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PLANT PHYSIOLOGY IN ADDRESSING GREEN ECONOMY

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23rd Malaysian Society of Plant Physiology Conference (MSPPC 2012)

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CHAPTER 1

PLANT GROWTH, DEVELOPMENT AND PRODUCTION

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Paddy Tiller Strength in Relations to Nitrogen Rate and Soil Nutrient Status: A Case Study at Kg. Gelam, MADA

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Introduction

The state of tiller strength is one of the factors that can prevent lodging phenomena in paddy. Lodging is permanent displacement of cereal stems from their upright position. There are two possible points of failure in the paddy plant's structure, the stem and the root (Nelson, 2001). The elongated stem consists of a series of jointed hollow internodes connected by solid swollen meristematic nodes. The stem is strengthened by lignin, but may fail due to bending or buckling of the lower stem internodes. Root lodging results from a failure in root-soil integrity so that straight, unbroken culms lean or fall from the crown (Baker et al., 1998).

Lodging seemed to be a physiological phenomenon rather than a varietal character. It is originated from structural weakness development in culm tissues and is caused primarily by deeper submergence of plants during vegetative growth aided by high nitrogen concentration in soil (Basak et al., 2006). Debate is continuing amongst scientists and growers as to whether stem lodging or root lodging predominates. Whatever the form of lodging, it is generally agreed that it is due to an interaction of the plant with the characteristics of the rain, wind and soil (Sterling et al., 2003). Their influence on lodging risk has been shown to be through their ability to alter crop structure by affecting certain plant characteristics. Understanding of the complex interaction between husbandry, weather and soil, which can result in lodging, has only recently begun, and consequently guidelines for reducing lodging are often based on perceived wisdom rather than comprehension (Sterling et al., 2003).

CREST and TRANS-pack (Urea 50kg/ha) production package applying the principle of balanced nutrition to reduce susceptibility for crop lodging has been proven in MARDI-MADA rice estate project (Suhaimi, 2007). In (Muda Agriculture Development Area) MADA, lodging usually occurs in wet season and this phenomenon may be influenced by inappropriate fertilizer application. Excessive nitrogen application may cause lodging due to weaker stem hardness and heavy panicle condition.

The objective of this study was to assess the relationship between the N fertilization rate and soil nutrient status on cellulose content the state of paddy tiller strength in Kg. Gelam, MADA, Kedah. Lodging phenomena in rice are contributed by many factors including environment, weedy rice and pest infestation. But fertilizer rate in this case Nitrogen also is one of the main factors that can cause lodging. Appropriate fertilizer rate is very important in order to get optimum plant growth without causing lodging.

Materials and Methods

The study was carried out at Kg. Gelam, a model of paddy field at MADA due to its reputation achieving high yield very season. The soil is Kangkung Series which is first class soil. (Suhaimi, 2007) Four treatments of N (Urea) fertilizer was used i.e control (T1), 50 kg (CREST-Pack recommendation) (T3), 100 kg (farmer's current practice) (T3) and last treatment consisted 200 kg (T4). The fertilizer type is straight fertilizer which is Urea 46%. In this experiment Complete Randomized Design (CRD) were used with 5 replications on each treatment. Each replicate consisted of 5 plants.

Urea as a source of N was applied during 35 day after sowing (DAS) and data were taken at 75 DAS. Plant height was measured from the ground to the highest shoot tip using 2 m ruler. Tiller size was measured every 7 days from 30 DAS at 3 to 4 cm from the ground and in field water depth level was maintain at 8 to 9 cm. To measure the chlorophyll content, chlorophyll meter (SPAD 502, Minolta Japan) was used, and data were taken

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at 10 a.m in the morning at flag leaf every 5 days starting from 30 DAS. Texture analyzer machine (TA-HD *Plus*) was used to analyze the tiller strength. To analyze the tiller strength, only 0.8 cm tiller size was used to get consistent result and the pressure was applied on internodes. The pressure weight will be set 5kg constantly for every sample. The pressure will stop once the paddy stem sample break. Once the pressure panel stops, the breakness data will be automated save in the software folder in the computer.

Results and Discussion

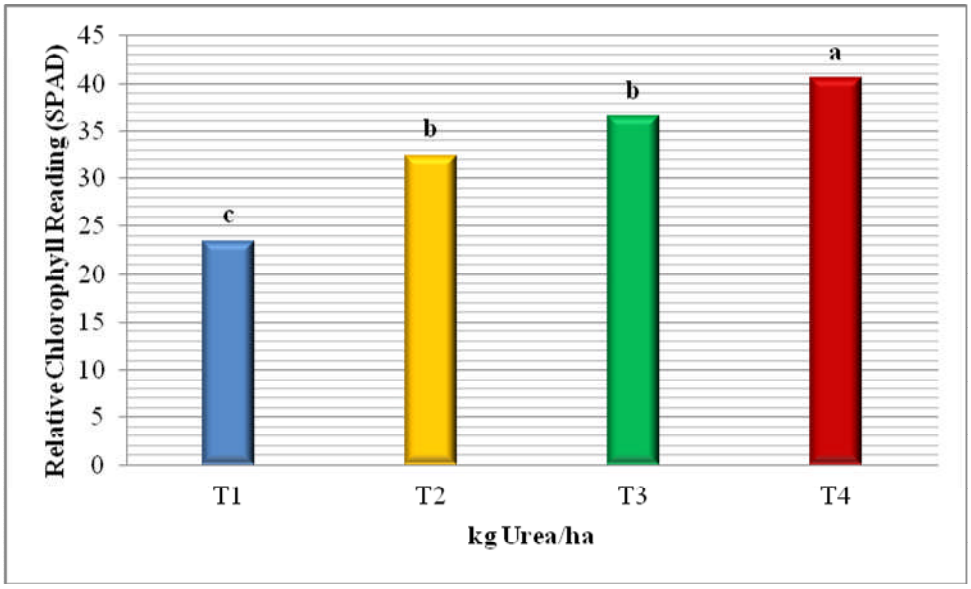

Figure 1. Comparisons on chlorophyll content. Means with the same letter(s) are not significantly different by LSD P≤0.05
T1= 0 kg Urea/ha (Control)
T2= 50 kg Urea/ha (CREST-Pack)
T3= 100 kg Urea/ha (Farmer's current practice)
T4= 200 kg Urea/ha

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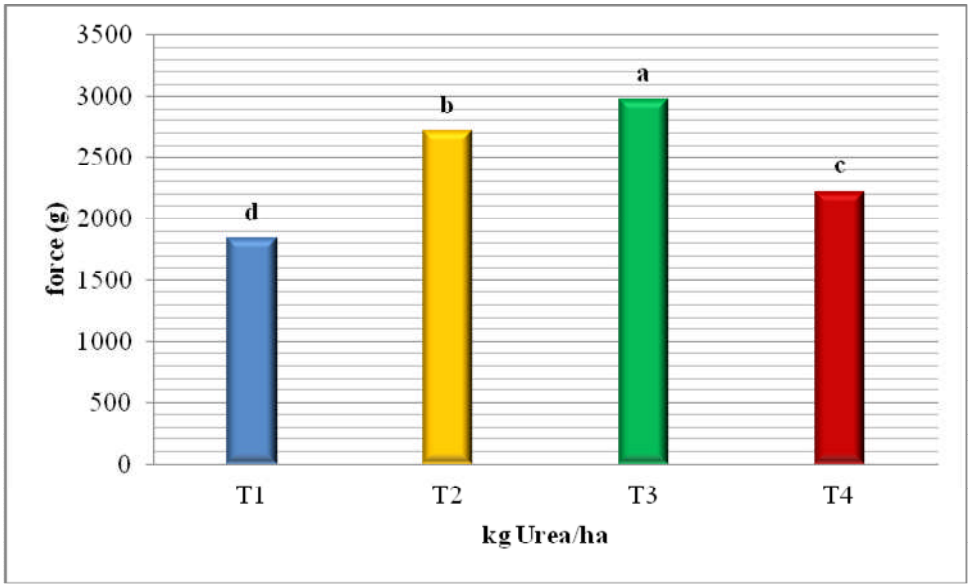

Figure 2. Comparison on breaking stress for tiller. Means with the same letter(s) are not significantly different by LSD P≤0.05
T1= 0 kg Urea/ha (Control)
T2= 50 kg Urea/ha (CREST-Pack)
T3= 100 kg Urea/ha (Farmer's current practice)
T4= 200 kg Urea/ha

Table 1. Cellulose content in paddy stem from nutrition analysis

Treatment (kg Urea/ha)	75 DAS (%w/w)	90 DAS (%w/w)	110 DAS (%w/w)
T1 (0)	**6.0bc**	**6.5b**	**9.1c**
T2 (50)	**6.8b**	**6.9b**	**10.5b**
T3 (100)	**8.7a**	**8.9a**	**13.3a**
T4 (200)	**6.7b**	**6.8b**	**11.0b**

Means with the same letter(s) are not significantly different by LSD P≤0.05

Table 2. Tiller size for lower part of tiller

Treatment (kg Urea/ha)	75 DAS (cm)	90 DAS (cm)	110 DAS (cm)
T1 (0)	**0.5ab**	**0.5b**	**0.5b**
T2 (50)	**0.6a**	**0.6b**	**0.6b**
T3 (100)	**0.6a**	**0.8a**	**0.8a**
T4 (200)	**0.5ab**	**0.5b**	**0.5b**

Means with the same letter(s) are not significantly different by LSD P≤0.05

From Table 1, the cellulose content in T3 is the highest. Cellulose is the important element in plant cell that give hardness to plant (Anne et al., 2000). The cellulose content is the highest in T3 because of 100 kg urea/ha give the optimum plant growth as we can see in tiller size (Table 2).

From Figure 2, the result in T3 was significant with the tiller size and cellulose content. The tiller in T3 was the hardest tiller among all the treatments. This is because 100 kg urea/ha give the optimum plant growth.

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From prediction, 200 kg urea/ha will produce the biggest tiller but this rate have caused increases in paddy tiller production. So the competition between paddies has caused small tiller paddy production (Yoshida, 1981).

Table 3. Soil nutrient analysis in Kg. Gelam, MADA

Description	Analysis Results
pH	4.7
Silica (SiO_2)	67.7 %w/w
Nitrogen (N)	0.27 %w/w
Phosphorus (P_2O_5)	0.88 %w/w
Potassium (K_2O)	0.16 %w/w
Magnesium (MgO)	726.8 ppm
Calcium (CaO)	1.07 %w/w
Cation Exchange Capacity	28.6 Meq/100g
Total Organic Matter	14.2 %w/w

From Table 3, soil nutrition analysis, it can be seen that average CEC and Silica is basically high at this area. Thus, it can improve nutrition uptake including silica which is high at this area (Savant et, al. 1996). Silica is a nutrient that can make rice stem harder than normal if planted with the same fertilizer rate at other granary area (Nader et al., 2012). From SPAD meter reading (Figure 1), treatment 50 kg urea/ha show the best rate of Nitrogen for chlorophyll content. According to CREST-Pack recommendation, SPAD meter reading should be between 30 to 33 to get the optimum growth and yield of paddy (Suhaimi, 2007). Although treatment with 100 kg urea/ha has achieved hardest tiller (SPAD meter reading is above 35), it showed N fertilizer has been excessive but it gave no significant different with 50 kg Urea/ha.

Appropriate fertilizer application increased chlorophyll content and increase tiller strength. There were significant changing in tiller strength in fertilizer rate between 50 kg to 100 kg, but obviously decreased in 200 kg fertilizer rate. This study shows that soil characteristics such as CEC have the capacity to increase the uptake of both silica and nitrogen. However, inappropriate fertilizer rate may affect the state of tiller strength although constantly increase the chlorophyll content.

Conclusions

In conclusion, 100kg Urea/ha is the best rate of nitrogen application because it's give the optimum growth of paddy and tiller strength in order to prevent paddy plant from lodging occurrence.

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Correlating the Absorption and Reflection Spectrum of Chlorophyll, Carotenoid and Anthocyanin Pigments Using Green Devices

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Introduction

Most of the leaves around us are green in colour, but if observed carefully there is some species of leaves that grows with different colours such as red and yellow. Generally, each pigment will give different colour to the plants. In previous study, most of the relations concerning leaf reflectance and pigment content have been extend and tested for only one or at most a few strongly correlated species (Chappelle, et al., 1992; Gamon and Surfus, 1999). Pigment molecules absorb light of different wavelengths so that our eyes see the colours of the spectrum that they reflect or transmit. Chlorophyll gives the basic green colour. Lichtenthaler (1987) suggested that, carotenoids are the major absorbers in yellow leaves, it produce yellow, orange and brown colour as observed in corn and banana. Anthocyanins give the pink-red colours of most flower petals, of most red fruits and almost all red leaves during the autumn. So for each type of pigments the absorption spectrum will also be different since each of them produces different types of colour.

The plant samples used in this study were selected based on their colour. Green and yellow sample were taken from *Codiaeum variegatum* and red sample were taken from Firebrand *Cordyline*. Spectrometer is used to obtain the reflectance spectrum of the plant sample. Different LEDs (which are green devices) are used as the light source. The reflection spectrum obtained from the spectrometer is compared with the absorption spectrum of the plant sample according to their colour or pigments. Therefore, the objective of this study was to determine the correlation between the absorption and reflection spectrum of chlorophyll, carotenoid and anthocyanin pigments.

Materials and Methods

In this project the main instruments that used was the fiber optic spectrometer (USB2000, Ocean Optics). Spectrometer is an instrument that is used to measure properties of light over a specific portion of the electromagnetic spectrum. Usually it is used in spectroscopic analysis to identify types of elements contain in a material. Frequently the variable measured was the light intensity, and the polarization state. This spectrometer was used with the OOI32 base operating software. This software was used to display the reflection spectrum obtain from the spectrometer. The light source in this experiment is white, green, red, and yellow LED. The other equipment used in this experiment was a power source, a box to hold the sample, the fiber optic probe (y-probe), and a computer to analyses the spectrum obtained. Figure 1 shows experimental set up for this project.

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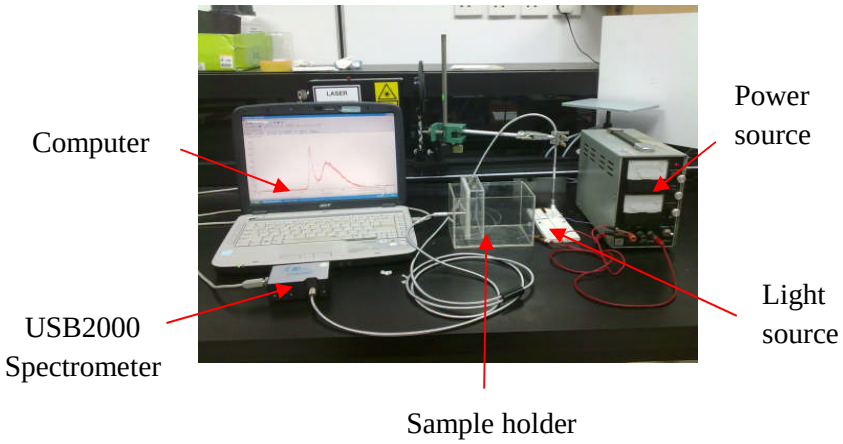

Figure 1. Experimental setup

Two species of plants had been selected, *Codiaeum variegatum* (green and yellow sample), and Firebrand *Cordyline* (red sample). The fresh leaves were cut and placed in the sample holder. The leaves sample was then stabilized at an appropriate angle for observation. An analysis was done by finding the value of the peak of the light intensity, and the area under the spectral line. There are four types of LED colour used as the light source. All those LED are used to obtain the reflection spectrum of the leaves and a white paper (as the reference). The reflection spectrum was then analyzed using OOIBase32 software. This software visualized the spectrum of the samples obtained from the spectrometer. The area under the spectrum peak was then calculated to compare the percentage of absorption for each sample.

Results and Discussion

The spectrums obtained by using white LED as the light source is showing two obvious peaks as shown in Figure 2. For each sample the peaks occur at the same wavelength (Figure 3). First peak (Peak 1) occurs at 443.77 nm and the second peak (Peak 2) at 541.41 nm. In order to calculate the area, the range of the wavelength covered the peak area was chosen it is between 400.01 nm until 474.86 nm for Peak 1 and for Peak 2 from 474.86 nm until 700.15 nm.

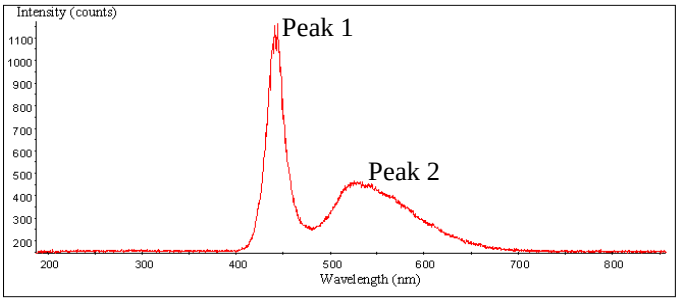

Figure 2. The spectrum obtained by using white LED as the light source

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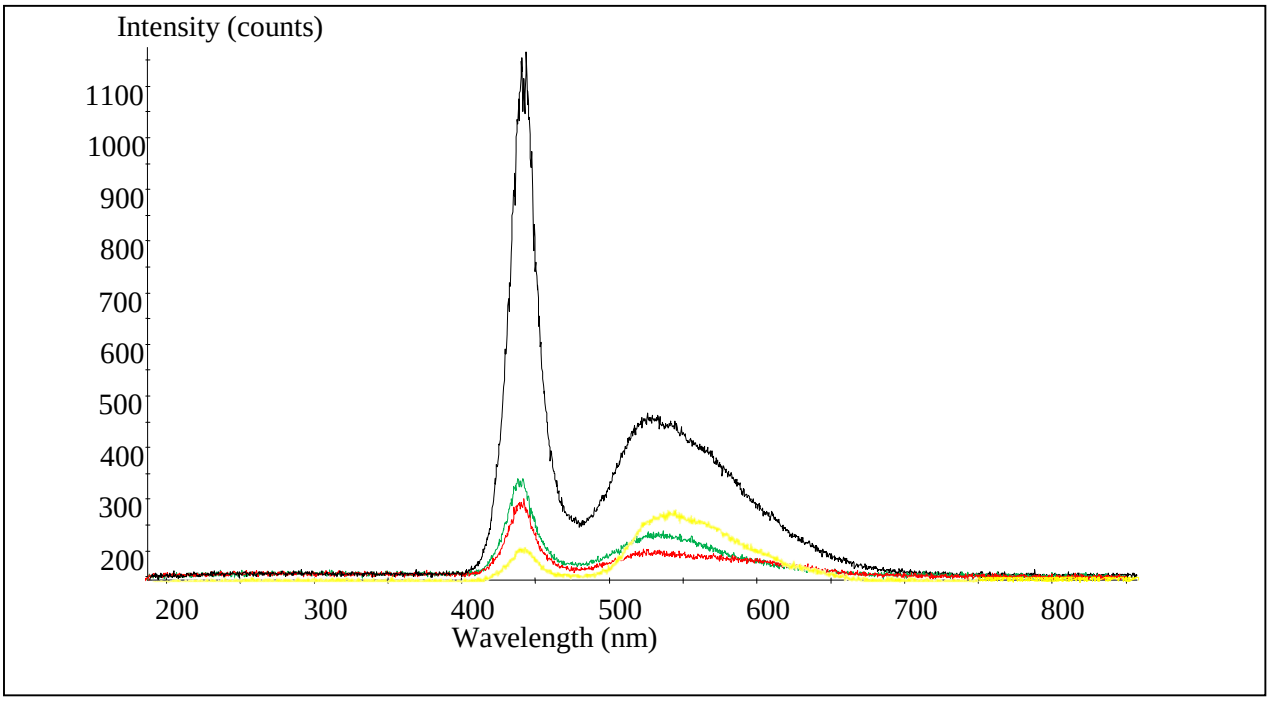

Figure 3. The spectrum of each sample using white LED as the light source

Table 1. The area under the peaks using the white LED as the light source and the percentage of the area compare to the area of the white sample.

Sample	Area under Peak 1		Area under Peak 2		Total Area	
	A_{p1} (±100) (counts.nm)	Percentage absorption (%)	A_{p2}(±100) (counts.nm)	Percentage absorption (%)	$A_{p1}+A_{p2}$ (±100) (counts.nm)	Percentage absorption (%)
White	36 600	-	69 600	-	106 200	-
Green	16 600	55 %	45 300	35 %	61 900	42 %
Red	15 400	58 %	42 700	39 %	58 100	45 %
Yellow	13 600	63%	49 600	29 %	63 200	40 %

Table shows the area under the peaks and the percentage of how much the area under the peak of the spectrum of the plant sample was reduced as compared to the area under the peaks of the white sample spectrum. The percentage was calculated using Equation 1:

$$\text{Percentage absorption} = 100 - \left(\frac{\text{area}_{\text{green,red,yellow}}}{\text{area}_{\text{white}}} \times 100\right) \quad (1)$$

In Table 1, the highest estimation of the absorption percentage under Peak 1 is 63%. It means that, in this region, the yellow wavelength was absorbed the most. While 58% for red sample and the least absorbed was green with 55%. While for Peak 2, red was absorbed the most with 39%, followed by green with 35%. The least absorb was yellow with 29%. Overall, the colour that was absorbed the most was red with the total percentage 45% followed by green with 42%, and the least absorbed is yellow with 40%.

