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A special issue devoted to Pest Management in the Tropics

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Law Yao Hua

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Preface

Towards the end of 2011, the human population surpassed 7 billion. The 8 billion mark will be crossed shortly after 2020[i], with most of the new population concentrating in the least-developed or developing countries. However, do we have enough food to feed this ever-growing population and to provide them with the necessary nourishment they need?

Agricultural yield has hiked leaps and bounds since the inception of industrial machinery, chemical fertilizers and pesticides. Crop losses to pests were once largely reduced with the use of pesticides. Yet, we know now that pesticides are not the silver bullets we have hoped for as pests have evolved and developed resistance to many of the pesticides we use. Pests, be they microbial pathogens like viruses, bacteria and fungi or insects and snails, are a huge threat to our food security. Very often, pests are also a natural component of the environment we live in. Gone is the notion that pests should be eradicated, for that goal is simply too costly, if not impossible. Instead, we need to manage pests and keep their impacts low. That is the reality; the goal is to manage pests well—sustainably and profitably.

In the tropics, where climate is relatively stable throughout the year, pests can often remain active year-long and maintain their economically-significant population. Furthermore, many developing countries with huge populations and severely limited arable lands are situated in the tropics, hence, aggravating the food security issue and augmenting the need for a more efficient pest management in tropical agriculture.

In this special issue, we published eight experimental studies and one review paper that addressed the various aspects of pest management in tropical agriculture. The taxa of pests and crops covered are diverse, as are the scientific approaches taken. Although most of the studies were conducted in Malaysia (due to the local research foci of the authors), we believe that the findings would be applicable to many other tropical regions. This is particularly true because most of the pests and crops reported here are ubiquitous throughout the tropics. Thus, we hope that this special issue will provide valuable insights and points of discussion for our colleagues, and contribute to better pest management in the tropics.

This issue is a concerted effort made possible with the help of many. Specifically, we thank Dr. Nayan Kanwal, the Chief Executive Editor and his dedicated Pertanika team, for their generous guidance and patience. We also acknowledge the relentless support given by Prof. Dr. Mad Nasir Shamsudin, the Dean of the Faculty of Agriculture, Universiti Putra Malaysia. We particularly congratulate and thank the authors for their full cooperation and understanding, despite their tight schedules. Last but not least, we are indebted to the reviewers — whose names are acknowledged at the back of this issue — for providing their critical and timely feedbacks.

Thank you.

Yao-Hua Law

Guest-Editor

December 2012

[i]Dept. of Economics and Social Affairs, United Nations. http://esa.un.org/unpd/wpp/Analytical-Figures/htm/fig_1.htm

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Review Article

Effect of Silicon on Rice Blast Disease

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ABSTRACT

Silicon is the second most plentiful element in soil and is beneficial for a large variety of plants. It is concentrated in plant tissues in quantities similar to that of macronutrients. Considerable damages to plants caused by abiotic stresses such as drought stress, salinity stress, heavy metal stress and nutrient imbalance, as well as biotic stresses like insect pests and pathogens and even herbivorous attacks, have been reported to be reduced significantly by silicon application. Among rice diseases, blast is recognized as the most devastating one. Silicon fertilization has been reported to be efficacious in controlling and mitigating rice blast severity. Two different hypotheses are proposed for the ability of silicon to lessen disease severity. The first hypothesis emphasizes on silicon function as a mechanical barrier against appressorial penetration, while the second hypothesis is based upon the belief that silicon has some physiological roles in disease resistance.

Keywords: Silicon, rice, *Pyricularia oryzae*, blast

WHAT IS SILICON?

Silicon (Si) is the second most abundant element in soil and it is considered as an absolutely useful element for a large variety of plants (Nakata *et al*., 2008). In fact, silicon forms 28% of the earth's surface (Rodrigues & Datnoff, 2005; Elawad & Green, 1979; Singer & Munns, 2005; Epstein, 1994). Although it is usually not classified as an essential element for higher plants (Hayasaka *et al*., 2008; Marschner, 1995; Epstein, 1994), silicon is concentrated at levels equivalent

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to those of macro nutrients (Kamenidou *et al*., 2009; Epstein, 1994). The positive impacts of silicon in improving plant growth and yield are significant, and these include increasing resistance against environmental stresses such as cold and salinity stress, insect attacks and penetration of pathogens (Jones & Handreck, 1967; Elawad & Green, 1979; Belanger *et al*., 1995; Savant *et al*., 1996). In addition to what has been mentioned above, using appropriate amount of silicon has been found to reduce the probability of plants being grazed by herbivores (Rodrigues & Datnoff, 2005; Belanger *et al*., 1995; Datnoff *et al*., 1997; Savant *et al*., 1996). Meanwhile, a large number of valuable investigations have been conducted on the positive effects of silicon in plants. Many plant species, especially those from the family of *Poaceae,* are able to accumulate silicon in their tissues (Hayasaka *et al*., 2008; Ma *et al*., 2006). Typically in soils with low amounts of silicon, silicon fertilization has been applied to increase both the quality and quantity of agricultural crops such as rice and sugar cane (Korndorfer & Lepsch, 2001). In addition, foliar applications of silicon have been confirmed to increase the resistance against pathogens in plant species that do not absorb silicon efficiently (Epstein, 1994; Bowen *et al*., 1992). Much of the research on silicon has been focused on rice, although its benefits to other crops such as wheat and corn have also been proven (Wang *et al*., 2001). The goal of this article is to review the research findings in relation to silicon and rice blast disease.

Rice is considered as one of the major crops in view of its vast consumption as a source of energy throughout the world (Hayasaka *et al*., 2008; Greenland, 1997) and its capability to actively absorb silicon in high amounts. Under silicon deficient conditions, some diseases such as blast, brown spot and sheath blight can be extremely threatening to rice cultivation (Rodrigues & Datnoff, 2005). Accumulated silicon in rice tissues enhances resistance against insects and diseases, increases erectness of leaves resulting in increased photosynthesis, improves water usage, and decreases toxicity of heavy metals and cuticular transpiration (Nakata *et al*., 2008; Epstein, 1994). Accumulation rates of silicon in different plants vary between 1-10 % (Elawad & Green, 1979; Epstein, 1994) and monocots store more silicon in comparison to dicots (Rodrigues & Datnoff, 2005; Jones & Handreck, 1967; Epstein, 1999; Rodrigues *et al*., 2001). The amounts of silicon accumulated in rice plants can be as much as 10% of plant dry weight. It is often several times higher than the rate of accumulation of other essential macro nutrients such as nitrogen, phosphorus and potassium (Nakata *et al*., 2008; Ma *et al*., 2006). In the tropics (where the soils are classified as Ultisols and Oxisols), high levels of weathering may result in nutrient deficiencies in soils, particularly silicon (Rodrigues & Datnoff, 2005; Juo & Sanchez, 1986). Silicon deficiency has also been observed in highly organic soils, as these soil types contain low amounts of minerals (Rodrigues & Datnoff, 2005; Savant *et al*., 1996).

It is clear that pests and diseases cause significant losses to rice plantings (Nakata *et al.,* 2008; Ou, 1985). Rice blast disease, caused by the fungus *Pyricularia oryzae* (Couch & Kohn, 2002), is particularly prevalent in all areas under rice cultivation and it also causes heavy yield losses (Nakata *et al.,* 2008; Ou, 1985). Development of efficient and cost-effective methods to manage the disease is one of the priority issues in rice production (Hayasaka *et al.,* 2008). There are many management methods that are being evaluated for rice blast disease management and these include, among others, breeding of resistant varieties (time consuming) and management methods to improve soil nutrients (Hayasaka *et al.,* 2008).

Rice productivity has been reported to be higher in temperate regions as compared to the tropics (Rodrigues & Datnoff, 2005; Savant *et al*., 1997) because the amount of silicon in the tropical soils is about 5 to 10 times lower than its amount in the temperate region soils (Rodrigues & Datnoff, 2005; Foy, 1992).

The minimum amount of silicon needed to control blast disease in rice is 3-5% (Datnoff *et al.,* 1997). Positive effects and importance of silicon as a nutrient element in rice plants have been reported in several studies (Hayasaka *et al.,* 2008; Ando *et al*., 2002; Ma *et al.,* 2006; Kamenidou *et al*., 2009; Deren *et al*., 1992; Ma & Takahashi, 2002; Gao *et al*., 2004) and a gene which plays a role in the transport of silicon has also been detected (Hayasaka *et al*., 2008; Ma *et al.,* 2006).

Silicon is an important element which is applied to paddy farms in the form of fertilizers (Ohta *et al.,* 1953; Snyder *et al.,* 1986). Plants absorb silicon in the form of mono silisic acid $Si(OH)_4$ (Rodrigues & Datnoff, 2005; Elawad & Green, 1979), which is accumulated in cell walls as silica gel. SiO_2-nH_2O is also referred to as 'Opals' or 'Phytolits' in leaves (Rodrigues & Datnoff, 2005; Yoshida *et al*., 1962; Lanning, 1963). The highest rate of silicon accumulation in leaves can be observed in epidermal cells (Rodrigues & Datnoff, 2005; Elawad & Green, 1979). Besides the positive effects which have been mentioned for silicon, its presence in plant tissue at high concentrations does not cause any toxicity or damage to the plant (Ma *et al.,* 2006).

BLAST SUPPRESSION BY SILICON APPLICATION

In the early twentieth century, many Japanese researchers stated that the use of various sources of silicon would increase resistance of rice to blast disease (Ishiguro, 2001). Similarly, subsequent studies have also confirmed that the application of silicon is an effective method to reduce and control rice blast disease (Datnoff *et al*., 1991; Hayasaka *et al*., 2005; Seebold *et al*., 2000; Seebold *et al*., 2001).

In retrospect, the authors found beneficial effects of silicon on growth of rice, as demonstrated by Sommer (1926). The usefulness of silicon application to confer resistance in rice plants against blast was demonstrated by Kawashima in 1927 as well. The amount of silicon applied to

soil and the concentration of silicon in rice husks and straw had a linear relationship and both were inversely proportional with blast severity. Similar findings were reported by Inokari and Kubota (1930), Ito and Hayashi (1931) and Miyake and Ikeda (1932), who confirmed the beneficial effects of silicon application in conferring resistance against blast. A significant reduction of blast severity due to various silicon sources applied to paddy fields was demonstrated in Japan (Suzuki, 1935; Kozaka, 1965). In 1997, a study was conducted at the Rice Research Institute of Iran (RRII) to assess the effects of application of silica on the growth and yield of a local rice variety. Silicon was applied as calcium silicate at 0, 400, 800 and 1600 kg/ha. The highest increase in dry matter and grain yield was observed with the application of 400 kg/ha of calcium silicate (Kumleh & Kavossi, 2003). Meanwhile, the application of calcinated serpentinit as a silicon source prior to rice planting has also been reported to be effective in reducing leaf blast severity and this reduction has a linear relationship with tissue sugar content. This indicates that the reduction of blast severity in leaves with increased rates of calcinated serpentinite is linear and can therefore be ascribed to decreased sugar levels (Prabhu *et al.*, 2007).

In the late 1950s, the first attempt to assess the possible uses of industrial by-products containing silicon as fertilizers was conducted in China. Subsequently, silicon application as fertilizers has increased steadily since 1970, and Si fertilizers have been applied repeatedly to improve rice production by enhancing resistance to diseases (Wang *et al.,* 2001) and increasing yields (Zhu & Chen, 1963).

Silicon fertilization was reported to have significantly reduced the occurrence of blast in rice plants (Qin, 1979; Zang, 1989). The degree of resistance increased in proportion to the amount of silicon accumulated in the plants (Chen *et al*., 1985; Chen, 1989). Shui *et al*. (1995) determined the efficacy of silicon fertilization on rice disease resistance to leaf and neck blast in silicon deficient soil. The results revealed that the resistance of rice to blast was enhanced by silicon application, and that disease index for leaf and neck blast was reduced by 50.5 and 26.8%, respectively. Maekawa *et al*. (2001) evaluated two sources of silicon on the blast occurrence in rice seedlings; one as silica gel and the other as potassium silicate. The silica gel application at 200 or 250 g per nursery bed (2.5 kg soil) significantly limited the blast occurrence to 10% of the control (without application). Potassium silicate application at 12 g per nursery bed was also effective in controlling the occurrence of blast. It was reported that silicon addition to soils increased rice yields by up to 10% and these increased yields could exceed 30% where leaf blast was severe (Yoshida, 1981; Korndorfer & Lepsch, 2001).

It has also been reported that rice blast severity is directly related with silicon deficiency in soils (Kim *et al.,* 2002; Rodrigues *et al*., 2003; Ranganathan *et al.,* 2006). In Colombia, experiments were conducted in soils classified as Oxisols (with

silicon deficiency) to assess the effects of silicon deficiency on favouring rice blast. Different levels and sources of silicon including Calcium Meta Silicate, as well as slag, were tested, and it was reported that silicon fertilization significantly reduced the severity of leaf (26%) and neck blast (53%) in non-treated plots to 15% in silicon-treated ones. It was also revealed that disease development could be further controlled due to the residual effect after years of silicon applications and this could be an efficient control method in rice production (Correa-Victoria *et al*., 2001).

Several researchers have demonstrated that silicon application has a suppressive effect on fungal diseases such as rice blast (*Magnaporthe grisea*, teleomorph, *Pyricularia oryzae*, anamorph), brown spot (*Cochliobolus miyabeanus*, teleomorph; *Bipolaris oryzae*, anamorph), leaf scald (*Gerlachia oryzae*), sheath blight (*Rhizoctonia solani*, teleomorph) and stem rot (*Sclerotium oryzae*, teleornorph) (Datnoff *et al*., 1991, 1992, 1997; Savant *et al*., 1997; Seebold, 1998; Winslow, 1992). Datnoff *et al*. (1991, 1992) reported decreases in blast severity ranging from 17 to 30% in the rice planting regions of Colombia on Histosols where silicon was applied. Disease severity tended to be reduced by increasing the concentration of silicon in tissues.

On the other hand, Okamoto (1957a, 1957b) observed that under silicon deficiency, the growth of rice was inhibited and this caused dying-off of leaf blades after heading, resulting in differences in the dry weights between the silicon-treated rice and non-treated ones. Moreover, dark brown spots were also observed on the stems and head of rice of the untreated plants. *Pyricularia oryzae* was isolated from the dark brown spots. It was concluded that silicon might not be an essential element for the growth of rice plants, since rice could ripen even without silicon, but silicon was considered as an agronomically essential element for rice production, since silicon deficiency causes significant reductions in grain yield.

In 2001, Ishiguro announced that silicon could alleviative rice blast. During cold summers, the occurrence of disease is usually serious. According to Ohyama (1985), silicon supplied to soil as compost (36 tonnes per hectare) and calcium silicate (1.8 tonnes per hectare) resulted in meaningful increases in the silicon content of leaf blades and a significant decrease in the severity of leaf blast as well as ear blast. Calcium silicate application caused an increase in the Si to N ratio, and resulted in the control of blast. In the year with a cold summer, the N content of rice plants was high, while the silicon content was low since silicon uptake decreased at low temperature. This resulted in a favourable silicon to N ratio, and thus explained why in a cold summer year, silicon application was more capable of reducing blast severity and damage.

Hayasaka *et al*. (2005) investigated the content of SiO_2 which was necessary for rice plants to be resistant against rice blast disease. Nine rice cultivars with different

complete resistance genes, as well as various degrees of partial resistance, were planted in nursery soils with different rates of silica gel amendment. At 28 days after planting, seedlings were inoculated with rice blast fungus (*Pyricularia oryzae*) to evaluate resistance of seedlings to blast. The results confirmed that the number of infections in all the cultivars was decreased significantly by increasing the silicon content of rice seedlings. The results showed that by increasing the SiO_2 content of the seedlings to 5%, a significant decrease could be observed in the number of blast lesions. Based on these findings, a 5% SiO_2 content in rice seedlings would be effective in controlling blast disease under a range of conditions.

Seebold *et al*. (2000) also studied the effects of different rates of silicon application as well as host resistance on the severity of blast in upland rice, and concluded that in soils with insufficient amounts of silicon, the application of silicon could reduce the severity of leaf and neck blast in partially resistant and susceptible cultivars. The reduction was dependent on the rates of silicon applied as well as disease pressure. Meanwhile, in areas where disease pressure is not very high, lower rates of silicon application can be sufficiently effective for disease control.

Providing appropriate amounts of silicon to the plants cultivated in silicon deficient soils could considerably improve the rate of plant growth as well as its resistance against biotic and abiotic stresses (Kamenidou *et al.,* 2009; Menzies *et al.,* 1991; Ma *et al.*, 2001; Savvas *et al.* 2002). Among the most controversial impacts and benefits of silicon is its ability to reduce transpiration and to increase photosynthesis rates in some plants such as rice (*Oryza sativa* L.), corn (*Zea mays* L.), soybeans (*Glycine max* L.), and wheat (*Triticum aestivum* L.) (Kamenidou *et al.*, 2009; Kupfer & Kahnt, 1992; Pandley & Yadav, 1999; Gao *et al.*, 2004). These effects may be related to the distribution of silicon in the cell wall as a double silicon-cuticle layer (Kamenidou *et al*., 2009; Ma & Takahashi, 2002), and also its role as a mechanical barrier against pathogens and pests (Kamenidou *et al*., 2009; Belanger *et al*., 1995). Under drought stress condition, silicon can further stimulate and increase photosynthesis (Kamenidou *et al.*, 2009; Matoh *et al.,* 1991; Trenholm *et al*., 2004). This may be caused by lower transpiration rates due to silicon accumulation in the leaves (Kamenidou *et al*., 2009).

POSSIBLE MECHANISMS FOR SILICON MEDIATED RISE RESISTANCE

There are different hypotheses on how silicon confers and induces resistance to some plants specially rice against diseases such as blast (Ishiguro, 2001). These hypotheses can be classified into two groups (Hayasaka *et al.,* 2008).

One hypothesis is based on the belief that silicon acts as a mechanical barrier against fungal penetration (Hayasaka *et al*., 2008) which is also known as physical resistance, while the second hypothesis is based upon physiological resistance. The

first suggests that resistance induced by silicon is the result of increased number of silicified bulliform cells in the epidermis of leaves that act as a physical barrier to impede penetration by *M. grisea* (Ishiguro, 2001; Snyder *et al*., 1986). This physical barrier hypothesis is further strengthened by the findings of Yoshida *et al*. (1962) who reported the existence of a layer of silica beneath the cuticle of rice leaves and sheath. These cuticles inhibit the penetration of *M. grisea,* and consequently decrease the number of infections on the leaves. Volk *et al*. (1958) suggested the formation of complexes with organic compounds with silicon in cell walls of epidermis cells, thereby conferring resistance to degradation by the enzymes released by *M. grisea.* In fact, silicon can be associated with lignin-carbohydrates complexes in the cell wall of rice epidermal cells (Inanaga *et al*., 1995).

The second suggests that the induced resistance is the result of silicon cuticle double-layer formation (Hayasaka *et al.,* 2008; Sakamoto, 1968). This kind of resistance could be related to the conservative action of Si-cuticle layer against NH_4-N volatilization loss from the surface of leaves (Park, 1982), since dissolving volatilized NH_4- N in dew drops or rain water can act as a nutrition source for the spores of pathogens.

On the other hand, the other group believes that the induction of resistance to plants by silicon is as a result of phenolics and phytoalexins accumulation and is related to the activity of P-R genes (Rodrigues & Datnoff, 2005). Rodrigues *et al*, (2003) investigated the ultrastructural changes of the rice-*M. grisea* interaction upon silicon application, which provided the first cytological evidence that silicon-mediated resistant to *M. grisea* was related with the deposition of the osmophilic material that occluded the epidermal cells. These amorphous materials contain phenolic compounds which play a crucial role in rice defence response against infection by *M. grisea*. Therefore, silicon could be acting as a modulator to positively amplified rice defence responses, i.e. by influencing the synthesis of antifungal compounds after the penetration peg of *M. grisea* into the epidermal cell. Rodrigues *et al*. (2004) further hypothesized that the alteration of the development of *M. grisea* in leaf tissue of rice plant amended with silicon could be associated with an enhanced production of phytoalexins. The evidence obtained from their research indicated that antifungal compounds (phytoalexins) were present 2 to 3 times more in the leaf extract from the plants amended with silicon and inoculated with *M. grisea* as compared to the lower levels observed in the leaf extract from non-inoculated plants amended with or without silicon. The rice plants that were not amended with silicon and inoculated with *M. grisea* were not protected against fungal colonization, in spite of releasing antifungal compounds. By contrast, the rice plants amended with silicon and inoculated with *M. grisea* released higher amounts of antifungal compounds, probably earlier in the infection process, benefited from a lower level of blast severity.

While little is known about the mechanism(s) of the resistance of rice plants amended with silicon in response to *M. grisea* infection, two mutually agreeable hypotheses must be considered. On the one hand, it is possible that in certain areas of heavy silicon deposition, delayed fungal ingress and colonization provide the rice plant enough time for synthesizing the antifungal compounds in response to infection by *M. grisea* to accumulate considerable levels and to express their fungitoxocity within the zone of the infection site. On the other hand, as proposed, the soluble silicon present in the plant cells may mediate some defense responses that are functionally similar to systemic acquired resistance. Evidence put forth by several researchers strongly suggest that silicon plays an active role in the resistance of rice plant to blast rather than simply forming physical barrier in leaf epidermis to impede fungal penetration.

FUTURE OUTLOOK

Although the function of silicon in rice plant is not wholly recognized, the application of this element would be an encouraging method to improve rice production in areas with soils poor in silicon, since it has been known to have the ability to lessen vulnerability of rice against different kinds of diseases, specifically those caused by fungal pathogens such as *Pyricularia oryzae*.

An important fact about silicon in addition of the aforementioned points is that it is an environmentally friendly element in relation to soils, fertilizers and plant nutrition. Hence, it can be beneficial in IPM programmes for those crops where silicon has been proven to have a positive impact. However, silicate slags are not only expensive sources of silicon to be applied as fertilizers, many of these are also high in heavy metals which may discourage their use due to environmental concern. Therefore, it is necessary to find more cost effective silicon sources especially in the tropics where soils are poor in silicon. Recycling rice straw and husks can be considered as alternatives that provide cheaper silicon sources. Meanwhile, the positive effects of silicon in plant nutrition and resistance against diseases have been well established. Thus, providing the knowledge about silicon to farmers and rice growers will help the agriculture industry to manage and control rice diseases effectively. Such efforts will ensure the production of safe food and provide for adequate environmental protection.

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Suppression of Basal Stem Rot Disease Progress in Oil Palm (*Elaeis guineensis*) after Copper and Calcium Supplementation

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ABSTRACT

The effects of copper and calcium nutrient supplementation on basal stem rot disease caused by *Ganoderma boninense* were evaluated in oil palm during a 6-month glasshouse study. Nutrients were supplemented when seedlings were at the 2- to 3-leaf stage. The aim of the study was to assess the effects of copper and calcium on the suppression of basal stem rot. Nutrient supplementation, with copper at 2.0 mg/L and calcium at 4000 mg/L alone or in combination, significantly reduced *G. boninense* infection in oil palm roots. The treated seedlings did not escape the disease, but it developed more gradually than in the untreated seedlings. The seedlings supplemented with a combination of calcium and copper remained free from any symptom for a longer period and developed the disease at a later stage than the controls. The supplementations of calcium and copper could have triggered oil palm mechanism of resistance by enhancing the production of peroxidase and lignin during fungal penetration. These findings suggested that copper and calcium supplementations could be used to reduce the severity of basal stem rot in oil palm.

