic reaction is controlled by an inducible enzyme known as nitratase. Among the isolates, only strain S1 and P13 were able to utilize nitrate. Although strain S1, 40 and 47 were suspected to be in the same group, S1 showed dissimilarity in the reduction of nitrate, since the presence of free oxygen might prevent nitrate reduction (Revsbech *et al*., 2006). Thus, this factor might be the major cause of the dissimilarity.

The results presented in Table 7 also show that the Gram-negative bacteria, P13, is able to degrade tyrosine and produce hydrogen sulphide. The capability of this strain to produce hydrogen sulphide from amino acid cysteine is dependent on the enzyme cysteine desulfurase, which works in conjuction with the coenzyme pyridoxyl phosphate. Hydrogen sulphide is the initial product of cystein deamination.

Table 8 shows the differences in the results gathered on the growth ability in 5% and 7% sodium chloride (NaCl). Amongst these strains, P1, P2, P3 and 23 showed the ability to grow in a medium which contained 7% NaCl. This shows that the strains are more resistant to the

TABLE 7
Results of biochemical tests

Test	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
Oxidase	-	-	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-	-	-
Hydrogen sulphite	-	-	-	-	-	-	-	-	-	+
Citrate	+	+	+	+	+	+	+	+	+	+
Tyrosine	-	-	-	-	-	-	-	-	-	+
Nitrate	-	-	-	+	-	-	-	-	-	+
MR	+	+	+	+	-	+	+	-	+	+
VP	+	+	+	+	+	+	+	+	+	-

+ positive reaction
- negative reaction

TABLE 8
Growth ability in NaCl and in anaerobic condition

Growth ability	Strain									
	P1	P2	P3	S1	22	23	40	44	47	P13
7% NaCl	+	+	+	-	-	+	-	-	-	-
10% NaCl	-	-	-	-	-	-	-	-	-	-
Anaerobic	+	+	+	+	+	+	+	+	+	+

+ positive reaction
- negative reaction

high osmotic pressure than the others. From the physical test, all the isolates were shown to be able to grow anaerobically.

CONCLUSIONS

From this study, 9 out of the 10 strains isolated from the different locations of rice rhizosphere were Gram positive rods bacteria. They are suspected to be *Bacillus* species due to the endospore-formation and series of presumptive test. According to the biochemical tests, strain P1, P2, P3 and 23 might be of the same species, while strain S1, 40 and 47 were listed in the same group. Meanwhile, Strains 22 and 44 are classified in another group. The only Gram-negative bacteria, P13, was presumably *P. mirabilis*, as it posed some important characteristics with this species of bacteria. The study indicated that bacterial diversity from the rhizosphere of paddy soil in Peninsular Malaysia is narrow but it is widespread throughout the geographical location of the country.

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