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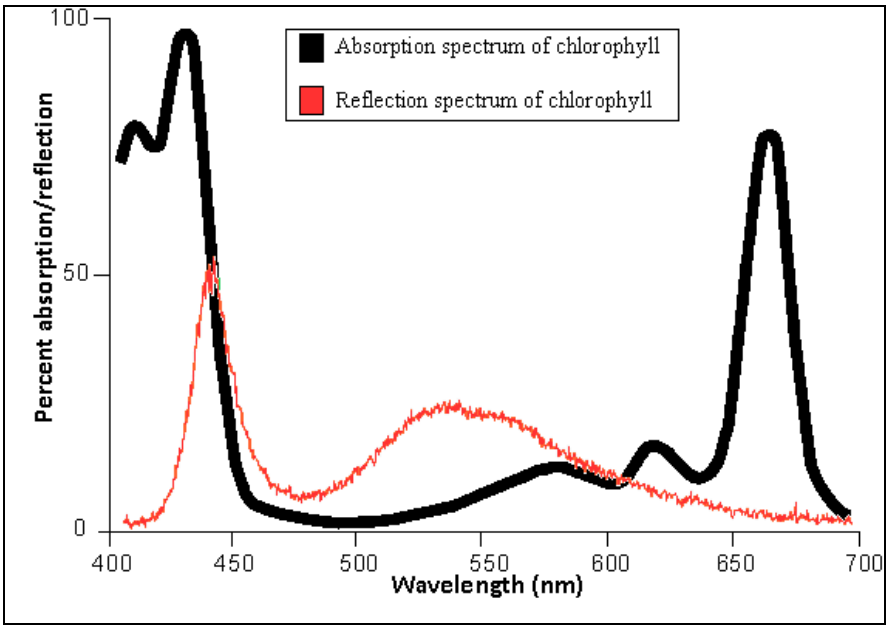

Figure 4. Combination of reflection and the absorption spectrum of green plant

As shown in Figure 4, green sample absorb less than the red (42%). This was due to the location of the peak outside the strong absorbing region of chlorophyll especially for Peak 2. While Peak 1, was partially inside the high absorbing area of the chlorophyll.

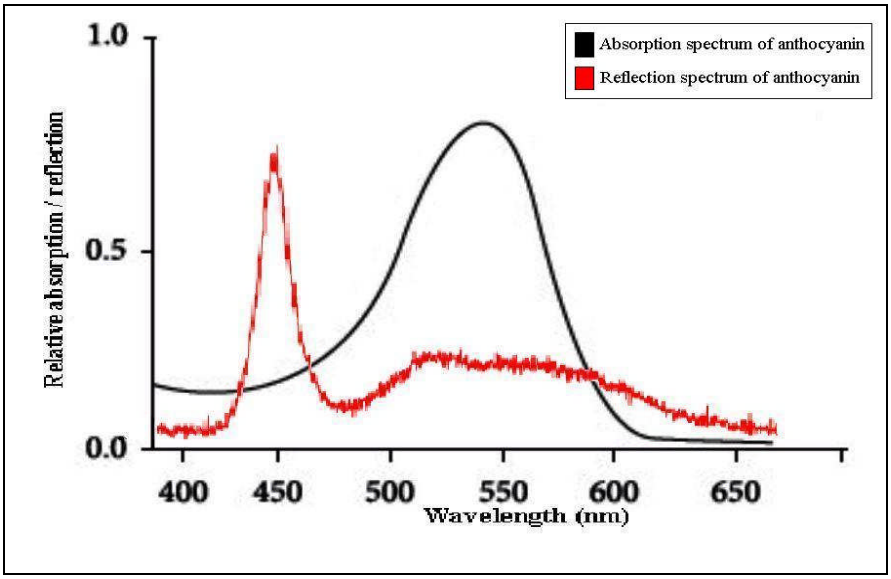

Figure 5. Combination of reflection and the absorption spectrum of red plant

In Figure 5, Peak 1 occurs within the low absorption region of the anthocyanin (400 nm to 500 nm) where the reflectivity was high and the absorption curve was at low intensity. The location of Peak 2 (470 nm to 600 nm) was within the absorbing region of anthocyanin where the Peak 2 was reduced 70%. Which means almost 70% of the white light was absorbed, giving low reflection intensity. The red sample absorbs the most (48%), compared to the other two samples.

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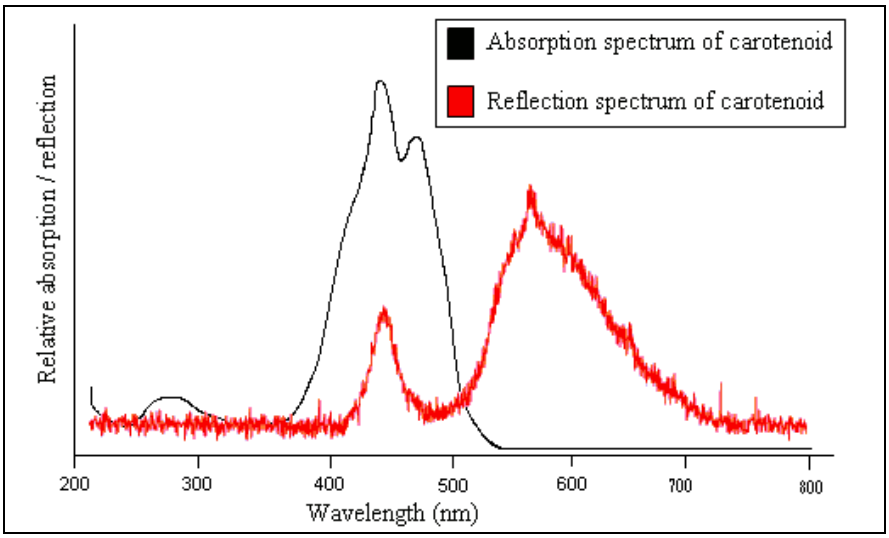

Figure 6. Combination of reflection and the absorption spectrum of yellow plant

Based on Figure 6, absorption intensity is the highest in between 400 to 500 nm, which explain the low intensity of Peak 1 where most of the white light had been absorbed by the yellow plant sample. The absorbance in the blue range for yellow leaves is mainly related to carotenoids pigments (Zur et al., 2000). Around 530 nm to 670 nm (Peak 2 region), light was absorbed the least. This can be explained by the high intensity of the reflection spectrum. Unlike other sample, the reflection spectrum of yellow sample was different since the intensity of Peak 2 is higher than Peak 1. This is because the absorbing intensity of carotenoid (yellow pigments) was the highest within the region of Peak 1.

Conclusions

In conclusion, there was a relationship between the absorption and the reflection spectrum. All of the reflection spectrum obtained can be explained by comparing it to their respective types of sample. This study also showed that both of the reflection and the absorption spectrum support each other properties.

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Response of Different Media Ratio on Growth and Biomass Production of *Andrographis paniculata* (Hempedu Bumi) Grown under Soilless Culture System

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Introduction

Andrographis is a large genus of herbs and shrubs and distributed mostly in the tropical and moist region. *Andrographis paniculata* (Burm. F.) Wallich ex. Nees (Family Acanthaceae) is one of the major medicinal plants in the tropics commonly known as Kalmegh or King of Bitters (Chauhan et al., 2009; Valdiani et al., 2012). In Malaysia, this herb is commonly known as hempedu bumi. It has several alternate uses and all its parts are useful, especially the aerial parts of the plant (stems and leaves). The mature leaves of *A. paniculata* contain maximum active principles like andrographolide, 14-deoxy-11, 12-didehydro-andrographolide and neo-andrographolide, which were reported to possess immunostimulant and anti-cancer (Kumar et al., 2004), antipyretic (Saxena et al., 2000), anti-inflammatory (Shen et al., 2002), anti-diarrhea properties (Valdiani et al., 2012) and as well as anti-malarial (Mishra et al., 2009). Andrographolide is the major constituent in leaves which is bitter substance (Gorte, 1911). The leaves of the herb were found to contain the highest amount of andrographolide and the seeds contained the lowest. In Malaysia, due to increasing popularity of alternative medication and natural health products, the demand for herbs has increased tremendously over the past few years. In 2009, a total of 578 hectares of land was used to produce 2,800 metric tonnes of herbs, increasing from 351 hectares of land used with the production of 1,317 metric tonnes in 2007 (DOA, 2011). Due to the extensive knowledge of herbs for treatment of various ailments and the recognization of biomedical values in the global scenario, the Malaysian Economic Transformation Program through NKEA Agriculture sector has identified hempedu bumi as one of the high value herbal crops that need to be commercially exploited for a new source of economic growth in the herbal industry.

Conventionally, hempedu bumi has been cultivated on beds of soil. The local herb industry is beset by importation, seasonality, low biomass production and quality. Yearly, Malaysia has to import 70% of 50 herbs utilized by local herbal industries were imported while the other 30% came from local sources (Mohamad Setefarzi and Mansor, 2001) hence interventions are necessary to enhance whole year production. Modern technologies such as protective cultivation, container growing and soilless culture are some cultural management practices that will enable year round production of hempedu bumi biomass. For sustainable commercial cultivation of this valuable herb, optimum agronomic practices under soilless cultivation need to be examined to enhance the sustainability of hempedu bumi biomass production. Many studies have revealed that soilless culture system can be a solution to overcome the problems of soil salinity, soil born pest, chemical residue and infertility of soil or accumulation of pest and disease in conventional cultivation. Several types of substrate can be used in soilless culture system as long as it is an inert media. Bistamam (2009) has reported that combination of 75% coconut coir dust (CCD) and 25% peat increased biomass of basil, while 100% CCD decreased dry weight of shoot, stem and root of chillies (Siti Suliza, 2008). Yahya et al. (2009) have found that good potting media for *Celosia cristata* was 70% CCD and 30% burnt rice hull (BRH). The plant was also reported to have highest plant height, total leaf area, heaviest stem, leaf, and root dry weight. In this study, five different ratios of CCD and BRH were used to evaluate their responses on the various morpho-physiological parameters and biomass production grown under soilless culture system.

Materials and Methods

Planting materials and maintenance

The experiment was conducted in the Control Environment Structure, Agro Technology Unit, Taman Pertanian Universiti, UPM, Selangor. The hempedu bumi seed coats were removed using the rough surface of

sandpaper (No. 120) to break the seed dormancy. Then, the seeds were soaked in the hot water (70°C) for 10 minutes before sowing in the petri dish and germinated in a germinator (28°C, 75% RH) at the Agrogene Bank, Faculty of Agriculture, UPM. Seedlings with expanded cotyledons were transferred into jiffy-pot until 6 to 8 leaf stage. The seedlings were then transplanted into poly-bags measuring 12" × 14" with different ratios of CCD and BRH to start the experiment. Fertilizer and water were supplied to the plants via drip irrigation system called fertigation technique. Fertigation supplied both water and plant nutrients at the same time as the fertilizers were dissolved in the water before added into fertilizer tank. In this study, fertilization was given by using Cooper formulation (Cooper, 1979). An automatic pump was used to supply the fertilizers from the tank by drip irrigation system. The plants irrigated four times a day for 10 minutes per cycle. Each poly-bag was installed with a pen dripper. In order to make sure nutrient was available for root uptake, electrical conductivity (EC) and pH of solution were maintained at the optimum range.

Experimental design and treatments

The experiment was conducted using the Randomized Complete Block Design (RCBD). There were 120 experimental units with five treatments and four replicates. The treatments were 100% CCD (T1), 75% CCD: 25% BRH (T2), 50% CCD: 50% BRH (T3), 25% CCD: 75% BRH (T4) and 100% BRH (T5). The parameters were taken at eight weeks after transplanting, including plant height, total leaf area (Automatic Leaf Area Meter, Model LI-3100), leaf and root fresh weight, leaf and root dry weight and root shoot ratio (RSR). Three young and fully expanded leaves per plant were used to measure relative chlorophyll content by using Minolta-Chlorophyll Meter (SPAD502). Net photosynthesis and stomata conductance were measured by using Portable Photosynthesis Machine Model LICOR (LI-6400). For each plant, three young and fully expanded leaves were used.

Statistical analysis

Data were analyzed using Analysis of Variance (ANOVA) by using Statistical Analysis System (SAS) Programme. The mean separations were carried out using Tukey's Honestly Significant Differences (HSD) with $P \leq 0.05$ for significant difference and $P \leq 0.01$ for high significant difference.

Results and Discussion

Plant height was significantly affected by different media ratios ($P<0.01$) as shown in Figure 1 (left). That with media of 100% CCD (control, T1) was significantly higher than that in T2 and T4. There was no significant change in plant height between T1, T3 and T5. This finding raised a question whether the media ratios can promote stem elongation in hempedu bumi due to insignificant difference between the control media and T3 media. However, T2 media significantly reduced plant height compared to control media. This finding obviously varied from the previous study done by Yahya et al. (2009). They found that 70% CCD incorporated with 30% BRH gave the highest plant height in *C. cristata* as compared to the media of 100% CCD. In this study, T2, however, gave the lowest plant height among all the under study treatments.

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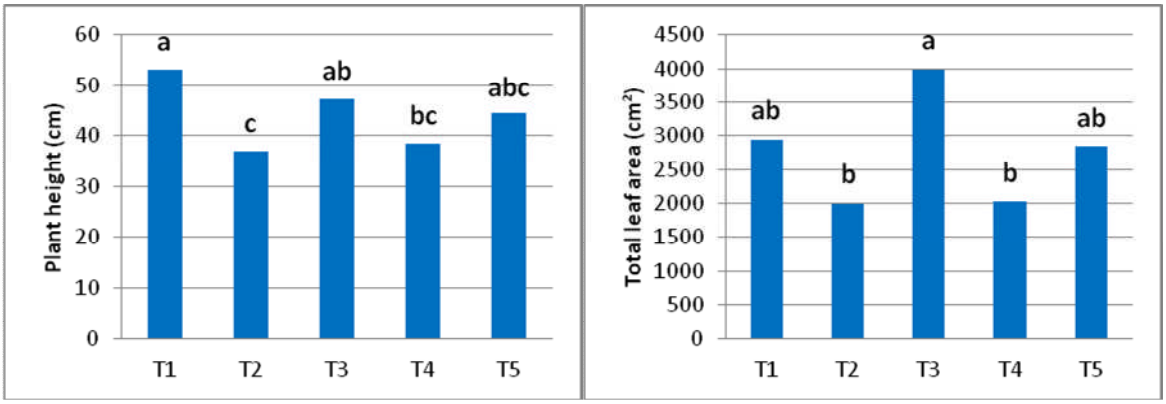

Figure 1. The effects of different media ratios on plant height (left) and total leaf area (right). Treatments applied were T1: 100% CCD, T2: 70% CCD: 30% BRH, T3: 50% CCD: 50% BRH, T4: 30% CCD: 70% BRH and T5: 100% BRH. Means followed by the same letter are not significantly different according to Tukey's Honestly Significant differences at 5% level.

Total leaf area of hempedu bumi was significantly ($P<0.05$) influenced by different media ratios as shown in Figure 1 (right). T3 media gave significantly highest total leaf area as compared to T2 and T4 media. A similar finding was reported in *Lactuca sativa* (lettuce) (Ananda and Ahundeniya, 2001). They found that total leaf of lettuce was higher in 50% CCD: 50% BRH media. Nevertheless, again these results varied from the finding obtained by Yahya et al. (2009). They reported that total leaf area of *C. cristata* was largest in the media ratio of 70% CCD: 30% BRH than that grown in 100% CCD. Although the reason for inconsistent results of media ratios is unknown, combination of CCD and BRH remained with very good influence on total leaf area of hempedu bumi as T3 media gave the highest total leaf area (3991.4 cm^2). In general, CCD was associated to have high water holding capacity but poor in aeration that would cause decreases in oxygen diffusion to the roots (Abad et al., 2002). The combination of both substrates can increase the diffusion of oxygen to the roots which leads to a good plant growth. At vegetative stage, leaves and roots scramble for carbohydrate assimilation. So, to gain an increase in leaf area, the growth of leaf and root should be in well balance.

The leaf fresh weight was affected by different media ratios. T3 media significantly increased leaf fresh and dry weight compared to T2 and T4 media (Figure 2, left). At early vegetative growth, roots were the source for all inorganic water and nutrient which were transported through xylem to the leaves. While the leaves produced carbohydrates through photosynthesis process and transported them to the roots via phloem. The reason for higher leaf fresh weight in T3 might be due to greater increase in nutrient uptake in that medium as compared to the others (Silber et al., 2003). Addition of BRH to CCD media probably improved the chemical properties of growing media. According to Suthamathy and Seran (2011), BRH has high percentage of potassium (K) which contributes as a driving force to solute flow in xylem and phloem. Accumulation of carbohydrates in leaves increases as K uptake in root system increases (Singh and Verma, 2001).

We discovered that there was high significant difference in leaf dry weight ($P<0.01$) among the treatments (Figure 2, right). The heaviest leaf dry weight was obtained from plant grown under T1, T3 and T5 media. Those treatments did not vary significantly to each other but significantly varied to T2 and T4 media. According to Akita (1995), dry matters of leaf were associated with the activities of photosynthesis and metabolism of protein. Clearly, this result has relationship with total leaf area of the plant. T1, T3 and T5 media resulted in the largest total leaf area among all the treatments. High leaf dry weight was related to high leaf area that possibly increased the photosynthetic process in the leaf and promoted higher plant assimilation.

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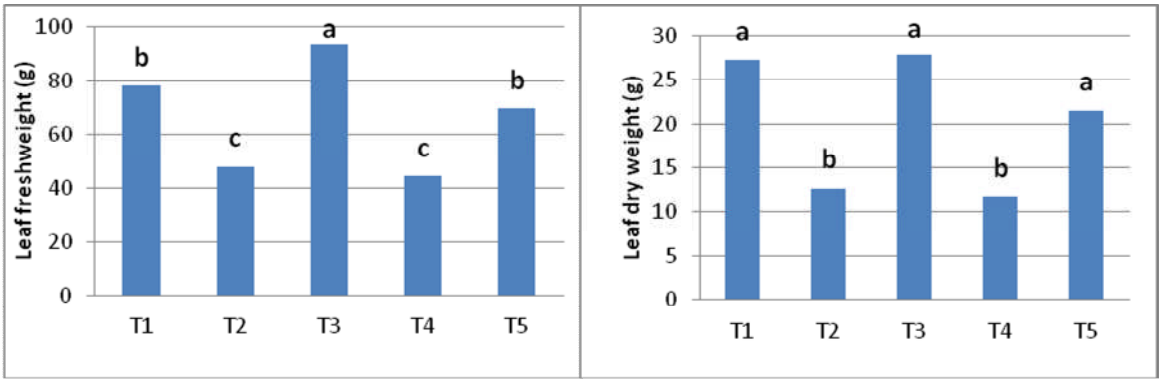

Figure 2. The effects of different media ratios on leaf fresh weight (left) and leaf dry weight (right). Treatments applied were T1: 100% CCD, T2: 70% CCD: 30% BRH, T3: 50% CCD: 50% BRH, T4: 30% CCD: 70% BRH and T5: 100% BRH. Means followed by the same letter are not significant different according to Tukey's Honestly Significant Differences at 5% level.

Root fresh weight was significantly affected ($P<0.01$) by different media rations (Figure 3, left). Based on the results, T1 and T3 media gave the heaviest root fresh weight among all the treatments and were not significantly different to each other. The lowest root fresh weight was obtained from plant grown in T2, T4 and T5 media. The reason of heavier root fresh weight in T1 and T3 media may be attributed to the high water absorption in CCD. In contrast, BRH was associated to have more spore space but lower water holding capacity than CCD (Tsakaldmi, 2006). This characteristic probably gave an effect to the root fresh weight. Since BRH could not hold water longer than CCD, water and nutrients given by drip irrigation were possibly leached out before the root had the time to up take all of them. This would give an effect on the water and nutrient uptake in the roots. Consequently, media with high proportion of BRH would have lower growth of roots than CCD.

Figure 3 (right) showed that different media ratio resulted in high significant ($P<0.01$) difference on root dry weight. Similar to the previous result of root fresh weight, at eight weeks after transplanting, the heaviest root dry weight was obtained in T1 and T3 media. These treatments gave significantly the heaviest root dry weight among all the under study treatments. Average root dry weight ranged from 3.5725 g to 8.4503 g. In comparison between root fresh weight and root dry weight, root dry weight would provide a better measurement for featuring total mass of root in the media. The nutrient supplied in T1 and T3 media might be enhanced by the physical properties of the media. Good aeration and high water availability within the media could be the factor for promoting good root growth. T3 media with the heaviest root dry weight perhaps had sufficient water and oxygen for plant growth, especially in the root system. According to Yahya et al. (2009), media that has good balance in air and water holding capacity would increase root dry weight of *C. cristata*. In addition, BRH has very fine texture like sand or dust particle. A compact media was probably attained which led to low favourable condition for root growth.

The results shown in Figure 4 (left) indicated that RSR was significantly different ($P<0.05$) among treatments. Average RSR ranged from 0.173 to 0.388. Unlike other parameters, the highest RSR was obtained in T2 media. However, it did not differ significantly from that with T1, T3 and T4 media. Increase in RSR gave an indication that photo-assimilation accumulated more in the root system (Muhammad, 2008). In another word, roots of plant grown under CCD-based media had greater priority in allocation of photosynthetic product than shoots. In contrast, assimilation in root system was lower when plant was grown under media containing 100% of BRH (T5).

Figure 4 (right) showed that the different media ratios significantly ($P<0.01$) affected the relative chlorophyll content at eight weeks after transplanting. Leaves of hempedu bumi grown in T1 and T3 media had the highest levels of relative chlorophyll content, whereas plant grown in T4 media had the lowest. Although the cause of reduction in relative chlorophyll content cannot be identified in this study, it is likely that the plant

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growth was attributed to heavy metals stress. According to Brahim and Mohamed (2011), reduction in chlorophyll content might be due to heavy metal stress such as cooper.

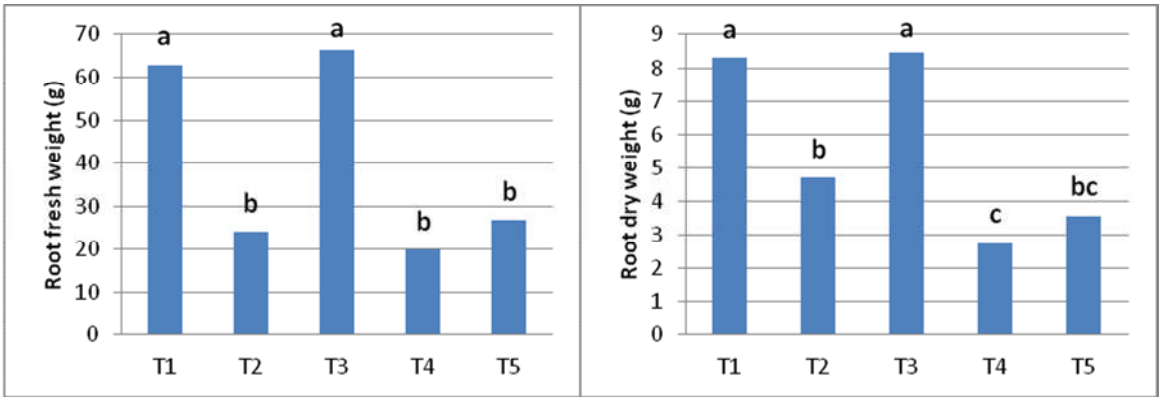

Figure 3. The effects of different media ratios on root fresh weight (left) and root dry weight (right). Treatments applied were T1: 100% CCD, T2: 70% CCD: 30% BRH, T3: 50% CCD: 50% BRH, T4: 30% CCD: 70% BRH and T5: 100% BRH. Means followed by the same letter are not significant different according to Tukey's Honestly Significant differences at 5% level.