Keywords: Copper, Calcium, Basal stem rot, *Ganoderma boninense*, *Elaeis guineensis*

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INTRODUCTION

Oil palm is of major economic importance in southeast Asia, where it is grown extensively in Malaysia and Indonesia for the production of vegetable oil used in foods, cosmetics, and biodiesel. Nonetheless, oil palm is

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vulnerable to various diseases, among which basal stem rot (BSR) which is caused by *Ganoderma*, is of particular significance, e.g., to Malaysia (Paterson *et al*., 2008). *Ganoderma boninense*, the causal pathogen of BSR disease, is a white rot basidiomycetous fungus. The fungus is spread by root contact between infected and healthy tissues. The pathogen that remains in the soil infects oil palm primarily through the roots and degrades the lignin component of wood, leaving white cellulose exposed. For this reason, it is called white rot fungus (Paterson, 2007). Eventually, the degradation of lignin will weaken the tree and make it susceptible to wind damage. Based on an understanding of the mode of infection of *G. boninense* on oil palm, the ideal solution to slow the emergence of basal stem rot disease is to enhance oil palm's natural defences through lignin manipulation resulting from the administration of plant nutrients.

Lignin is believed to be one of the products of the evolution of phenol metabolism. More pecifically, it is considered as a product of phenylpropanoid pathway. This secondary metabolic pathway is hypothesised to be important in the adaptation of plants to land. It is considered to provide several benefits, including protection against UV light and pathogens (Mazza *et al*., 2000). Peroxidase (POD) within the cell wall has been shown to be involved in monolignol polymerisation. Therefore, it appears to participate in lignification (Fry, 2004). Laccase is responsible for the early stage of lignin biosynthesis. Subsequently, laccase and POD function cooperatively in lignin biosynthesis (Lin *et al*., 2005) and become important enzymes during lignification. Copper (Cu) has been reported to be involved in the enhancement of peroxidase in rice leaves (Fang & Kao, 2000), lignin biosynthesis in soybean roots (Lin *et al*., 2005), and enhancement of peroxidase activity and lignin content in *Raphanus* (Chen *et al*., 2002). Cu in low concentrations may not cause significant damage to cell membranes but it may also lead to accumulation of peroxide compounds (Weckx & Clijsters, 1996). In contrast, calcium (Ca) augmented soluble POD activity (Kolupaev *et al*., 2005) or reduced phenolic compounds (Ruiz *et al*., 2003). Meanwhile, deposition of Ca pectate is crucial for the plant to undergo lignification, which later provides a barrier against pathogen attack (Willats *et al*., 2001). In view of these findings, this study was conducted to evaluate the effects of Cu and Ca on the suppression of *G. boninense* infection in oil palm seedlings.

MATERIALS AND METHODS

Planting Materials and Experimental Design

Three-month-old oil palm seedlings were used for the infection studies. The seedlings were DxP crosses supplied by Sime Darby Sdn. Bhd., Banting, Selangor. The seedlings were grown in 12 x 15 cm white polythene bags in standard plantation soil, mixed with river sand in a ratio of 7:3. In Cu and Ca supplementations, 2 mg/L Cu (as $CuSO_4.5H_2O$) and 4000 mg/L Ca (as $CaNO_3$)

were amended into the basal fertilizer (Table 1). These concentrations were chosen as the optimum levels of Cu and Ca required for oil palm under field conditions (Goh & Hardter, 2000). Each supplementation was replicated five times (with four seedlings per replicate) and arranged in a Completely Randomised Design (CRD). The seedlings were watered twice daily, and 7g 15/6/4 NPK fertilizer per seedling was applied once a month to serve as basal fertilizer (BF). The seedlings received Cu and Ca supplementations at monthly intervals for three months.

TABLE 1: Treatments with Cu and Ca applied to oil palm seedlings inoculated with *G. boninense*

Treatment	Descriptions
T1	Control *(BF)
T2	BF + 4000 mg/L $CaNO_3$
T3	BF + 2 mg/L $CuSO_4$
T4	BF + 4000 mg/L $CaNO_3$ + 2 mg/L $CuSO_4$

**Basal Fertilizer*

Preparation of Ganoderma Inoculum on Rubber Wood Blocks

Rubber wood blocks measuring 12 cm x 12 cm x 12 cm were obtained from Kilang Kayu Getah Wah Heng Sdn. Bhd., Semenyih, Selangor. These blocks were sterilised (121°C; 1.04 kg cm^{-2} pressure) for 30 min. The blocks (one block per bag) were placed in heat-resistant polypropylene bags (15 cm x 33 cm x 0.05 mm thick material), and 100 mL of molten malt extract agar (MEA) was added as a supplementary nutrient for *G. boninense*. The bags, containing a rubber wood block and molten MEA, were autoclaved at 121°C for 30 minutes. After sterilisation and cooling, the rubber wood block in the polypropylene bag was rotated to ensure that it was well covered with the agar before the latter solidified. After the agar had been solidified, the rubber wood blocks were inoculated with 14-day-old *G. boninense* culture at a half plate per block and incubated for four weeks under dark conditions at room temperature (28 ± 2°C) until they were fully colonised with *G. boninense*. The *G. boninense* isolate was obtained from a basidiomata of an infected oil palm trunk growing in Banting, Selangor, Malaysia. Ganoderma-selective medium (Ariffin & Idris, 1991) was used to obtain the isolate. The identification was confirmed based on spore morphology and cultural characteristics.

Inoculation of Oil Palm Seedlings with G. boninense*-infected Rubber Wood Blocks*

Three months after Cu and Ca supplementation, the seedlings were challenged with *G. boninense*-colonised rubber wood blocks placed in contact with the roots (Sariah *et al*., 1994). The plant-cum-inoculum was placed in a polythene bag filled with one-third soil mixture (3:2:1 v/v/v topsoil:peat:sand). More soil (10 kg) was then added to cover the roots and inoculum. The progress of colonisation over 6 months was monitored based on the development of the macromorphological symptoms of BSR.

Disease Assessment

Disease development was monitored monthly by measuring the percentage of Disease Incidence (DI). The DI is the number of visibly diseased seedlings (chlorosis and necrosis of leaves, with or without sporophore production) relative to the total number of seedlings and is assessed using the following formula (modified from Campbell & Madden, 1990):

$$DI(\%) = \frac{\text{Number of seedlings infected}}{\text{Total number of seedlings assessed}} \times 100$$

A reduction in the disease incidence compared with the control would be a measure of the effectiveness of the treatment in suppressing the disease. This value was assessed by plotting the data in the form of a disease progress curve. The Area under the Disease Progress Curve (AUDPC) was calculated using the following formula from Campbell and Madden (1990):

$$AUDPC = \sum_{i}^{n-1} \left(\frac{Y_i + Yi_{i+1}}{2} \right) \left(t_{i+1} - t_i \right)$$

where n is the number of assessment times, Y is the disease incidence and t is the observation time. The slope of the curve was obtained by transforming the DI data with the monomolecular model (Monit) in Campbell and Madden (1990).

The development of disease in the seedlings was also rated in terms of Disease Severity (DS). DS refers to the total area or volume of plant tissue that is diseased (Kranz, 1988). DS is calculated based on the symptoms appearing on the foliage, root and bole on a 0-4 assessment scale (modified from Breton *et al*. 2005), as follows:

0 = Healthy; 1 = Yellowing of lower leaves and formation of rhizomorph at base of bole; 2 = Necrosis of lower leaves and emergence of button-like sporophore at the base of bole; 3 = > 50% necrosis of leaves and production of sporophore at the base of bole; 4 = Total necrosis and production of sporophore at the base of bole.

The DS for the foliar symptoms was calculated using the formula derived from Liu *et al*. (1995), as follows:

$$DS_{(external)} = \frac{\text{Number of seedlings in the rating} \times \text{rating number}}{\text{Total number of seedlings assessed} \times \text{highest rating}} \times 100$$

At the end of the experiment (i.e. 6 months after the seedlings were inoculated with *G. boninense*), the internal symptoms were recorded. The seedlings were uprooted carefully, split longitudinally and visually assessed for the severity of the internal symptoms based on the rating of the bole-tissue damage produced by *G. boninense*. This assessment was based on the following scale (modified from Breton *et al*., 2005):

0- Healthy; 1- Up to 20% rotting of bole tissues; 2- From 20% to 50% rotting of bole tissues; 3- Over 50% rotting of bole tissues; 4- Over 90% rotting of bole tissues.

Meanwhile, Disease Severity (DS) for the internal symptoms of the bole tissues was calculated based on the following formula derived from Liu *et al*. (1995):

$$DS_{(internal)} = \frac{\text{Number of seedlings in the rating} \times \text{rating number}}{\text{Total number of seedlings assessed} \times \text{highest rating}} \times 100$$

Statistical Analysis

Statistical analysis was performed using SAS (version 9) software. The significance of the differences between means was determined with a one-way analysis of variance (ANOVA) at 95% confidence level. Products with the same letter have no significant difference with $p>0.05$.

RESULTS AND DISCUSSION

The external symptoms of BSR infection were observed. These symptoms included the progressive yellowing of the lower leaves, the subsequent desiccation from the oldest to the younger leaves, and the rapid development of the sporophore, which assumed a button-like form. The symptoms followed the typical pattern of infection, which had previously been described by Sariah *et al*. (1994) (see Fig.1).

The Disease Incidence (DI) based on foliar symptoms in seedlings pre-supplemented with Cu, with Ca and with a combination of Cu and Ca progressed more slowly than in the control. The appearance of disease symptoms between treatments

Fig.1: Progressive development of the BSR symptoms in Ganoderma-inoculated oil palm seedlings; (A) yellowing of lower leaves, (B) dessication of lower leaves, (C) extensive necrosis, and (D) dead seedling with sporophore production.

was observed 3 months after the inoculation of the seedlings with *G. boninense*. Disease suppression is indicated by a lower DI value. Three months after the inoculation of the seedlings with *G. boninense*, the DI was found to be 0% for the seedlings pre-supplemented with Treatment 4 (a mixture of 2 mg/L Cu and 4000 mg/L Ca). This indicated that the seedlings had achieved partial or complete disease suppression (Fig.2). Meanwhile, the yellowing of the leaves was observed 4 months after the inoculation of the seedlings. The formation of a sporophore was only observed 6 months after the inoculation of the seedlings. At the end of the experiment, the DI was only 28.3%. This result suggested that the combined supplementation of Cu and Ca produced a good level of disease suppression.

The seedlings pre-supplemented with 4000 mg/L Ca alone (Treatment 2) and 2 mg/L Cu alone (Treatment 3) also showed a DI reduction relative to the control. However, the amount of disease suppression was less than that produced by Treatment 4. At six months after the inoculation of the seedlings with *G. boninense*, the DI values for Treatment 2 and Treatment 3 were 43.3% and 48.5%, respectively. These values were not significantly different. As expected, the control seedlings (Treatment1) recorded the highest DI of 88.3% six months after they had been inoculated with *G. boninense*. Although the foliar symptoms in the infected seedlings were clearly distinguishable, foliar symptoms could not indicate the extent of damage to the roots and in the bole region.

The Cu and Ca supplementation was considered effective in suppressing the onset of BSR infection because the DS was significantly reduced. The DS, assessed from foliar symptoms, developed rapidly in the absence of Cu and Ca supplementation and reached a level of 4 (on a 0-4 scale) in the control 6 months after the inoculation of the seedlings with *G. boninense*. At 6 months after they had been inoculated with

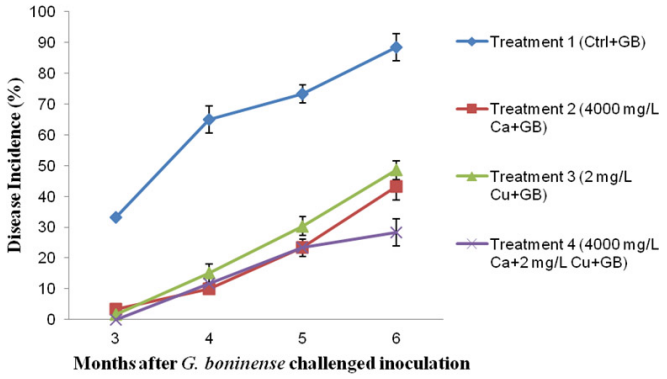

Fig.2: Basal Stem Rot Disease incidence (DI) on *G. boninense* inoculated oil palm seedlings based on chlorosis and necrosis of leaves, with and without production of sporophore. Values are the means of five replicates. Bars represent standard deviation.

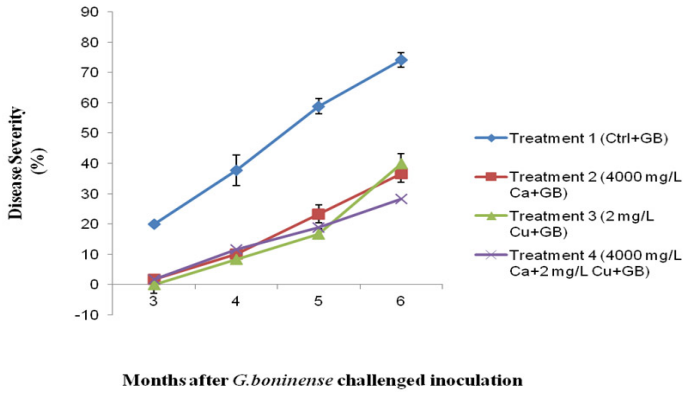

Fig.3: Disease severity (DS) over time observed on *G. boninense* inoculated oil palm seedlings based on foliar symptoms. Values are the means of five replicates. Bars represent standard deviation.

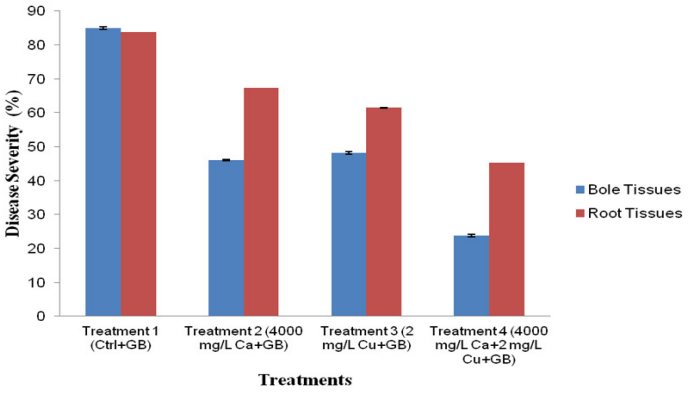

Fig.4: Disease severity (DS) observed on root and bole tissues of oil palm seedlings 6 months after *G. boninense* inoculation. Values are the means of five replicates. Bars represent standard deviation.

G. boninense, the seedlings in Treatment 2 (4000 mg/L Ca) had an external DS of 36.7% (DS scale ≥ 2). Treatment 3 (2 mg/L Cu) showed a DS of 40.0% (DS scale ≥ 2), and Treatment 4 (mixture of 2 mg/L Cu and 4000 mg/L Ca) showed a DS of 28.3% (DS scale ≥ 1). In contrast, Treatment 1 (control) showed a DS of 74.2% (DS scale ≥ 3) (Fig.3) at six months after the inoculation of the seedlings with *G. boninense*.

Destructive sampling was carried out at the end of the experiment (i.e. 6 months after the inoculation of the seedlings with *G. boninense*) to assess the extent of root rot and bole decay. The highest level of root rot and decay, with extensive colonisation of fungal masses on the root surface, was observed in Treatment 1 (control), in which 83.8% of the primary roots showed brown discolouration as compared to Treatment 2 (67.3%), Treatment 3 (61.5%) and Treatment 4 (45.3%). Meanwhile, the longitudinal sections of the infected boles showed brown lesions marked by an irregular, darker zone.

The disease severity, based on the extent of lesions in the bole, was also affected by Cu and Ca supplementation (Fig.4). In the control seedlings, the severity was significantly higher (87.0%) than in the supplemented seedlings. The seedlings that were pre-supplemented with the combination of Cu and Ca (Treatment 4) showed the lowest percentage of disease severity (23.8%). Treatment 2 (46.1%), with 4000 mg/L Ca, did not differ from Treatment 3 (48.2%), with 2 mg/L Cu. However, both the treatments differed significantly from the control. These results suggest that Treatment 4 effectively slowed the penetration and spread of *G. boninense* to the vascular system.

The Area under the Disease Progress Curve (AUDPC) is a quantitative summary of the disease intensity over time and can be used to compare management strategies. The percentage of disease reduction (%DR) was derived from the values of the Area under Disease Progress Curve (AUDPC), as shown in Table 2. AUDPC was calculated based on the DI or DS of the foliar symptoms or the DS of the bole tissues. Higher AUDPC values indicated a greater susceptibility of the seedlings to the disease. Seedlings pre-supplemented with 4000 mg/L Ca + 2 mg/L Cu (Treatment 4) showed the lowest AUDPC values for DI (49.17), DS_{foliar} (48.06) and DS_{bole} (489.09). Therefore, the mixture of Cu and Ca was the most effective in slowing down the infection of *G. boninense* because it also showed the highest percentage of disease reduction based on DI (71.67%), DS_{foliar} (71.66) and DS_{bole} (76.17) as compared to the other treatments. In addition, it also showed the lowest epidemic rates based on DI (0.545 unit/month) and DS_{foliar} (0.548 unit/month).

The lower DI%, DS%, AUDPC and ER and higher values of DR in the seedlings pre-supplemented with Ca and Cu singly or as a mixture (Treatment 2, Treatment 3 and Treatment 4) relative to the control suggested that the nutrient supplementation had caused the former seedlings to acquire a level of tolerance to the physical damage caused by *G. boninense*. In this study, the combination of Cu and Ca supplementation yielded the best disease control of all the supplementation treatments. Disease suppression has been associated with the activation of plant defence mechanisms such as the induction of phenolic compounds (Gross, 1980), the presence of laccase and POD and the alteration in physical barriers, where the latter is associated with the formation of lignin (Walter, 1992) and suberin (Espelie & Kolattukudy, 1986) in roots. The result of the current study has also shown that the presence of H_2O_2 and the enzymatic activities of PODs and laccases are strongly correlated with the onset of lignification in the roots of oil palm seedlings that were treated with Cu and Ca (Nur Sabrina *et al*., 2012).

Cu has been reported to influence many plant diseases, primarily by decreasing the spread of the disease (Evans *et al*., 2007). A study by Chmielowska *et al*. (2009) showed that pepper plants stressed by Cu were less symptomatic if challenged with *Verticillium dahlia*; wilt pathogen. In addition, their

study also showed that plants stressed by Cu had fewer numbers of wilted leaves and lower reduction in the length of stem, which were the symptoms of Verticillium disease, than plants which were not supplemented with Cu.

Meanwhile, Cu plays an essential role in photosynthesis, respiration, antioxidant activity, cell wall metabolism, hormone perception (Pilon *et al*., 2006) and the induction of POD (Ros Barceló, 1995). POD is known to be induced by both abiotic and biotic stresses, including heavy metal stress and pathogen attack (Passardi *et al*., 2005). POD may play several roles in the plant, such as the functions related to resistance to pathogens. In addition, POD can produce massive amounts of reactive oxygen species (oxidative burst) that are involved in plant cell signalling and that also create a highly toxic environment for pathogens. Moreover, POD is involved in the deposition of materials such as lignin and suberin, which strengthen the cell wall by forming a mechanical barrier against pathogenic agents. A higher value of DR% in the plants pre-supplemented with Cu, singly or in combination, suggested that Cu plays an important role during lignification by producing laccase and POD, which later act as an important substrate for monolignol polymerisation. These lignified cell walls subsequently serve as a barrier to *G. boninense* penetration. It has previously been demonstrated that the induction of POD activity in pepper by Cu stress is related to lignin accumulation (Diaz *et al*., 2001) and that lignification confers tolerance to *Verticillium dahlia* in pepper plants (Pomar *et al*., 2004). Finally, another possible role for Cu-induced POD in plant defence is a direct and intrinsic antifungal activity, which has been reported for POD from several plant sources (Caruso *et al*., 2001; Ghosh, 2006). Even though Cu is often used as an active ingredient in fungicide, in this study, there was no direct assessment made on the growth of Ganoderma as monitoring was carried out on disease development on inoculated plants.

Ca significantly suppressed disease incidence and delayed the onset of Phytophtora stem rot in soybean (Sugimoto *et al*., 2008). These results indicate that Ca-rich areas may be more resistant to invasion by *P. sojae* and that calcium crystals may play an important role in Ca ion storage and its availability to allow the plant tissues to maintain long-term field resistance. In fact, Ca may harden plant primary cell walls by cross-linking of pectic polymers and confer resistance to pathogen attack (Akai & Fukutomi, 1980). For lignification to be successful, the middle lamella and the cell wall corners must be rich in calcium pectate because these regions are the primary sites of lignification (Lewis & Sarkanen, 1999). Our preliminary observations showed that Ca supplementation increased the production of the lignin-related enzymes POD and laccase and thus amplified lignin production. Sufficient Ca content decreases the rate of breakdown in pathogenic disease so that when *G. boninense* attempts to breach and invade the plant cell, the barrier reinforces the wall. This effect can be

observed if the DR% is higher in plants pre-supplemented with Ca singly or in combination. Furthermore, Ca naturally slows the reactions involving pathogenic enzymes during cell decomposition. The beneficial effects of Ca also include the improvement of the structure of the soil, the stabilisation of the cell membranes, and an increase in the pH of the soil. These effects decrease the probability of *G. boninense* attachment.

CONCLUSION

The interactions between Cu and Ca have profound effects that serve to suppress disease progression in oil palm seedlings. The suppression of disease, as indicated by the observations of DI, DS, AUDPC, and ER, showed that the mixture of 2 mg/L Cu and 4000 mg/L Ca is the best supplementation treatment for slowing the emergence of BSR. However, field studies are still needed to explain the effectiveness of Cu and Ca supplementation against *G. boninense* attack by conducting analysis on the increase of the cell wall components. Moreover, it could be more useful to integrate the use of ergosterol as a tool to measure infection, as proposed by Mohd As'wad *et al*. (2011). There is also a need to determine the best method of application for the management of BSR.

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Dispersion Pattern and Sampling of *Diaphorina citri* Kuwayama (Hemiptera:Psylidae) Populations on *Citrus suhuiensis* Hort. Ex Tanaka in Padang Ipoh Terengganu, Malaysia

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ABSTRACT

An understanding of the dispersion patterns of a pest is an important pre-requisite for developing an effective pest management program. In this study, fifty five (55) citrus trees were surveyed for adult *Diaphorina citri* once every four week for a period of ten months (March 2011 – December 2011). Analysis of spatial-distribution pattern using various indices of dispersion and regression models showed that *D. citri* exhibited an aggregated distribution on *Citrus suhuiensis*. Taylor's power law (a = 0.897, b = 1.267, R^2 = 0.74) fitted the data better than Iwoa's regression modal (α = 0.376, β = 0.196, R^2 = 0.409). The optimal sample sizes needed for fixed precision levels of 0.10, 0.15 and 0.25 were estimated using Taylor's regression coefficients, and the required sample sizes increased dramatically with increased levels of precision. Therefore, these sampling-plan presented should serve as a tool for an efficient estimation of *D. citri* population density in citrus orchard for pest management decision.

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INTRODUCTION

Diaphorina citri Kuwayama (Hemiptera: Psyllidae) is regarded as one of the most important pests of citrus worldwide (Boina

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et al., 2009; Sule *et al*., 2012a) because it is known to be the most efficient vector of citrus greening disease. It is believed to be of Far Eastern origin (Tsai *et al*., 2002), and has been reported in Malaysia since 1970 (see Abdullah *et al*., 2009; Sule *et al.,* 2012a). A recent report has also showed that most of the cultivated citrus varieties in Peninsular Malaysia, Sabah and Sarawak have been infected with citrus greening disease (Sijam *et al*., 2008).

Feeding by adult and immature *D. citri* can result in curled and notched leaves, defoliation, flower drop, irregular-shaped canopies and branch death (die back), especially when their populations are high (Yang *et al.,* 2006). In addition, honeydew excreted by nymphs leads to blemishing of foliage and fruit with subsequent reduction in photosynthesis (Wang *et al.,* 2001). Besides that, citrus tree infected with greening diseases produce bitter, inedible, misshapen fruits and eventually die within 5–10 years of infection (Mann *et al*., 2010; Sule *et al.*, 2012b).

Dispersion or distribution and abundance of organisms are important attributes of insect population and vital ecological properties of species (Siswanto *et al*., 2008). Knowledge about dispersion pattern of an organism is essential in understanding population biology, resource exploitation and dynamics of biological control agents (Fauvergue & Hopper, 1994). It provides a better understanding of the relationship that exists between organism and its environment which may be helpful in designing efficient sampling programs for population estimates, development of population models (Soemargono *et al*., 2008) and pest management strategy.

There are many methods used to describe the dispersion of arthropod populations, but most estimates are based on sample means and variances (Bisseleua *et al*., 2011), while the relationships between the variance and mean are used as indices of aggregation (Arnaldo & Torres, 2005). The models of Taylor's and Iwao's also depend on the relationship between the sample mean and the variance of insect numbers per sampling unit. The slope of the regression models are used as an index of aggregation. Designing sampling plans based on these indicators has been reported to reduce sampling effort and minimize variation of sampling precision (Kuno, 1991; Payandeh *et al*., 2010).

Despite the economic importance of *D. citri* to citrus growers, little is known about its dispersion pattern in Southeast Asia where *D. citri* still remains a major threat to citrus cultivation. Thus, there is an urgent need for such information as it will provide citrus pest managers, researchers, extension officers, and citrus farmers with a cost-effective sampling method for *D. citri.* Therefore, this study was undertaken to determine dispersion pattern of *D. citri* on *Citrus suhuiensis* (variety limau madu) in order to develop a suitable sampling plan for the pest.

METHODOLOGY

Study Site

The study was carried out from March 2011 to December 2011 at a pesticide-free

citrus orchard at Pusat Pertanian Padang Ipoh, Kuala Berang, Terengganu, Malaysia (N 05 02' 55.6" E 103 00' 54.6"). The orchard has an area of 20.4 hectares, which is divided into sixteen blocks. Crops planted in the plantation were citrus (seven blocks), vegetables (five blocks), rambutans (three blocks) and bananas (one block). Two plots measuring 1.2 hectare and containing 596 citrus trees were selected for the study. The citrus trees were planted at 5m x 4m spacing with agronomic practices such as weeding and application of manure are given to the citrus plants at regular intervals.

Population Sampling

Fifty five citrus trees of similar size (1.6m – 1.7m high) making 1/12 of the total trees planted in the two blocks were selected randomly as the sample trees. One sampling visit was made every four weeks for a total of ten sampling visits to survey the population of *D. citri* in the selected trees. The canopy of each tree was partitioned into upper and lower strata, and thereafter, each stratum was divided into four quadrants, namely, north, south, west and east. From each quadrant, three young shoots with newly expanded leaves (mostly light yellow in colour) were randomly selected during each sampling visit for observation, counting and recording the number of *D. citri* (adults).

Analysis

Distribution Pattern

Based on the *D. citri* counts from the two canopy strata and the four cardinal points, a mean number of *D. citri* per tree over time was calculated to be used in calculating the various dispersion indices. Furthermore, the spatial distribution of *D. citri* was determined by using different methods. The simplest method is the variance to mean ratio $\frac{S^2}{m}$, where the value of $\frac{S^2}{m} < 1$ indicates a uniform dispersion, while $\frac{S^2}{m} = 1$ indicates random dispersion and $\frac{S^2}{m} > 1$ indicates an aggregated dispersion.

Lloyd's index of patchness is described as ratio of the mean of mean crowding (m^*) to mean density (m). The mean crowding was calculated as described by Southwood (1978) using the following formula:

$$m^* = x + \left[\left(\frac{S^2}{x}\right) - 1\right]$$

where x is the mean density and S^2 is the variance, where Lloyd's index =1 indicates a random dispersion, Lloyd's index > 1 indicates aggregated dispersion, and Lloyd's index < 1 indicates regular dispersion.

The degree of aggregation was determined by using the three most commonly used dispersion indices, i.e., the Green coefficient (Cx), Taylor's power law and Iwao's patchiness regression. The Green coefficient was calculated as described by Green (1979) using the following formula:

$$Cx = \frac{\left(S^2 / m\right) - 1}{\Sigma x - 1}$$

where S^2 = variance of mean, m = mean number of *D. citri* per shoot and Σx = total number of *D. citri,* where $Cx = 1$ the coefficient indicate a random dispersion; where $Cx > 1$, it indicates aggregated dispersion; and where $Cx < 1$ indicating a regular dispersion.

Taylor's power law describes the regression between logarithm of the population variance and logarithm of population mean according to the following equation:

$$LogS^2 = \log a + b \log m$$

where S^2 is the population varience, m is the population mean, a is the Y intercept and b is the slope of the regression, which is an index of aggregation. When b =1, it indicates a random dispersion; when it is > 1, it indicates aggregated dispersion; and when it is < 1, it indicates regular dispersion.

Iwao's Method: The Iwao's patchiness regression method quantifies the relationship between the mean crowding index (m^*) and the mean (m) by the following formula: $m^* = \alpha + \beta m$, where m^* was determined as $[m(S^2/m-1)]$. The intercept (α) is the index of the basic component of a population or basic contagion (where α <, =, and > 1 represent regularity, randomness, and aggregation of populations in spatial patterns, respectively), and the slope (β) is the density contagiousness coefficient interpreted in the same manner as b of Taylor's regression.

Sampling Plan

Based on the sample counts, the optimal sample sizes (n) was calculated with *a* and *b* from Taylor's Power Law to develop the enumerative sampling plan of Green (1970), with precision levels of 0.10, 0.15, and 0.25 for ecological and pest management purposes, as recommended by Southwood (1978), using the following formula:

$$n = am^b / D^2$$

where *a* and *b* are Taylor's power law coefficients, *m* is the *D. citri* density and *D* is the desired precision. The sampling stop line was calculated as suggested by Pedigo and Buntin (1994) and Namvar *et al*. (2012) using the following formula:

$$T_n = \left[\frac{an^{1-b}}{D^2} \right]^{\frac{1}{(2-b)}}$$

where, T_n, n and D are the cumulative total for sample n, the maximum number of sampling units, (sample size) and the fixed level of desired precision. The parameters *a* and *b* were determined from Taylor's power law (Southwood & Henderson, 2000).

RESULTS AND DISCUSSION

Distribution Pattern

The distribution patterns of *D. citri* on *C. suhuiensis* were established in accordance with the various indices of dispersion. The result of the current study reveals the dispersion patterned of *D. citri* to be highly aggregated within *C. suhuiensis*.

In all the trees sampled, the variance to mean ratio ($\frac{S^2}{x}$) was greater than the one with the values ranging from 4.67 to 1.24 (see Table 1). The Lloyd's Patchiness mean crowding ($\frac{m^*}{m}$) was also greater than one, indicating an aggregated distribution of the psyllid within *C. suhuiensis.* Similarly, the Green coefficient (Cx) values were greater than one, confirming the distribution of *D. citri* on *C. suhuiensis* to be aggregative in nature. However, the Lloyd's mean crowding (m^*) reveals a variable distribution pattern (Table 1), with 63.63% of the sampled trees showing aggregated distribution, while 20.0 and 16.36% showing regular and random distribution, respectively. These results corroborate with the previous finding by Soemargono *et al.* (2008), who showed the distribution of *D. citri* to be spatially aggregated on both the *Citrus reticulate* variety madu and *Murraya paniculata*. In addition, other results similar to ours were reported by Van den Berg *et al.* (1991) on other psylla species (*Trioza erytreae* Del Guercio and *Cacopsylla mali* Schmidtb).

Many authors have reported that an aggregated distribution pattern is a characteristic of arthropods and regular distributions are rarer, which are mainly found in the population where there is strong competition between individuals (Agrov *et al*., 1999). The aggregated distribution pattern display by *D. citri* in the present study might be attributed to food source and mate, since *D. citri* was reported to be more attracted to flush leaves for feeding and

TABLE 1

Distribution statistics and dispersion indices of *Diaphorina citri* on *Citrus suhuiensis*

Tree No.	x	S^2	S^2/x	m^*	m^*/m	Cx
1	0.78	1.73	2.22	2.00	2.35	6.93
2	0.67	0.89	1.33	1.00	1.17	4.56
3	0.78	1.51	1.94	1.71	1.98	6.16
4	0.33	0.89	2.67	2.00	5.33	8.11
5	0.67	1.56	2.33	2.00	2.67	7.22
6	0.22	0.40	1.78	1.00	3.72	5.74
7	0.89	1.21	1.36	1.25	1.30	4.63
8	0.89	2.32	2.61	2.50	2.70	7.96
9	0.22	0.40	1.78	1.00	3.72	5.74
10	0.44	0.91	2.06	1.50	2.82	6.48
11	0.22	0.40	1.78	1.00	3.72	5.74
12	0.33	0.44	1.33	0.67	1.33	4.56
13	0.33	0.44	1.33	0.67	1.33	4.56
14	0.33	0.44	1.33	0.67	1.33	4.56
15	0.89	3.65	4.11	4.00	4.39	11.96
16	0.33	0.44	1.33	0.67	1.33	4.56
17	0.33	0.89	2.67	2.00	5.33	8.11
18	0.78	3.51	4.51	4.29	5.29	13.02
19	0.22	0.40	1.78	1.00	3.72	5.74
20	0.33	0.89	2.67	2.00	5.33	8.11
21	0.33	0.44	1.33	0.67	1.33	4.56
22	0.33	0.44	1.33	0.67	1.33	4.56
23	0.44	0.91	2.06	1.50	2.82	6.48
24	0.33	0.89	2.67	2.00	5.33	8.11
25	0.33	0.44	1.33	0.67	1.33	4.56
26	0.44	0.91	2.06	1.50	2.82	6.48
27	0.44	0.91	2.06	1.50	2.82	6.48
28	0.33	0.89	2.67	2.00	5.33	8.11
29	0.56	0.91	1.64	1.20	1.72	5.39
30	0.56	0.91	1.64	1.20	1.72	5.39
31	0.44	0.91	2.06	1.50	2.82	6.48
32	0.56	0.69	1.24	0.80	1.00	4.32
33	0.22	0.40	1.78	1.00	3.72	5.74
34	0.33	0.44	1.33	0.67	1.33	4.56
35	0.44	0.69	1.56	1.00	1.69	5.13
36	0.56	1.14	2.04	1.60	2.44	6.45
37	0.56	2.47	4.44	4.00	6.76	12.85
38	0.67	2.44	3.67	3.33	4.67	10.78
39	0.33	0.44	1.33	0.67	1.33	4.56
40	0.78	1.51	1.94	1.71	1.98	6.16
41	0.33	0.89	2.67	2.00	5.33	8.11
42	0.78	2.17	2.80	2.57	3.08	8.45
43	1.11	1.88	1.69	1.80	1.73	5.50
44	0.56	0.91	1.64	1.20	1.72	5.39
45	0.44	0.91	2.06	1.50	2.82	6.48
46	0.44	1.58	3.56	3.00	6.19	10.48
47	0.67	0.89	1.33	1.00	1.17	4.56
48	1.00	4.67	4.67	4.67	4.67	13.44

TABLE 1 (continue)

49	0.67	1.11	1.67	1.33	1.67	5.44
50	0.22	0.40	1.78	1.00	3.72	5.74
51	0.67	1.56	2.33	2.00	2.67	7.22
52	0.44	0.91	2.06	1.50	2.82	6.48
53	0.67	1.56	2.33	2.00	2.67	7.22
54	0.22	0.40	1.78	1.00	3.72	5.74
55	0.33	0.44	1.33	0.67	1.33	4.56

x = mean, S^2 = variance,
m^* =Lloyd's mean crowding,
m^*/m =Lloyd's Patchiness mean crowding and
Cx = Green index

oviposition (Sule *et al.*, 2012b), or to either active aggregation on the part of this psyllid or to some variations of the environment such as microclimate, preferred part of plant, and natural enemies (Tsai *et al.,* 2000).

Taylor's power law analysis appeared to illustrate the distribution of *D. citri* well by showing highly significant relationships between the variance and mean of *D. citri* population (Fig.1), and the model was found to be significantly different from 0 (t = 12.35, p < .0001). The slope values of Taylor's power law for the psyllid on *C. suhuiensis* (b = 1.267) was significantly greater than 1 (t = 9.71, df = 54, p < .0001), indicating an aggregated or clumped distribution pattern for *D. citri* on *C. suhuiensis*. However, Iwao's patchiness regression based on the same sampled trees did not show any significant relationship between the mean crowding index (m^*) and the mean (m) of *D. citri* (Fig.2) (t = 1.69, p = 0.896). It also produced a slope value (β = 0.196) below 1 (t = 0.13, df = 55, p = 0.13), indicating a regular distribution. Nevertheless, the constant α in the Iowa's model indicates the tendency to crowding when it is positive (+) or repulsion when it is negative (-) as it is the 'Index of Basic Contagion' defined by Iwao (1970).

Based on the higher value of R^2 produced

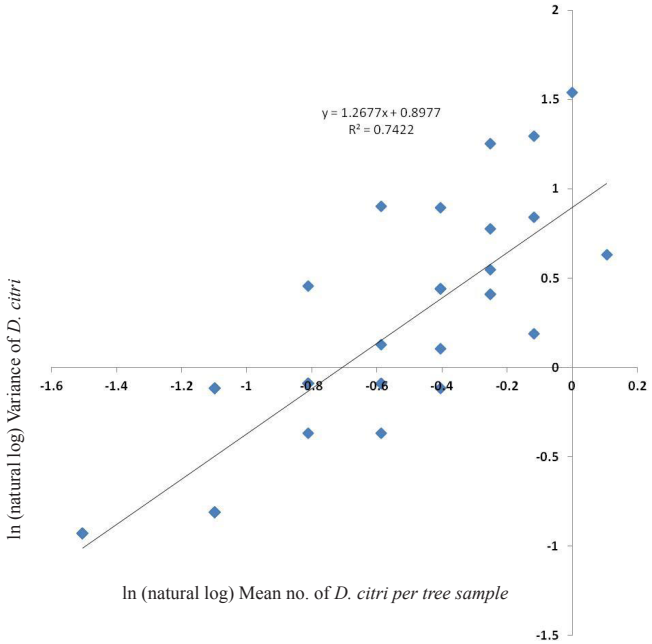

Fig.1: Regression analysis of Taylor's power law for *Diaphorina citri* populations on *Citrus suhuiensis*

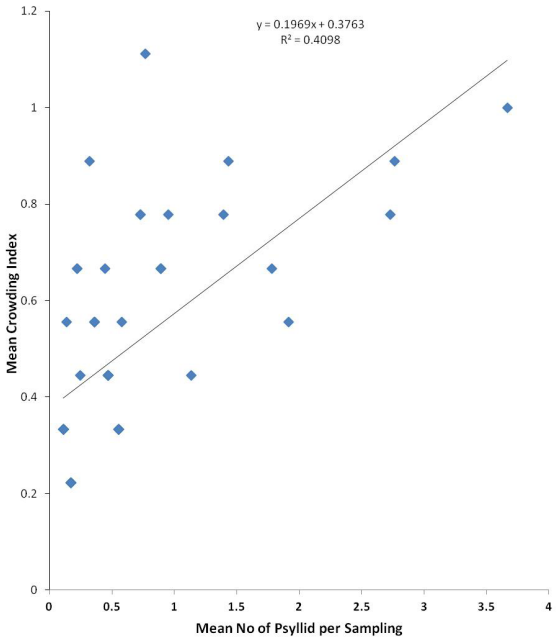

Fig.2: Regression analysis of Iwao's mean crowding index (m^*) on mean density (m) for *Diaphorina citri* populations on *Citrus suhuiensis*

by Taylor's power law compared to Iwao's patchiness regression, it could be stated that Taylor's model fitted the data better than Iwao's modal. Furthermore, Taylor's power law provides a more even distribution of the points along the line than Iwao's model. In spite of Iwao's model inability to fit the data very well, it could still give an insight into the interpretation of implication of ecological parameters (Kuno, 1991). For instance, the positive value of α of Iwao's patchiness regression in the present study is indicative of a mutual attraction (positive interaction) between the individuals even at a very low density.

Sampling Plans

The relationship between the mean psyllid density and the required sample size for the fixed precision levels of 10, 15 and 25% is shown in Fig.3. The stop line of the fixed precision level of 25% of the mean for sequential sampling is presented in Fig.4. Since the variance mean regression in Taylor's model provided a good description of the data (Fig.1), the regression variability would only have a minor effect at very low mean density.

In order to achieve high fixed precision levels of 10 and 15% for precise density estimate, quite a large number of samples are required (Fig.3). Thus, the level of the precision needed is a choice made based on the purpose of a sampling plan. From the results of the present study, the optimal sample size for a precision of 25% ranged from 14 to 443 trees, depending on the mean. However, the sample sizes increased

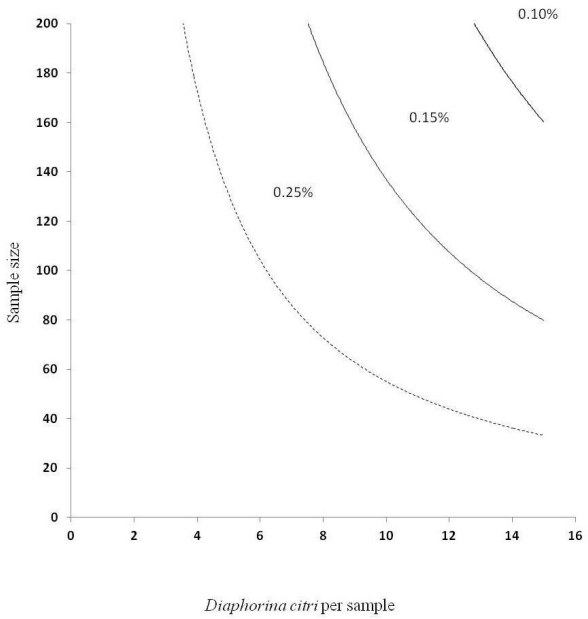

Fig.3: The relationship between required sample size and mean density for achieving fixed precision levels of 0.10, 0.15 and 0.25% for *Diaphorina citri* populations on *Citrus suhuiensis*

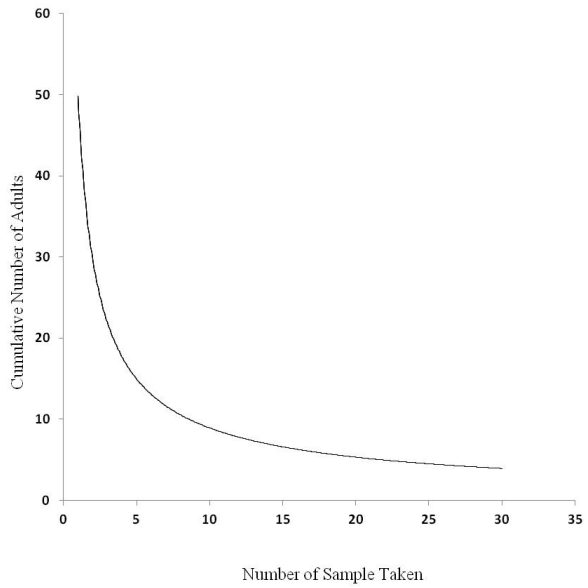

Fig.4: Sampling stop line at a fixed precision level of 25% for *Diaphorina citri* populations on *Citrus suhuiensis*

considerably when the precision level required also increased. For instance, for the precision level of 10%, the sample size ranged from 90 to 2772 trees.

Furthermore, considering a desired accuracy of 25% in the present study, the number of sample trees needed for mean densities of around fourteen *D. citri* per tree (assuming this number to be the action threshold for *D. citri* in the present condition) was approximately 35. This number of trees is considerably lower than the actual 55 trees sampled during our survey. However, if the level of precision was raised to 10%, the number of the samples required became 215 trees, for the same density. In their work, Setamou *et al*. (2008) used forty citrus orchards with the sampling of ten trees per orchard and twenty new flush per tree, and recommended using eight flush per tree and ten trees per orchard to provide a density estimate of *D. citri* with a percentage relative precision of 25%. Meanwhile, Dharajothi *et al*. (1986) recommended a sample size of nineteen flush per tree for a sampling plan that is based on one tree per orchard to achieve a 25% precision level. Although Setamou *et al*. (2008) and Dharajothi *et al*. (1986) recommended small number of trees per orchard and shoots per tree than the present study, these differences might be attributed to the total number of trees sampled, the number of sampling unit and abundance of psyllid. For instance, it has been reported that a decrease in the mean number of insects sampled normally leads to an increase in the sample size and vice versa (Naranjo & Flint, 1994). Furthermore, sampling a higher number of trees with a low number of sampling unit per tree will yield smaller sampling error than sampling a small number of trees with a higher number of sampling unit per tree. Nevertheless, the findings of this study will go a long way in alleviating the problem faced by growers on decision making with respect to pests.

CONCLUSION

The distribution analyses using various indices of dispersion and regression models have shown that *D. citri* was spatially aggregated on *C. suhuiensis* variety limau madu. The fixed precision sampling plan developed in this study will provide useful insights into efficient estimation of *D. citri* population density in citrus orchard. Furthermore, information on the density level of *D. citri* provides a sound base for selecting appropriate decision making in designing IPM programmes for this particular pest.

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Isolation, Fruiting and Pathogenicity of *Marasmiellus palmivorus* (Sharples) Desjardin (comb. prov.) in Oil Palm Plantations in West Malaysia

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ABSTRACT

Malaysia's golden crop, oil palm (*Elaies guineensis*), is susceptible to bunch rot disease caused by *Marasmiellus palmivorus* (Sharples) Desjardin (comb. prov.). Nonetheless, there is no published information on the morphology and pathogenicity of the species found in local oil palm plantations in Malaysia. Rhizomorphs and basidiocarps found on dead fronds or trunks were randomly sampled from the plantations located in the states of Perak and Selangor. Isolates were identified based on the morphology and molecular methods as *Marasmiellus palmivorus* and pure cultures subsequently produced similar fruit bodies (basidiocarps) by *in vitro* methods. Hyphal morphology was examined by light and scanning electron microscopy and found to be septate and produced clamp connections. White spore prints were obtained from each pileus. Naturally grown and induced basidiocarps were similar with diameter of pileus ranging from 1.0-2.8cm, slightly depressed at the centre, smooth, convex, with involute margin, orange-white fading to white and possessed a central, solid, cylindrical, tough, overall whitish stipes with length ranging from 0.8-2.6cm. The gills were adnate, distant and have a non-distinctive odour. Basidiospores were ellipsoid in shape and spores were found to be viable with percentage germination of 80-85%. Upon germination, they produced germ tubes ranging from 64.3 – 82.5 µm after 24 h incubation at ambient temperature (27 ± 2°C) on water agar. Pathogenicity test of six isolates of *Marasmiellus* sp. positively produced necrotic symptoms on wounded leaves of oil palm seedlings.