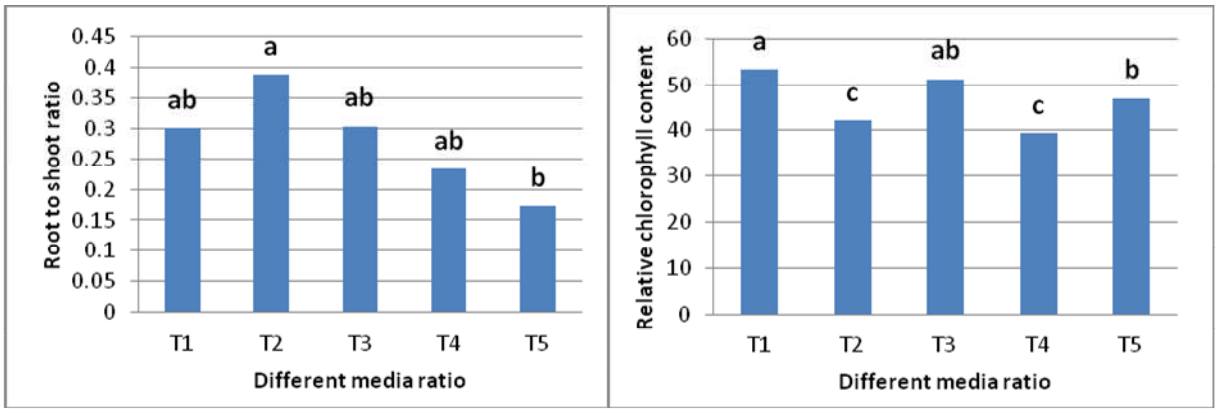

Figure 4. The effects of different media ratios on RSR (left) and relative chlorophyll content (right). Treatments applied were T1: 100% CCD, T2: 70% CCD: 30% BRH, T3: 50% CCD: 50% BRH, T4: 30% CCD: 70% BRH and T5: 100% BRH. Means followed by the same letter are not significant different according to Tukey's Honestly Significant differences at 5% level.

Conclusions

In the present study, it was found that different media ratios gave influence on growth and physiological attributes of hempedu bumi. Combination of CCD and BRH with the same fraction (T3) gave very good influence on growth of hempedu bumi. Based on the growth and physiological measurement, it was found that T3 media resulted in the highest values in most parameters, i.e. plant height, total leaf area, fresh and dry weight of shoot and root, and relative chlorophyll content. Incorporation of these two substrates created a good media condition for growing hempedu bumi. However, in all cases, T3 media was not significantly different from T1 media except in shoot fresh weight. As the objective of this study was to evaluate the effects of each media ratio on hempedu bumi growth, T1 was also concluded to be a favourable condition for good growth of hempedu bumi.

Although it may have some weaknesses in physical properties like poor air-water relationship that may lead to low aeration within the media, the result in this study revealed that hempedu bumi was capable to tolerate this problem. The selection of media should consider other aspect as well. The production cost is the main aspect that should be discussed as soilless culture system is associated with high technology production that costs a

lot at the initial stages. Unlike BRH, CCD is cheaper, relevant, and available at all time in our country. The extraction process of fibre from the coconut husk is also simpler than the production of BRH. BRH is more expensive than CCD because the burning process of rice husk acquires further increase in production cost. Above all, the application of CCD alone will minimize the cost of production of hempedu bumi grown under soilless culture system. Therefore, 100% CCD would be the most suitable growing media for high biomass yield of hempedu bumi as it gave large mean values in all parameters under study.

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Regulating Plant Organ Growth: The Enigmatic Role of Expansins

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Introduction

Expansins are a group of extracellular matrix proteins which can non-hydrolytically trigger the relaxation of the cell wall for cell expansion (Cosgrove, 2005). Despite their importance in plant development, the exact mechanism on how the proteins act at the interface between the cellulose and hemicellulose remains unclear. Furthermore, expansins are encoded by a large gene family. This has confounded the functional study of expansins due to gene redundancy, which might mask the developmental effect from the loss of single gene function (Li et al., 2002; Schipper et al., 2002). There are 38 genes in the model plant thale cress *Arabidopsis thaliana* which can be divided into 4 subgroups in expansin superfamily (Sampedro et al., 2005). To date, only a limited number of expansin genes were reported with developmental roles in different plant species. These studies mainly focus on fruit softening, internode elongation and petal expansion, highlighting the correlations between expansin gene expression and growth (Li et al., 2003). However, the number of experiments in which expansin gene expression is modulated through transgenic approach that result in a clear growth response is limited.

Here, I focus on investigating the role that expansins play during leaf development and compare that with the growth of hypocotyl and root (data not shown) by using Arabidopsis as a study system.

Materials and Methods

An expression study was carried out to identify which expansin genes are expressed during different stages of leaf development. Transgenic approach using an inducible gene expression system was then adopted to allow spatial and temporal flexibility in the modulation of transgene expression, either to over-express a heterologous expansin gene or to down-regulate a set of expansin genes through artificial microRNA (amiRNA) strategy. A series of experiments was then performed using these molecular tools to observe the outcomes of altered expansin gene expression on growth at whole-plant, organ and cellular levels.

Results and Discussion

Data show that both suppression of the expression of multiple expansin genes and overexpression led to a marked reduction in leaf growth when found at high levels (Figure 1). Within the leaf, the restriction of petiole extension was much more pronounced than the degree of repressed lamina expansion (Figure 2). This suggests a possibility for a differential sensitivity of the two tissues to the same manipulation of expansin gene expression, which would fit with other observations where repression of expansin gene expression to a particular phase of plant development resulted in growth repression only in leaves at a particular developmental stage (Goh et al., 2012).

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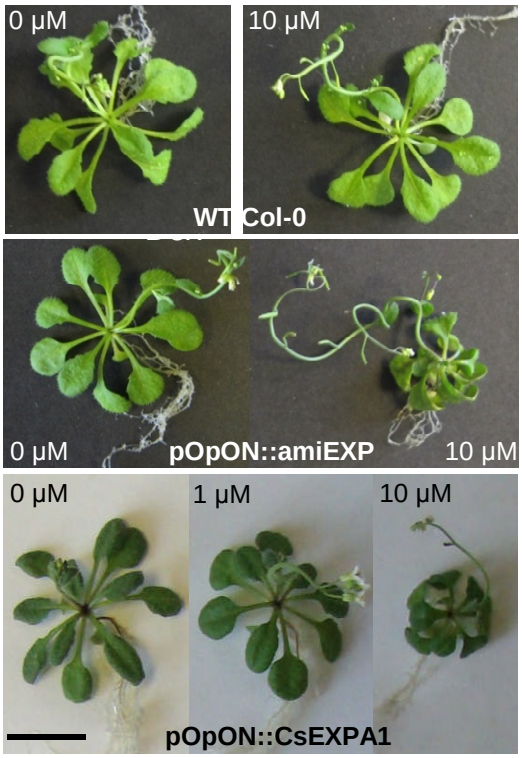

Figure 1. Comparison of pOpON::amiREXP, pOpON::CsEXPA and wild-type plants grown with Dex or control medium at 31 days after sowing (DAS). Scale bars = 10 mm.

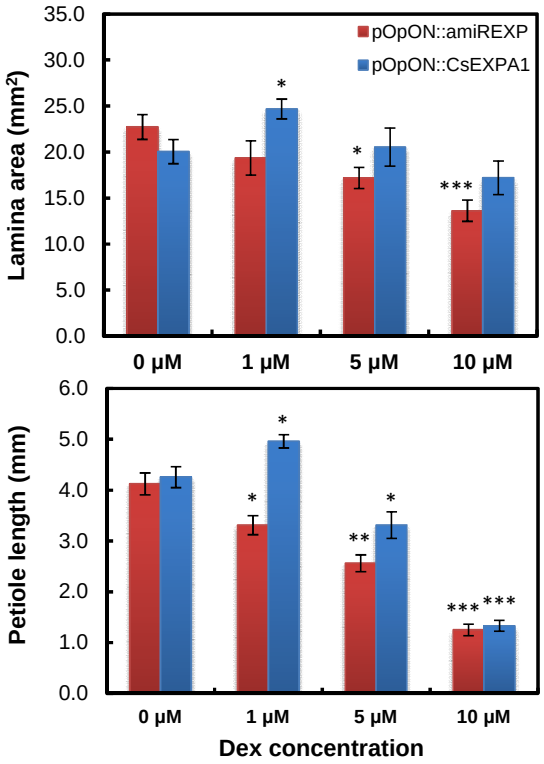

Figure 2. Comparison of leaf 6 lamina and petiole of pOpON transformant plants grown on medium supplemented with different concentrations of Dex at 27 days after sowing. Values are means ± SE (n = 10-13). One-way ANOVA, Tukey tests compared to 0 µM control: * indicates $p<0.05$, ** indicates $p<0.01$, ***indicates $p<0.001$.

Interestingly, these experiments also indicated a broader window of sensitivity to expansin gene repression in the petiole relative to the lamina, again consistent with the idea that the outcome of manipulation of expansin gene expression is dependent on the phase of development and the tissue being observed. However, we cannot discount another possibility that this reflects a different ability of the two distinct parts of the leaf to counter the imposed alteration of expansin gene expression by, for example, up- or down-regulating alternative expansin genes.

Histological analysis of leaves and petioles in which expansin gene expression was repressed or up-regulated revealed an interesting counter-intuitive response in terms of mean cell size (Table 1). For example, mean cell size following repression or over-expression of expansin gene expression was significantly increased in the lamina epidermis, although the overall lamina area was decreased. A similar trend was also observed in the lamina mesophyll. Thus, following repression and over-expression of expansin gene expression a smaller lamina was generated consisting of fewer but larger cells. This reflects that plants can undergo a growth compensatory process whereby the constituent cell size can, to a certain extent, accommodate counteract manipulations which lead to altered final organ size (Tsukaya, 2002).

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Table 1. Histological analysis of the cell size and cell number at the middle region of leaf 6 lamina and petiole of transformant plants induced with Dex 17 days after sowing. Values are mean ± SE. Bold italic text indicates statistical significance at $P<0.05$.

Transgenic plants	pOpON::amiREXP		pOpON::CsEXPA1		
Dex concentration (μM)	0	10	0	1	10
Lamina adaxial epidermis (n = 10)					
Cell area (μm^2)	2377 ± 98	***2933 ± 144***	2326 ± 186	2552 ± 105	***2797 ± 145***
Pavement cell number	88 ± 6	***63 ± 11***	90 ± 4	84 ± 3	***71 ± 5***
Stomata number	32 ± 4	***21 ± 5***	31 ± 1	28 ± 1	***23 ± 2***
Petiole mesophyll (n = 20)					
Cell area (μm^2)	621 ± 57	734 ± 70	666 ± 37	669 ± 46	***873 ± 31***
Number of cell file	20 ± 0.6	***17 ± 0.6***	20 ± 0.8	19 ± 0.4	***16 ± 0.6***

Conclusions

The results showed that the outcome of altering expansin gene expression on plant development may depend on the tissue being targeted and the degree of modulation, which led to tissue/organ-specific growth responses. These results provide functional evidence of expansins' role in leaf growth. Furthermore, this study highlights that the outcome of altered expansin gene expression is dependent on the organ developmental context, which could also be separated at the cellular and organ levels. Therefore, attempts to alter a specific plant organ size have to take in consideration of developmental context and possible confounding effects.

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Effect of Empty Fruit Bunch and Rice Husk Biochar on Sweet Corn (*Zea mays* L.) Growth

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Introduction

Recently, there has been increased research interest on biochars due to the ability of these amendments to store carbon in the soil. These carbonaceous materials are produced from biomass through the pyrolysis process without or with minimal supply of oxygen. Biochars which are strong aromatic compounds are stable materials, promising existence of long-term carbon pool in the soil system. Research has found that biochars improved soil properties (Glaser et al., 2002; Yeboah et al., 2009) and plant growth performance (Julie et al., 2010; Uzoma et al., 2011) when used as soil amendments. The high surface area, internal porosity and the presence of charges on surface sites enable the biochar to absorb associated nutrients. The high surface charge density of biochar enables the retention of cations by cation exchange (Cornelissen and Gustafsson, 2005). However, from our knowledge, there are minimal reports on application of empty fruit bunch (EFB) and rice husk biochars in agriculture. Thus, this study was undertaken to investigate the effect of these biochars on nutrient uptake and corn growth performance.

Materials and Methods

Two types of biochars, EFB and rice husk were characterized according to their physical and chemical properties. The pH and electrical conductivity (EC) of biochars were determined in deionized water at a ratio of 1:5. Total carbon was measured using a LECO CR-412 Carbon Analyzer and total nitrogen was determined according to the Kjedahl method. The cation exchange capacity (CEC) and exchangeable bases of biochars were determined by the leaching method with ammonia acetate (NH_4OA_c) buffered at pH 7.0. Scanning electron microscopy (SEM) was used to observe the morphologies of the biochars.

The pot experiment was conducted at the glasshouse unit of the Faculty of Agriculture, Universiti Putra Malaysia. The polybags were filled with 15 kg muck soil and EFB and rice husk biochar according to treatment rates. For both biochars, the same control and rates of C (2.5, 5, 10, 20 ton C ha^{-1}) were used as treatments with 5 replicates making a total of 45 polybags. The treatments were arranged according to randomized complete block design in the glasshouse. Five sweet corn seeds were sown in each polybag and thinned to three uniform seedlings after 2 weeks of planting. The soil was fertilized with N, P_2O_5 and K_2O at the rate of 60, 60 and 40 kg ha^{-1}, respectively, after 7 days of planting. The plants were watered twice daily with deionized water to maintain the soil moisture content at field capacity. After 56 days of planting, the aerial plant part was harvested and thoroughly cleaned. The fresh weight of plant biomass was recorded before drying in the oven at temperature of 65°C. Once a constant weight was obtained, the dry weight was recorded, and the tissue sample was ground and sieved at 1-mm size. The prepared foliar samples were then subjected to elemental analysis of P, K, Ca, and Mg (dry ashing method) using the atomic absorption spectrophotometer (Perkin Elmer 5100). Kjedahl digestion was used to extract N and it was then analysed using the auto analyzer.

Results and Discussion

There are many pores with regular and homogenous shape for EFB biochar when observed through the SEM. The ash particles were found scattered on the surface of the rice husk biochar. The biochars were alkaline with

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pH ranging from 9 to 10. EFB biochar was also found to have higher total carbon content and CEC compared to the rice husk biochar. The chemical properties of EFB and rice husk biochar are summarized in Table 1.

Table 1. Chemical properties of EFB and rice husk biochar

Properties	Biochar	
	EFB	Rice husk
pH (H_2O)	9.45	9.99
Total C (%)	54.08	7.78
CEC (cmolc/kg)	63.93	13.45
Total N (%)	1.63	0.23
P (%)	0.21	0.36
K (%)	5.32	0.72
Ca (%)	0.11	0.02
Mg (%)	0.13	0.08

After 56 days of planting in the glasshouse, the effect of biochar on the plant dry biomass was recorded (Figure 1). The plant dry biomass significantly increased ($P<0.05$) with the increasing rates of rice husk biochar application. The addition of EFB biochar at 10 tons C ha^{-1} application has significant effect on dry biomass as compared to the control.

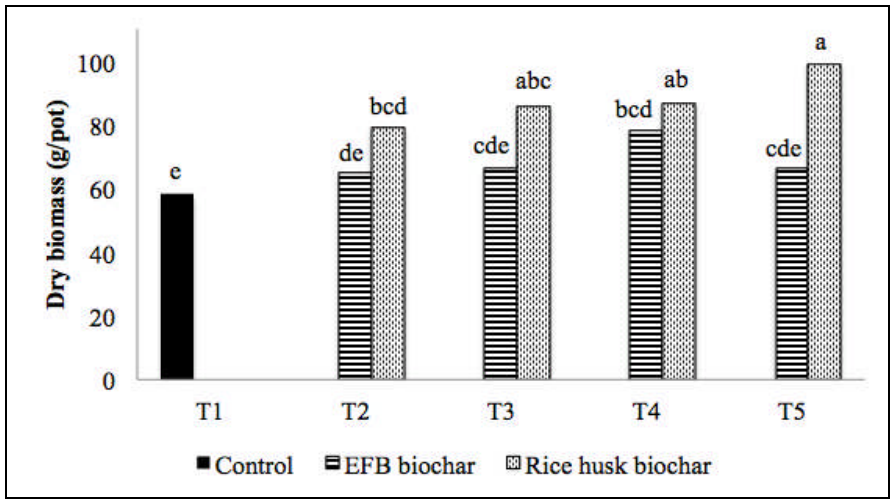

Figure 1. Effect of EFB and rice husk biochar on plant dry biomass. Means with the same letter(s) are not significantly different by Tukey's Honestly Significant Difference (HSD) Test at 5% level of significance.
T1 = Control
T2 = biochar at 2.5 tons C ha^{-1}
T3 = biochar at 5 tons C ha^{-1}
T4 = biochar at 10 tons C ha^{-1}
T5 = biochar at 20 tons C ha^{-1}

From Table 2, the N and Ca content slightly decreased with the addition of both amendments to the soil. The K content increased significantly with the application of biochars and this can be attributed to the richness of K in the biochars. Rice husk biochar has effect on the Mg uptake, which was higher as compared to the control. The high CEC of biochar might help to retain the cations in the soil system and hence increase their uptake by sweet corn (Liang et al., 2006; Laird et al., 2010).

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Table 2. Effect of EFB and rice husk biochar on nutrient uptake by sweet corn

Treatment	Rate (ton C ha^{-1})	%				
		N	P	K	Ca	Mg
Control	0	1.82 a	0.120 b	0.025 d	1.32 abc	0.053 e
EFB biochar	2.5	1.86 a	0.130 ab	0.041 cd	1.47 abc	0.060 de
	5	1.80 a	0.129 ab	0.105 cd	1.52 ab	0.066 cde
	10	1.86 a	0.122 ab	0.239 b	1.42 abc	0.071 cde
	20	1.81 a	0.163 a	0.478 a	1.08 bc	0.062 de
Rice husk biochar	2.5	1.48 b	0.102 b	0.048 cd	1.56 a	0.078 cd
	5	1.17 c	0.120 b	0.126 c	1.48 abc	0.089 bc
	10	0.85 d	0.124 ab	0.256 b	1.36 abc	0.103 ab
	20	0.59 e	0.126 ab	0.397 a	1.02 c	0.114 a

Numbers followed by the same letter within a column are not significantly different at 5% level of significance based on Tukey's Honestly Significant Difference (HSD) test.

Conclusions

Biochar showed positive response on the nutrient uptake and dry biomass of corn plants. Thus, EFB and rice husk biochars can be potentially used as soil amendments to improve soil properties and plant growth performance. However, the results might not be the same for other biochar feedstocks which might have different properties and pyrolysed at different temperature.

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The Root Physiological and Hydrological Enhancement for Green Slope Bioengineering Approach

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Introduction

Landslide is a major geological hazard to most regions of the world. The soil covered by vegetation, known as green bioengineering technique, arguably, is a suitable technique for preventing shallow slope failures (Ji et al., 2012). In soil bioengineering techniques, the use of vegetation is highly recommended as a structural element to stabilize natural and man-made slope due to its eco-friendly solutions (Bischetti et al., 2010). Additionally, vegetation helps to stabilize the masses of soil by holding soil particles in place and assists in preventing soil erosion by the root systems and root-soil matrix (Stokes et al., 2009). However, propagation by seeds is sometimes difficult in harsh slope conditions resulting in complexities of growing up and undesirable physiological characters.

In bioengineering techniques, stem cutting is used in stabilizing burned hill-slopes, road-cuts and fill-slopes and river banks (Bischetti et al., 2010). When stem cuttings are grown, the roots are developed along their stems and this increases the root-soil matrix, reinforcement act and pullout resistance too. Opuni-Frimpong et al. (2008) and Kraiem et al. (2010) documented that the establishment and growth rate of the stem cutting depend upon age variation, position in stem and diameter of stem. In addition, many researchers recommended that the survival rate and root initiation can be increased by applying endogenous rooting hormones before planting (Mori et al., 2011). Indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) are common auxins, which can control many aspects of plant development including root initiation and elongation (Shan et al., 2012). These exogenous hormones normally act by signaling the proteins to stimulate new cell and resulting in the initiation of numerous lateral roots (Durbak et al., 2012). In view of the increasing demand of soil bioengineering techniques, there is a need to select new species and effective rooting hormone for vegetative propagation through stem cutting.

Therefore, the specific aims of this study are to (i) assess the suitability of tropical woody species for the vegetative propagation via stem cutting and (ii) determine the suitable cutting position and rooting hormone to induce adventitious roots and improve root-soil matrix.

Materials and Methods

Plant Sample Collection and Auxin Treatments

The experiment was conducted under glasshouse conditions at Institute of Biological Sciences, University of Malaya, Malaysia, in February-August, 2012. Disease-free and evenly matured *Leucaena leucocephala* (LL) and *Peltophorum pterocarpum* (PP) legume trees were selected for experimentation. The branches were cut (24 cm) into two pieces namely basal and apical position. Two types of auxins i.e., IBA and NAA solution (100 mg/L) were used as exogenous hormones and distilled water was used as control. Individual stem cutting of both species was submersed (about 15 cm stem portion) into respective hormone formulations and kept for 24 hr at room temperature. Then, the treated stem cuttings were vertically planted in polyvinylchloride (PVC) pots having slope soil. After 2 days of growth, bud initiation, bud number and initial budding length of the each cutting were measured. The survival rate was recorded at the 6th months of growth.

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Matric Suction, Soil Moisture Content and Leaf Area Index (LAI)

Matric suction, soil moisture content and LAI were recorded using the soil moisture tensiometers (Model 2100F, Soilmoisture Equipment Corp.), a moisture probe (HH2 Moisture Meter, England) and leaf area instrument (AccuPAR-LP80, UK), respectively.

Biomass and Water Absorption Rate (WAR)

The biomass (oven-dried at 80°C for 48 hours) were determined using a balance (Model-Mettle PJ3000, Japan) at the end of experiment. The WAR was obtained based on the Baker's theory (Baker, 1984).

Root Profiles

The root profiles were analyzed by using the WinRHIZO Pro Software at the end of experiment. This software was used to assess the total root length and root volume at different root diameter ranging from 0.0 to 4.5 mm.

Statistical Analysis

Statistical analysis was performed using SPSS software. Two-way ANOVA was applied to evaluate significant (LSD: $p < 0.05$) differences among the treatments.

Results and Discussion

Auxins-induced Bud Initiation and Survival Rate

The days taken to bud initiation of PP were significantly longer compared to LL cuttings. In the case of basal and apical cutting position of PP, the highest bud number (per stem cutting) was noticed in IBA and the lowest was observed in control. Initial bud length of LL and PP was 6 and 10 cm, respectively, in both cutting position, irrespective of the different rooting hormones (Table 1). The 0% survival rate was observed in both positions of LL stem cutting treated with IBA and NAA. Thus, the LL was not suitable for vegetative propagation by stem cutting, although cuttings were treated by different rooting hormones. In the case of survival rate of PP, basal position of cuttings treated with IBA, NAA and the control cuttings showed 100, 83 and 66% survival, respectively, but apical position cutting showed poor survival.

Table. 1. The effect of different cutting positions and hormone treatments on bud initiation, bud number and surviving rate of different species

Species and cutting position	Treatments	Bud initiation (days)	Bud number (per cutting stem)	Initial budding length(cm): at 2nd week	Survival rate at the 6th month (%)
LL Basal	IBA	2±0.5 ns	4.4±0.2 ns	6.4±0.4 ns	0
	NAA	2±0.4 ns	4.8±0.3 ns	6.8±0.4 ns	0
	Control	2.2±0.3 ns	4.8±0.2 ns	6.8±0.4 ns	0
LL Apical	IBA	2.2±0.3 ns	5±0.2 ns	6.4±0.5 ns	0
	NAA	1.8±0.3 ns	4.4±0.4 ns	5.4±0.2 ns	0
	Control	2.2±0.5 ns	4.6±0.4 ns	6.2±0.3 ns	0
PP Basal	IBA	7±0.4 ns	3.4±0.2a	10.8±0.4 ns	100a
	NAA	7±0.5 ns	3.2±0.2ab	10.6±0.6 ns	83b
	Control	7.4±0.7 ns	1.2±0.2c	10.8±0.3 ns	66c
PP Apical	IA	7.60.6 ns	2.2±.2a	0.8±0.5 ns	50a
	NAA	7.4±0.7 ns	2±0.0ab	11±0.4 ns	30b
	Control	7.2±0.3 ns	1±0.0c	10.8±0.5 ns	30b

Means (means ± standard error) with different letters within the same column, same species and same cutting position were significantly different ($p < 0.05$), ns not significant.

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Biomass, WAR and LAI

In terms of root biomass production, basal position had significantly ($p<0.05$) higher root biomass than apical position. Studies have found that the amount of root biomass was influenced by different auxin hormones. The weight of shoot biomass (new branches and leaves) was observed to be the highest in IBA treatment. Thus, it can be said that the shoot growth is favored by triggering enough root growth by IBA hormone than both NAA and control (Table 2). In addition, WAR was higher in IBA treated cuttings, implying that a greater increase in root biomass can absorb more water by utilizing a greater volume of soil. This observation may be due to the hormonal action to regulate several physiological activities in proper way such as transpiration and plant development including root-shoot initiation and elongation (Ezekiel et al., 2010; Shan et al., 2012). Basal cuttings had higher LAI compared to apical stem cuttings. The LAI values of IBA treated cuttings were higher in 6^{th} months than those in the NAA treated cuttings. This observation may reflect that IBA has initiated more to biomass production than NAA.

Table 2. Biomass, WAR and LAI of PP stem cuttings with different treatments

Cutting position	Treatments	Shoot biomass (g)	Root biomass (g)	WAR	LAI at 6^{th} month
Basal	IBA	212±6.6a	61.3±3.5a	24.2±0.6a	0.7±0.02a
	NAA	196.6±8.8b	28.3±2b	21.2±2.2ab	0.57±0.02b
	Control	107.6±4.3c	21±1.5c	19.4±0.5c	0.45±0.07c
Apical	IBA	166±3.4a	34±2.3a	20.3±1.6a	0.47± 0.01a
	NAA	151.3±3.7b	29±2b	17.4±0.6ab	0.38± 0.01b
	Control	70.6±3.1c	16±1.8c	14±0.8c	0.26± 0.04c

Means (means ± standard error) with different letters within the same column and same cutting position were significantly different ($p<0.05$).

Interaction of Stem Cutting with Soil Moisture and Matric Suction

It was observed that uses of basal position are more effective for the reduction of soil moisture content than the apical position. Moreover, IBA rooting hormone has more promising effects on the reduction of soil moisture than NAA and control treatments (Table 3). Additionally, it was observed that the matric suction in basal position of control stem cuttings rooted soil was 35% greater than that with apical position cutting. A higher matric suction was also found in the soil having IBA treated cuttings than that having NAA and control cuttings. This may be due to the presence of a higher root biomass and a lower moisture content of soil (Huat and Kazemian, 2010). Higher LAI, vegetative growth and root biomass were observed in both hormonal treated basal cuttings, implying that higher LAI, vegetative growth and root biomass increased both the water absorption capacity and matric suction.

Table 3. The soil moisture content (%) and matric suction at 6^{th} months of growth

Parameters	Basal position			Apical position		
	IBA	NAA	Control	IBA	NAA	Control
Moisture content (%)	13.6±0.3c	17.9±0.5b	21.3±0.6a	16.4±0.7c	18.2±0.8b	23.6±0.6a
Matric suction (KPa)	30±0.8a	26±0.5b	23±0.6c	26±0.8a	24±0.5b	20±0.5c

Means (means ± standard error) with different letters within the same row and same cutting position were significantly different ($p < 0.05$)

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Root Length and Volume

The responses in root length and root volume varied with different types of hormone and positions of stem cutting. The highest total root length was noticed in basal position cutting with IBA treatment and the lowest was observed in apical position with control treatment (Figure 1). In addition, root volume was significantly ($p<0.05$) higher within the root diameter ranging from 0.5 mm to 2.5 mm in each treatment (Figure 2). These roots (from 0.5 to 2.5 mm) are categorized as fine roots which are important for root-soil interaction, water absorption and soil fixation (Stokes et al., 2009). Therefore, it was assumed that higher root length and volume due to presence of fine roots (0.5-2.5 mm) increased the root-soil matrix area, which ultimately increased its capability to absorb water. Consequently, removal of excessive water would lead to drying of soil.

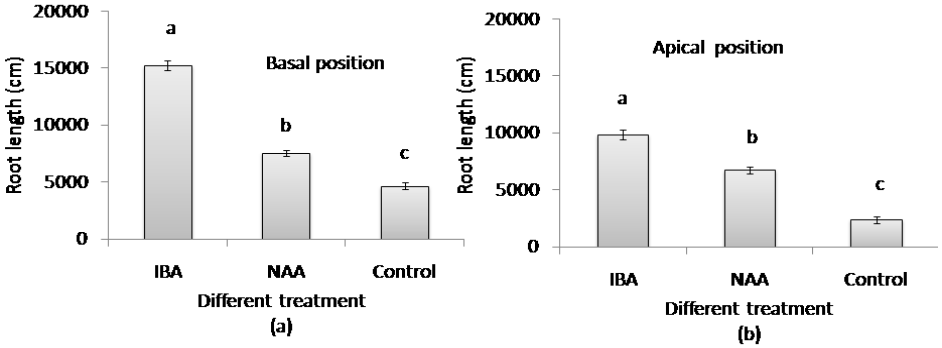

Figure 1. Effects of different treatments on the total root length; (a) Basal position and (b) apical position. Different letters were significantly different ($p < 0.05$).

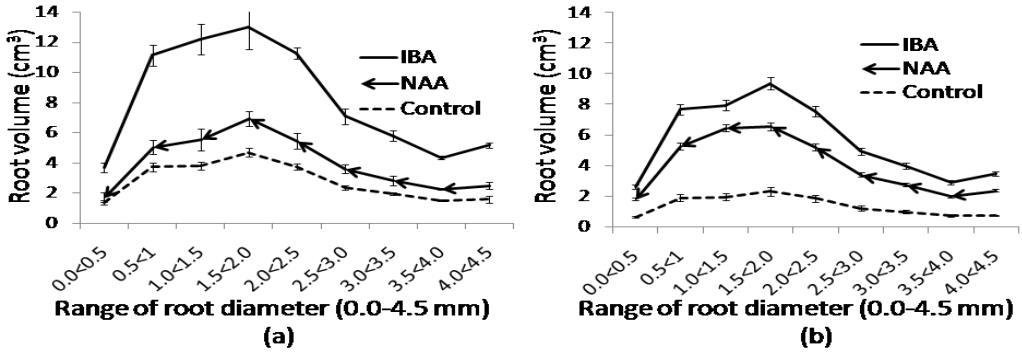

Figure 2. Effect of different treatments on the root volume. (a) Basal position and (b) apical position. Root classification: fine roots (0.0-2.0 mm) and thin roots (2.0<4.5 mm).

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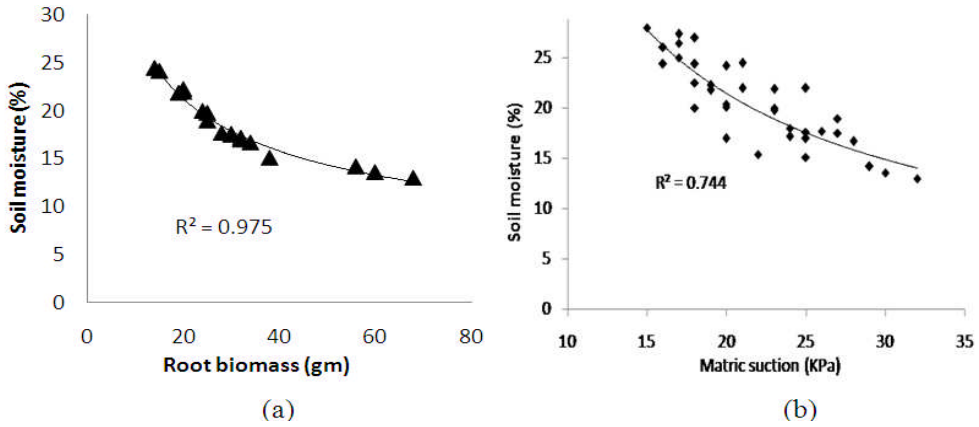

Figure. 3. Negative correlation among parameters

On the other hand, soil moisture content and root biomass are negatively correlated (R^2=0.97), implying that the higher the underground biomass, the lower soil moisture content (Figure 3a). Current results also indicated that soil moisture content was strongly (R^2=0.74) correlated with matric suction, i.e., high matric suction was attributed to low soil moisture content (Figure 3b).

Conclusions

In conclusion, vegetative propagation through stem cuttings was suitable for *P. pterocarpum* but not for *L. leucocephala*. Basal position of stem cuttings and IBA hormone were the most effective ways in initiating adventitious roots and enhancing root-soil matrix. However, more stringent screening will be conducted on *P. pterocarpum* to examine further their potential as a soil bioengineering materials on slope condition.

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Profiling of Flavonoid Compounds and Alteration of Their Production and Antioxidant Activity during the Growth Period in Young Ginger (*Zingiber officinale* Roscoe)

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Introduction

Plants and herbs consumed by humans may contain thousands of different phenolic acid and flavonoid components. The effect of dietary phenolics is of great current interest due to their antioxidative and possible anticarcinogenic activities (Malencic et al., 2007). Phenolic acids and flavonoids also function as reducing agents, free radical scavengers, and quenchers of singlet oxygen formation (Ghasemzadeh and Jaafar, 2011). Antioxidant compounds that scavenge free radicals help protect against degenerative diseases (Amin and Tan, 2002). Phenolic components have important role to control cancer and diseases in human body. For example, *Zingiber officinale* Roscoe has long been used in traditional medicine as a cure for some diseases including inflammatory diseases (Chan et al., 2008). At present, flavonoids are extracted from leaves of *Ginkgo biloba* (Feng et al., 2002), roots of *Pueraria lobata* (Liao and He, 2003), leaves of *Nelombo lutea* (Chen et al., 2002) and rhizomes and leaves of *Z. officinale* (Ghasemzadeh et al., 2010). *Zingiber officinale* is an important horticultural crop in tropical Southeast Asia. It produces a pungent, aromatic and bioactive constituent's rhizome that is valuable all over the world either as a spice or herbal medicine. *Zingiber officinale* is known as a resource with higher phenolic components, wide source and low price (Rozanida et al., 2003), and therefore it can serve as a cheap and important material in food. It is a natural food component with many active phenolic compounds such as gingerol and shagaol, and it has been shown to have anti-cancer and antioxidant effects (Surh, 2003). In Malaysia only rhizomes of *Z. officinale* are being consumed as food flavoring and the leaves are thrown away. Information on the flavonoid contents of plant foods and plant parts commonly consumed in Malaysia is still scarce. In the present study, we identified some important phenolic components (flavonoids) in both leaves and rhizome of two varieties of *Z. officinale* of Malaysia and antioxidant activities in these varieties were considered. The alteration of flavonoids concentration as a function of growth period of young *Z. officinale* was also evaluated.

Materials and Methods

Plants Material

Seed rhizomes of two varieties of *Z. officinale* (Halia Bentong and Halia Bara) were germinated in peatmoss for two weeks in small pots and then transferred to polyethylene bags containing soilless mixture of burnt rice husk and coco peat. The plants were grown under glasshouse conditions at the glasshouse complex of University Putra Malaysia (UPM). The plants were harvested at 16 weeks, with the leaves, stems, and rhizomes separated. Once dried (freeze dried), they were all kept at -80 ºC for future analysis.

Analysis of Flavonoid Composition by HPLC

Reversed-phase High Performance Liquid Chromatography (HPLC) was used to assay compositions of flavonoids. Agilent HPLC system (Tokyo, Japan) consisted of a Model 1100 pump equipped with a multi-solvent delivery system and a L-7400 ultraviolet (UV) detector. The column type was a Agilent C18 5 um, 4.0 × 250 mm. The mobile phase composed of (A) 2% acetic acid (CH_3COOH) and (B) 0.5% acetic acid – acetonitrile. The operating temperature was maintained at room temperature (Wang et al., 2007). Identification of the flavonoids was achieved by comparison with retention times of standards, UV spectra and

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calculation of UV absorbance ratios after co-injection of samples and standards. Commercial standards were purchased from Sigma–Aldrich (USA).

Determination of Antioxidant Activities (*DPPH Radical Scavenging Assay*)

In order to determine the radical scavenging ability, the method reported by Mensor et al. (2001) was used. Briefly, 1 ml from 0.3 mM alcohol solution of DPPH was added to 2.5 ml samples containing different concentrations originating from extracts of different parts of the varieties under study. The samples were first kept at room temperature and their absorbance was read at 518 nm after 30 minutes using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). The antiradical activity (AA) was determined using the following formula:

Percent (%) inhibition of DPPH activity = [(A0 – A1) / A0)] × 100 %

Where A0 is the absorbance value of the blank sample or control reaction, and A1 is the absorbance value of the test sample. The dose-response curve of DPPH radical scavenging activities of the samples were compared with the standards BHT and α-tocopherol.

Results and Discussion

HPLC Analysis Results

The results showed Halia Bara had more flavonoids content in the leaves and rhizomes as compared with Haila Bentong. What is interesting in this data is that in both varieties the concentration of flavonoids in leaves decreased with increasing growth period but in rhizome increased with increasing growth period. The decreases in Halia Bentong leaves were 10% for quercetin, 64.5% for rutin, 19.7% for catechin, 18% for epicatechin, 9.3% for naringenin and 15.7% for kaempferol. In Halia Bara, they were 24.8% for quercetin, 2.9% for rutin, 24.4% for catechin, 42.2% for epicatechin, 2.5% for naringenin and 21.2% for kaempferol. In rhizome, increases of these components in Halia Bentong were 37.5% for qurcetin, 29.0% for rutin, 20% for catechin, 36.4% for epicatechin, 32.7% for naringenin and 48.9% for kaempferol. In Halia Bara, they were 25.5% for quercetin, 45.5% for catechin, 78.0% for epicatechin, 25.0% for naringenin and 20.0% for kaempferol. In the family Zingiberaceae, especially *Z. officinale*, it is generally believed that secondary metabolites produced by the plants are transported to the rhizomes where they are accumulated (Habsah et al., 2000; Katsube et al., 2004). Results of this research showed that flavonoids are important components of this plant, and that some of their pharmacological effects could be attributed to the presence of TF and TP valuable constituents. High content of quercetin was obtained in leaves of Halia Bara at 16 weeks after planting. During the growth period, variable contents of rutin and naringenin were found in the leaves and rhizomes of both varieties. Kaempferol is a rare flavonoid in plants. It was detected in the leaves and rhizomes of Halia Bara and Halia Bentong with low concentration (0.023-0.06 mg/g DW). However, as compared with *Capsicum annum* (0.039 mg/g DW), *Pachyrhizus* (0.037 mg/g DW), *Raphanus sativus* (0.0383 mg/g DW) and *Centella asiatica* (0.02 mg/g DW), both ginger varieties recorded higher contents of kaempferol.

DPPH Radical Scavenging Activity

It was observed that methanolic extracts of the leaves in both varieties had higher activities than those of the rhizomes at different harvest times. The free radical scavenging activities also decreased in the leaves and increased in the rhizomes from 8 to 16 weeks after planting. Leaves of Halia Bara at 8 weeks after planting showed high antioxidant activities but high DPPH activity has been observed in the rhizomes of Halia Bara at 16 weeks after planting. The IC_{50} of rhizomes extracts observed just at 16 weeks after planting. Positive relationship between phenolic components and flavonoids with free radical scavenging had been reported in previous studies (Pongsak and Parichat, 2010; Praven et al., 2007). Similar observations have been reported

by Chan et al. (2008) and Ghasemzadeh et al. (2010) who observed the leaves of ginger with high level of TF had high antioxidant activities as compared to rhizomes. The antioxidant study showed that the ginger extracts have the good proton-donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

Conclusions

Flavonoids consist of enormous collection of biologically active compounds that are ubiquitous in plants, many of which have been used in traditional eastern medicine for thousands of years. *Zingiber officinale* has long been used for the treatment of much pathology. Therefore, its use in the medicine industry should be stimulated in order to develop new pharmaceuticals with particular antioxidant or antimicrobial pharmacological profiles. Herein, we investigated the properties of two varieties of *Z. officinale* in Malaysia. Currently, the advance in analytical techniques such as HPLC allows one to gain insight on their composition, and to study the activity of their components. In this work, we have identified seven important flavonoid components in two varieties of *Z. officinale*. We have also found that Halia Bara leaves and rhizomes contained more flavonoid components as compared to Halia Bentong and that the leaves of both varieties can be used for food flavouring and in traditional medicine because of good concentration of flavonoid components. Results of leaves analysis showed that the concentration of flavonoids in leaves decreased during growth period with subsequent flavonoid increases in the rhizomes and the level of medicine components in leaves were high at 8 weeks after planting. On the other hands, this study revealed for the first time the medical potential of leaves in Halia Bentong and Halia Bara.

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Productivity of Grass-Legume Mixtures under Different Shade Levels

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Introduction

The need to meet the nutritional requirement of the ever increasing world population, especially that of protein from animal origin, has over the years become an issue of serious concern. This is even more so, given the fact that the world population is predicted to increase to nine billion people by 2050 and by then both crop and livestock production need to double to meet the demand (Godfray et al., 2010). Livestock are important historical components of most small holder farming systems in Southeast Asia providing draught power, manure, meat and milk for human consumption and cash income (Stur et al., 2002). The level of ruminant production in Malaysia is generally low compared to non-ruminant production. In the light of this, it is therefore imperative to adopt strategies that may lead to increase in supply of food of animal origin to meet the increasing human demand (Davendra, 2006). One of the ways to improve animal production is through intensive commercial ruminant production under plantation crops. This will require the sowing of productive high quality forage species that are able to persist under grazing in shaded environment (Stur and Shelton, 1990). Malaysia has an estimated land area of 4.48 million hectares under palm oil cultivation. This area of land could be used for integrated system involving ruminants, pastures and plantation crops. Some of the benefits of integration include, increased soil fertility and conservation, improved thermal comfort for the animal and increased quality in forage and animal production.

The attainment of the benefits of pasture, trees and livestock integration depends on the balance among the trio as competition for growth resources such as radiation, water and nutrients can render the system unsustainable, of utmost importance is the shade tolerance of the forage species as shade imposes serious limitation to pasture growth and development. Therefore, the objective of the experiment was to evaluate the productivity of mixtures of *Panicum maximum* (guinea grass) with each of the following legumes of contrasting growth habit; *Stylosanthes guianensis* (stylo), *Arachis pintoi* (*Arachis*), *Centrosema pubescens* (centro) and *Macropitilium bractaetum* (burgundy bean) under 3 radiation levels of full sun (100%, 50% and 30%) and the suitability of the mixtures for plantation livestock integration.