Keywords: Basidiocarps, basidiospores, bunch rot disease, *Marasmiellus palmivorus*, oil palm

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INTRODUCTION

The oil palm industry is a major financial contributor to the economy of Malaysia. With cultivated areas of 5 million ha in 2011, RM80.4 billion revenues from the export of oil palm products were made (MPOB, 2012). Nonetheless, oil palm is susceptible to fungal diseases including bunch rot disease caused by *Marasmiellus* spp. This fungus belongs to the class of basidiomycetes in the order Marasmiaceae (Wilson & Desjardin, 2005). *Marasmiellus palmivorus* was previously known as *Marasmius palmivorus*. Hemmes and Desjardin (2002) found it more suitable to be grouped under the genus *Marasmiellus* based on its close morphology to other *Marasmiellus* species such as *Marasmiellus troyanus* and *Marasmiellus semiutus*. In addition, Wilson and Desjardin (2005) also revised the genus and tentatively accepted it as *Marasmiellus palmivorus* (Sharples) Desjardin comb. prov. until further phylogenetic analysis was done to support its accurate identification (D.E. Desjardin, personal communication, August 12, 2011).

Meanwhile, Sharples (1928) reported the first local outbreak of this disease as causing significant losses to oil palm. However, there has been no published information on the morphology and pathogenicity of this fungal species found in oil palm plantations in Malaysia. This paper reports the investigations made on the isolation, morphological characteristics of vegetative structures and fruit bodies, as well as fruiting behaviour of induced basidiocarps and pathogenicity of *Marasmiellus* isolates from oil palm plantations.

MATERIALS AND METHODS

Isolation of the Fungus

Random samplings of rhizomorphs and basidiocarps of fungus, associated with diseased oil palm fruits, fronds or trunks, were made in the states of Perak and Selangor (see Fig.1). For this purpose, four samples of the isolates from Selangor and two from Perak were obtained (Table 1). Fresh basidiocarps and rhizomorphs collected were washed in three changes of sterile distilled water. They were then

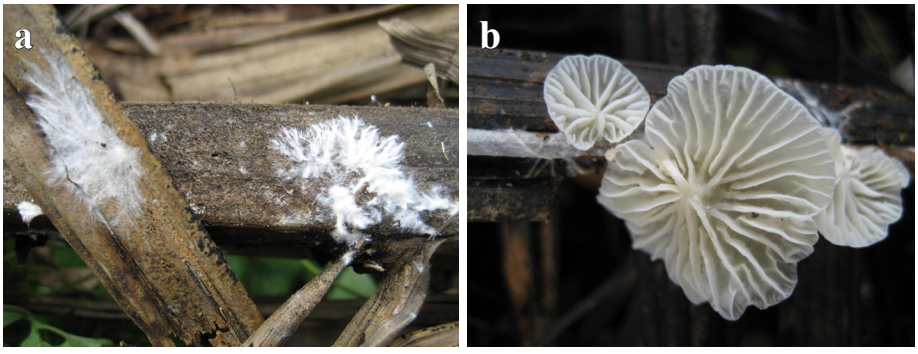

Fig.1: Random samplings of fungus associated with diseased oil palm. a. White rhizomorphs on dead oil palm fronds in Teluk Intan, Perak; b. Basidiocarps produced on dead oil palm trunks in Bangi, Selangor

TABLE 1
Marasmiellus isolates, collection information and GeneBank accession no.

Isolate	Location	Sample Type	GeneBank Accession no.
Bangi	Bangi, Selangor	Basidiocarp	JQ654222
Bangi1	Bangi, Selangor	Basidiocarp	JQ654223
Bangi3	Bangi, Selangor	Basidiocarp	JQ654224
UPM42	Serdang, Selangor	Basidiocarp	JQ654219
OP2	Teluk Intan, Perak	Basidiocarp	JQ654220
OP4	Teluk Intan, Perak	Rhizomorph	JQ654221

immersed in 20% Chlorox® solution for two minutes to eliminate contaminants and rinsed with three changes of sterile distilled water. Pieces of surface treated tissues (0.2cm x 0.2cm) were cut from the edge of the pileus and placed on Malt Extract Agar (MEA, Merck) plates and incubated at ambient temperature (27±2°C) for four days. The margins of vigorously growing colonies were subcultured and transferred onto fresh MEA slants and maintained at 4°C prior to further studies.

Morphology and Molecular Identification of Fruit Bodies and Vegetative Structures

Visual assessment of basidiocarps was carried out. Size, colour, shape and texture of pileus, stipe and gills were studied and identified using Sharples (1928), Turner (1981), Hemmes and Desjardin (2002) as source of references. Vegetative structures of isolates were described by examining hyphal structures on culture plates using light microscope and scanning electron microscope. Molecular identification of fungus was confirmed by extracting genomic DNA using the CTAB procedure and a large subunit (LSU) region was amplified and sequenced using LR07/LR7 primers (B.S.A. Almaliky, personal communication, July 20, 2012) (see GeneBank Accession No. shown in Table 1).

In vitro Production of Basidiocarps

The *Marasmiellus* sp. isolates were first cultured for use as the source of spawn. Wheat grains of 0.5kg obtained from a wholesale market were soaked in water overnight, drained, transferred into a 1 L Scott bottle, tightly-plugged and autoclaved at 121°C, 1.05 kg/cm^2 for 20 mins. Upon cooling, 5mm mycelial discs of three-day old pure cultures were inoculated onto the grains aseptically for spawn-run.

Following the methods used to induce the fruit bodies of *Marasmiellus inoderma* (Sabet *et al.*, 1970) and *Marasmiellus scandens* (Ooi, 1987), empty fruit bunch (EFB) fibres from a local palm oil mill were used as substrates. The fibres were ground to pieces, autoclaved for one hour and cooled. Sawdust was similarly prepared and utilized as substrate for comparison. During the preparation, 2 kg of substrate was mixed thoroughly with 0.2 kg rice bran and 0.02 kg calcium carbonate (CaCO3) (90% substrate:9% rice bran:0.9% CaCO3). The mixture was then dispensed into polypropylene bags (15.2 cm x 33 cm) at 100g of substrate per bag, tightly capped with a stopper and autoclaved for 20 mins. After cooling, the substrate inoculated with a table spoon of fungal spawn and incubated in a glass chamber (92 cm x 46 cm x 30 cm) at ambient temperature

(27 ±2°C) for 30 days in total darkness. After that, the polypropylene bag was removed. Sterile distilled water was sprayed twice a day (at 0800h and 1700h) to maintain relative humidity of more than 85%. The substrate was exposed to 24 h of continual light (fluorescent white 20 W tubes), with normal alternating day and night conditions in the laboratory to study the effects of light on fruiting. There were four replications for each isolate, type of substrate and light exposure treatments. Meanwhile, fruiting was monitored by counting the number of basidiocarps produced over a period of four weeks. The experiment was a complete randomized treatment combination in a three factorial design. Spore print was prepared by placing overnight a freshly produced basidiocarp with the gills facing downward in a Petri dish containing a piece of black paper.

Viability of Basidiospores

Basidiospores collected from basidiocarps were dispensed into a vial containing 50 µL of sterile distilled water. One µL drop of spore suspension was placed on a glass slide layered with water agar, overlaid on a glass rod in a sterile Petri dish, sealed with a parafilm and incubated at 27 ±2°C. Basidiospore germination was assessed after 24 h of incubation by staining in lactophenol cotton blue. The percentage germination of basidiospores was recorded and germ tube growths were also measured.

Pathogenicity Tests

The tests were done on three-month old oil palm seedlings in a glasshouse at Universiti Putra Malaysia. First, a wound was created across each leaf by cutting a straight line using a needle. Basidiocarps that were borne on EFB substrate were supported and placed over each wounded leaf (see Fig.2). Transparent polypropylene bag was used to cover each seedling and inoculum. The tests were repeated for the basidiocarps produced by six isolates and replicated three times with three seedlings each. The relative humidity surrounding each seedling

Fig.2: Placement of *Marasmiellus* basidiocarps over wounded leaf of oil palm seedling in the pathogenicity tests

was monitored using a relative humidity meter at more than 85 % by spraying sterile distilled water twice at 0800 h and 1700 h daily. A period of two days was given to allow the natural dispersal of basidiospores and inoculation of the wounded leaf. The infectivity of the leaves was monitored and assessed over a period of four weeks. Fungal hyphae re-isolation from diseased leaves was obtained to confirm the presence of *Marasmiellus* sp. The percentage of disease incidence (I) was determined as follows:

$$I = \frac{\text{Number of plants infected per isolate}}{\text{Total number of plants per isolate}} X100\%$$

Statistical Analysis of Data

The data of the yield of basidiocarps, as well as the diameter of pileus and length of stipe, were subjected to the Analysis of Variance (ANOVA) test. The mean comparisons were done by using the Duncan Multiple Range of Test (DMRT). Data analysis of pathogenicity test was based on the transformed value obtained from arc-sine transformation of disease incidence percentages.

RESULTS

Morphology and Molecular Identification of the Fruit Bodies and Vegetative Structures

Pure cultures of *Marasmiellus* sp. isolates produced dense, whitish, fan-shaped cottony mycelia with feathery edges on MEA plates when incubated at ambient temperature (27 ± 2 °C). The diameter of pilei ranged from 1.0-2.8cm and the length of the stipes were in the range of 0.8-2.6cm. Each pileus was slightly depressed at the centre, smooth, convex, with involute margin, orange-white fading to white. Stipe was central, solid, cylindrical, tough, and whitish, while lamellae were adnate, distant and have a non-distinctive odour (see Fig.1). Hyphae were septate and produced clamp connections under observations by light and scanning electron microscope (Fig.3).

All the isolates deposited in the GeneBank Accession (Table 1) showed

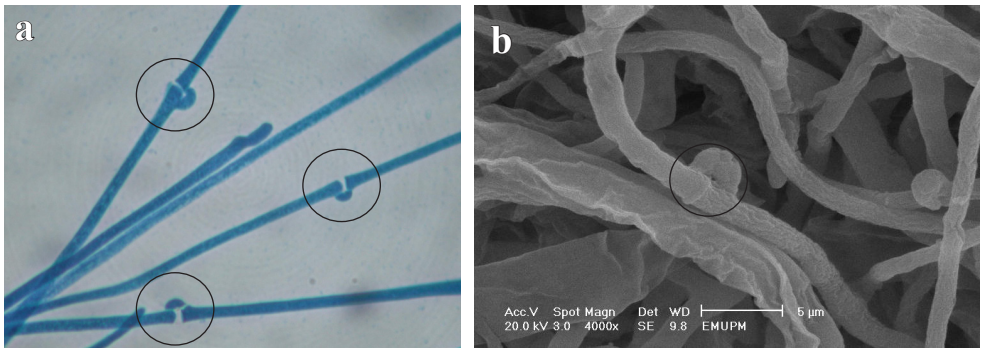

Fig.3: Hyphae of *Marasmiellus* sp. isolates; a. Appearance of septal walls and clamp connections (in circles) under the observation of light compound microscope; b. Presence of septate wall and clamp connection as shown (circle) by scanning electron microscope

sequences that were identical to each other and 99% similar to a *M. palmivorus* sequence deposited in the NCBI database (Accession No. AY639434) (Wilson & Desjardin, 2005).

In vitro Production of Basidiocarps

All six *Marasmiellus* sp. isolates from Perak and Selangor fruited on both EFB and sawdust substrate medium (see Fig.4). The statistical analysis showed that the yield of basidiocarps on EFB substrate was significantly higher than on the sawdust substrate under the conditions of complete darkness and normal alternate day night condition (Table 2). Nonetheless, fruiting did not occur on both the substrates with 24 h exposure to continuous white light. The diameter of the pileus on the EFB substrate was significantly larger than those found on the sawdust substrate (Table 3). However, there was also significant difference in stipe length of basidiocarps, where they were found to be longer on EFB compared to sawdust substrate (Table 4).

The morphology of *in vitro* produced basidiocarps of isolates on EFB and sawdust substrates was similar. Each pileus was slightly depressed at the centre, smooth, convex, with involute margin, orange-white fading to white, while stipe was central, solid, cylindrical, tough, and overall whitish. Lamellae were adnate, distant and have a non-distinctive odour (Fig.5*)*. Spore prints were white, as shown in Fig.6.

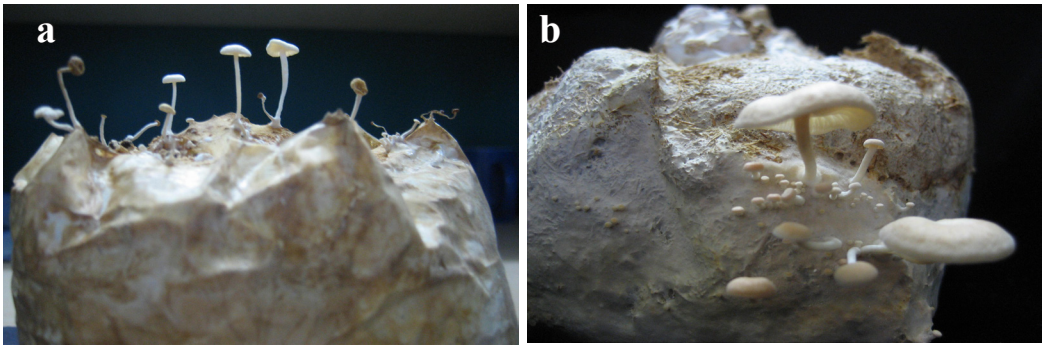

Fig.4: Fruiting of *Marasmiellus* sp. isolate; a. Smaller *Marasmiellus* basidiocarps on sawdust substrate; b. Larger sized *Marasmiellus* basidiocarps on oil palm empty fruit bunch substrate

Fig.5: A general appearance of basidiocarp (*Marasmiellus*) (bottom view) produced *in vitro* on the EFB substrate

Fig.6: Whitish spore prints (left) obtained from basidiocarp (*Marasmiellus*) (right)

TABLE 2
The effects of substrate on the number of basidiocarps produced by *Marasmiellus* isolates

Light exposure	Substrate	The mean number of basidiocarps produced according to light exposure of isolate per 100 g of substrate*					
		Bangi	Bangi1	Bangi3	OP2	OP4	UPM42
Complete darkness	EFB fibre	24a	24a	23a	20a	21a	22a
	Sawdust	9b	10b	8b	8b	9b	11b
Normal alternate day / night	EFB fibre	23a	23a	24a	22a	22a	21a
	Sawdust	10b	10b	9b	8b	8b	9b
24 h of continuous white light	EFB fibre	0c	0c	0c	0c	0c	0c
	Sawdust	0c	0c	0c	0c	0c	0c

*Mean of four replicates
Mean values with the same letters in the same column are not significantly different at 5% by DMRT

TABLE 3
The effects of substrate on the diameter of pileus (cm) of *Marasmiellus* isolates

Light exposure	Substrate	The mean diameter of pileus (cm) according to light exposure of isolate per 100 g of substrate*					
		Bangi	Bangi1	Bangi3	OP2	OP4	UPM42
Complete darkness	EFB fibre	2.60bc	2.56c	2.63bc	2.58c	2.66abc	2.59c
	Sawdust	0.74def	0.61g	0.58g	0.77de	0.60g	0.59g
Normal alternate day / night	EFB fibre	2.74ab	2.73ab	2.72ab	2.73ab	2.77a	2.73ab
	Sawdust	0.807d	0.67efg	0.65gf	0.67efg	0.675efg	0.66efg
24 h of continuous white light	EFB fibre	0h	0h	0h	0h	0h	0h
	Sawdust	0h	0h	0h	0h	0h	0h

*Mean of four replicates
Mean values with the same letters in the same column are not significantly different at 5% by DMRT

TABLE 4
The effects of substrate on the length of the stipe (cm) of *Marasmiellus* isolates

Light exposure	Substrate	Mean length of stipe (cm) according to light exposure of isolate per 100 g of substrate*					
		Bangi	Bangi1	Bangi3	OP2	OP4	UPM42
Complete darkness	EFB fibre	2.35ab	2.35ab	2.34ab	2.34ab	2.36ab	2.34ab
	Sawdust	1.32cd	1.23cb	1.20d	1.24cd	1.20d	1.23cd
Normal alternate day / night	EFB fibre	2.22b	2.48a	2.23b	2.22b	2.23b	2.23b
	Sawdust	1.45c	0.87c	1.12d	1.16d	1.17d	1.15d
24 h of continuous white light	EFB fibre	0f	0f	0f	0f	0f	0f
	Sawdust	0f	0f	0f	0f	0f	0f

*Mean of four replicates
Mean values with the same letters in the same column are not significantly different at 5% by DMRT

Viability of Basidiospores

The basidiospores observed under the light microscope were ellipsoid, with a size range of 6.2-8.7 µm (Fig.7a). They were viable with a percentage germination of 80-85% and germ tube growths recorded between 64.3 and 82.5 µm (see Fig.7b).

Pathogenicity Tests

The results of pathogenicity tests showed positive results with no disease incidence recorded in the uninoculated (control) oil palm seedlings and between 33 to 55% incidence of disease on all the seedlings inoculated by six *Marasmiellus* sp. isolates (Table 5). However, the uninoculated oil palm seedling showed no development of disease as compared to the formation of necrotic lesions on the wounded leaf of inoculated seedlings (Fig.8)

TABLE 5
Disease incidence percentage recorded on control (uninoculated) oil palm seedlings and six isolates of *Marasmiellus* sp.

Test isolate	Disease incidence*(%)
Control (uninoculated)	0[b]
Bangi (Bangi, Selangor)	55.5[a]
Bangi1 (Bangi, Selangor)	44.4[a]
Bangi3 (Bangi, Selangor)	44.4[a]
OP2 (Teluk Intan, Perak)	33.3[a]
OP4 (Teluk Intan, Perak)	33.3[a]
UPM42 (Serdang, Selangor)	44.4[a]

*Mean of three replications. Percentage values arc-sine transformed and analyzed for significance. Means followed by the same letter in the same column are not significantly different by DMRT at P≤0.05

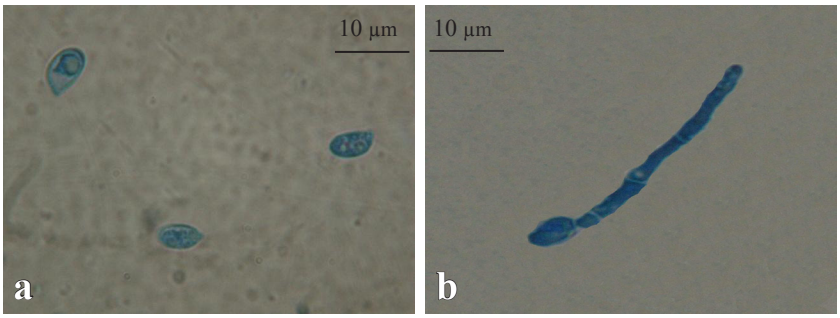

Fig.7: Basidiospores (*Marasmiellus*) observed under light microscope; a. Ellipsoid in shape; b. Germ tube growth on water agar at 24 h incubation at ambient temperature (27 ± 2 °C)

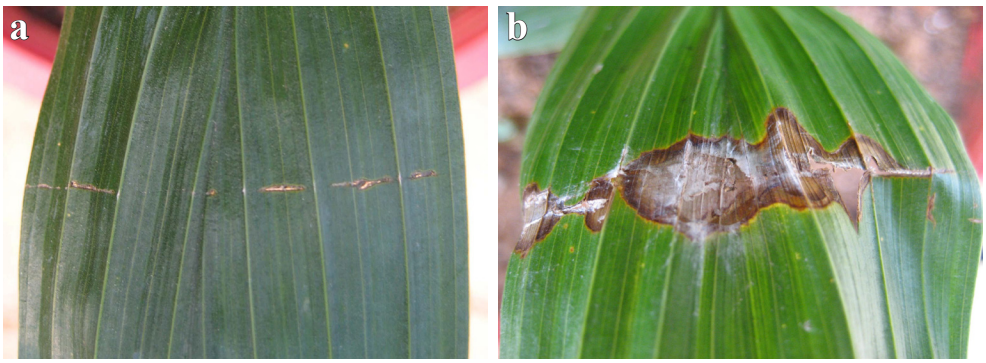

Fig.8: (a) Uninoculated oil palm leaf showing no development of disease; (b) Formation of necrotic lesions on wounded oil palm leaves at 13 days after inoculation with *Marasmiellus* sp. isolate

DISCUSSION

The morphology of rhizomorphs and basidiocarps of *Marasmiellus* sp. observed was found to be almost similar to those described by Sharples (1928), Turner (1981) and Hemmes and Desjardin (2002). Meanwhile, the presence of clamp connections in the hyphae of isolates used in this study were confirmed to have similar features with those described by Singer (1973) for *Marasmiellus* sp. Basidiocarps found in nature, while the *in vitro* produced in this study was shown to be generally smaller (1.0-2.8 cm) as compared to the sizes of natural basidiocarps (2.5-7.5 cm) described in the literature. This may be attributed to the amount of substrate available and the variability in the environment (Turner, 1981).

The fewer fruit bodies produced on the sawdust medium than the oil palm EFB substrate could be due to higher nitrogen content in the sawdust (1.64 %) compared to that in the EFB fibres (0.2-0.7 %) (Do, 1999; Mahlia *et al.*, 2000; Segura *et al*, 2001). According to Hawker (1971), nitrogen is an important element involved during fruiting and a minimum amount of nitrogen will allow sporulation. On the contrary, excess amounts of nitrogen will promote active vegetative growth and inhibit fruiting. Carbon compounds have been known to play important roles in influencing fungal reproduction (Hawker, 1971). Although there could be higher carbon content in sawdust compared to EFB fibres (Do, 1999; Amal Nafissa *et al*, 2008; Khor *et al*, 2009), lower fruiting number in the former could be due to lower carbon-to-nitrogen (C/N) ratio (50-75:1) in the EFB fibre compared to sawdust (Schuchardt *et al.*, 2002).

Carbon derived from cellulose, hemicellulose and lignin are the most abundantly utilized. In this study, the lower C/N ratio could be improved by addition of rice bran into the substrate medium used.

Meanwhile, some environmental factors such as light and nutrient availability were known to contribute to *in vitro* morphogenesis (Schwalb, 1978; Suzuki, 1979; Manachère, 1980). Light was found to be substantial in fungal fructification process and pileus differentiation (Plunkett, 1961; Kitamoko *et al.*, 1968, 1974; Perkins, 1969; Perkins & Gordon, 1969; Morimoto & Oda, 1973; Schwalb & Shanler, 1974; Raudaskoski & Yli-Mattila, 1985; Kaneko & Sagara, 2001). Since prolonged white light exposure has been shown to inhibit fruiting by *Marasmiellus*, the continuous illumination appeared to give a negative effect to the formation of primodia. Okwujiako (2001) found that light may inhibit the vegetative growth of some agarics but exposure of appropriate light duration is essential for the formation of basidiocarps. Dark condition has been shown to favour spawn run, while 12h of alternate light was found to be optimum for certain agarics during fruiting (Datta & Chakraborty, 2002).