Materials and Methods

Experimental Location

The experiment was conducted in Agronomy Field 2, Faculty of Agriculture Universiti Putra Malaysia, located at latitude 3^0 02'N longitude 101^0 42'E and altitude 31m above sea level. Mean monthly rainfall from January to July 2012 was approximately 139.4 mm. Mean monthly minimum and maximum temperatures were 25.5 °C and 33.6 °C respectively while average monthly relative humidity was 93.4%. The experimental plot was previously sown to forage grass-legume mixtures. The soil is a clay loam with pH of 5.04, N of 0.04%, P of 29 mg/L and K of 19 mg/L.

Experimental Materials and Field Culture

The experimental materials consisted of mixtures of guinea-*Arachis*, guinea-burgundy, guinea-centro and guinea-stylo. Guinea grass and *Arachis* were established by vegetative propagation. Stylo and burgundy seeds were scarified by soaking in hot water at 80 °C for 3 minutes in order to break seed dormancy prior to sowing, while centro seeds were treated by soaking in cold water for 1 hour. The species mixture were sown in small plots (2.5 m X 2 m) covered with plastic sheets that served as mulch. Grass and legumes were sown in

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alternate rows. Distances within and between rows were 0.5 m and 0.4 m respectively, while those between plots and blocks were 0.5 m and 2 m respectively. Each block consisted of 12 plots. Fertilizer was applied at sowing at a rate of 10 kg N ha^{-1}, 50 kg P ha^{-1} and 50 kg K ha^{-1} in the form of urea, triple super phosphate and muriate of potash. Each mixture was subjected to 3 radiation levels (100%, 50% and 30%) in a randomized complete block design at 3 weeks post sowing. This was achieved through the imposition of shade structures which consisted of 4 wooden frames (2.5 m high) pegged at the four corners of each plot. The frames were covered at the top and all the four sides by shade cloth made up black plastic net of varying mesh size but not covered at the base about 0.25 m to the ground to facilitate air movement.

First harvest was done at 8 weeks post sowing; subsequent ones were carried out at 5 weeks interval. Parameters such as height of grass and legumes, internode length of grass (1^{st} internode) and tiller numbers were measured on plants within 0.4 m X 0.5 m at the centre of individual plots. Thereafter grass and legumes were cut separately to a stubble height of 5 cm to the ground. Fresh weight of each was measured using a digital balance. A representative grab sample was taken, separated into leaf and stem, weighed and oven dried in order to determine leaf to stem ratio. The remaining forage material was oven dried at 65 °C for 3 days. Dry weight was taken and the yield on dry matter basis was calculated. Prior to harvest, measurements of physiological parameters such as leaf area index (LAI), photosynthesis, leaf chlorophyll and photosynthetically active radiation (PAR) were taken using LICOR LAI-2000 Plant Canopy Analyzer USA, LICOR LI-6400 portable photosynthesis system Lincoln Nebraska USA, LICOR Minolta SPAD-502 chlorophyll meter Japan and Light meter model LI-250 USA respectively. PAR was measured on very clear days (cloudless) at solar noon while chlorophyll, photosynthesis and leaf area index measurements were taken between 8 and 11 am in the morning. The experimental design was a 4 X 3 X 3 factorial in a randomized complete block design. Data were analyzed by analysis of variance using (SAS) 9.2 while treatment means were compared by Duncan's Multiple Range Test.

Results and Discussion

Cumulative dry matter yield is shown in Table 1. Cumulative dry matter yield of guinea over 5 harvest was not significantly different irrespective of mixture combination but highly significant differences ($p<0.05$) were observed among radiation levels. The cumulative dry matter yield of guinea in guinea-legume mixtures decreased with decreasing radiation level. In contrast, cumulative dry matter yields of legumes in mixtures were significantly different. Centro and stylo produced greater yield ($p<0.05$) than *Arachis*; centro produced the highest dry matter yield (4 tons ha^{-1}). Cumulative dry matter yields of legumes were also significantly ($p<0.05$) affected by radiation levels; legumes did better ($p<0.05$) under 100% radiation as compared to 50% and 30% radiation. Significant interaction ($p<0.05$) was observed between legume species and radiation levels. Centro and burgundy recorded greater cumulative dry matter yield at 30% radiation than at 50% while the reverse was the case in the dry matter yield of *Arachis* and stylo. Centro was significantly superior ($p<0.05$) to other legumes at 50% radiation level. Mixtures were not significantly different in terms of cumulative total dry matter yield, however, guinea-centro tended to record higher cumulative total dry matter yield than other mixtures in absolute values. The cumulative total dry matter yield of mixtures was equally affected by radiation levels with 100% greater ($p<0.05$) than that with 50%, which was in turn higher than that with 30%. Dodd et al. (2005) in an experiment in New Zealand reported that the proportion of sown species (perennial rye grass) and Yorkshire fog decreased under increased shade level adding that the proportion of sown legume species (white clover and lotus) also decreased significantly under shade with the legumes recording greater losses. Mean percentage guinea in mixtures was observed to be highest in guinea-*Arachis* mixture and superior ($p<0.05$) to guinea in guinea-centro and guinea-stylo mixtures. The mean percentage was affected by radiation levels, 50 and 30% radiation recorded significantly higher percentage of guinea than 100% radiation. Centro and stylo in mixtures with guinea had significantly higher ($p<0.05$) mean percentages than that with *Arachi*s. Legumes were generally depressed at low radiation levels, i.e. percentage of legumes was higher ($p<0.05$) at 100% radiation than at 50% and 30% radiation.

Morphological characteristics of pastures revealed that mean tiller number and length of the first internode of guinea grass were not significantly different among mixtures; however, both were significantly and highly affected by radiation levels. Mean tiller number and internode length decreased with decrease in radiation

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levels. Peri et al. (2007) reported a reduction in tiller population in cocksfoot (*Dactylis glomerata* L.) under 10 years old *Pinus radiate* as shade level increased. Grass height differed among radiation levels, guinea appeared to be taller at 50% and 30% radiation levels as compared to 100% radiation, while legumes were taller ($p<0.05$) at 100% radiation than 50% and 30% radiation. Significant interaction ($p<0.05$) was observed between legume height and radiation level.

Table 1. Mean square values of cumulative dry matter yields of grass, legumes and total with the mean of species and radiation

Source	Df	Mean Square				
		CDMYG t ha^{-1}	CDMYL t ha^{-1}	CTDMY t ha^{-1}	Grass %	Legume %
Rep	2	3947334.6	1138942.24	6764853	13.18	13.17
Radiation	2	296935125.1**	44380249.96**	551393546**	267.76*	267.76*
Species	3	11236107.3	13225636.99*	10334662	190.94*	190.95*
Species*radiation	6	6093643.9	7255926.42*	4812414	82.84	82.84
Error	22	12328075	2498016.6	13866350	42.17	42.17
CV		25.67	61.61	22.92	7.64	44.42
Species				Species Mean		
GA		15.12a	1.39c	16.51a	90.91a	9.09b
GB		13.54a	1.75bc	15.29a	87.24ab	12.76ab
GC		13.64a	4.00a	17.64a	80.61b	19.39a
GS		12.40a	3.12ab	15.52a	82.77b	17.23a
Radiation (%)				Radiation Mean		
100		18.94a	4.79a	23.73a	80.1b	19.89a
50		13.02b	1.45b	14.47b	89.2a	10.79b
30		9.06c	1.46b	10.52c	86.8a	13.17b

Means with similar letters within columns are not significantly different. CDMYG=Cumulative dry matter yield grass, CDMYL=Cumulative dry matter yield legume CTDMY=Cumulative total dry matter yield, GA=Guinea-*Arachis*, GB=Guinea-burgundy, GC=Guinea-centro GS=Guinea-stylo

100% radiation level received significantly higher PAR ($p<0.05$) above and below canopy compared to 50% and 30%; 50 and 30% were not significantly different in PAR. Leaf area index (LAI) for guinea was not different among mixtures, in the case of legumes, centro recorded significantly greater LAI ($p<0.05$) than *Arachis*. Differences among radiation levels were highly significant. LAI values for both grass and legumes were found to increase with decreased radiation, the LAI was observed to be higher ($p<0.05$) at 50% and 30% radiation than at 100% radiation. Huawei et al. (2010) recorded increase in LAI with shading in winter wheat (*Triticum aestivum*). Light extinction coefficients (k) for guinea and legumes in mixtures were similar. 100% radiation resulted in higher ($p<0.05$) extinction coefficients for guinea and legume, as compared to 30% radiation. Stomata conductance in centro was observed to be significantly higher ($p<0.05$) than *Arachis* and stylo. Chlorophyll contents of grass and legumes among mixtures were not significantly different but differences among radiation levels were highly significant. In the case of guinea, chlorophyll content was significantly higher ($p<0.05$) at 30% radiation than at 100% and 50% radiation. Cavagnaro and Trione (2007) reported increased chlorophyll content in *Trichloris crinita* with increasing shade level adding that significant increment was found under higher shade level but not under full sun and 40% shade. In contrast, legumes had higher chlorophyll content at 100% radiation, though similar to that at 30% radiation but superior ($p<0.05$) to that at 50% radiation. Significant interaction ($p<0.05$) was observed in chlorophyll content among species and radiation levels. No significant differences were observed among species and radiation levels in leaf temperature and photosynthesis

Conclusions

The suitability of any pasture species for inclusion in plantation livestock integration depends on its shade tolerance characteristics. The cumulative dry matter yields of grass-legumes mixtures declined with

decreasing radiation level. The percentage decline varied with the type of species, thus the dry matter loss recorded by guinea at lower radiation levels (50% and 30%) was lower than that of legumes indicating the susceptibility of legumes to low radiation. Guinea-centro mixture seemed to hold some promise considering the higher productivity of centro in the mixture compared to other legumes and the physiological advantage of greater LAI and stomata conductance that centro had over other legumes. However, the success of the mixture under plantation will very much depend on the dynamics of light reaching the lower canopy level as any light level below 50% could prove detrimental to the pastures. In this regard, it is recommended that guinea-centro mixture should be planted between wide row spacing of palm trees such that the mixtures under the tree canopy will have guaranteed access to light, or better still, the mixture could do well under coconut plantation which allows a better light penetration through its canopy as the tree ages.

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Nutritional Content of Ridged Gourd (*Luffa acutangula*) Using Different Types of Manure

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Introduction

Luffa acutangula is a climbing green vegetable that is listed under Cucurbitaceae family whose fruit has high nutritional value. It is known as patola, Angle Luffah and Luffah ridge gourd. It was grown mainly for its immature fruits as it can be eaten cooked or raw. As the vegetable is important in maintaining a well-balanced diet, its nutritional information is used by the public agencies and agricultural industries to promote their fresh product. The Food and Agriculture Organization (FAO) report which summarized the worldwide production of some cucurbit fruits (pumpkins, squash and gourds) noted a significant increase cucurbit production due to high demand and consumer awareness on the health benefits of cucurbit fruits (Nurul Aqilah et al., 2011). In Malaysia, this plant was commercially planted in Johor, Pahang and Kelantan. *Luffa acutangula* is best grown in the well-drained sandy loam soil. The productivity of the plants and its nutritive quality are influenced by the supply of nutrients in the soil. Composted manure is widely used in organic farming as an affordable source of fertilizer (Lyimo et al., 2012). Fertilizer application is an important factor in determining horticultural crop yield, quality and nutritional content (Martinez-Ballestra et al., 2008). The most important nutrients that are needed for the plant growth and development are nitrogen (N), phosphorus (P) and potassium (K). N helps the plants with rapid growth, increasing the seed and fruit production and also enhances the quality of leaves. P helps in proper plant maturation and confers the plant with the ability to withstand stress while K helps in the building of proteins that increase the fruit quality and reduce disease infection. Therefore, it is important to evaluate the effects of different types of manure on the yield and nutritional content of *L. acutangula*. The objective of this study is to compare the effects of three types of manure on the nutritional composition of *L. acutangula*. Indeed, the nutritional data are important parameters to defining the quality of a food.

Materials and Methods

This experiment was conducted under the rain shelter with six replicates using a randomized complete-block design (RCBD). *Luffa acutangula* seeds were immersed in distilled water overnight to enhance the seed germination. The plants of *L. acutangula* were planted in polybags and the trellis system was used to support the plants. Three types of manures were used which were chicken manure, cattle manure and goat manure. During the first manure application at planting, each soil medium was mixed with 200 g of manure and this was followed with applications of 20 g of manure each at 3, 6 and 8 weeks after planting. The fruits were harvested at 10 weeks after planting.

Proximate Analysis

The nutritional composition of the fruits was determined after harvest. These included moisture, ash, crude protein (CP), fat and carbohydrate. Moisture content was determined after oven drying at 65 ^{o}C for 24 hours. Ash content was determined using the muffle furnace at 500 ^{o}C for 2 hours. CP was determined using the Kjeldahl method where samples were digested in concentrated sulphuric acid and the dissolved N was measured with an auto analyzer. Fat content was determined by the Soxhlet method where samples were refluxed in ether. Carbohydrate content was estimated by subtracting all other components from the dry matter.

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Statistical Analysis

All the data were analyzed using Analysis of Variance (ANOVA). Differences between treatments means were compared using Duncan's Multiple Range Test (DMRT). The analysis was carried out using SAS.

Results and Discussion

Edible vegetables are important component in human diets as they contain essential biochemicals responsible for the human metabolism (Aliyu, 2006). The result of proximate analysis of biochemicals (carbohydrate, fat and protein) and other contents (ash and moisture) is shown in Table 1. The moisture content in the fruits of *L. acutangula* showed significant differences between fruits applied with chicken manure and cow manure. The percentage of moisture in the fruits with cow manure (94.81%) was significantly higher than that applied with chicken manure (90.22%).

Table 1. Mean percentages of moisture, ash, CP, fat and carbohydrate content in the fruit of *L. acutangula*

Treatment	Moisture (%)	Ash (%)	CP (%)	Fat (%)	Carbohydrate (%)
Chicken manure	90.22^{b}	0.12^{a}	2.39^{a}	0.30^{a}	6.97^{a}
Cow manure	94.81^{a}	0.04^{c}	0.69^{c}	0.20^{a}	4.27^{a}
Goat manure	92.86^{ab}	0.06^{b}	0.88^{b}	0.22^{a}	5.98^{a}

The ash content, which is an index of the mineral content, showed that there were significant differences between the three different types of manure applied to *L. acutangula* fruits. The percentage of ash in fruits of plants applied with cow manure was the lowest, which was only 0.04%. Chicken manure gave the highest percentage of ash (0.11%) followed by fruits produced with application of goat manure (0.06%).

The percentage of CP was highest in the fruits following application of chicken manure (2.39%). It was followed by the fruits produced after application of goat manure (0.88%). The fruits of plants applied with cow manure showed the lowest CP percentage (0.69%). As the chicken manure had highest N content among the organic manures (Azeez and Van Averbeke, 2010), the fruits from the plants applied with chicken manure therefore had highest percentages of CP due to the function of N content in the fertilizer. Poultry birds feed mainly on grains with higher protein and fat content may also contribute to the nutrient enrichment of their faeces (Azeez et al., 2010). The chemical characteristics of the manures are shown in Table 2. The N and P are significantly higher in poultry manure compared to other manures. The K element of the manure was in order of magnitude: goat manure > poultry manure > cattle manure (Azeez and Van Averbeke, 2010).

Table 2. Chemical characteristics (N, P and K) of the manures

Manures	N (g/kg)	P (g/kg)	K (g/kg)
Poultry manure	37.1 (0.10)	14.65 (0.30)	17.95 (7.45)
Cattle manure	17.0 (0.001)	4.19 (0.12)	16.80 (0.30)
Goat manure	22.2 (0.75)	4.27 (0.12)	41.15 (1.85)

Values in parenthesis are standard error of the means (SEM), n=3
Source: Azeez and Van Averbeke (2010)

Fat contents were not significantly different in the fruits of *L. acutangula* subjected to the different types of manure ranging from 0.22 to 0.30%. The different types of manure on the crops of *L. acutangula* did not have

impact on the percentage of fat in the fruits of the crop. Sudjaroen (2012) reported that the fat content in *L. acutangula* was about 0.20%. The percentage of carbohydrate in the fruits of *L. acutangula* ranged from 4.27 to 6.97% and was not significant different among different types of manure.

Conclusions

The nutritional values of *L. acutangula* following application of different types of manure were analyzed using the standard AOAC methods for evaluation of proximate parameters of moisture, ash, CP, fat and carbohydrate. The application of chicken manure gave the highest CP and ash content which makes it the most preferable organic fertilizer among those used in the study.

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Domestication of *Aquilaria* (Karas) Tree Species for Agarwood and Oil Production: Reaping the Wealth

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Introduction

In Malaysia, there are more than five main local species of *Aquilaria* belonging to the Family Thymelaeaceae which are known to produce agarwood or *gaharu* . The resin impregnated heartwood when burnt, releases rich soothing aroma, sometimes considered a scent from heaven (Ng et al., 1997; Baden et al., 2000). *Aquilaria malaccensis* and *Aquilaria crassna*, locally known as *karas*, are primarily associated with world agarwood trade. However, the local *A. malaccensis* is more widely distributed, domesticated and studied here. In Malaysia, the exported agarwood has been reported to come from at least six species, *A. malaccensis*, *Aquilaria hirta*, *Aquilaria beccariana*, *Aquilaria microcarpa*, *Aquilaria rostrata* and *Aetoxylon sympetalum* (Zich and Crompton, 2001; Chang et al., 2002; Tawan, 2003). Due to existing discriminate and destructive felling, it is presently listed as an endangered species in the World List of Threatened Trees and Convention on International Trade of Threatened, Endangered Species of Wild Fauna and Flora (CITES- under Appendix II) (Oldfield et al., 1998).

Recent strong global demands and the attractive prized commodities has strongly supported the planting of *Aquilaria* as a potential forest plantation crop, for it has also been recognized as an integral part of sustainable forest management and complementary to natural forests. There is currently an urgent need to cultivate, understand and manage the species better for trade benefits derived from agarwood and oil. Local agarwood traders and individuals are now also more receptive to plant *Aquilaria* as a new economic plantation crop. Although effective inducement techniques are still being developed and evaluated to suit these locally plantation-grown trees, already some large-scale plantations, comprising not only *A. malccensis*, have been established within the last five-six years with a total of about 2,177 ha.

Ecology and Distribution of Aquilaria

Aquilaria trees are native trees to Asia, widely distributed in countries such as Bangladesh, Bhutan, India, Indonesia, Malaysia, Myanmar, Philippines, Singapore and Thailand (Oldfield et al., 1998). There are about 25 species of *Aquilaria* with 15 species reported to form agarwood (Barden et al., 2000). In Malaysia, about five known genera viz., *Aetoxylon*, *Aquilaria*, *Gonystalus*, *Ekleia* and *Wikstroemie* and five species from *Aquilaria* (*A. malaccensis*, *A. hirta*, *A. beccariana*, *A. rostrata*, and *A. microcarpa*) were reported to produce agarwood (Burkill, 1966; Chang *et al*., 2002). However, the most popular and important source of agarwood trade species are *A. malaccensis* and *A. crassna* (not indigenous sp.). There is no specific information on the distribution of some of these species available in Peninsula Malaysia and domestication of these species may be found within several known main producing states like Kedah, Perak, Kelantan, Terengganu, Pahang, Johor and Sarawak. CITES, and the International Union for Conservation of Nature and Natural Resources (IUCN) classify these species as vulnerable bases on the reduction of at least 20% over three generations caused by levels of exploitation and declined in population (Hilton-Taylor, 2002). To overcome this diminishing resources, recently an export quota for agarwood at 200,000 kg for 2012 has been imposed by the Ministry of Natural Resources and Environment (NRE) which also acts as the Scientific Authority of CITES in Malaysia (MASKAYU, 2011). *Aquilaria* species were reported to occur in various habitats, including those that are rocky, sandy or calcareous, well-drained slopes and ridges and even land near swamps. They can grow at latitudes up to 1000 m with an average daily temperature between 24-32 ^{o}C and annual rainfall of 2,000-4,000 mm/year (Wiriadinata, 1995).

Planting Systems and Growth Performance

Several successful attempts have been undertaken to investigate the potential of domesticating *Aquilaria* in the open and plantation conditions. Among the earliest FRIM's attempts were in 1928 where the natural mortality in the original population density of 833 trees/ha was reduced to 31 trees/ha by 1995 (Lok and Zuhaidi, 1996). Current practices suggest planting can be done under agro-forestry system by inter-planting with other tree species (e.g. Rubber, *Eucalyptus* spp.), mono-cropping (single) and even with short term cash crops such as tongkat ali, bananas, serai wangi to help generate income while waiting for production of agarwood/oil. The lowest and highest average diameter growth rate of *A. malaccensis* for native forests was 0.33 cm and 0.81cm/year respectively (La Frankie, 1994). In a 67 years old experimental plot, the mean diameter and height of 38.2 cm and 26.7 m were obtained (Lok and Zuhaidi, 1996) while early growth results for trees planted under partial shades of 40%, using tissue culture plantlets/seedlings, showed promising growth rates. For up to 24 months old, average initial and final height of these plantlets were 43.1 cm and 136.6 cm while those in the seedlings were 27.9 cm and 114.8 cm respectively (Lok et al., 1999). In another study of inter-planting with *Azadirachta excelsa* (sentang), survival rate of 93% with average initial and final height of 86.2 cm and 114.4 cm respectively was obtained after six months (Lok et al., 1999).

Inducement and Inoculation Techniques

Agarwood inducement is the main determinant to cultivate *Aquilaria.* Effective inducement techniques or inoculants can only be derived through more intensive research and development programme. There are many existing known methods used to induce agarwood production. Some of these are the traditional method using nails, slashing, FRIM's Preliminary Inducement Research Method, the Patented Technology of using CAKit (also known as "Vietnam Method"), "Taiwan Method", "Somkit Method" (Thailand), "Pheerapan Method" (Thailand and Malaysia) and other on-going research methods either produced by researchers or individuals (Lok et al., 2008; Chang, 2009; Lok and Ahmad Zuhaidi, 2010).

Conclusions

Based on the results obtained, it was concluded that domestication of this species is possible due to the following reasons:

Environmental conditions and natural habitat - suitable and promising
Need new successful/innovative R&D–biotechnogical techniques-reveal resin mechanism
Ex-situ conservation/gene bank -all agarwood producing species in Malaysia
Improved planting stocks – growth performance and disease resistant clones and there are Ample opportunities for investment-prospects-both in local agarwood industry and marketed products.