The development of necrotic symptoms on leaves of oil palm seedlings after inoculation with basidiospores indicated that the *Marasmiellus* sp. isolates were pathogenic. However, the findings of this study showed variations in the incidence

of disease. Therefore, it is suggested that the quantity of inoculum of isolates should be quantified in order to obtain uniform infectivity in future work.

CONCLUSION

Bunch rot is an important disease of oil palm in Malaysia. This research has provided the first detailed documentation of the morphology *Marasmiellus palmivorus* (Sharples) Desjardin (comb. prov.) in oil palm plantations in Malaysia. In particular, the fruiting behaviour and pathogenicity of the local species on oil palm were elucidated.

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Prey Preference of Four species of Forest Spiders to *Spodoptera litura* and *Plutella xylostella*

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ABSTRACT

The prey preference of *Heteropoda garciai*, *Olios mahabangkawitus*, *Ctenus floweri*, and *Pardosa apostoli* was examined on *Spodoptera litura* and *Plutella xylostella* in laboratory. These spiders showed a clear preference for *P. xylostella* compared to *S. litura*. However, there was no significant difference observed between the second and third instars larvae of *P. xylostella*. Similarly, the size of prey was not an important factor in the prey selection by *H. garciai*, *O. mahabangkawitus* in this study. *P. apostoli* showed higher preference on smaller sized *S. litura* larvae.

Keywords: Heteropoda garciai, *Olios mahabangkawitus*, *Ctenus floweri*, *Pardosa apostoli, Plutella xylostella*, *Spodoptera litura*, prey preference test

INTRODUCTION

The armyworm, *Spodoptera litura* (Fab.), is a polyphagous insect pest of many economically important crops (Cab International, 2002). In particular, this insect pest causes heavy yield loss in groundnut and soybean productions, depending on the infestation level and crop stage (Dhir *et al*., 1992; Dhaliwal & Koul, 2010). In Malaysia, this insect pest has become the main pest of *Musa sapienthum* L., *Citrullus vulgaris* L. and cucurbit vegetables (Badri *et al*., 2009). Meanwhile, diamondback moth (DBM), *Plutella xylostella* L. (Lepidotpera: Plutellidae), another serious insect pest in Malaysia, is a well-known insect pest of Brassica crops. It has caused 99% (1992) and 80% (1994) cabbage loss in Jiangsu if no spray was applied for DBM control (Zhao *et al*., 1996). Over dependent on chemical insecticides for *S. litura* and DBM control has resulted in pest resistance development to chemical insecticides. *S. litura* has been reported resistant to conventional chemical

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insecticides such as organochlorines, organophoshates, carbamates and synthetic pyrethroids (Kranthi *et al*., 2001; Shi *et al*., 2003), while DBM resistance to all classes of synthetic insecticides has been documented as well (Grzywacz *et al*., 2009). The annual cost of managing DBM worldwide is estimated to be about US$1 billion (Talekar & Shelton, 1993).

Formulations based on *Bacillus thuringiensis* (Bt) have been used as alternative means to control insect pests with pesticide resistance problem. Development of resistance to Bt products or Bt-crop has been documented in several insect pests. For example, *B. thuringiensis* resistance has been reported in *P. xylostella* and is strain-dependent (Kao & Cheng, 1994). *Spodoptera* species such as *Spodoptera exigua* (Hübner) (Lepidotpera: Noctuidae) has been reported to be resistant to *Bacillus thuringiensis* Cry1C (Moar *et al*., 1995), while *Spodoptera fruigiperda* (Lepidotpera: Noctuidae) has been shown to have low sensitivity to *B. thuringiensis* Cry1F (Storer *et al*., 2010). Other microbial insecticides such as baculoviruses and entomopathogenic fungi have been reported to pathogenic to *S. litura* (Sajap *et al*., 2000; Vijayavani *et al*., 2009) and also *P. xylostella* (Wang *et al*., 2009; Dezianian *et al*., 2010). However, it is crucial to note that these microbial agents have slow killing rate compared to Bt.

Spiders are unique arthropods which prey on living organism and not fussy about their prey. They are important natural enemies of many insect pests in the agricultural ecosystem (Maloney *et al*., 2003). Many studies have been conducted to study the potential use of spiders as a predator (Amalin *et al.*, 2001; Larsson , 2007). Our research team conducted a preliminary assessment on local spiders in agricultural lands such as oil palm and rubber plantation, and tropical forests such as National Park, Sungai Dusun Wildlife Reserve, Fraser Hill Wildlife Reserve, Tasek Bera, Ayer Hitam Forest Reserve, Pangkor Island and Jerejak Island from 2008 to 2010. Based on the research findings, a rich spider fauna exists in Malaysia. Meanwhile, the current study was conducted to evaluate the efficiency of forest spiders as the predators of *S. litura* and *P. xylostella* under laboratory condition.

MATERIALS AND METHODS

Insects

The larvae of *P. xylostella* and *S. litura* were used as prey to evaluate the feeding activity and food preference of these spiders. They were obtained from MARDI, Serdang, and maintained in plastic aquariums under 12L:12D photoperiod, at 26±1°C and 60-70% relative humidity. The larvae were supplied with chemical- and disease-free cabbage plants while the adults were supplied with 10% honey-water solution.

Spiders

Sampling activity was carried out from the 1st to 4th of April 2011 in Pulau Tioman forest and also from 27th to 30th June 2011 at Penang National Park. All the spiders were brought back to the laboratory and

maintained under laboratory condition. The adult spiders were identified to species level using available literature (Barrion & Litsinger, 1995; Richman *et al.*, 2006). A total of ten adults (mixture of males and females) of four spider species were used in the prey preference test.

Prey Preference Test

Adult spiders were kept individually in plastic containers under laboratory condition and starved for three days before predation test was carried out to ensure that they would be adequately hungry to hunt. The predation test was conducted in clear plastic containers measuring 10 cm x 10 cm x 1.5 cm, with chemical- and disease-free cabbage leaves as food for larvae and wet tissue to provide moisture. The larvae were prepared in a similar manner for the no-choice and choice prey preference tests. The larvae with body length measured at 3 mm (early second instar *S. litura*; second instar *P. xylostella*) and 5 mm (late second instar *S. litura*; third instar *P. xylostella*) respectively were used in the predation test. All the experiments were conducted in laboratory at 12L:12D photoperiod, at 26 ±1°C and 60-70% relative humidity.

In the no-choice prey preference test, each spider was offered with one insect species of the same size larvae. Treatments were replicated 9 times and each replicate contained 10 larvae for each spider. The number of survival (larvae) was recorded daily. The same procedure was also used for the choice prey preference test, except that each spider was offered with two different insect species of the same size larvae (namely, five *P. xylostella* larvae and five *S. litura* larvae). All the data collected were analyzed using the analysis of variance (ANOVA), followed by means separation using Tukey test analysis ($P<0.05$).

RESULTS AND DISCUSSION

A total of 489 individual spiders were collected from Pulau Tioman forest and Penang National Park. Spiders such as *Heteropoda garciai*, *Olios mahabangkawitus*, *Ctenus floweri*, and *Pardosa apostoli* have been identified as the most abundance spider species in both the sampling sites (Fig.1 and Fig.2). In particular, *H. garciai* and *O. mahabangkawitus* are the Sparassidae spiders which can be mostly found on leaf litter. Meanwhile, *C. floweri* has a similar habitat, while *P. apostoli* (Lycosidae spider) can be found in sandy area. These spiders are ground hunting spiders. Other species of spiders were insufficient for prey preference test due to the low number of collections or they died during handling.

The results of the prey preference test under laboratory condition are shown in Tables 1 and 2. The preference of these spiders was towards *P. xylostella* larvae in the no-choice prey preference tests, except for *C. floweri*. Meanwhile, *H. garciai* and *O. Mahabangkawitus* preferred *P. xylostella* than *S. litura* and no significant difference was observed between the second and third instars larvae of *P. xylostella*. *C. floweri,* and *P. apostoli* preferred both the insect species. However, *P. apostoli* did not consume much on the 5 mm *S. litura* larvae. When two

Fig.1: Spiders used in the prey preference test; (A) Female *Heteropoda garciai* (Bar = 2mm), (B) Male *Olios mahabangkawitus* (Bar = 2mm), (C) Female *Ctenus floweri* (Bar = 2mm), and (D) Male *Pardosa apostoli* (Bar = 2mm)

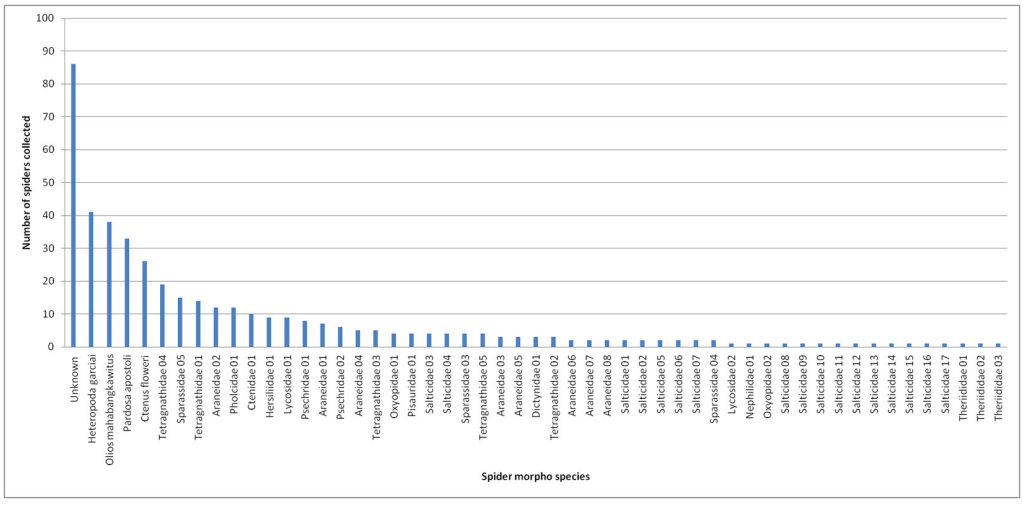

Fig.2: Total collection of spiders from two sampling sites (Pulau Tioman forest and Penang National Park)

TABLE 1
The mean number (± S.E.) of insect pest larvae consumed in 5 days by four species of spiders under no choice prey preference test

Type of larvae	Spider species			
	Heteropoda garciai	*Olios mahabangkawitus*	*Ctenus floweri*	*Pardosa apostoli*
3 mm *P. xylostella*	$10.0^{a} \pm 0.0$	$9.7^{a} \pm 0.9$	$10.0^{a} \pm 0.0$	$10.0^{a} \pm 0.0$
5 mm *P. xylostella*	$10.0^{a} \pm 0.0$	$9.3^{a} \pm 1.6$	$10.0^{a} \pm 0.0$	$9.8^{a} \pm 0.4$
3 mm *S. litura*	$2.6^{b} \pm 1.4$	$2.8^{b} \pm 1.3$	$9.4^{a} \pm 1.6$	$7.7^{a} \pm 1.9$
5 mm *S. litura*	$2.4^{b} \pm 2.5$	$5.8^{b} \pm 1.8$	$7.6^{a} \pm 2.6$	$3.6^{b} \pm 3.4$

Means followed by the same letters within a column are not significantly different (P≤0.05) according to Tukey test analysis

TABLE 2
The mean number (± S.E.) of insect pest larvae consumed in 5 days by four species of spiders under the choice prey preference test

Size of larvae	Type of larvae	Spider species			
		Heteropoda garciai	*Olios mahabangkawitus*	*Ctenus floweri*	*Pardosa apostoli*
3 mm	5 *P. xylostella* + 5 *S. litura*	$4.7^{a} \pm 0.5$	$4.7^{a} \pm 0.5$	$5.0^{a} \pm 0.0$	$5.0^{a} \pm 0.0$
		$2.4^{b} \pm 1.4$	$2.2^{b} \pm 0.8$	$5.0^{a} \pm 0.0$	$4.9^{a} \pm 0.3$
5 mm	5 *P. xylostella* + 5 *S. litura*	$4.8^{a} \pm 0.6$	$4.4^{a} \pm 1.6$	$5.0^{a} \pm 0.0$	$5.0^{a} \pm 0.0$
		$2.0^{b} \pm 1.9$	$1.7^{b} \pm 0.9$	$5.0^{a} \pm 0.0$	$1.0^{b} \pm 0.8$

Means followed by the same letters within a column are not significantly different (P≤0.05) according to Tukey test analysis

different insect species of the same body length were provided as food sources in the choice prey preference test, *H. garciai* and *O. mahabangkawitus* still preferred *P. xylostella* than *S. litura*, while the maximum predation was observed with *C. floweri* on both the insect species. A similar trend of prey preference was also observed for *P. apostoli* in the choice and no-choice prey preference tests. Prey preference is evident in *H. garciai* and *O. Mahabangkawitus* as they consumed significantly more *P. xylostella* than *S. litura* under both the choice and no-choice conditions regardless of the prey size.

Therefore, the present study has shown that *C. floweri* has no significant preference between *S. litura* and *P. xylostella* but the maximum predation was observed on both the insect species. In particular, *C. floweri* used in this study was two times bigger in size compared to other tested spiders, resulting in a higher consumption rate of prey. In other words, the size of a predator is important for feeding capacity. Larsson (2007) reported that bigger Salticidae spiders consumed significantly more *P. xylostella* compared to those smaller Salticidae spiders.

The size difference between the prey and predator clearly affects the killing capacity because the predators usually attack prey that is smaller than themselves (*Cogni et al*., 2002). Miranda *et al.* (2011)

reported a clear trend for individual predators to have a higher mean killing rate on smaller sized prey (second instars) than larger sized prey (third instars). Ma *et al.* (2005) also reported a similar result of predator preferences towards smaller prey when *Nabis kinbergii* preyed on smaller *P. xylostella* at increasing rates while the number of prey consumed by *N. kinbergii* decreased as the body size of prey increased. This is also evident in *P. apostoli* predation on *S. litura* larvae. However, when preying on the same prey species, this feeding behaviour was not observed in *H. garciai* and *O. Mahabangkawitus*, which have similar body length with that of *P. apostoli.* This finding demonstrates that the prey size preferences will be affected when there is a prey species preference.

CONCLUSION

Four species of the spiders collected from Pulau Tioman forest and Penang National Park had demonstrated predation towards *P. xylostella* and *S. litura* larvae under laboratory conditions. The present study is an initial laboratory study undertaken to examine the preference of *Heteropoda garciai*, *Olios mahabangkawitus*, *Ctenus floweri*, and *Pardosa apostoli* under the most simplistic conditions. Nonetheless, the actual effect of predation on prey population is hard to measure without experimental field studies when environmental factors become complicated. The question on whether or not these spiders are effective predators of *S. litura* and *P. xylostella* in vegetable farms cannot be conclusively answered as yet. Thus, a further study is needed to evaluate the actual potential of the forest spider species as biological control agents for *S. litura* and *P. xylostella* in vegetable farms.

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Histological Study of the Interaction between *Exserohilum Longirostratum*, Barnyard Grass, and Rice var. MR219

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ABSTRACT

The course of infection and the development of *Exserohilum longirostratum* (Subramanian) Sivanesan on barnyardgrass (*Echinochloa crus-galli* (L.) Beauv. spp. *crusgalli*) and rice (*Oryza sativa* L. var. MR219) were studied under light microscopy (LM) and scanning electron microscopy (SEM). Observation under SEM indicated similarity of the gross anatomy of both rice and barnyard grass leaves. Meanwhile, germination of the conidium of *E. longirostratun* was found to be not influenced by inoculation time as the conidia started to germinate 4 hours after the inoculation on both leaf surfaces. However, the patterns and number of germ tubes and appressoria formation were influenced by host plants. On barnyard grass, the primary infection process consisted of the conidial germination, elongation of the germ tube, formation of the appresorial initials, maturation of the appressoria, and formation of secondary hyphae. Successful penetration was followed by an extensive colonization of the invaded epidermal cell wall. Observation of the cross section revealed that the infection hyphae expanded into a spherical vessel and colonized the cells, causing the collapse of the epidermal cells and resulting in the formation of necrotic lesions of infected and adjacent tissue. Although fungus successfully grew and produced germtube on rice, both the primary infection process and the successful penetration of the cuticle were not observed on rice. The conidia germinated and produced slender and thin germ tube, with occasional appressorium formation. Germ tubes and appressoria formation on the barnyard grass (70% and 92%, respectively) were significantly higher as compared to rice leaves (51% and 10%). It was observed that the mycelium infected barnyard grass much faster (less than 24 h) than the conidia as it immediately formed

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appressorium without having the need, like the conidia, to germinate first. These results suggest that it may be possible to utilize *E. longirostratum* as a bioherbicide to control barnyard grass under rice production in Malaysia.

Keywords: Barnyard grass, biological weed management, *Exserohilum longirostratum*, rice

INTRODUCTION

In Malaysia, wetland rice is facing increasing weed problems due to the change in its planting method, i.e. from transplanting to direct-seeding. Among the grassy weeds, barnyard grass (*Echinochloa crus-galli* complex) has become one of the most troublesome. Its genetic similarity and similar growth requirements to rice make it a formidable competitor to the crop causing untold losses in terms of yield by its competition. It has been reported that barnyard grass reduced rice yield by 21-40% (Azmi, 2000; Tjitrosemito, 1994; Tarif *et al.,* 2004). To compound the problem, the weed is also host to many insect pests and pathogens, which will indirectly cause yet more losses in the yield and produce quality.

The present method of weed control is by herbicides, but the method, which was once economic and effective, has now begotten its own problems. In particular, the continued use of chemicals has induced resistance in the weeds and the chemical residues wrought environmental havoc. Nevertheless, herbicides continue to be used for want of other choices. It is, therefore, imperative that safer and more environmentally-friendly alternatives be developed for greater effectiveness and to minimize the wanton destruction of the environment.

Exserohilum longirostratum has been investigated as a bioherbicide to control barnyard grass (Juraimi *et al.,* 2006; Ng, 2007; Ng *et al*., 2010), with a particular attention given to the influence of environmental factors on its bioherbicidal activities. However, the histology of the pathogen-host interaction has not been elucidated, especially to understand the resistance/susceptibility of the host to the pathogen. Slight inherent or induced differences in the morphology, biochemistry or physiology between the plants can have a major effect on their resistance/susceptibility to a pathogen. In addition, different inocula of *E. longirostratum,* conidia or mycelium, may have different modes of infecting the host plant.

The main pre-infection action of the fungus is to attach itself to the host – usually on the leaf surface - by forming a germ tube to penetrate the surface, before differentiating into infection structures. Stomata are the most important natural openings for the fungus to enter. However, direct penetration through an intact epidermis can also occur, and this usually happens through formation of an infection structure at the tip of the germ tube (appressorium) or arising from the mycelium (hypopodia or infection cushion) (Vandyke & Trigiano, 1987).

As fungi thrive in warm, moist conditions, the herbicidal activity is greatly

enhanced by the presence of dew on the surface to be infected. An oil emulsion can help to overcome the adverse effects of dry conditions by preserving any available moisture for the fungus propagules to germinate. Oil emulsions have therefore been evaluated for formulating bioherbicides (Shabana, 2005). Nevertheless, there remains a dearth of information at the ultra structural level on the fungal penetration and other early events in pathogenesis by *E. longirostratum,* such as whether more or less oil in the emulsion is better for infection. Understanding the interactions should shed light on the mechanism of host death and the effect of the oil emulsion on the infection process by *E. longirostratum.* The objectives of this study were, therefore, to examine the germination and growth of *E. longirostratum* on barnyard grass and rice and to assess the subsequent infection process through light (LM) and scanning electron microscopy (SEM).

MATERIALS AND METHODS

Plant Production

Oryza sativa var. MR219 (rice) was used in this experiment as this variety is widely planted in Malaysia. The seeds of rice and barnyard grass (*Echinochloa crus-galli* (L.) Beauv. spp. *crusgalli*) were collected from a rice granary in Tanjung Karang, Malaysia, and were produced in flats in the glasshouse, before they were transplanted after the emergence of coleoptiles into round pots (10 cm diameter x 10.6 cm height; 5 seedlings/ pot) containing a potting medium (3:2:1 top soil:sand:organic matter). The plants were watered to soil saturation twice daily and allowed to grow until the 3- to 4-leaf stage (LS).

Inoculum Production

Inoculum of *E. longirostratum,* which was isolated from *Rottboellia cochinchinensis* (Lour.) W.D. Clayton (Kadir *et al*., 2007), was produced using a biphasic culturing technique (Chandramohan *et al*., 2002) with several modifications. Five mycelium plugs were transferred into 100 ml of V8 broth in 250 ml flask. The inoculated flask was then shaken (100 rpm) for 2 days at 28^{o}C and then transferred into 500ml of V8 broth in 1-L flask. The culture was allowed to grow further in 1-L flask for 2 days. The contents of the flask were blended in a Waring blender at low speed for 30-60 sec and 25 ml of this suspension was poured onto a layer of V8 agar (250 ml) in trays measuring around 35 x 26 x 2.5cm. The trays were exposed continuously to 24 h light at 30±2^{o}C. The conidia were gently scraped off with rubber spatula into sterile water and filtered through cheesecloth. The remaining conidia were then rinsed off the agar surface with sterile water. The conidial suspensions were pooled and the concentration of conidia was determined and adjusted to the required concentration with a hemocytometer. The inoculum in the form of mycelial suspension was produced using the above method with a slight modification. After blending the content of the flask, the suspension was stored in the refrigerator at 4^{o}C before it was used for plant inoculation.

Plant Inoculation

Groups of five seedlings each of rice or barnyard grass, at the three- to four-leaf stages, were inoculated with a suspension of either conidia (10^5 conidia mL^{-1}, 20 % vegetable oil, 0.05% Maxigreen [nonionic spreader and sticker] v/v/v) or mycelia (1:5 ratio of 5 day-old mycelium:V8 juice, 20% vegetable oil, 0.05% Maxi green v/v/v) of *E. longirostratum*. Eight inoculated leaves were excised and placed on a moist filter paper in a glass humidity chamber (Mason jar) and incubated in an incubator (Memmert GTR0124) at 28±2°C for 4 hours. Similarly, other batches of eight leaves were incubated for 8, 16 and 24 hours, respectively. After the incubation, each leaf was trimmed to a disk (about 0.5 cm x 0.5 cm). Of the eight disks, four were examined under a light microscope (LM) at 40x magnification, while the other four under a scanning electron microscope (SEM) at 600x, 850x, and 2000x magnification, respectively.

Light Microscopy (LM)

Four leaf disks were fixed on a filter paper saturated with a formalin/ alcohol/ acetic acid (FAA) solution (1:18:1 v/v/v) on a petri dish, which was then sealed with a parafilm and left for 2 hours. The leaf disks were cleared for 42-48 hours by soaking in a solution of choral hydrate (200 g), dH_2O (80 mL), ethanol (250 mL) and four drops of Tween-20 (Celio & Hausbeck, 1997). The leaf disks were transferred to glass slides, and a drop of lacto phenol (20% phenol, 20% lactic acid, 40% glycerol, and 20% water) containing 0.1% cotton blue was also added (Bailey *et al.,* 2000). The percentages of the conidia germinated and appressoria formed were determined by counting 200 conidia per leaf (taken at random) under LM with a 4X objective. A conidium was considered germinated if its blue-staining germ tube was visible with the length of the germ tube at least equal to the width of the conidium.