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CHAPTER 2

BEST PRACTICES AND CURRENT TECHNIQUES

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Enhancing Quality of Export Grade Carambola by Methyl Jasmonate Induced Stress

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Introduction

Malaysia is a leading country in the export of carambola, a tropical fruit with a star shaped cross-section. However, these fruits require careful handling due to the characteristic shape. In addition, carambola is highly susceptible to rib browning. Various postharvest technologies have been developed for carambola, the most significant being modified atmosphere storage and low temperature storage. Both of these technologies are capable of retarding both softening and water loss of the fruits (Ali et al., 2004). However, low temperature storage, which is the most common commercial practice, leaves this tropical fruit susceptible to chilling injury. Chilling injury was found to be pronounced in fruits stored at 10 ^{0}C and transferred to 28 ^{0}C after 10 or 14 days (Ali et al., 2004).

Methyl jasmonate is the methyl derivative of jasmonic acid, a fatty-acid derivative stress hormone. It plays an important role in fruit ripening and stress responses (Cao et al., 2010). This occurs through the activation of the oxidative stress response and secondary metabolic pathways of the stressed fruit (Chen et al., 2011).

It is postulated that through the exposure of carambola to methyl jasmonate and the induction of stress response, the bioactive content of the fruit will be enhanced. Thus, the aim of this study was to assess the effect of methyl jasmonate induced stress on the quality of cold stored carambola.

Materials and Methods

Experimental Material, Design and Treatments

150 pieces of small (110-130 g) green-mature carambola were purchased from a commercial farm in Broga and selected for uniformity in size, shape and colour and free of defects. The fruits were washed with 0.05% sodium hypochlorite, rinsed with distilled water and air-dried at ambient temperature.

The experiment was arranged in a completely randomised design (CRD) with four replications. The fruits were subjected to methyl jamsonate (MeJA) vapour treatments of 0 (control), 0.01 (trt 1), 0.1 (trt 2), 0.2 (trt 3) and 0.5mM (trt 4), by incubating in a 45L air-tight storage box for 16 hours at room temperature (22^{0}C). After ventilation for 2 hours, the fruits were removed from the boxes and stored at 10 ^{o}C for 17 days, during which the quality parameters and bioactive content were measured twice a week.

Quality Parameters

Weight loss was analysed according to the AOAC International (Association of Official Analytical Chemists) method. The maximum force required to penetrate through the rib of the starfruit was measured as the firmness property of the fruit. This was measured using Instron Universal Testing Machine (USA) equipped with an 8.0mm plunger tip single-column, set at maximum compression at a speed of penetration of 20mm/min.

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Bioactive Content

Ascorbic acid was determined by DCPIP (2,6-dichlorophenol-indophenol dye) titration method. The DCPIP dye was prepared by dissolving 42mg ± 0.1 of sodium bicarbonate and 50mg ± 0.1 of DCPIP sodium salt in 200ml ± 0.1 of distilled water. Starfruit juice was extracted using 3% metaphosphoric acid (metaphosphoric acid) as the extracting medium and titrated against the DCPIP dye. Total phenolic content was determined using Folin Ciocalteu spectrophotometric method. A 10 ml mixture, containing 7.9ml ± 0.01 distilled water, 0.5ml ± 0.01 of undiluted Folin Ciocalteu reagent, 0.1ml ± 0.01 of starfruit methanolic extract and 1.5ml ± 0.01 of 7% sodium carbonate solution, was prepared and incubated at 37 °C for two hours. Absorbance readings at 765nm were then obtained using Varioskan Flash microplate reader and the results were expressed as mg gallic acid equivalent (GAE)/ 100g fresh weight.

Statistical Analysis

All the experiments conducted were arranged in a completely randomised design (CRD) with four replications. The data was subjected to analysis of variance (ANOVA) and means were separated by least significant difference ($P < 0.05$) using SAS® (9.1, SAS Institute Inc., USA).

Results and Discussion

Quality Parameters

Percentage weight loss (Figure 1A) increased linearly throughout the experiment. The increase was observed at a significantly slower rate for Trt 4 (0.5mM MeJA) ($P<0.05$). However, amongst the other treatments the difference in weight loss was not significant ($P>0.05$). Additionally, the differences in firmness between the different treatments were not significant ($P>0.05$), although there was a general decline during the storage period (Figure 1B).

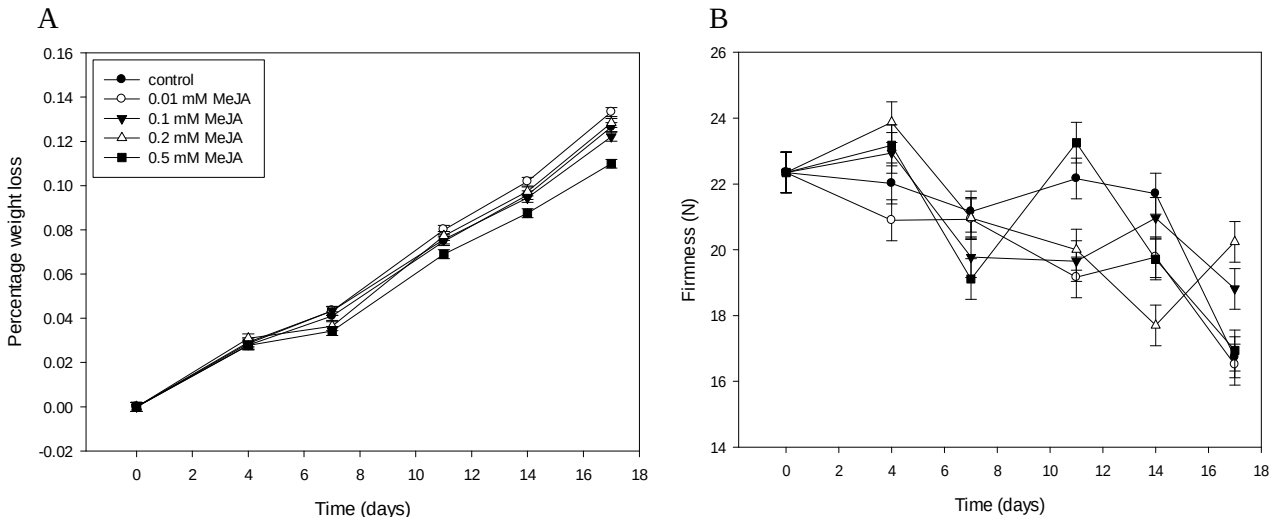

Figure 1. Effect of different concentrations of methyl jasmonate (MeJA) on (A) percentage weight loss; (B) firmness of carambola stored at 10^0C. Values are the means ± SE.

With the exception of weight loss for the highest MeJA treatment, the results for the physical quality parameters indicate that the MeJA treatment at 10^0C did not have a significant effect on the physical quality parameters of carambola. Percentage weight loss was retarded for the highest MeJA treatment, suggesting a positive effect of that treatment on moisture retention.

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Bioactive Content

Ascorbic acid content increased linearly up to day 14 for all treatments (Table 1). The highest content was reported for Trt 2, Trt 3 and Trt 4 (0.1mM, 0.2mM and 0.5mM, respectively) with no significant difference between the values reported for these treatments ($P>0.05$). Meanwhile, the control was significantly lower than all treatments ($P<0.05$). The total phenolic content fluctuated throughout the storage period, but a peak was observed for all treatments (with the exception of trt 4) on day 14 (Table 2). The highest content was observed for Trt 1 (0.01mM MeJA) at a value of 22.79 mgGAE/100gFW, followed by the control and Trt 2 (0.1 mM MeJA). As the concentration of MeJA increased, the phenolic content for day 14 declined (Trt 2, 3 and 4). These results are not in par with the results for ascorbic acid content.

As the fruit ripens, the organic acid content has been found to decline, mainly oxalic and malic acid or tartaric (O'Hare, 1993). Ascorbic acid content was measured in this experiment, and it was found to significantly increase as the fruit ripens. Methyl jasmonate reduces chilling injury by increasing activity of antioxidant enzymes and accumulation of ascorbic acid (Cao et al., 2010). This is in compliance with the results observed, as the highest ascorbic acid content were observed for Trt 2, Trt 3 and Trt 4. Meanwhile, the decline in total phenolic content could lead to a decline of rib browning incidence.

Table 1. Ascorbic acid content of carambola in response to methyl jasmonate (MeJA) treatment

Trt	Day 1	Day 4	Day 7	Day 11	Day 14	Day 17
Control	1.83 ± 0.05 a	3.50 ± 0.05 b	3.61 ± 0.11 ab	4.06 ± 0.23 a	6.44 ± 0.33 c	5.07 ± 0.19 c
0.01 mM MeJA	1.83 ± 0.05 a	3.23 ± 0.05 c	3.28 ± 0.11 bc	3.89 ± 0.14 a	7.06 ± 0.21 b	5.53 ± 0.07 bc
0.1 mM MeJA	1.83 ± 0.05 a	3.73 ± 0.05 a	3.56 ± 0.32 abc	3.89 ± 0.14 a	7.81 ± 0.31 a	5.87 ± 0.57 b
0.2 mM MeJA	1.83 ± 0.05 a	3.45 ± 0.08 b	3.84 ± 0.11 a	4.17 ± 0.38 a	7.88 ± 0.14 a	6.53 ± 0.23 a
0.5 mM MeJA	1.83 ± 0.05 a	2.73 ± 0.07 c	3.22 ± 0.11 c	4.06 ± 0.11 a	7.63 ± 0.16 ab	6.73 ± 0.13 a
SE	0.049	0.060	0.113	0.162	0.205	0.196
P	1	<0.0001	0.0093	0.7031	0.0007	0.0001

Ascorbic acid in (mg/100gFW) Values are the means ± SE. Means separation within columns by Least Significant Difference ($p<0.05$).

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Table 2. Total phenolic content of carambola in response to methyl jasmonate (MeJA) treatment

Trt	Day 1	Day 4	Day 7	Day 11	Day 14	Day 17
Control	10.62 ± 0.02 a	9.93 ± 0.35 c	12.11 ± 0.15 a	13.11 ± 0.38 a	17.66 ± 0.69 b	12.10 ± 0.73 b
0.01 mM MeJA	10.62 ± 0.02 a	12.30 ± 0.45 b	12.07 ± 0.4 a	10.90 ± 0.29 b	22.79 ± 0.47 a	15.08 ± 0.92 a
0.1 mM MeJA	10.62 ± 0.02 a	12.73 ± 0.47 b	12.90 ± 0.37 a	12.86 ± 0.41 a	16.84 ± 0.70 b	13.55 ± 0.59 ab
0.2 mM MeJA	10.62 ± 0.02 a	14.86 ± 0.65 a	8.59 ± 0.28 b	8.59 ± 0.65 c	14.77 ± 0.36 c	12.41 ± 0.68 b
0.5 mM MeJA	10.62 ± 0.02 a	15.41 ± 087 a	11.98 ± 0.60 a	11.83 ± 0.56 ab	13.19 ± 0.58 c	11.95 ± 0.98 b
SE	0.016	0.586	0.389	0.476	0.573	0.793
P	1	<0.0001	<0.0001	<0.0001	<0.0001	0.0511

Total phenolic content in mgGAE/100gFW. Values are the means ± SE. Means separation within columns by Least Significant Difference ($p<0.05$).

Conclusions

The results suggest that methyl treatment at 0.5mM can serve as an economically viable technology for maintaining the quality of carambola during transportation and storage. Further studies are required to understand the physiological changes occurring within carambola.

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Effects of Sucrose on the Quality of Dendrobium '*Sonia red*' Flowers Stored at Ambient Temperature

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Introduction

Orchids are found throughout the world and become one of the largest families which have about 800 genera with 25,000 species (Sheehan and Sheehan, 2009). Cut flower industry of Malaysia is very promising due to increase in household usage and foreign market demand. *Dendrobium* is one of the important species being a major contributes for total cut flowers production. The problem with the distribution is that flowers have to be transported all over the world and the quality loss of flower may result rejection in the marketplace thus becoming major factor influencing profitability in the flower trade (Reid, 2009). Generally, florists practice placing cut flowers in water to retain its freshness until they reach the customers but placing the flowers in water only, it cannot get the food source as it's already detached from the mother plant. Some of them use holding solutions which contain sucrose to act as a substrate for respiration and other metabolic activities. Sucrose alone can do more harm to the flowers as it allows the growth of microorganisms causing blockage of vascular system leading to water stress then was expressed in the form of early wilting of flowers. The study of sucrose in holding solution for cut flowers had been carried out by previous researchers and it was agreed that it can extend the shelf life of several cut flowers, however, no reports on the biochemical changes that occur in cut flowers during storage was obtained. Therefore, this study aims to see the effects of sucrose on the quality of *Dendrobium* 'Sonia Red' flowers stored at ambient temperature.

Materials and Methods

Inflorescences of Dendrobium '*Sonia Red*' were purchased from a commercial grower in Rawang, Selangor, Malaysia. Inflorescences were harvested in the morning and were selected for freshness and uniformity with 4-5 open flowers and 3-5 floral buds. Peduncles of orchid flowers were slantingly cut into 15 cm length from the lower most of open flower. Individual inflorescence was held in graduated test tube containing 30 ml of distilled water (control) or sucrose solution. Each vase mouth was covered with aluminium foil to prevent evaporation. All flowers were held in a room at ambient temperature and humidity (31.6ºC and 68.9% RH) without replacing the solutions. Fresh and dry weight, number of days required for bud opening, water uptake (ml), total anthocyanin, sugar and protein content of different flower parts (upper flower part, middle flower part, lower flower part and floral bud) were determined at three days interval for fifteen days of storage. Anthocyanin content of the flowers was determined using the method from Bhartil and Khurana (2003) with some modification. The protein contents were measured at absorbance 595 nm using Bovine Serum Albumin as standard (Bradford, 1976) while total sugar content was measured using method from Somogyi (1952) using glucose as a standard. In all experiments, inflorescences was arranged in a completely randomized design (CRD) and replicated five times. Data obtained were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test at 5% of significant level to determine the best treatment. The statistical programme used was Statistical Program for Special Science (SPSS) version 20.0.

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Results and Discussion

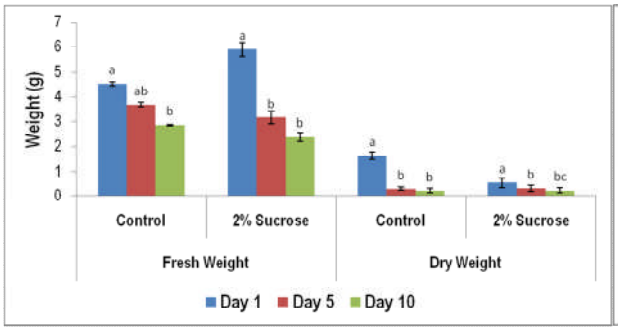

Figure 1. Changes in floral bud fresh and dry weight of *Dendrobium* inflorescence held in distilled water (control) and 2% sucrose. Means with the same letter(s) are not significantly different at P≤0.05

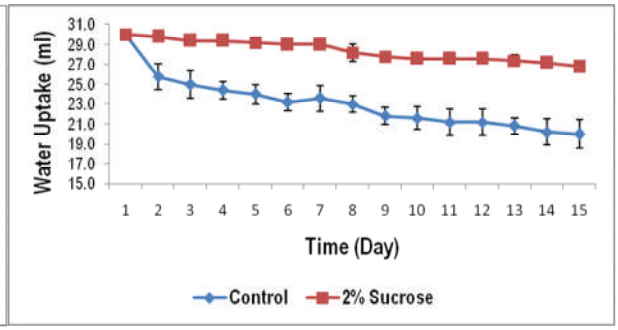

Figure 2. Changes of water uptake on *Dendrobium* inflorescence held in distilled water (control) and 2% sucrose.

The studied of fresh weight of floral bud placed in sucrose treatment decreased substantially during five days of storage (Figure 1). It may be due to bud fails to open and abscise while competing with open flowers in the same inflorescence for the amount of available sucrose (Arrom and Munné-Bosch, 2012). It appears that water uptake of orchid flowers held in sucrose solution decrease slowly than control (Figure 2). According to Van Doorn (2011), air embolism, plugging of bacteria, plant debris, and poor water quality is the major reasons that reduce the uptake of holding solution. The results are in agreement with studies carried out on Dendrobium '*Jew Yuay Tew*' by Rattanawisalanon et al. (2003).

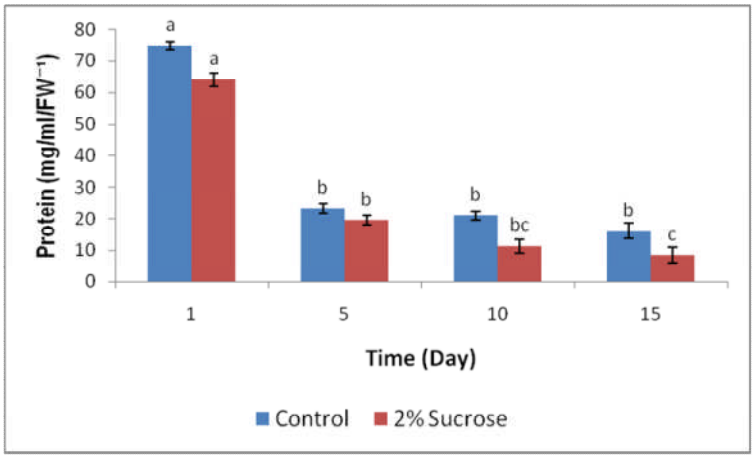

Figure 3. Changes of protein content in *Dendrobium* inflorescence held in distilled water (control) and 2% sucrose solution. Means with the same letter(s) are not significantly different at P≤0.05.

Total protein for control and sucrose treatment was significantly decreases during fifteen days of storage (Figure 3) but the sharp declining trend can be seen at day 5. Flower held in sucrose treatment shows lower significant value compare to control until day 15. It is somehow related to protein degradation due to flower senescence and failure of nutrient uptake cause by microorganism occlusion in solution containing sugars. According to Ling and Subramaniam (2007), the higher protein content in particular orchid plant, the higher amount of amino acids accumulation, thus, the better the plant quality and vice versa. Decrease in protein content at day 10 and 15 indicates buds and flowers in sucrose treatment have already senescence and abscised.

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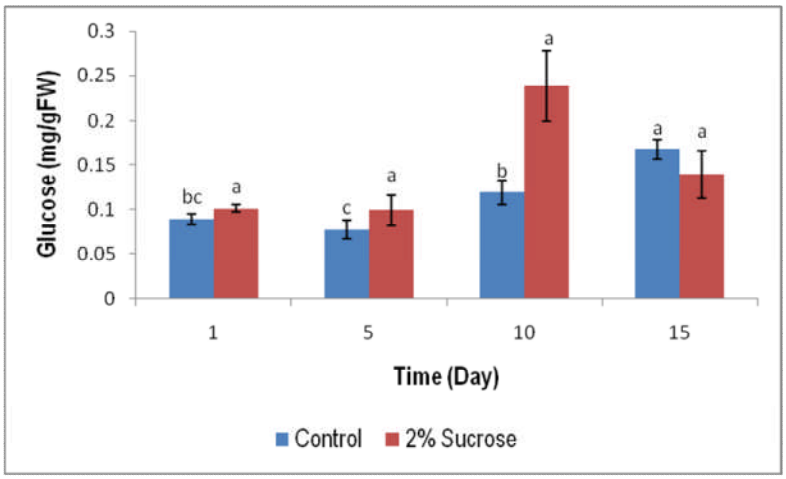

Figure 4. Changes of glucose content of *Dendrobium* inflorescence held in distilled water (control) and 2% sucrose solution. Means with the same letter(s) are not significantly different at P≤0.05.

Analysis of the sugar content in Figure 4 demonstrated the presence of increase concentrations of glucose in both treatments but sucrose treatment shows higher significant value as compared to control. Ling and Subramaniam (2007) reported that flower emergence was fasten when large amount of sugar content present in the flower. At day 10, flowers held in sucrose solution shows highest glucose content compared to control. The higher levels of glucose suggest rapid conversion to reducing sugars to facilitate bud opening at this position. Glucose serves to provide osmotic potential for the expansion of the petal cells and partly responsible for the improved opening of the flowers in preservative solution (Cho et al., 1999). In control, flowers accumulated higher levels of reducing sugars at day 15. This indicates the flower already reached the maximum full bloom. Sood et al., (2006) in their study stated sugar content was found to be an important nutrient that significantly contributed its part for the flower synthesis and blooming.

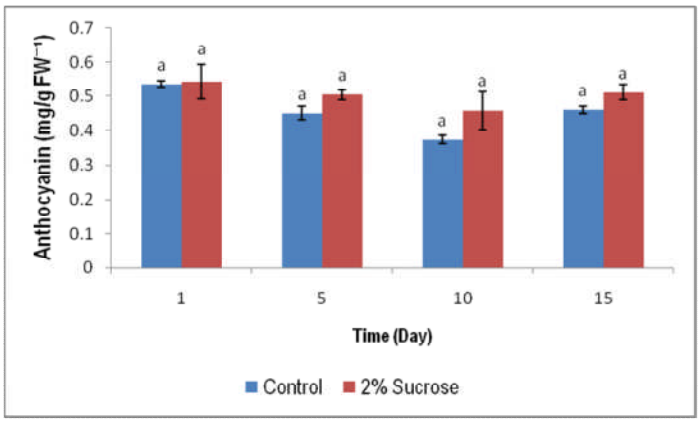

Figure 5. Changes of anthocyanin content of *Dendrobium* inflorescence held in distilled water (control) and 2% sucrose solution. Means with the same letter(s) are not significantly different at P≤0.05.

Figure 5 shows that anthocyanin content in Dendrobium cut flowers were no significant different for both treatment when compared by day. Control exhibit lower anthocyanin value and decrease at day 10 may be due to bud begun to senescence as it did not receive enough nutrient uptakes from the flower stem. This data indicates, flowers held in distilled water affects anthocyanin production as the flower start to senescence at day five of storage.