Scanning Electron Microscopy (SEM)

Four leaf disks were fixed in 3% glutaraldehyde buffer for 2 hours and post-fixed in 1% osmium tetroxide for 2 more hours and dehydrated by passing through a graded ethanol series (10%, 20%, 30%, 50%, 70%, 90% and 100%) prior to critical point drying with CO_2 as the transition medium (Spencer, 2001). The samples were then mounted on stubs and coated with gold-palladium, as well as viewed and photographed with a JEOL 5610LV SEM.

Data Analysis

All the percentage data were transformed to arcsine before analysis (Gomez & Gomez, 1984). Data points were curve fitted using linear regression. Analysis of variance (ANOVA) using the general linear model was also used wherever appropriate (SAS Institute, Cary, NC) to analyze the effect of each factor individually and their interactions. Mean separation was performed using Tukey's HSD test based on the variance if the treatments showed significant differences.

RESULTS

Light Microscopy

The conidia germinated on both the rice and barnyard grass leaves as soon as 4 hr after the inoculation. Most of the conidia germinated monopolarly, with occasional bipolar germination. All the germinating conidia initially formed appressoria, arising from the primary germ tube, which was slightly lobed to lobed.

After 4 hr of inoculation, about 25% of the conidia germinated on the barnyard grass (Fig.1). The most prolific production of germ tubes occurred 8 hr after inoculation, presumably when suitable penetration sites on the leaf surface had prevailed. Few appressoria (12% of the germinated conidia) were formed by the conidia germinated between 4 and 8 hr after the inoculation; however after 8 hrs, more appressoria (79%) formed directly on the epidermal cells or over the junctions between them. The appressoria formation reached 92% after 24 hours (Fig.1).

On rice leaves, the initial germination (9%) of the conidia occurred 4 hr after the inoculation. The germination increased

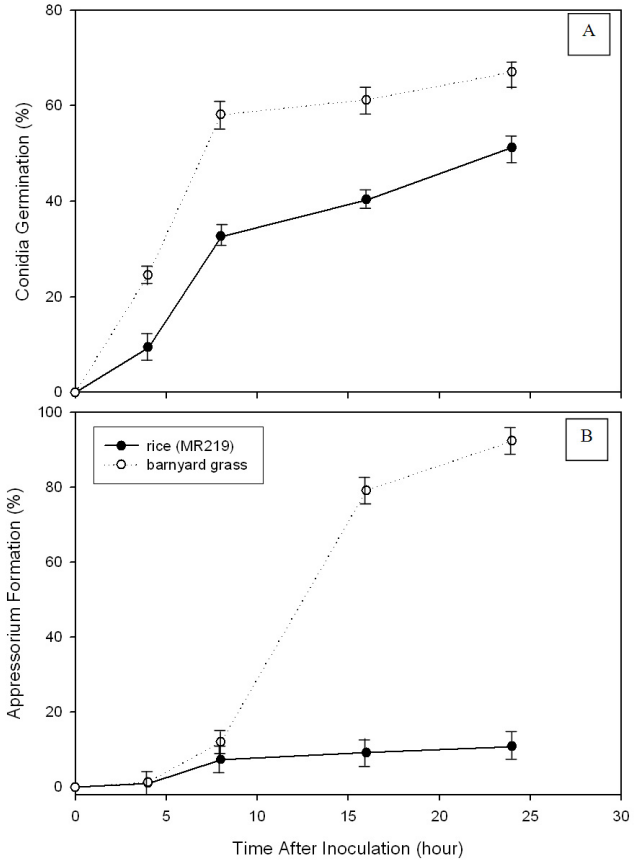

Fig.1: The percentage of conidia germination (A), and percentage of appressoria formation (B) on rice and barnyard grass. Vertical bars represent standard errors of the means. Mean percentage of conidial germination were calculated based on 200 observations taken from a repeated experiment

quickly to reach 33% after 8 hrs, and then more gradually to 40% in 16 hrs, and finally 51% after 24 hrs (Fig.1). Fewer appressoria were formed on the rice leaves. Only 7% appressoria formed 4 hr after inoculation. The respective parallel figures for 16 and 24 hrs after inoculation were 9% and 11%. Earlier in the penetration period, there were no measurable differences in the numbers of appressoria formed on the rice and barnyard grass leaves. Eight hours after the inoculation, a significant increase in the appressoria formation was recorded with preferential appresorium formation on the barnyard grass.

The relationship between spore germination and appressorium formation on barnyard grass is best described by a linear regression of the first order polynomial (see Fig.3). On the barnyard grass, the

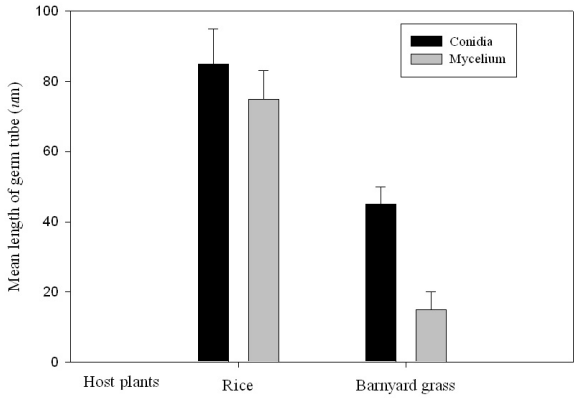

Fig.2: Length of germ tube of *E. longirostratum* on Rice var. MR219 and on Barnyard grass at 24 hrs after inoculation. Vertical bars represent standard errors of the means. All the experiments were repeated twice with 200 observations in each experiment

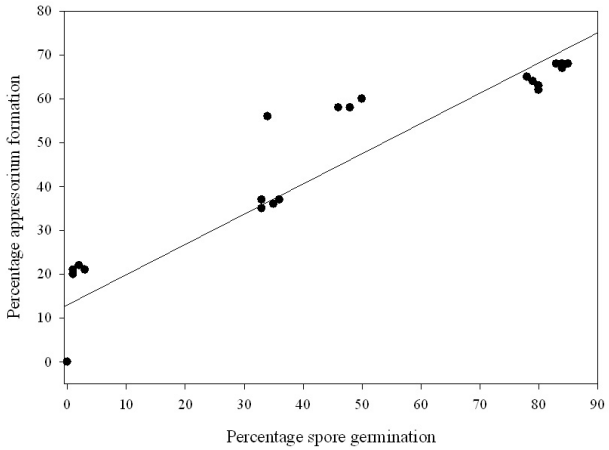

Fig.3: The correlation between percentage conidia germination and percentage appressorium formation of *E. longirostratum* on barnyard grass at 24 hrs after inculation. The regression was fitted using percentage data. The regression obtained is Y= 13 + 0.69X (R^2=0.87).

numbers of appressoria formed were positively correlated with the numbers of the germinating spores. Higher numbers of appressoria were formed after 24 hrs and this was found to be correlated with the higher numbers of germinating spores. This observation suggests that barnyard grass has many infection sites where the appressoria could penetrate the surface of barnyard grass leaves. Nonetheless, such a relationship was not manifested on rice leaves. These results indicate a random formation of appressorium on rice and is therefore not related to the number of germinating spores.

Scanning Electron Microscopy

The salient features of the anatomy of rice and barnyard grass leaves showed a similarity in both the leaves having vascular bundles in between thick-walled, non-living sclerotized sclerenchyma cells, most of which were fibrous. This observation has earlier been reported by Hau and Rush (1982). Both the leaves are characterized by the presence of papillae - epidermal cells projected in rows along the axis of the leaf.

The primary infection process consists of conidial germination, germ tube elongation, formation of appressorial initials, maturation of appressoria, and formation of secondary hyphae. In the barnyard grass leaves inoculated with conidia, the infection hyphae were produced after the appressoria had formed over the bulliform cells. Germ tubes emerged mostly from the end cells of the conidia. Throughout this study, germ tube length refers to the first length of germ tube from the conidium to the first appressorium. The germ tube length varied with the host leaves. The length of the germ tubes formed by the conidia and the mycelia on rice was significantly longer (84µm and 78µm) as compared to the germ tubes on barnyard grass (42µm and 18µm), respectively (Fig.2). Many of the germ tubes extended on the surface along the junctions of the epidermal cells (Fig.4). In the intracellular penetration of the barnyard grass leaves, the infection hyphae distended into spherical vessels in colonizing the cells

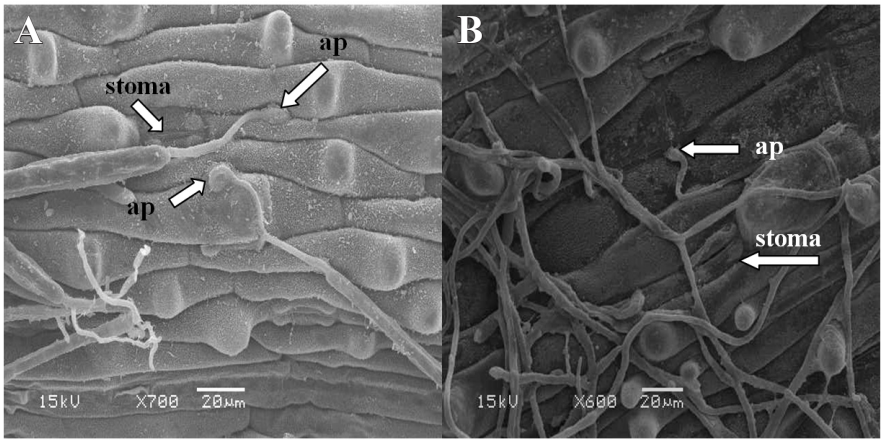

Fig.4: The SEM of the appressoria (ap) formed directly on the epidermal cells from conidial (A) and mycelium (B) based suspensions on barnyard grass

(Fig.5) leading to the collapse of the infected and adjacent epidermal cells and causing necrotic lesions.

In the mycelium inoculation, the formation of appressoria occurred before the formation of infection hyphae. The appressorium formed at the end of a massive germ tube deposited over the leaf surface. The infection hyphae then enlarged into spherical vessels and colonized the cells (Fig.5). Colonization was more severe with the mycelial inoculation than with the conidial inoculation due to more infection hyphae being formed within 24 hrs of inoculation. It was very rarely that penetration was accomplished without a well-defined appressorium. The SEM images indicated that the fungus did not

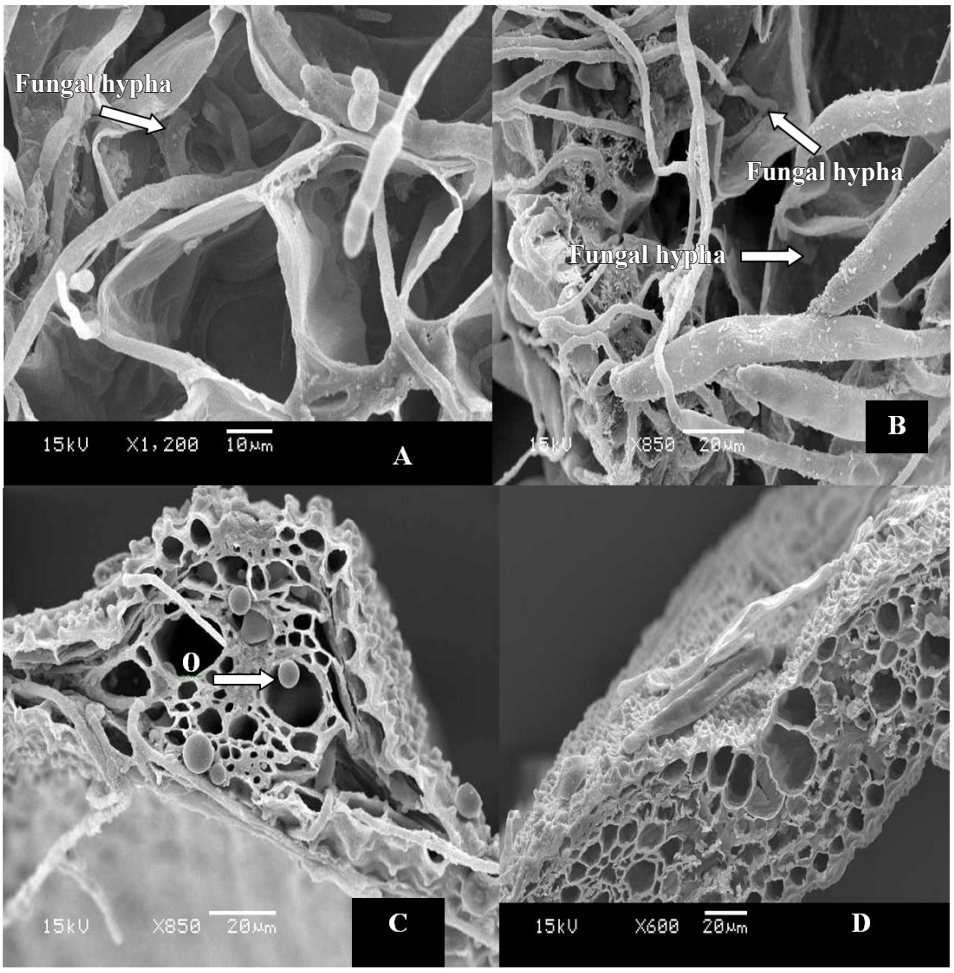

Fig.5: The cross-section of leaf showing infection process of *E. longirostratum* on the barnyard grass (A, B) and rice (C, D) leaves. In the barnyard grass, hyphae colonized the cells by mycelium (A) and conidia (B) -based suspensions. In rice, hyphae (C) and conidia (D); expansion and colonization of the cell did not occur even at 24 hr after inoculation. Some oil microdrops (O) are seen adhering to the rice cell

form appressoria over the stomata and trichomes nor entered the leaves through them.

The infection process in rice differed from that in barnyard grass, where the hyphae neither distended into vessels nor colonized the cells in rice, whether inoculated with conidia or mycelium. Meanwhile, the epidermal cells remained firmly rigid and did not collapse (Fig.5). Although the conidia were able to germinate on the rice leaves, they formed significantly fewer appressoria compared to barnyard grass leaves. The orientation of the germ tubes was also random, suggesting that rice var. MR219 is not susceptible to *E. longirostratum*.

During the infection, the resistant host defends itself through a number of physical and chemical factors. *Exserohilum longirostratum* on the barnyard grass leaf surface is often associated with a sheath-like structure (Fig.6), mostly at the distal end of the appressoria in contact with the cuticle,

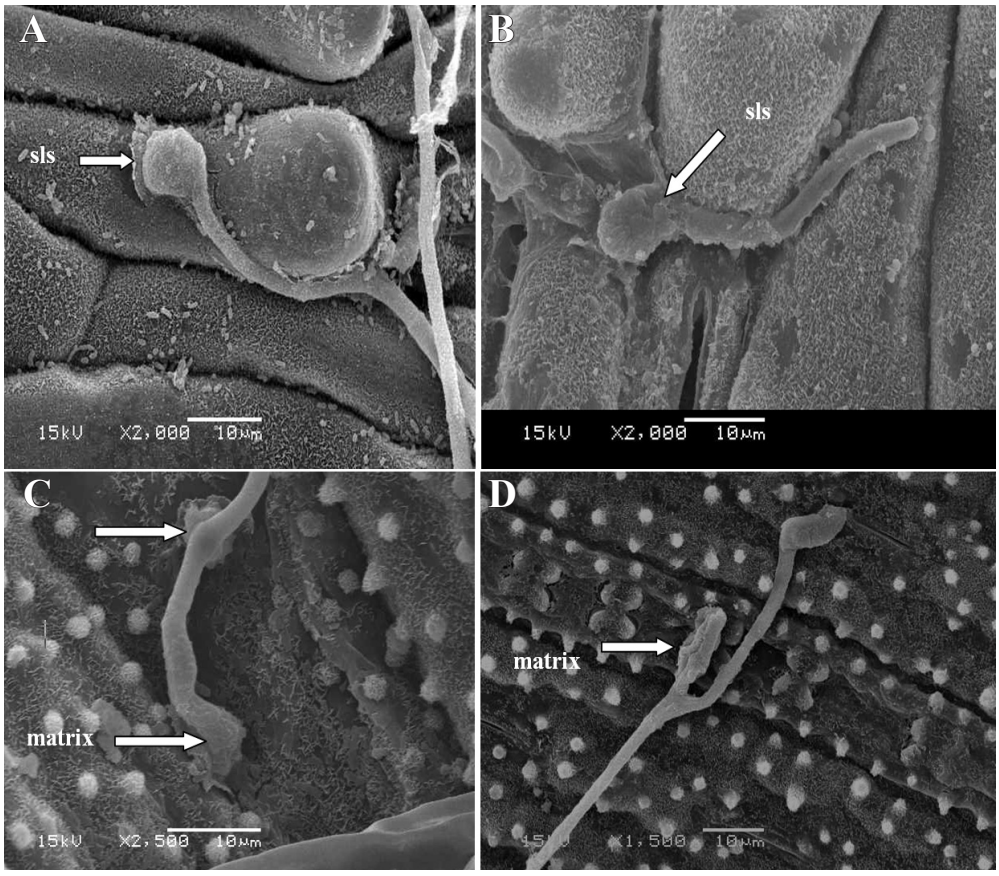

Fig.6: The appressorium of *E. longirostratum* adhering to the waxy surface of barnyard grass leaf by an extracellular sheath-like substance (A); The mycelium on the barnyard grass leaf surface often have sheath-like substances (sls) associated with them (B); The matrix formed outside the germ tube on rice (C and D)

suggesting the formation of the sheaths is induced by the presence of the pathogen. However, such sheaths were not observed on rice but instead a dense matrix of amorphous substance was formed outside the germ tube by the germinating conidia. This matrix accumulation may be the non-host response of rice to the pathogen conferring it (host) the resistance to infection.

DISCUSSION

Exserohilum longirostratum has a similar infection process as other fungi. The conidium first attaches to a suitable site and then germinates in about 4 hrs after inoculation. The germ tube forms an appressorium, which in turn produces infection hyphae in the sub-epidermal cells. The appressorium forms directly on the epidermal cells or over the junctions between them so that the hyphae can directly penetrate the cuticle through the weak joints. Tsukamoto *et al*. (1999) observed that the appressoria of *E. monoceras* penetrated directly through the epidermal cells as well as through stomata of *Echinocloa oryzicola.* However, this observation was not noted by Hau and Rush (1982); instead, they reported that *Helminthosporium oryzae* infection process of susceptible rice plants started at the juncture between epidermal cells and penetrated the bulliform cells. This study concurs with both reports, with the exception that the penetration was never observed over the stomata as reported by Tsukamoto *et al*. (1999).

Barnyard grass was found to be infected faster by the mycelia than conidia inocula. The mycelium of *E. longirostratum* infected barnyard grass much faster than the conidia as it immediately formed appressorium without having the need, like the conidia, to germinate first. Using mycelium, almost all the cells of the barnyard grass were colonized by infection hyphae within 24 hrs of inoculation. At the same time, with conidia, the infection hyphae had only just begun colonizing the host cells. The efficacy of mycelium, as a component of bioherbicidal formulation, was further justified by Shabana *et al*. 2010, who reported that the formulations containing 30% Sunspray 6E and *Drechslera gigantea* mycelium (10 g), causing 88 to 100% injury on tropical Signalgrass, Crabgrass, Smutgrass, and Torpedograss in greenhouse trials.

Rice var MR219 appeared to be resistant to both the conidia and mycelium of *E. longirostratum*. Longer germ tubes but with fewer appressoria and poor hyphal growth were observed on the rice – possible indicators of the resistance mechanism at work.

The percentages of germinating conidia and the fungus subsequently forming appressoria on barnyard grass inoculated with oil emulsion were higher than those reported by Chia (2005), who inoculated barnyard grass with *E. longirostratum* without oil emulsion. The oil emulsion was therefore effective in enhancing the efficacy of the inoculum. Similar results were also reported by Auld (1993) with vegetable-oil emulsions to formulate the fungus *Colletotrichum orbiculare* (Berk. and

Mont.) for controlling *Xanthium spinosum* L., and also by Daigle *et al.* (1990) who used invert emulsion formulations of *Alternaria cassiae* Jurair and Khan to control sicklepod [*Senna obtusifolia* (L.) Irvin and Barneby]. The oil emulsion not only retained water for the conidia to germinate, but might also have attached the conidia more strongly to the leaf surface. The oily phase of the emulsion rapidly penetrated the leaf surface into the intercellular spaces, and water diffused in from the neighbouring cells into the oil to form micro drops, and in effect, an invert emulsion. Greaves and Macqueen (1990) reported that water drops evenly diffused throughout the oily phase to form a water film on the leaf surface providing for the ideal environment for a microbial herbicide to function.

Bulliform cells have been reported as the usual infection point for *Pyricularia oryzae*, the causal agent of rice blast of rice (Hau & Rush, 1982). In Poaceae, bulliform cells are unusual epidermal cells, where the radial walls of these cells are thin and the outer walls remain in a pectic-cellulosic state long after the epidermal cells have become lignified (Clark & Lorbeer, 1976). Meanwhile, Whitney (1977) showed that a high degree of penetration in bulliform cells was correlated with low degree of mechanical toughness of the outer wall. In addition, Whitney (1977) also reported that lower concentration of chlorogenic acid (a fungitoxin) in the bulliform cells could explain the lower resistance of bulliform cells, which perhaps making them less resistant to penetration. The present investigation showed that *E. longirostratum* preferentially formed appresoria over the bulliform cells of barnyard grass as compared to rice, especially over the junctions between them. Hau and Rush (1982) stated that the junctions between bulliform cells offer a chemical environment and groove-like topography that may stimulate the formation and maturation of appressoria.

An extracellular sheath was formed to cover the fungal structure on barnyard grass. This observation was first reported by Wheeler (1977), who had noticed that *Helminthosporium maydis* and *H. victoriae* have extra cellular sheaths associated with hyphae. The finding of the present study confirms the result of his study and also the observation by Locci (1969), who reported that under low resolution SEM, adhesive matrix associated with hyphae and appressoria and the absence of stomatal penetration was observed. The adhesive matrix adhered to the cuticle might have enabled the fungus to attach to the leaf surface and facilitated infection that caused the cell membranes of barnyard grass leaves to drastically alter and the ultra structure of cells to have severely deformed. Wheeler (1977) reported such sheaths to be common in the species within the genus *Helminthosporium (Exserohilum)* and this observation further concurred by the observation of Hau and Rush (1982), who reported that the sheath like substance was often associated with susceptible rice variety infected with *H. oryzae*. The absence of the extra cellular sheath on the rice var MR219

indicates a resistant reaction toward *E. longirostratum.* The extra cellular sheath is required for the appresorium to adhere to wax crystal and facilitate infections. Thus, this study has indicated that rice var MR219, a commonly cultivated rice variety in Malaysia, is resistant to *E. longirostratum.* In more specific, *E. longirostratum* has the potential to be used in controlling barynyard grass for rice production in Malaysia.

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Bioefficacy of Controlled Release Formulations of Diuron on *Brassica rapa*

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ABSTRACT

Controlled-release formulations (CRF) of diuron were prepared in laboratory using the concept of physical matrix utilizing sodium alginate and kaolin. The release rates of diuron from the formulations were determined by chemical assay its efficacy on plant was tested on *Brassica rapa*. In the chemical assay using high performance chromatography with UV detector, significant differences in the release rate between formulations were observed from 3 to 7 days after the granules had been placed in distilled water. The formulation having a 1:1 ratio of alginate to kaolin with 1mm granule size showed the fastest release of diuron, while release from the 2mm granules was slower. Increasing the proportion of kaolin to sodium alginate in the CRF reduced the release rate of active agent. The bioefficacy using *Brassica rapa* as a bioindicator showed that CRF released slower than the conventional formulation at the beginning of the treatments. In the 3rd week after the treatment (WAT), there was no significant difference in the mortality as compared to the conventional formulation at 16 WAT. The same results were also observed up to 24 WAT, the CRF caused between 40-70% mortality, while the conventional formulation treatment caused only 6% mortality. Among the CRF, the AK-2 with 1:1 ration of alginate:kaolin was found to have given the best result with the highest percentage mortality of the seedlings.

Keywords: Sodium alginate, slow release, herbicide, bioefficacy, chemical assay

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INTRODUCTION

Weeds are one of the most important pests, while herbicide is the main method used in

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controlling this particular pest. In order to achieve control of weeds, it is necessary to maintain an appropriate concentration of chemical in contact with the plants for a sufficient amount of time for absorption to take place. In order to counter environmental losses and maintain the concentration above the minimum threshold of activity, applications of excessive amounts of conventional formulation of the herbicide are generally required. Nonetheless, a disadvantage of the conventional herbicide is the immediate release of active ingredient, which will partly be lost to environment consequences from the processes such as chemical and biodegradable, photolysis, evaporation, surface run-off and percolating to groundwater (Tomlin, 2002). These loses of the pesticide affectivity could reach as high as 30% (Cespedes *et al.,* 2007). The increase in the application rate, however, is likely to increase the potential for adverse impact on the environment. One of the effective approaches to reduce environmental loss while providing more efficient weed control is through the use of controlled release formulation (CRF).

Controlled release pesticide technology has received increased attention for the last two decades due to the growing awareness that pesticides may produce undesirable environmental effects when applied in conventional formulations at the levels required for activity (Kydonieus, 1980). The controlled release pesticides are better than conventional formulation in reducing leaching (Fernandex-Perez *et al*., 1999), volatilization (Dailey, 2004) and its prolonged activity (Bahadir, 1987). Numerous types of matrices have been tested for the preparation of CRF herbicides, while different mechanisms of release rate are often involved (Murphy & Barrett, 1990). Alginate has been used due to the unique properties they possess such as high biocompatibility and biodiegradbility (Pasparakis & Baouropoulus, 2006). It has been reported to be used in medical applications (Wan, Heng, & Chan, 1992), as well as in agriculture (Kulkarni *et al.*, 2000). For the use in herbicide it has been studied on diquat and dichlobenil (Barret & Logan, 1982), 2, 4-D (Connick, 1982), thiobencarb (Hussain *et al.,* 1982), as well as alachlor and atrazine (Gerstl, 1994). Many research on controlled release has emphasized on different sorbents and kinetic release on pesticide from controlled release formulations (Garrido-Herrera *et al*., 2006; Kumar *et al*., 2010; Flores-Cespedes *et al*., 2007); however, little is known about the efficacy of the controlled release formulation pesticide in controlling the target pest. Therefore, the objective of the study was to determine the bioefficacy and the prolong efficiency of CRF formulations of diuron on the bioindicator, *B. rapa*.

MATERIALS AND METHODS

The alginates CRF diuron was prepared based on the gelling properties of the alginate with kaolin as filler. It was made up of the formulation in water containing 20% of diuron with three different ratios of kaolin over sodium alginate. Sodium alginate of 2% viscosity and kaolin are both obtained

from Sigma Chemicals. The solution was homogenized with magnetic stirrer for 1 hour. The alginate mixtures were dropwise added into 0.25 M calcium dichloride ($CaCl_2$) by Easy-Load Masterflex® peristaltic pump. Materflex Pharmed® tubing, size numbers 13 and 14, were used to give 1 and 2 mm granule size, respectively. The 1 mm granules were coded as AK-1, AK-2, AK-3 for 4:0, 1:1 and 1:3 ratio of alginate to kaolin, respectively. The 2 mm granules were coded as AK-1b, AK-2b and AK-3b for the same ratio. The beads were filtered and dried at room temperature.

Herbicide Release from CRF by Chromatographic Analysis

The release rate for diuron from formulated CRFs was analysed by reverse-phase high performance liquid chromatography. For this purpose, 0.25 g of CRF (equivalent to 0.02 g a.i) was added for each sample (five replicates) and placed in volumetric flasks containing 25ml distilled water. The flasks were capped and stored in dark environment at room temperature. Samples of 30 uL from each flask at 3, 6, 10, 15 and 20 days were taken and then injected into liquid chromatography for the quantification of diuron.

A Waters 501® liquid chromatography with Nova Pak HR C 18 reverse phase colume (3.9 x 300 mm) was operated at a flor rate of 1 ml/min using mobile phase of acetonitrile:distilled water (60:40). The UV-VIS Waters® 484 Tunable absorbance Detector set at 254 wavelengths was used to detect diuron. Each of the 20 uL samples was injected into the pump by using the 100 uL Hamilton® syringe. Peaks were recorded by the computerize Waters System Interface Module (SIM) and analyzed by the Baseline programme.

Bioefficacy of CRF on the Bioindicator Brassica rapa

The granules of CRF and wettable powder CF were applied at a mass application rate of 1 kg ai/ha. The granules of CRF were applied by spreading them evenly in the pots and the amount applied per pot was 38mg. The treatments were applied to the soil one day before sowing one hundred *Brassica rapa* per pot. The soil was watered twice daily throughout the experiment. The seeds were germinated at 5 days after sowing (DAS) and symptoms of leaf chlorosis, leaf tip burning and death of the seedlings were only observed at 8 DAS.

The percentage mortality of the seedlings was recorded at 10 and 14 DAS, followed by removing all the seedlings at 20 DAS. 100 seeds were sown again at 3 weeks after the treatment (WAT). Similar procedures and data collections as the first batch of seeds were followed. The subsequent batches of seeds were sown at 6, 8, 9, 10, 16 and 24 (WAT). The seedling mortality was assessed visually based on a qualitative scale (Burril *et al.,* 1976). The experiment was a completely randomized design (CRD) with six replications. All the data were subject to the analysis of variance and the treatments were compared using Tukey's Test.

RESULTS AND DISCUSSION

Release Rate of CRF by Chromatographic Analysis

The retention time of diuron for the chromatographic condition described earlier was 3.8 minutes. Fig.1 shows the cumulative release of diuron into water from various formulations which occurred in a controlled manner. After 3 days, the AK-2 formulation was the fastest to release the diuron. The amount detected was 16.1% of the original content of diuron in the formulation. During the same period, AK-1, AK-3, AK-1b, AK-2b and AK-3b released 11.1, 15.5, 8.10 and 10.7% of diuron, respectively. More diuron was released from all the formulations after 6 days. The increase compared to the release at 3 days was more than 100% for AK-1, AK-1b, AK-2b and AK-3b, 60% for AK-2 and 48% for AK-3. The amount of diuron released reached the maximum on day 15, as the amount recorded for 20th days is the same as that of 20th days . The release on the 20th day showed that the 1 mm granules released higher amount of diuron compared to the 2 mm granules, with the following increasing order: AK-1b<AK-2b<AK-3b<AK-3<AK-1<AK-2. The release of diuron from CRF did prolong as compared to conventional formulations pesticide. Fernandez-Perez *et al.* (1998) used conventional formulation of imidacloprid to compare it with CRF and found the technical imidacloprid was completely dissolved within 2 hours, while Garrido-Herrera *et al*. (2006) discovered that 100% technical grade of isoproturon and imidacloprid were fully dissolved within 3 days and 3 hours, respectively. As technical grade of pesticide would loss up to 5% during the preparation (Cotteril *et al*., 1996), the release of diuron from this experiment therefore did not reach the full amount of its content. The results showed a distinct effect of granule size on the rate of release. As expected, the smaller the granule diameter, the faster the rate of release. Thus, it is apparent that the use of sodium alginate

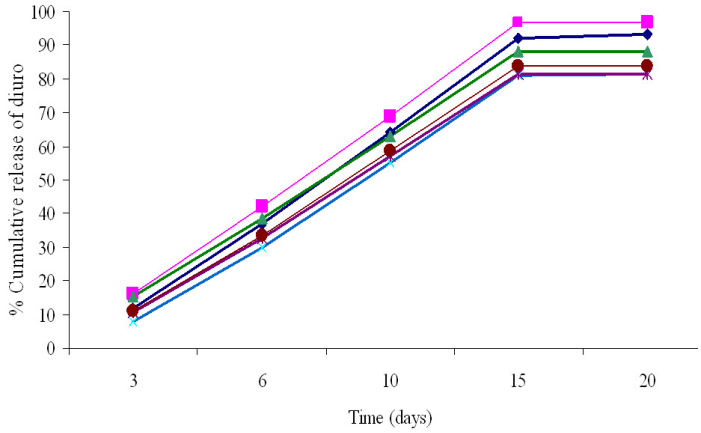

Fig.1: Cumulative release of diuron from alginate-kaolin controlled release formulation into distilled water. AK-1=◆; AK-2=■; AK-3=△ ; AK-1b=✕; AK-2b=◇; AK-3b=□

increased the crosslinking density of the formulation, while kaolin (as filler) slowed down the release of herbicide from the CRF. Similar responses have also been reported by some earlier researchers (e.g. Hussain *et al.,* 1992; Gerstl *et al.,* 1994).

Determination of CRF Release Rate by Bioassay

The percentage mortality of *Brassica rapa* seedlings exposed to CF and CRF is shown in Tables 1-3. Based on the assessment made at 10 and 14 DAS, a significantly higher mortality was observed for the CF treatment with 18.7% and 65%, respectively, as compared to other formulations after 1 week of the treatment (WAT) (see Table 1). This indicated the immediate availability of diuron from the CF. The highest mortality obtained from the CRF treatments was only 10% at 10 DAS and this was obtained from the AK-1, AK-2 and AK-2b formulations. At 14 DAS, the AK-1 formulation showed 35% mortality, which was significantly higher compared to AK-1b and AK-3b formulations, where the percentage of mortality was recorded at only 15%. This could be due to CRF released diuron slower and hence, insufficient doses were available to produce similar effects of CF. At 3 WAT (see Table 1), no significant difference was observed between the treatments based on the mortality at 10DAS, except for AK-2b. At 14DAS, there was no significant difference between all the treatments. This showed that the release of diuron from the CRF had increased, and hence, sufficient doses were released to show similar result as the CF treatment. A similar result was also obtained at 6 WAT.

At 8 WAT (Table 2), the AK-1b and AK-2b formulations showed significantly lower mortality compared with that of the CF, while other formulations indicated no significant difference with the CF. This could be due to uneven release of active ingredient from bigger granules, causing inconsistent results on the percent mortality. There was no significant difference on the mortality of the seedlings at 14 DAS for

TABLE 1
The effects of CF and CRF on the percentage mortality of *Brassica rapa* seedlings at 1, 3 and 6 weeks after the treatments (WAT)

Treatments	Percentage Kill **					
	1 WAT		3 WAT		6 WAT	
	10 DAS	14 DAS	10 DAS	14 DAS	10 DAS	14 DAS
CF	18.7±1.25 a	65±2.88 a	100±0.00 a	100±0.0 a	85.0±2.88 a	95.0±2.04 a
AK-1	10.0±0.0 b	35.0±2.88 b	90.0±3.53 a	98.7±1.25 a	85.0±2.88 a	93.7±1.25 a
AK-2	10.0±2.04 b	32.5±6.29 bc	67.2±10.33 a	100±0.0 a	62.5±4.78 b	88.8±3.75 a
AK-3	6.25±1.25 b	22.5±2.50 bc	80.0±7.90 a	95.0±2.04 a	55.0±6.45 b	81.3±7.73 a
AK-1b	6.25±1.25 b	15.0±2.88c	63.7±16.63 ab	86.2±10.48 a	52.5±4.78 b	76.3±8.98 a
AK-2b	10.0±2.04 b	25.0±5.0 bc	23.7±6.88 b	87.5±4.78 a	50.0±4.08 b	76.3±3.75 a
Ak-3b	6.25±1.25 b	15.0±1.25c	62.5±9.24 ab	85.0±5.00 a	62.5±4.78 b	82.5±4.33 a

Note: Values for the mean percentage (± SE) in a column, followed by the same letter, are not significantly different ($P \geq 0.05$) according to the complete randomized design procedures based on Tukey's test.
DAS= Days after sowing; WAT= Weeks after treatments

TABLE 2
The effects of CF and CRF on the percentage mortality of *Brassica rapa* seedlings at weeks 8, 9 and 10 after the treatments

Treatments	Percentage Kill **					
	8 WAT		9 WAT		10 WAT	
	10 DAS	14 DAS	10 DAS	14 DAS	10 DAS	14 DAS
CF	15.5±4.78a	90.0 ± 0.00 a	67.5±4.78ab	92.5±2.50 a	67.5±4.78 ab	92.50±2.5 a
AK-1	50.0±5.77 ab	75.0± 6.45 ab	75.0±2.88 a	97.5 ± 2.50a	75.0 ± 2.88 a	97.5 ± 2.30a
AK-2	27.5±6.29 ab	72.5± 2.59abc	60.0 ± 4.08ab	90.0± 4.08 a	60.0 ± 4.08ab	90.0 ± 4.08 a
AK-3	27.5±10.30ab	75.0 ± 2.88 ab	42.5 ± 8.53 b	87.5 ± 2.50a	42.5 ± 8.53 b	87.5 ± 10.4 a
AK-1b	17.5 ± 2.50 b	50.0 ±0.00 c	52.5± 7.50 ab	75.0 ± 10.4 a	52.5 ± 7.50 ab	75.0 ± 10.4 a
AK-2b	30.0± 9.12 ab	60.0 ± 10.8 bc	52.5 ± 4.78 ab	70.0 ± 5.77 a	52.5 ± 4.78 ab	70.0 ± 5.77 a
Ak-3b	40.0±8.53 ab	80.0 ± 4.08 ab	47.5 ± 7.50 ab	80.0 ± 9.12 a	47.5 ± 7.50 ab	80.0 ± 9.13 a

Note: Values for the mean percentage (± SE) in a column, followed by the same letter, are not significantly different ($P \geq 0.05$) according to the complete randomized design procedures based on Tukey's test.
DAS= Days after sowing; WAT= Weeks after treatments

all the treatments from evaluation at 9 and 10 WAT (see Table 2). All the treatments showed more than 70% mortality. Based on the evaluation at 10 DAS, higher mortality was observed at 9 WAT and 10 WAT as compared to the earlier treatments. This could be due to the release of diuron reaching the optimum and sufficient does was also available to give immediate effects on the mortality of the seedlings.

The immediate effect on seedlings' mortality declined at 16 WAT and 24 WAT (Table 3), indicating that time and cumulative effects of diuron from CRF played important roles in killing the seedlings. Meanwhile, the effect of CRF on the mortality of *Brassica rapa* seedlings was observed to be better than the CF treatments at 16 WAT. At 14 DAS, the 1mm granules with a ratio of 1:1 alginate and kaolin (AK-2) gave the highest mortality, i.e. 70%, as compared to the CF which gave 37.5% mortality. Only the AK-2b formulation gave the same level of mortality as that of the CF. This indicated that smaller granules release the diuron faster than the larger granules, giving it a better performance. This was primarily due to the larger surface area of the smaller granules. A similar result was also reported by Yousefzadeh *et al.* (1994) for the polyacrylamide hydrogel formulation, whereby smaller granules released active agent much faster than the bigger granules.

At 24 WAT (Table 3), the persistency of diuron was significantly prolonged in the soil when CRF was used. Only the CF showed significantly lower mortality at 14 DAS as compared to all the CRFs. Among the CRF, the mortality produced by AK-2 formulation (72.5%) was significantly higher than the AK-1 formulation (42.5%). This was clearly proven by the conventional diuron which had reduced its effectiveness with less than 50% of *B. rapa* being killed. However, all the CRF formulations gave better control than CF. The prolonged effectiveness of CRF is due to the diuron entrapped in alginate polymer which is protected against degradation agents such as sunlight, water, oxygen, and hydrolysis.

Similar results with CRF metribuzin by Kumar *et al.* (2010) showed that CRF stayed longer in soil than CF. The result indicated that the ratio of 4:0 alginate to kaolin in AK-1 released the active agent faster and presumably at greater quantity, depleting the diuron in the formulation. The present of kaolin could control the release of diuron because it could serve as a binder of the active ingredients (Gerstl *et al.,* 1994). The above results have shown that the 1mm granules of alginate and kaolin in a 1:1 ratio were the most suitable formulation and this combination was selected for further evaluation in subsequent experiments.

CONCLUSION

The release of diuron from alginate kaolin CRF was determined by a bioefficacy study which showed no significant influence of the size of granules. All the CRFs showed slower activity at the beginning of the experiment. This was no surprising as the CRF released their active agent over time, while in the CF treatment the active ingredient was immediately available upon application. At 15 and 24 WAT, AK-2 and AK-3 CRF showed higher percentages of the killed seedlings as compared to CF. Diuron in the CRF also persisted longer than the CF formulation. The increased persistence was attributed to the gradual release of the active ingredient, which at the same time, ensured minimal environmental loss due to leaching, volatilization and photo-degradation.

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Molecular Identification and Phylogenetic Analysis of *Pseudoperonospora cubensis* Isolates in Peninsula Malaysia

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ABSTRACT

Thirteen isolates of *Pseudoperonospora cubensis*, the causal agent of downy mildew, were collected from cucurbit fields in five states of the western part of Peninsular Malaysia during its growing season between November 2008 and March 2009. The host range of these isolates was determined previously using leaf disc assay and the results indicated that there were 12 pathotypes among these isolates. The objective of this study was to analyze the 13 isolates for phylogenetic relationship using internal transcribed spacers (ITS) of ribosomal DNA (rDNA) and mitochondrial COX-II regions. A high sequence similarity among the 13 isolates and similar sequences from GenBank were detected in ITS (>99%) and COX-II (>98%) regions. Phylogenetic analysis of the 13 isolates based on Minimum Evolution method performed on ITS and COX-II regions revealed five and three groupings, respectively. However, no relationship was found between the phylogenetic groupings using both genes and pathotypes in this study.

Keywords: Pathotype, genetic diversity, cucumber downy mildew

INTRODUCTION

Downy mildew is one of the more common diseases of cucurbits, caused by the obligate biotrophic oomycete, *Pseudoperonospora cubensis* (Berk. et Curt.) Rostow. Recently in Europe and the United States, a severe infection by *P. cubensis* in cucumber plants was reported and the pathogen was demonstrated to have high variability in pathogenicity over 60 cucurbit species (Lebeda & Cohen, 2011). The importance of internal transcribed spacers (ITS) sequence

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analysis of ribosomal DNA (rDNA) and mitochondrial encoded cytochrome c oxidase 2 (COX-II) genes have received increasing attention for their role in evaluating the taxonomic and phylogenetic relationships of species with different degrees of intraspecific diversity (Choi *et al.*, 2006).

To date, there has been little information on intraspecific molecular variation among the *P. cubensis* samples collected from the different areas in Peninsular Malaysia. The aims of this study were to examine the genetic diversity of *P. cubensis* isolates belonging to different pathotypes based on the ITS and COX-II polymorphic regions and to determine which of these two regions could be applied to differentiate isolates belonging to the various pathotypes. A total of 13 isolates were collected from five states of Peninsular Malaysia and their pathotypes were identified previously using the leaf disc method (Salati *et al.*, 2010).

MATERIALS AND METHODS

The isolates of *P. cubensis* identified and characterized previously (Salati *et al.*, 2010) were used for the phylogenetic study. The extraction of the total genomic DNA from leaves dried in silica gel was conducted based on the modified CTAB method. As for the amplification of complete ITS1-5.8S-ITS2 and COX-II regions, the universal primers, ITS5-P2/ITS4 (Voglmayr and Constantinescu, 2008) and COX-II specific primers (Hudspeth *et al*., 2000) were respectively used. The amplified regions were directly sequenced at both strands using an automatic sequencer. DNA sequences were analyzed for similarity using the basic logical alignment search tool (BLAST), while multiple sequence alignment was performed using the CLUSTAL W2 programme. The evolutionary history inferred using the Minimum Evolution method available in MEGA4 (Version 4.0.2) software.