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Conclusion

Effects of sucrose on *Dendrobium* 'Sonia Red' flowers stored at ambient temperature were carried out to determine the quality of the flower during it shelf life. The amount of protein, sugar and anthocyanin obtained varied during day of storage. Such variation can be due to the factor like quality of flowers during harvest, environmental temperature and fungal occlusion in holding solution. As the results from this research, the use of sucrose in controlling senescence in orchid cut flowers is of potential, however, the concentration of sucrose needs to be optimized.

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Oxidative Stress Response of *Colletotrichum Capsici* Induced by Postharvest Treatment with Ozone

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Introduction

Proliferation of anthracnose disease among economically important crops, such as pepper, papaya, banana and mango, has reduced the storage life of crops and their profitability, and has highlighted *Colletotrichum* species to be the most important pathogen for postharvest treatments (Zahid et al., 2012). *Colletotrichum capsici* is one of the economically significant species that causes anthracnose disease and its proliferation on chilli has resulted in 80% yield loss in Thailand and losses of up to US$ 100 million per year in Korea and US$ 1.33 million per year in India (Mahasuk et al., 2009). The prevalence of fungal disease on fruits is currently control by the application of fungicides. The fungicides are however possess well-known hazards to humans and the environment. This has forced the development of more effective and safe postharvest treatments to control the fungal proliferation.

Ozone, a potent oxidizing agent produces reactive oxygen species which capable in preventing microbial proliferation on fruit tissues as well as sanitizing fruit surfaces from toxic chemicals by converting the hazardous form of fungicides to non-harmful components (Gabler et al., 2010). Therefore, ozone technology has been used as a postharvest treatment to prevent disease proliferation and chemical contamination on fruits such as grapes (Palou et al., 2002).

Ozone efficacy on fungal growth has been studied on several fungal species such as *Penicillium* sp. (Palou et al., 2003), *Botrytis cinerea* (Ozkan et al., 2011), *C. acutatum* (Yun et al., 2006) and *C. gloeosporioides* (Barbosa-Martinez et al., 2002). The fungal response towards ozone however has been reported to vary. The variation is largely due to fungal species which respond differently towards oxidative stress. Therefore, it has come to our interest to study the ozone oxidation effects on *C. capsici* and to reveal its behaviour towards ozone oxidative stress.

Materials and Methods

Design of Ozone Fumigation Chambers

Polycarbonate chambers (112 x 47.5 x 42.5 cm) were constructed to supply ozone treatment to fruits at desired concentrations. Each chamber contained six 12 V fans to achieve uniform circulation of ozone molecules generated by a MedKlinn Professional Series Ozone Generator. The ozone concentration inside the chamber was controlled using an ozone sensor (Model OEM-2 Eco-Sensor, Inc.) that was synchronized with a process controller (Model K3MA-J, OMRON Corp.) and calibrated against an ozone analyser (Model IN2000-L2-LC, In USA, Inc.). Temperature and relative humidity of the chambers were monitored using a data logger (Model U14 LCD Logger, HOBO®, USA) and were maintained at 18 - 20°C and 90% RH respectively.

Evaluation of the Effects of Ozone on Radial Growth of C. Capsici Mycelium

In vitro analysis was carried out according to Minas et al. (2010) with some modifications. Mycelial plugs (5.0 mm diameter) from the periphery of actively growing *C. capsici* cultures (isolated from anthracnose infected bell pepper) were inoculated onto Potato Dextrose Agar (PDA, Merck, pH 7.0) and the mycelium was exposed to ozone at 0 (control), 1.0, 3.0, 5.0, 7.0 and 9.0 ppm for 72 hours at 18 - 20°C, 90% RH with Petri plate lids removed to allow air circulation. Following the exposure, the cultures were incubated in duplicate

clean air chambers (0 ppm of ozone) at 18 - 20°C until the end of incubation period (total of 9 days). Radial mycelia growth was recorded at the end of the incubation period and inhibition percentage was calculated by comparing the growth of ozonated mycelium with the control. There were three replicate Petri dishes for each treatment and the experiment was run thrice.

Evaluation of the Effects of Ozone on Sporulation Rate of C. Capsici

C. capsici sporulation was determined according to Antony-Babu and Singleton (2009) with some modifications. Following the period of treatment and clean-air incubation (total of 9 days), agar plugs (42.52 mm^2) adjacent to the mycelial colonies were carefully sampled using a 5.0 mm cork borer. The plugs were incubated in 5.0 ml of distilled water with continuous agitation at 250 rpm for 18 hours to produce spore suspensions. Concentration of the spore suspensions was estimated using a haemocytometer. Three replicates of each treatment were set up and the experiment was run thrice.

Microscopic Evaluation of Mycelium Morphology Using Environmental Scanning Electron Microscopy (ESEM)

Following the period for treatment and clean-air incubation (total of 9 days), agar plugs (7.07 mm^2) adjacent to the mycelium colonies were carefully removed using a cork borer without disturbing the fungal structure. The culture plugs were viewed under scanning electron microscope (Model: Quanta 400F ESEM, FEI, USA) using ESEM mode to determine the effects of ozone on fungal mycelium development (Antony-Babu and Singleton, 2009).

Statistical Analysis

The experiment was carried out with a Completely Randomized Design (CRD) with 3 replicates per treatment. The results were analysed using Statistical Analysis Software (SAS, version 9.1.3, SAS Institute Inc., USA). Analysis of variance (ANOVA) was performed with $P < 0.05$ significance level and differences in data means were analysed using Duncan's Multiple Range Test (DMRT). Data were presented with standard errors generated using SAS.

Results and Discussion

Effects of Ozone on the Radial Growth of C. Capsici Mycelium

Radial mycelial growth was found to be significantly ($P<0.05$) reduced by ozone exposure (Figure 1) where exposure to 7.0 and 9.0 ppm of ozone resulted in the highest mycelial inhibition.

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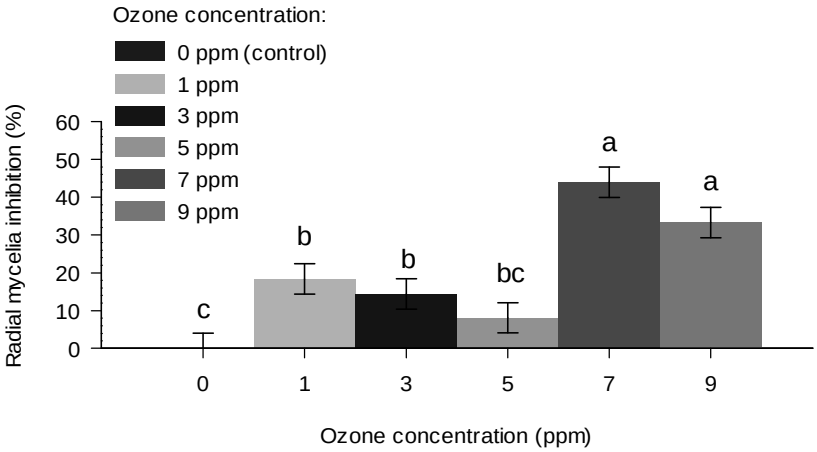

Figure 1. Effects of ozone exposure on *C. capsici* radial mycelia growth. Error bars are the standard error values. Bars with the same letters are not significantly different using Duncan Multiple Range Test (DMRT), (n = 3, P < 0.05). The treatments illustrated that exposure to 7 ppm of ozone for 72 hours has the highest inhibition on the *C. capsici* radial mycelia growth.

Effects of Ozone on Sporulation Rate of C. Capsici

Sporulation rate of *C. capsici* was found to be significantly (P<0.05) influenced by ozone exposure as exposure to 3.0 and 5.0 ppm of ozone has significantly induced sporulation. The exposure to 7.0 and 9.0 ppm of ozone however did not significantly (P<0.05) induce sporulation (Figure 2).

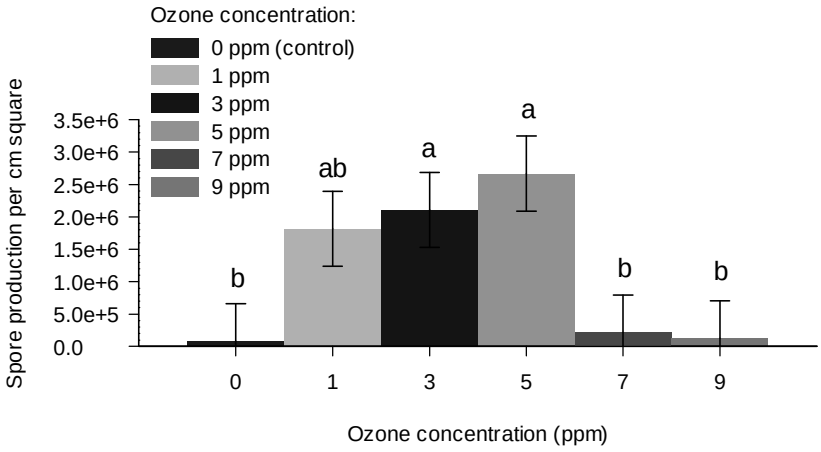

Figure 2. The effects of ozone on *C. capsici* spore production. The error bars are the standard error values. Bars with the same letters are not significantly different using Duncan Multiple Range Test (DMRT), (n = 3, P < 0.05). The treatments illustrated that exposure to 3.0 and 5.0 ppm of ozone has significantly induced fungal spore production but further increase of ozone concentration did not significantly induce sporulation.

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Effects of Ozone on Micro-Morphology of C. Capsici

Ozone was found to have significant effects on *C. capsici* mycelia as the mycelia was found to be compressed and highly branched as indicated in Figure 3.

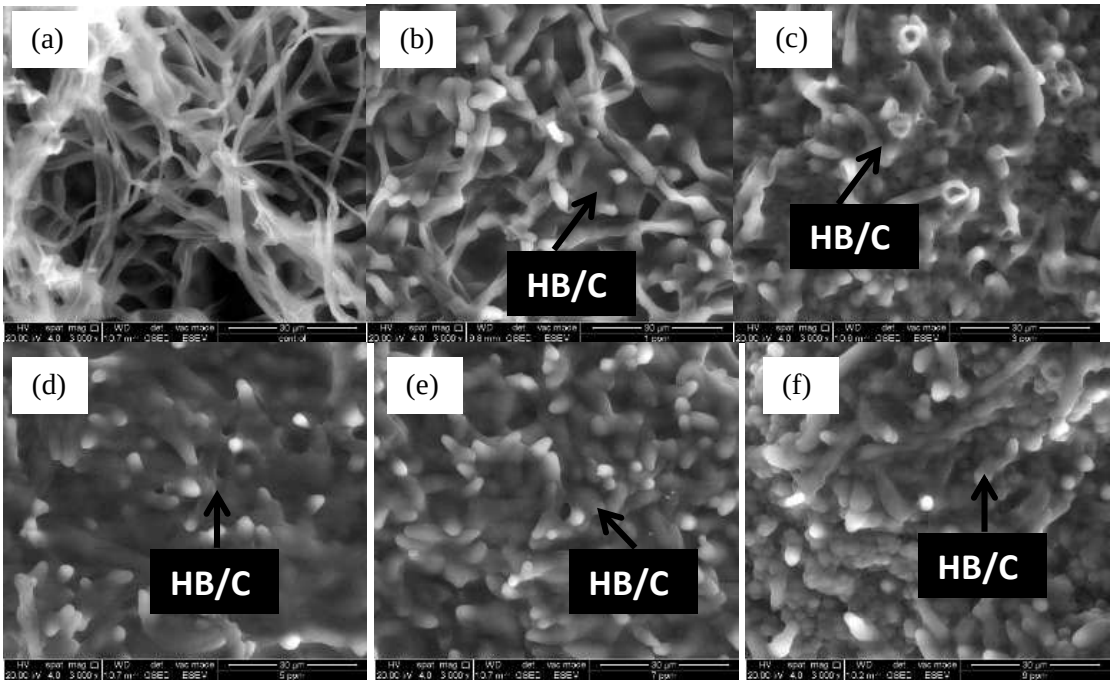

Figure 3. Scanning electron micrograph of *C. capsici* mycelia exposed for 72 hours to (a) 0 ppm (control) (b) 1 ppm (c) 3 ppm (d) 5 ppm (e) 7 ppm and (f) 9 ppm of ozone. Note the highly branched and compressed (HB/C) mycelium caused by ozone treatments.

Results obtained in this study demonstrated that ozone affects both *C. capsici* mycelium radial growth and sporulation rate. *C. capsici* mycelium radial growth was found to be significantly ($P<0.05$) reduced by ozone exposure (Figure 1) where exposure to 7 ppm of ozone resulted in the highest mycelial inhibition (43.96%). Compared to other fungal species, *C. capsici* was found to be more resistant towards ozone oxidation as exposure of *Botrytis cinerea* to only 0.3 ppm of ozone for 24 hours has inhibited mycelial growth by 30% (Minas et al., 2010).

Ozone was also found to affect *C. capsici* sporulation rate (Figure 2) as exposure to 3.0 and 5.0 ppm of ozone has significantly ($P<0.05$) induced the sporulation rate. This modification in fungal behaviour is coherent with Kleb's theory that fungal cultures shift to sporulation as their vegetative development is hampered by environmental stress such as nutrient starvation (Johnson et al., 2003). Further increase of ozone concentration to 7.0 and 9.0 ppm of ozone, however, did not significantly ($P>0.05$) induce the fungal sporulation rate.

ESEM analysis revealed that ozone has significantly affected ozone micro-morphology as ozonated mycelia were found to be contorted, compressed and highly branched. The response of shorter hyphae was also observed by Kreiner et al. (2003) when *Aspergillus niger* was subjected to oxidative stress caused by hydrogen peroxide. The production of shorter hyphae was possibly due to the highly extensive branching network which results in slower hyphal elongation hence could explain the inhibition of mycelium radial growth.

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Conclusions

Ozone has affected both *C. capsici* mycelium radial growth and sporulation rate. The effects are however concentration dependent as different degrees of ozone resulted in different fungal behaviour. In conclusion, the results demonstrated that exposure to 7.0 and 9.0 ppm of ozone is the most effective treatment as it resulted in the highest mycelia inhibition without inducing fungal sporulation rate.

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Effect of Modified Packaging on Postharvest Quality of 'Frangi' Papaya

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Introduction

Papaya is a climacteric fruit that has short shelf life once harvested. Postharvest technology such as modified atmosphere packaging (MAP) and low storage temperature have been widely used to delay fruit ripening. The principle of MAP is after packing a product using a selected film, a desired atmosphere develops naturally as a consequence of the products' respiration and the diffusion of gases through the film (Hu et al., 2005). The gaseous from product respiration (O_2 and CO_2) exchange with atmosphere through packaging film. Horticultural produce that have been packed using film is often stored under low temperature to regulate respiratory metabolism. This is because permeability of package film may not change to the same extent as respiration with temperature, and lead to change in atmospheric composition inside the package (Exama et al., 1993).

Study using combination of MAP and low temperature has been carried out in ciku (Suhaila et al., 1996), fresh ginseng (Hu et al., 2005) and melons (Rodov et al., 2002). Combination of MAP and low temperature storage for papaya has also been studied by Mohd. Salleh et al. (2007). The cultivar of papaya used was Eksotika. Currently, there is a new hybrid of papaya known as Frangi. From our knowledge, information using MAP on this new hybrid is not available. Therefore, a study was conducted to study the effects of MAP on postharvest quality of Frangi papaya during low temperature storage.

Materials and Methods

Mature green fruits of 'Frangi' papaya (*Carica papaya* L. var. Frangi) were obtained from Lanchang, Pahang. Papayas were pre-treated with fungicides at the farm after harvesting. Upon arrivals, fruits were selected based on the uniformity in fruit size and colour with defect and disease free. Then, total of 108 fruits were selected randomly and divided into three lots. Divided fruits were wrapped with paper (bottom half as control), shrink film and Xtend® film. After wrapping, fruits were placed in carton boxes with the floral end parts facing upwards. Fruits were then stored in a cold room of 12 °C for 0, 1, 2 and 3 weeks. At the end of every storage week, 9 fruits from each treatments were transferred to a room temperature of 27 °C with 70% of relative humidity (RH), allowing them to ripen naturally.

Fruits were analyzed at day 0, 3 and 6, using three fruit samples per treatment for each ripening day. Damaged or deteriorated fruits that identified along ripening were merely discarded and not being analyzed in the daily data collection. Pulp firmness was evaluated using a Bishop Penetrometer FT 327 (Alfonsine, Italy). The force required for an 11-mm probe to penetrate the cut surface in two opposite locations to a depth of 8 mm was recorded. The penetration force was expressed in newtons. Soluble solids concentrations (SSC) was measured using juice extracted from pulp samples using a refractometer (Model N1, Atago Co., Ltd., Tokyo, Japan) to obtain the %SSC. The remainder of the juice from the SSC determination was used to measure titratable acidity (TA) by titrating with 0.1 $molL^{-1}$ NaOH. The results were calculated as a percentage citric acid. The pH of the juice was measured using a glass electrode pH meter (CRISON GLP 21, Barcelona, Spain). The vitamin C content was determined according to Ranganna (1977).

The experimental design was a randomised complete block design. The treatments were a 3 x 4 x 3 factorial arrangement with three types of wrappings stored for four weeks and three ripening days after removing from storage chamber with three replicates per treatment. Data were analysed using ANOVA (SAS 9.1) and separation of means was carried out using Duncan's multiple range test.

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Results and Discussion

Generally, interaction between factors did not affect fruit quality except for pH where it was affected by storage week x ripening day (Table 1). Flesh firmness was affected significantly by packaging materials where shrink film showed higher firmness than Xtend® film. For Charentais-type melons, MAP bag-in-box using Xtend® liners delay fruit softening (Rodov et al., 2002). As expected, flesh firmness decrease as storage week and ripening day progressed (Table 1). Firmness loss was relatively gradual as fruit ripened at ambient temperature and it was markedly retarded in fruit stored at 10 °C (Geetha and Thirumaran, 2010). According to Paull et al. (1999), the firmness of papaya decreases quickly as the fruit color changed from 40 to 60% yellow.

SSC were significantly affected by different packaging materials (Table 1). The SSC of control fruit was significantly higher than Xtend® wrapped fruit, indicating Xtend® did not delay fruit ripening. There was a significant decrease on papaya SSC during storage however, no significant changes in SSC were found during ripening. Most probably the low starch content in papaya fruit (Selvaraj et al., 1982) causes the fruit do not have significant amounts of starch to be hydrolyzed during ripening. Zhou and Paull (2001) also reported that the papaya sugar content remains constant during ripening.

The pH of papaya was affected by packaging materials (Table 1). A similar finding was reported in pomegranate (D'Aquino et al., 2010). They found that increase in pH was generally higher in wrapped pomegranate fruit than in control ones. TA and vitamin C content of Frangi papaya fruit was not affected by packaging materials, storage duration and ripening day (Table 1). Similar result was reported by Selvaraj et al. (1982), where the citric acid in papaya showed little changes in the ripening phase. High vitamin C was found in newspaper wrapped papaya at ripe stage (Alam et al., 2010). In this study, TA and vitamin C content of Frangi papaya was retained throughout storage and ripening regardless of packaging materials.

Table 1. Main and interaction effects between packaging materials, storage weeks and ripening days on flesh firmness, soluble solids concentration (SSC), pH, titratable acidity (TA) and vitamin C of Frangi papaya fruit.

Factor	Firmness (N)	SSC (% SSC)	pH	TA (% citric acid)	Vitamin C (mg/100g)
Packaging material (P)					
Control (paper)	13.63 ab[z]	13.37 a	5.38 b	0.16 a	45.13 a
Shrink film	16.07 a	13.19 ab	5.54 a	0.15 a	44.29 a
Xtend®	12.69 b	12.86 b	5.53 a	0.14 a	42.46 a
Storage week (W)					
0	22.49 a	13.48 a	5.37 b	0.17 a	47.69 a
1	12.92 b	13.56 a	5.47 b	0.15 a	47.25 a
2	11.35 bc	12.84 b	5.45 b	0.14 a	42.13 a
3	9.48 c	12.67 b	5.69 a	0.15 a	37.53 a
Ripening day (D)					
0	17.65 a	13.06 a	5.62 a	0.15 a	43.01 a
3	12.63 b	13.38 a	5.57 a	0.17 a	45.11 a
6	11.86 b	12.92 a	5.26 b	0.15 a	43.56 a
Interaction					
P x W	NS	NS	NS	NS	NS
P x D	NS	NS	NS	NS	NS
W x D	NS	NS	**	NS	NS
P x W x D	NS	NS	NS	NS	NS

NS, *, ** Non-significant, significant or highly significant difference at P≤0.05. [z] Means separation within column by DMRT at P≤0.05.

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Conclusions

Although Frangi papaya fruit quality among control, shrink and Xtend® films did not vary a lot, control fruit showed much more decay than films wrapped fruit during 3 weeks of storage. In conclusion, wrapping Frangi papaya with film, especially shrink film, combined with low storage temperature could prolong the shelf life of papaya. In future, water loss, disease incidence and respiration rate of the fruit should be evaluated to draw a better conclusion for films wrapped Frangi papaya fruit.

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Determining Harvesting Maturity of Frangi Papaya Using Non-destructive Method

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Introduction

In papaya, stage of fruit for harvesting is usually performed on the basis of color and starch content. The ripening development of the papaya fruit is separated into five stages, beginning with the hint of yellow at the blossom end: the color break, quarter-ripe, half-ripe, three quarters-ripe and fully-ripe stages (Calegario et al., 1997). Skin color does not allow a fully reliable identification of fruit maturity stage, in particular for the cultivars which are precociously and extensively covered by yellow. Starch content can be assessed simply and rapidly to determine papaya fruit maturity too, but, fruits have to be destruct in order to take the reading.

Recently, a non-destructive method by using visible/near infra-red (VIS-NIR) was developed to determine fruit maturity. DA-meter is developed based on the principle of Vis/NIRwhereby maturity stage is calculated as the difference in absorbance between two wavelengths near the chlorophyll *a* peak (known as Index of Absorbance Difference, I_{AD}). The I_{AD} is related to the time course of change in ethylene emission, fruit quality traits and transcription of ripening-related gene which accompany the progression of ripening. This technology has been used to determine harvesting stage for apples (Nyasordzi et al., 2013) and peach (Ziosi et al., 2008). To author knowledge, no study has been carried out to determine papaya fruit maturity using Vis/NIR. Therefore, a study was carried out to verify the capability of DA meter in monitoring papaya fruit maturity for harvesting.