RESULTS AND DISCUSSION

Fragments of 802 bp were obtained from all 13 isolates, whose ITS region was studied (Table 1) and divided into three, namely, ITS1 (1-216 bp), 5.8S rDNA (217-378 bp) and ITS2 (379-802 bp). The length of the ITS sequences is consistent with the finding of Choi *et al.* (2005) from Korea and Sarris *et al.* (2009) from Greece and the Czech Republic. The results of the BLAST analysis comparing ITS sequences in GenBank confirmed that the detected pathogen was *Pseudoperonospora cubensis.* The greatest variations in the whole ITS1-5.8S-ITS2 sequence was observed in ITS2 region (1.9%), followed by ITS1 (1.3%), similar to that found in a study using *Albugo candida* isolates (Choi *et al.*, 2006). All 5.8S region of the 13 isolates studied did not show any polymorphism in nucleotide which could be considered a conserved region. In the phylogenetic analysis, the optimal tree with a branch length sum of 0.04092469 was calculated for the full length of ITS regions (Fig.1). The result revealed five groupings (A, B, C, D and E) among the 13 isolates. Isolates EU660054 (Voglmayr *et al*., 2009), AY608618 (Choi *et al*.,

TABLE 1

GenBank accession numbers of internal transcribed spacers (ITS) and mitochondrial encoded cytochrome c oxidase 2 (COX-II) regions of *P. cubensis* isolates

Isolate	Length (bp)		Accession number in GenBank	
	ITS	COX-II	ITS	COX-II
A1	802	556	HM208310	HM988994
A7	802	556	HM208311	HM988995
A9	802	559	HM208312	HM988996
A10	802	556	HM208313	HM988997
B1	802	557	HM208314	HM988998
B2	802	553	HM208315	HM988999
C1	802	556	HM208316	HM989000
C2	802	556	HM208317	HM989001
C4	802	556	HM208318	HM989002
D1	802	556	HM208319	HM989003
D2	802	556	GU233293	HM989004
D3	802	556	HM208320	HM989005
E1	802	555	HM208321	HM989006

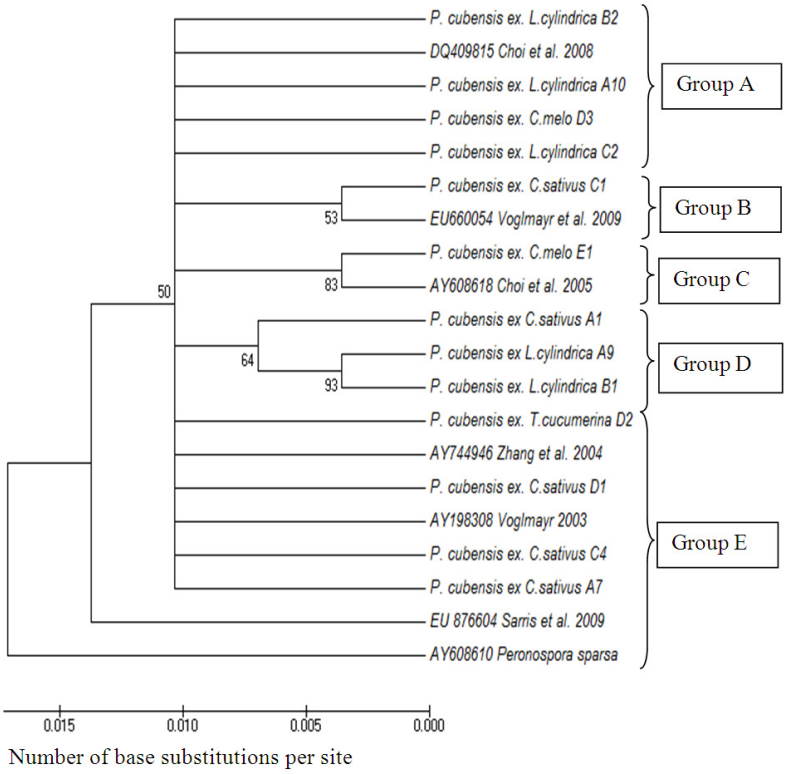

Fig.1: Phylogenetic tree based on the sequencing results on ITS regions of *P. cubensis* isolates (A1, A7, A9, A10, B1, B2, C1, C2, C4, D1, D2, D3 and E1) in comparison with the reference isolates. *Peronospora sparsa* is an out group isolate. The bootstrap number more than 50 was selected in Minimum Evolution tree

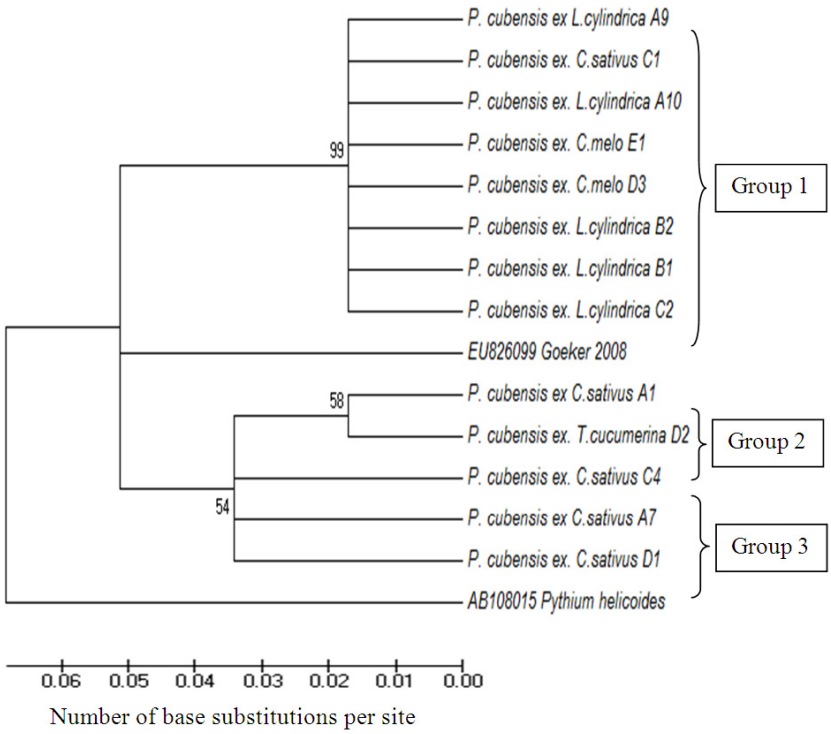

Fig.2: Phylogenetic tree based on the sequencing results on COX-II region of *P. cubensis* isolates (A1, A7, A9, A10, B1, B2, C1, C2, C4, D1, D2, D3 and E1) in comparison with the reference isolate, EU826099, obtained from GenBank. *Pythium helicoides* was selected as an out group species. The bootstrap value more than 50 was selected in Minimum Evolution tree

2005), AY744946 (Zhang *et al*., 2004) and AY198308 (Voglmayr, 2003) from various geographical regions used as comparisons were placed within the five groupings, whereas isolate EU876604 (Sarris *et al*., 2009) placed in a separate branch and *Peronospora sparsa* (AY608610) was shown to be an outgroup. The minimum sequences similarity within the five groupings was detected at 99.5%, 99.5%, 100%, 99.7% and 99.9%, respectively. Two isolates, with the same pathogenic factor and pathotype number (namely, D1 and D2), were placed together in one group (E). In the present study, the ITS sequence homology among the isolates from various hosts was very high similar with that of Choi *et al.* (2005), suggesting that *P. cubensis* is a homogenous taxon.

The COX-II region was amplified from all the 13 isolates with sequences ranging from 553-559 bp (Table 1). Results of the BLAST analysis showed that all the 13 isolates shared 98-99% similarity with *P. cubensis* COX-II sequences in the GenBank. In the phylogenetic analysis, the optimal tree with a branch length sum of 0.14932510 was calculated for COX-II region (see Fig.2). All the 13 isolates were delineated into three groupings (1, 2 and 3), where isolate EU826099 (Goeker, 2008) used as a comparison was placed in

a separate branch. Meanwhile, *Pythium helicoides* (AB108015) was shown to be an outgroup. The minimum sequences similarity within the first, second and third grouping was detected at 99.8%, 99.6% and 100%, respectively. The similarity among the isolates based on the COX-II region was higher than those based on the ITS region indicated by lesser groupings. In contrast to the phylogeny based on the ITS region, two isolates (D1 and D2) with the same pathogenic and pathotype number were placed in different groups of one cluster.

The results of the current study indicated that nuclear locus, demonstrated by the ITS phylogram (five groupings), was more diverse than mitochondria locus, as shown by COX-II phylogram (three groupings) and these findings corroborate with those of Choi *et al.* (2006) and of *Phytophthora capsici* (Quesada-Ocampo *et al*., 2011). Interestingly, the results of the present study also showed that there was no correlation between the genetic clusters of both nuclear and mitochondria loci and the pathotypes of the isolates studied (Salati *et al.*, 2010) similar to that reported of *Phytophthora capsici* (Quesada-Ocampo *et al*., 2011). As a conclusion, the present study demonstrated that the utilization of ITS and COX-II regions was useful for the identification of *P. cubensis* at species level but was rather inadequate for the differentiation of *P. cubensis* pathotypes. Thus, the information suggests that multi-locus analyses and the association of virulence/pathogenicity with particular clusters should be used to obtain higher phylogenetic resolution among the isolates from Peninsula Malaysia and for host resistance screenings (Runge *et al*., 2011; Quesada-Ocampo *et al*., 2011).

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Ultrastructural Features of *Catharanthus roseus* Leaves Infected with Cucumber Mosaic Virus

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ABSTRACT

Catharanthus roseus var. *rosea*, infected with Malaysian isolate of cucumber mosaic virus (CMV-MP), exhibited leaf mosaic, leaf deformation and malformed flowers. Electron microscopic examination of the infected leaf cells revealed significant alteration of the chloroplasts in the mesophyll cells. Large starch grains in necrotic zones and disorganized thylakoid system were the most prominent modifications observed within the chloroplasts of the infected tissues. Meanwhile, membrane-bound vesicles were detected in the vacuoles of the CMV-MP-infected leaf cells. A crystalline array of phytoferritin macromolecules was detected in the chloroplast at 40 days post-inoculation. However, neither single nor aggregate of CMV-MP particles was detected in the cytoplasm due to difficulties in differentiating them from the ribosomes. Nonetheless, structure resembling the inclusion bodies, commonly produced after virus infection, could not be found in the infected leaf cells. Similarly, structure abnormality in the nucleus or mitochondria was also not observed.

Keywords: Catharanthus roseus, cucumber mosaic virus, leaf cells, chloroplast abnormalities

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INTRODUCTION

The cucumber mosaic virus (CMV) has isometric particles about 28-30 nm in diameter and is composed of a capsid (coat) protein shell that encapsidates a single-stranded, positive-sense RNA genome. The virus carries a tripartite genome containing four single stranded positive – sense RNAs

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and has a very wide host range (Palukaitis *et al.,* 1992).

Madagascar periwinkle, *Catharanthus roseus* (L.) G. Don (which is also known as 'kemunting cina' in Malaysia), is an ornamental plant that produces very important anticancer drugs such as vincristine and vinblastine (Manganey *et al*., 1979; Svoboda, 1983; Cragg & Newman, 2005), as well as antihypertensive compounds, ajmalicine and serpentine (van de Heijden *et al*., 2004). The plant has been recorded as one of the hosts for CMV infection (Ong & Ting, 1977; Inon *et al*., 1999). Recently, studies on the aetiology of mosaic symptoms in *C. roseus*, grown wild or cultivated in pots, revealed that an isolate of CMV was the causal agent of the disease (Mazidah *et al*., 2012). The diseased leaves exhibited light and dark green patches and were deformed in shape. The flowers were malformed with slight colour-breaking on the petals. The CMV isolate was characterized at molecular level (Mazidah *et al*., 2012) and its complete coat protein gene was cloned and sequenced (Accession number EU726631). This isolate was identified and designated as Malaysian periwinkle isolate (CMV-MP).

Cytopathological data can reflect viral characteristics which stand independent from particle morphology, particle serology and possibly host reactions in the sense of symptoms, transmission in plants and resistance phenomena (Lesemann, 1988). Studies on the cytopathic effects of CMV infection in leaf cells of host plants with mosaic symptoms (Misawa & Ehara 1966; Honda & Matsui, 1973) and local lesions (Ehara, 1979; Ishihara *et al*., 2002) have been reported. However, no ultrastructural study has been conducted on the leaves of virus-infected *C. roseus.* Thus, this paper reports on the ultrastructural changes in *C. roseus* leaf cells systemically infected with CMV-MP.

MATERIALS AND METHODS

Source of the Virus Isolate

CMV-MP was isolated from the leaves of *C. roseus* var. *rosea,* which exhibited leaf mosaic, leaf deformation and malformed flowers grown in a field in Serdang, Selangor. The inoculum was prepared by grinding symptomatic leaves in 0.01 M phosphate buffer, pH 7.0, containing 0.25% DIECA and carborundum (600 mesh). The extract was mechanically inoculated onto the leaves of healthy *C. roseus* seedlings at four-leave stage. The plants were then kept in an insect proof glasshouse. Ultrastructural observations were made on the second leaves and these showed distinct mosaic symptoms at 12, 25 and 40 days post-inoculation (dpi). The leaf tissues from mock-inoculated plants (inoculated with buffer only) were used as uninfected controls.

Tissue Processing and Electron Microscopic Examination

Small tissue sections (about 2 mm x 2 mm) were excised from the mosaic area of the CMV-MP-infected leaves. Symptomless leaf from the mock-inoculated plant was used as an uninfected control. The selected

tissue samples were fixed in buffered 4% glutaraldehyde solution (pH 7.0) for 48 hours. The fixed specimens were washed three times (at 30 min intervals) with 0.1 M sodium cacodylate buffer, followed by two hours of post-fixing in 1% osmium tetroxide at 4°C. The samples were washed again three times (at 30 min intervals) before they were dehydrated in ascending concentrations of acetone solution, sequentially followed by acetone: resin (1: 1 and 1: 3 v/v) to facilitate the resin entry into the tissues, and finally embedded in epoxy resin. The resin was polymerized at 60°C for 48 hours. Ultrathin sections were made using a diamond knife in an ultramicrotome mounted in copper grids. These sections were then stained with uranyl acetate, followed by lead citrate. The sections were observed under a Hitachi H-7100 transmission electron microscope.

RESULTS AND DISCUSSION

The development of the mosaic symptoms, caused by viral infection, is related to drastic metabolic changes that occur in the host tissue (Kato & Misawa, 1974). Distinct mosaic symptoms were evident on the leaves of CMV-MP-inoculated *C. roseus* as compared to the mock-inoculated ones (Fig.1).

The ultrastructural changes in the *C. roseus* leaf cells were observed in this study following inoculation with CMV-MP. At 12 dpi, the chloroplasts in the infected leaf cells became swollen (Fig.2C and Fig.2D) compared with those in the uninfected leaf cells (Fig.2A and Fig.2B). At 25 dpi, alteration of the chloroplasts was detected (Fig.3A and Fig.3B) compared to those in the uninfected mesophyll cells (Fig.3C and Fig.3D). The alterations were in the form of large starch grain formation in the necrotic

Fig.1: Appearance of mosaic symptom on the CMV-MP-infected leaf (left) compared with the mock-inoculated one (right).

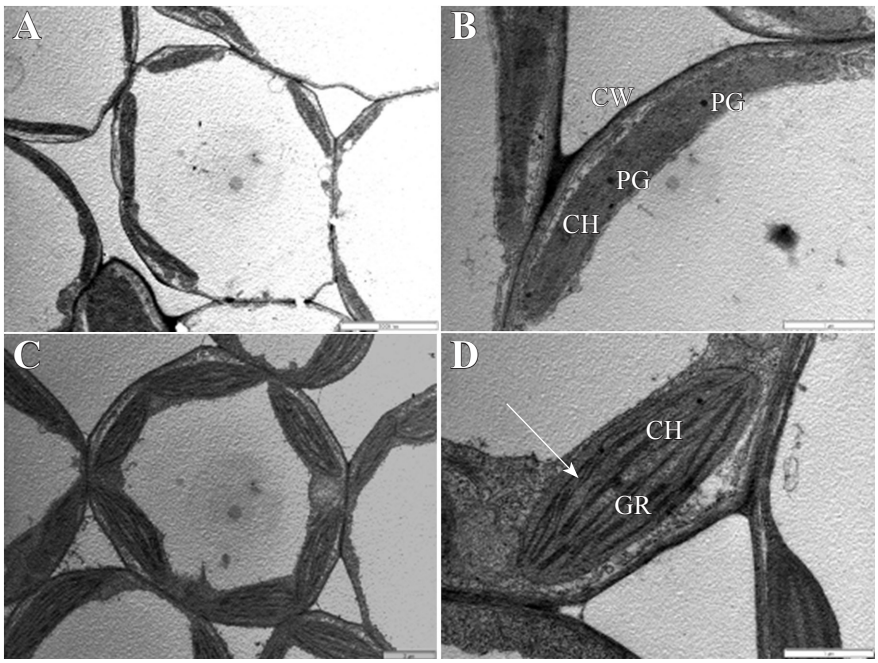

Fig.2: Electron micrographs of the leaf cells from uninfected and CMV-MP-infected *C. roseus* at 12 days post-inoculation. (A and B) Chloroplasts in the mesophyll cells of uninfected leaf tissues at X4,600 and X21,500, respectively. (C and D) Chloroplasts in the mesophyll cells of CMV-MP-infected leaf tissues at X7,700 and X21,500, respectively. A white arrow shows stroma lamellae. PG=plastoglobuli, GR=grana, CW=cell wall, CH=chloroplast

zones and disorganized thylakoid system within some of the chloroplasts in the infected leaves. The stromatic lamellae and grana were disintegrated by the presence of the large starch grains. Similar chloroplast alterations, induced by CMV infection, have been reported by Poolpol and Inouye (1986) who found that the mesophyll cells of a cucumber leaf infected with CMV alone showed deformed chloroplasts with an abnormal thylakoid system and large starch grains.

Vesicular bodies were detected in the vacuoles of the CMV-MP-infected leaf cells (Fig.3E) which were absent in the uninfected leaf tissues. Similar structures have been observed in the vacuoles in the cells surrounding necrotic local lesions of CMV-infected cowpea leaves (Ehara, 1979) and *Nicotiana glutinosa* leaves (Ishihara *et al*., 2002). These membrane-bound vesicles are associated with tonoplasts and may be the sites of viral RNA synthesis (Hatta & Francki, 1981).

Arrays of phytoferritin macromolecules (iron-containing molecules) were detected in the chloroplast stroma of CMV-MP-infected leaf cells at 40 dpi (Fig.4A and Fig.4B). These macromolecules were not detected in the uninfected leaf tissue sections. Wildman and Hunt (1976) detected phytoferritin particles in the chloroplasts of yellow leaf pinnae from coconut palm (*Cocos nucifera* L.). They concluded that these particles

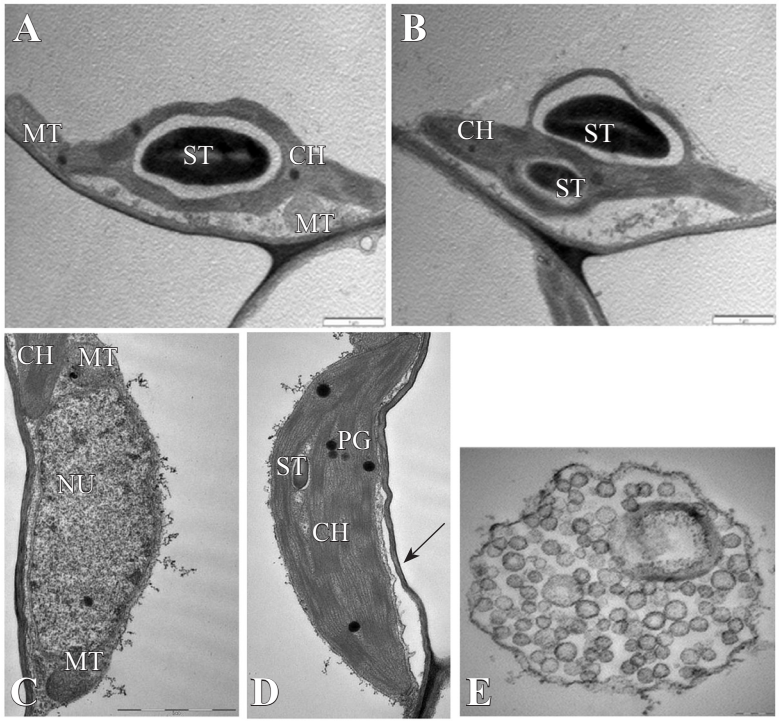

Fig.3: Electron micrographs of the leaf cells from uninfected and CMV-MP-infected *C. roseus* at 25 days post-inoculation. (A) and (B) show the altered chloroplasts with large starch grains and abnormal thylakoid systems in the CMV-MP-infected leaf cells of *C. roseus*. (C) Mitochondria and nucleus in the uninfected leaf cells of *C. roseus*. (D) A structure of chloroplast with a starch grain and a distinct cell wall (arrow) in the uninfected leaf cells of *C. roseus*. (E) Vesicular bodies were detected in the cytoplasm of CMV-MP-infected *C. roseus* leaf cells. (A) and (B) X16,500; (C) X70,000; (D) X17,000; (E) X70,000. CH=chloroplast, ST=starch grain, MT=mitochondrion, NU=nucleus, CW=cell wall, PG=plastoglobuli

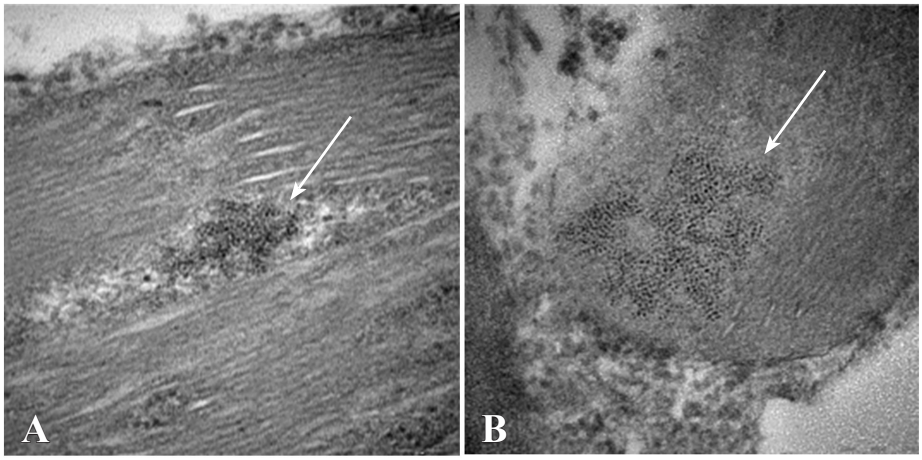

Fig.4: Arrays of phytoferritin macromolecules (white arrows) were detected in the chloroplast stroma of CMV-MP-infected leaf cells at 40 days post-inoculation. (A) X50,000;(B) X100,000.

were a breakdown product associated with the disruption of the chloroplast structure and photosynthetic activity in *C. nucifera,* but were not specifically associated with disease. Tomenius and Oxelfelt (1982) also discovered phytoferritin in the chloroplasts stroma in the chlorotic regions of pea leaf cells which had been infected with red clover mottle virus (strains N and S). They suggested that the phytoferritin molecules were associated with viral infection in the plant.

Starch and phytoferritin are the main energy and iron reserves in plant, respectively. Accumulation of starch and phytoferritin in the CMV-MP-infected *C. roseus* chloroplasts suggested that the metabolism of carbohydrate and iron-containing compounds were affected by viral infection. Meanwhile, alteration of the chloroplasts may have contributed to the mosaic symptom development in CMV-MP-infected *C. roseus.* Nonetheless, no clear evidence of structure abnormality was found in the nuclei or mitochondria. Infection by CMV-MP may not affect these organelles. Inclusion bodies are intracellular structures produced as a result of viral infection, which may contain virus particles, virus-related materials or ordinary cell constituents in a normal or degenerating condition (Sofy *et al.,* 2007). In this study, the inoculated plants were detected positive to CMV infection by DAS-ELISA analysis but no inclusion body was detected in the organelles and in the cytoplasm of the CMV-MP-infected leaf cells under TEM examination. CMV-MP appeared as densely stained particles with the sizes around 20 to 23 nm in diameter and was difficult to be distinguished from cytoplasmic ribosomes (Hatta & Francki, 1979).

CONCLUSION

The present investigation showed that CMV-MP infection in *C. roseus* had induced ultrastructural changes in the leaf cells which were parallel to the morphological alteration of the infected leaves. These ultrastructural changes seemed to be restricted in the chloroplasts. Meanwhile, enlargement of starch granules and accumulation of phytoferritin macromolecules in the infected leaf cells suggest that CMV-MP infection may have interfered the metabolism of carbohydrate and iron-containing compounds in *C. roseus*. Modification of the chloroplast structure following CMV-MP infection may have attributed to mosaic symptoms development.

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REFEREES FOR THE PERTANIKA JOURNAL OF TROPICAL AGRICULTURAL SCIENCE

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While every effort has been made to include a complete list of referees for the period stated above, however if any name(s) have been omitted unintentionally or spelt incorrectly, please notify the Executive Editor, *Pertanika* Journals at nayan@upm.my.

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Journal of Tropical Agricultural Science

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Revised: September 2012

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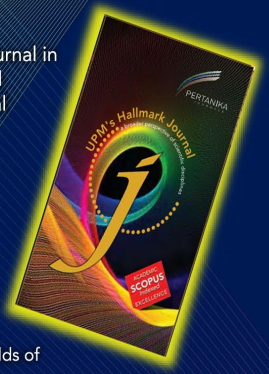

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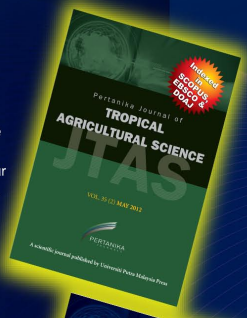

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