Materials and Methods

The DA index (I_{AD}) is obtained through the portable spectrophotometer DA-meter brand Turoni-Italy. The light beams are emitted at wavelength of 670 and 720 nm (infra-red), in which the product obtain the signals of interactance (I) and absorbance (A) of the fruits and the index calculation is based, in the Lambert Beer law ($A = \log_{10} I^{-10}$) being calculated as: $I_{AD} = A_{670} - A_{720}$.

A_{670} and A_{720}, were the absorbance values of fruits in wavelength of 670 and 720 nm, respectively. The equipment consists of a light source composed of six LEDs, positioned around a photodiode. Three diode LEDs emit wavelengths of 670nm and other three in length 720nm. The fruits are submitted to light of short duration with two monochromatic sources and within each, the amount of light remitted from the fruit is captured and measured by the central photodiode. The light received then is converted in one “Adc converter” (“analog to digital converter”) and elaborated by a micro-controller for the DA index calculation.

Ten Frangi papaya plants were chosen randomly in a commercial farm, Lanchang, Pahang. The fruits of these plants were monitored for its I_{AD} for 6 weeks. Each plant considered as a replication. Nine fruits facing east and west, respectively, were selected and divided into upper, middle and lower zones with each zone consisted of 3 fruits. Fruits at upper zone of trunk are the youngest fruit among three zones investigated. While the fruit at lower zone were the most mature among three zones studied and the fruits are readied for harvestingby week 6 of observation period. A DA meter was used to determine skin $I_{AD}$with one reading taken from floral end, equatorial and stem end of a fruit. Means of three readings was obtained. The collected data was subjected to analysis of variance and means was separated using LSD at $P \leq 0.05$.

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Results and Discussion

There was no significant interaction between weeks of observation x fruits orientation whether facing east or west (Table 1). The I_{AD}values of fruits grow at three different positions on a trunk which was categorized as upper, middle and lower zones, showed significant differences as observation weeks progressed. However, fruits facing east or west did not affect the I_{AD} values of fruit, reflecting the fruit received similar amount of sun light at both orientations.

Table 1. Analysis of variance for I_{AD} of Frangi papaya fruits positioned at upper, middle and lower zones of a trunk with fruits facing east and west observed for 6 weeks in field.

Source	df[z]	I_{AD} at upper zones	I_{AD} at middle zones	I_{AD} at lower zones
Observation week (W)	5	**[y]	*	**
East-West orientation (O)	1	ns	ns	ns
W x O	5	ns	ns	ns

[z]df, degrees of freedom
[y]ns,* and ** indicate non-significant and significant at $P \leq 0.05$, 0.01 and 0.001, respectively.

Fruits grow at upper and middle zones of trunk showed increasing values of I_{AD} as observation week progressed (Figure 1). In contrast, fruits grow at lower zone showed decreasing values of I_{AD} as duration of observation progressed. Frangi papaya fruits grow at lower zone of trunk are the most mature as compared to the other two zones of fruit. Furthermore, the fruits at lower zone were harvested by workers at week 6 of observation. The decreasing I_{AD}values as observation week progressed indicated the chlorophyll content of skin decreased since I_{AD} is the difference in absorbance between two wavelengths of 670 and 720 nm. This finding is accordance to the finding by Paull et al. (2008) where chlorophyll in papaya skin tissue is degraded at the final phase of fruit maturation. The increasing I_{AD} values of papaya fruits situated at upper and middle zones of trunk indicated chlorophyll accumulation is still actively being carried out. Thus, the fruits are not mature enough to be harvested. Even if harvested, the fruit quality is not acceptable by market.

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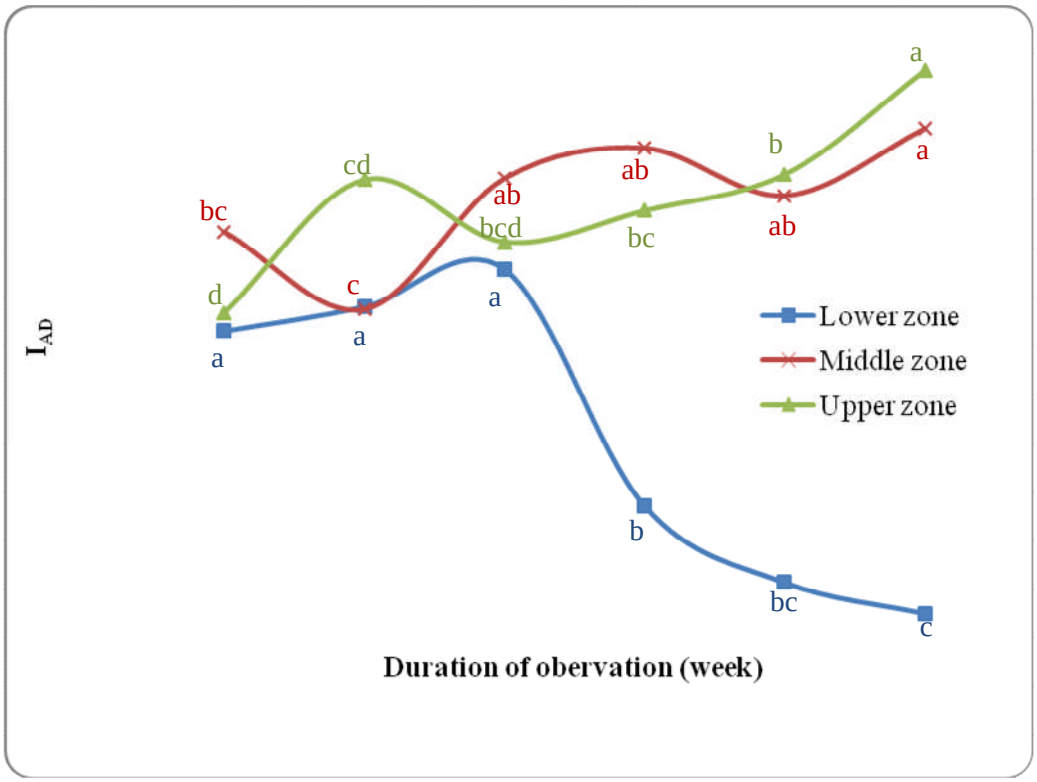

Figure 1. I_{AD} of Frangi papaya positioned at upper, middle and lower zones of a trunk observed for 6 weeks in field. Duration of observation not connected by the same letters are not significantly different at P = 0.05.

Conclusions

From the data reported, DA meter can be used as non-destructive tool to determine harvesting stage for papaya. In future, papaya fruit quality attributes such as ethylene production, firmness and colour pigments contents should be carried to correlate with I_{AD} values to draw a better conclusion as these quality attributes have been widely used in determining papaya fruit maturity.

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Effect of Pre-harvest Application of Calcium on Physicochemical Characteristics of Papaya (*Carica papaya* L. cv. Eksotika II)

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Introduction

Papaya, which belongs to family Caricaceae, is a large perennial plant with rapid growth (Paull and Duarte, 2010). It is an important fruit in Malaysia, ranking third after durian and banana. The Eksotika II cultivar is a high yielding and good quality F_1 hybrid released by Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. The cultivar has gained popularity in the domestic and export markets (Shukor and Shokri, 1997). Calcium, a major plant nutrient, affects cell wall and plasma membrane formation and plays a key role in growth and biomass production in plants. It can decrease fruit decay, and increase firmness and storage duration. In addition, to increase fruit quality, it can be added as soil or foliar applications (White and Broadly, 2003). Therefore, this study was conducted to look at the effect of pre-harvest application of calcium chloride on postharvest changes of fruits in storage.

Materials and Methods

Fifty-two papaya (*Carica papaya* L. cv. Eksotika II) plants from six month-old orchard at Agro-Tech Unit, University Agriculture Park (TPU), Universiti Putra Malaysia, Serdang, Selangor were randomly selected for experiment. The experiment conducted between May to July, 2012. Calcium chloride at various concentrations, 0, 0.5, 1.0, 1.5 and 2.0% calcium were sprayed 21 days after flower anthesis and Tween 20 was used as surfactant. Sprayings were done every two weeks for six times until 10 days before harvesting. Uniform size and shape fruits were harvested at index 2 (green with trace of yellow), washed with water and allowed to air dry before randomly divided into five different lots. Each lot contained 52 fruits in four replications. Fruits for each treatment were packed in commercial boxes and stored at 12 ± 2^{o}C and 85-90% relative humidity. After three weeks, physico-chemical characteristics of the fruits in storage were determined. Four fruits per treatment were used for parameter determinations. Respiration rate and ethylene production were measured using the method of Saltveit (1982). Soluble solids concentration (SSC) and titratable acidity (TA) were measured by the methods of Ranggana (1986). The SSC was determined by a hand refractometer. Titratable acidity was determined by titrating with 0.1 N NaOH to pH 8.2, and results were expressed as percentage of citric acid per 100g fresh weigh. Fruit firmness was measured by Instron Universal Testing Machine and results expressed in Newtons. For determining weight loss, fruits were weight with a digital balance before storage and after 21 days in storage and results were expressed as percentage loss of initial weight. For sensory evaluation, a panel of six judges was selected based on reliability of judgement. Sensory evaluation of all fruits for texture, flavour and aroma was performed using the Hedonic scale by allotting the number from 0 to 5, where 0 for very poor and 5 for excellent.

Statistical Analysis

The experiment was designed in Randomised Complete Block Design (RCBD) with four replications. Data were subjected to Analysis of Variance (ANOVA) using Statistical Analysis System (SAS) version 8.2 (SAS Institute Inc., Cary, NC, USA). The means were compared by the Duncan's Multiple Range Test (DMRT) at significance level of 0.05.

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Results and Discussion

Results showed that with increasing calcium concentration, respiration rate and ethylene production decreased (Table1). Our results are in agreement with other researchers who found that calcium contributed to decrease ethylene production in peach (Agusti et al., 2004). There were no significant differences among 0, 0.5 and 1% calcium chloride for SSC, while there were significant differences between control with 1.50 and 2%. The effect of calcium in reducing SSC of fruits was probably due to slowing down respiration and metabolism activity hence reducing ripening. Lower respiration slows down the synthesis of metabolites that results lower SSC due to slower change of carbohydrate to sugars (Rohani et al., 1997). Similar results were obtained for titratable acidity. Similar results were reported in peach treated with pre-harvest calcium treatment (Manganaris et al., 2005). It was observed that pre-harvest spray with 1.50 and 2.0% calcium showed higher retention of firmness compared to the control fruits (Table2). This is probably due to added calcium in peel and pulp that help in maintaining the structure and function of cell wall. Similar result was observed in kiwi fruit (Dimitrios et al., 1996). Weight loss among treatments was significantly reduced with increasing concentrations of calcium. While the lowest weight loss was in 1.50 and 2.0% calcium chloride, the highest in the control. The increased in weight loss in untreated fruits might be due to increased breakdown that was related to higher respiratory rate (Mandal et al., 2010).

The fruits treated with 1.50 and 2.0% calcium attained maximum score by the panel in all tested parameter, while untreated fruits had lowest scores (Table3). Calcium treatments seemed to increase sensory traits of fruits. This would suggest that the benefits of calcium were from the process that affected detoriation. Increased firmness was related to increase in cell wall structure and integrity.

Conclusions

Based on results obtained, calcium is an applicable method for increasing postharvest attributes of papaya fruits. In this experiment both 1.5 and 2.0% calcium increased postharvest characteristics of fruits. So, 1.5% calcium chloride can probably be recommended to be applied in the field as pre-harvest calcium treatment for papaya.

Table 1. Effect of pre-harvest calcium chloride treatments on respiration rate, ethylene production, soluble solids concentration (SSC) and titratable acidity (TA) in papaya fruits after three weeks of storage at 12 ± 2 °C.

Calcium chloride concentrations (%)	Respiration (ml/kg/h)	Ethylene(µl/kg/h)	SSC (%)	TA (%)
Control	22.80a[z]	11.20a	11.62a	0.13b
0.5	22.00ab	11.10a	11.30a	0.14b
1.0	20.27ab	7.57b	9.75ab	0.16ab
1.5	19.90 b	5.50b	9.25b	0.17a
2.0	19.90b	5.45b	9.15b	0.17a

[z]Means followed by the same letter in the same column are not significantly different at ($p \leq 0.05$) at DMRT

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Table 2. Effect of pre-harvest calcium chloride treatments on firmness and weight loss of papaya fruits after three weeks of storage at 12 ±2 C.

Calcium chloride concentrations (%)	Firmness (N)	Weight loss (%)
Control	3.42c[z]	12.30a
0.5	4.45c	11.00b
1.0	11.20b	6.45c
1.50	15.6a	5.60d
2.0	15.15a	5.55d

[z]Means followed by the same letter in the same column are not significantly different at ($p \leq 0.05$) at DMRT

Table 3. Effect of pre-harvest calcium chloride treatments texture, flavour and aroma in papaya fruits after three weeks of storage at 12 ±2 C.

Calcium chloride concentrations (%)	Texture	Flavour	Aroma
Control	2.21c[z]	3.05c	2.70bc
0.5	2.08c	3.03c	2.60c
1.0	2.80b	3.16b	3.00b
1.50	3.26a	3.31a	3.40a
2.0	3.28a	3.35a	3.48a

[z]Means followed by the same letter in the same column are not significantly different at ($p \leq 0.05$) at DMRT

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Effectiveness of Selected Mulches Improving Postharvest Quality of Field-Cultivated Leaf Lettuce

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Introduction

Lettuce (*Lactuca sativa* L.) is a leading leafy vegetable worldwide with an estimated production of 23 million tons in 2007 (Food Agriculture Organization of the United Nations, 2007). In Malaysia, the planted acreage for vegetables was 53,057 ha, with a production of 874,602 tons in 2010. For lettuce, the acerage was 2,297 ha with a production of 38,790 ton (Mokhtar, 2008). Lettuce is eaten fresh and commonly grown as a hydroponic crop, but the local leaf lettuce is grown on soil. Naturally, weeds become the main problem of growing lettuce on soil because lettuce is slow to establish, thus causing weeds to outgrow the crop. Also, weed control with application of chemical herbicides are hazardous to the environment and human as it is eaten fresh. For this reason, the use of allelopathic mulch to control weed is an alternative to herbicide control in sustainable vegetable production.

For a leafy vegetable like lettuce, the practice of mulching would help to control weeds as well as conserve moisture and nutrients in the soil. These cultural practices influence nutrient and water supply which affect the postharvest quality characteristics, such as appearance, texture, taste and aroma, of the harvested vegetable (Kader, 2002). Besides, mulching obstructs the reflection of soil heat and the produce has a longer keeping quality. Mulching has been widely recommended in vegetable production worldwide. Since some of the benefits derived from mulching include increase water and nutrient availability and improved soil structure and porosity, it has become important to identify cost-effective biological mulch materials that could be used by vegetable growers to improve crop production and reduce weed growth. The objective of this study was to evaluate the effect of two different mulch materials, sunflower and cucumber, on improvement nutrient postharvest quality.

Materials and Methods

Leaf lettuce (*Lactuca sativa*) seeds used as planting material were purchased from a Commercial Seed Company. The field experiment was conducted at Taman Pertanian Universiti, Universiti Putra Malaysia (2^{o} 59.225'N, 101^{o}42.439'E, 45 m from sea level) on a clay loam soil. The treatments involved two types of mulches which were sunflower and cucumber plant residues. The plant residues were collected from organic farm, chopped and air dried. Four rates of allelopathic mulch residues used were 0 (water as control), 5, 10 and 15 t/ha. Allelopathic mulch residues were applied manually on the soil surface after transplanting. The experimental design was a randomized complete block design (RCBD) with four replications. The total experimental units comprised 28 raised beds: (3 rates x 2 residues x 4 replications) + (control x 4 replication).

The seeds of leaf lettuce were sown in a 220-cell seed tray using peat moss as the substrate. The seeds were allowed to germinate in the nursery with watering done twice a day. After 15 days in the nursery, the seedlings were transplanted into raised planting beds at the rate of 1 seedling/hole, with each bed measuring 1 m wide x 1 m long and 20-30 cm high. The distance between beds was 0.5 m in a field plot of 11 m wide x 6.5 m long. The distance between planting rows on each bed was 20 cm and between plants within a row was 20 cm, giving a total of 25 plants per replication. After transplanting, mulching with an allelopathic mulch residues was applied on each raised bed and the plants were watered. General agronomic practices carried out in the field were mulching, watering, pest control management, and soil fertilisation. The plants were watered twice a day as required, by using sprinkler irrigation. Weeding was not carried out in order to determine the

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effects of allelopathic mulch residues on weed growth. Ten ton/ha chicken manure was incorporated into each bed, 3 days before transplanting. Organic compound NPK fertilizer (8: 8: 8) was also applied at the rate of 100 kg N/ha at 10 days after transplanting. The fertilizer was applied along each plant row on the raised bed. Fresh leaf lettuce was hand-harvested by up-rooting the whole plant after 49 days of transplanting. All samples were cleaned under running water and excessive water was drained off with a tissue paper. Then, the vegetables were brought to the Postharvest Laboratory, Faculty of Agriculture, Universiti Putra Malaysia for postharvest quality analysis.

Determination of firmness

Lettuce firmness was measured using an Instron (Model 5543 load frame, Instron Corp., USA) with a 6 mm diameter cylindrical probe at a speed of 20 mm/min. The Instron was used simultaneously with an Instron Merlin Software version M12-13664-EN. Two readings per plant were recorded in Newton (N) and the mean was calculated.

Determination of titratable acidity

Three whole lettuce plants were chopped and 10 g of the chopped sample was blended with 40 mL distilled water using a blender (MX-798S, National, Malaysia) and filtered through cotton wool. Citric acid was determined according to the titration method by Ranganna (1977). Five millilitres of the filtrate was transferred into a conical flask and 2-3 drops of % phenolphthalein was added as the indicator. The sample was titrated with sodium hydroxide 0.1 N to pH 8.2. The titre volume was recorded and the result was expressed in percentage of citric acid (equivalent weight of citric acid=64 g) as the predominant acid present.

Determination of soluble solids concentration

The SSC was determined using a digital refractometer (Model N-1 α, Atago, Japan) according to Dadzie and Orchard (1997). About 1-2 drops of the balance of juice from titratable acidity determination was placed on the glass prism of the refractometer. The percentage soluble solids concentration was recorded. The reading was corrected according to dilution and a standard temperature of 20^0 C by adding 0.28% to obtain %SSC (Bourne, 1980)

Determination of pH

The remaining of juice from titratable acidity determination was used to measure pH. The pH was determined by using a pH meter (GLP 21, Crison, Barcelona). Before being used, the glass electrode was calibrated with buffer at pH 4.0, followed by buffer at pH 7.0. Then, the glass electrode was immediately placed in the filtrate to measure the pH. The stabilized pH reading was recorded.

Determination of ascorbic acid contents

Ten gram of lettuce was blended with 40 mL 3% metaphosphoric acid (HPO_3) using a blender (MX-798S, National, Malaysia) and filtered through cotton wool. Ascorbic acid content was determined according to the titration method by Ranganna (1977). Five millilitres of the filtrate was titrated with 2,6-diclorophenol-indophenol dye until the filtrate changed to a pink colour which persisted for 15 seconds. The titre volume of dye solution used was recorded and ascorbic acid content was calculated

Experimental design and data analysis

The experimental design was a randomized complete block design (RCBD) with four replications. Data were analyzed using the analysis of variance and regression (SASVersion 9.1; 2003).

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Results and Discussion

Results indicated that cucumber and sunflower allelopathic residues used for mulching during field production of leaf lettuce did not significantly affect firmness, pH, TA and AA of leaf lettuce (Table 1). The postharvest quality characteristics of leaf lettuce grown using allelopathic mulches showed no significant differences compared to control treatment.

The firmness of crops is important for eating quality and is a factor in withstanding shipping stresses (Kader, 2002). Firmness was caused by the increase of mineral Ca in the plant. Ca interact with the pectin in the cell wall and causes the cell wall to become rigid (Bartley and Knee, 1982). Firmness depended on the rigidity of cell wall, amount of SSC and amount of starch in the vegetables. SSC could be used as an indicator of sweetness because sugar (sucrose, glucose and fructose) are major components of soluble solids. The increase in SSC could be attributed to hydrolysis of starch into simple sugars.

Table 1. Firmness, pH, soluble solids concentration (SSC), titratable acidity (TA), and ascorbic acid (AA) of leaf lettuce that was mulched with allelopathic residues from sunflower and cucumber at rates of 0 (distilled water control), 5, 10 and 15 ton/ha.

Factors	**Firmness (N)**	**pH**	**SSC (%)**	**TA (%)**	**AA (mg/100 g)**
Allelopathic mulching (AM)					
Sunflower	13.69 a[z]	6.01 a	1.77 a	0.05 a	26.50 a
Cucumber	12.18 a	6.09 a	1.91 a	0.05 a	24.10 a
Rate (tonne/ha) (R)					
0	10.68 a	6.01 a	2.00 ab	0.07 a	22.56 a
5	13.39 a	6.09 a	2.29 a	0.05 a	22.50 a
10	13.00 a	6.01 a	1.67 bc	0.05 a	27.22 a
15	13.41 a	6.03 a	1.51 c	0.04 a	25.83 a
AMxR	ns[y]	ns	ns	ns	ns

[z]Means followed by the same letter are not significantly different using LSD at P=0.05.
[y]ns is non-significant at P=0.05.

Different cells cause different texture to the vegetable. Young plants rich with the parenchyma cells are succulent. With the differentiation of cells, collenchyma and sclerenchyma cells produce different texture to the vegetables (Pantastico, 1975). In addition, water content affect quality of vegetables. Moderate water stress could cause toughness and poor appearance but increase SSC, acidity and ascorbic acid content of vegetables (Kader, 2002). However, excessive water cause turgidity of plant cells, leading to increase susceptibility to physical injury and reduced firmness. Mulching conserve soil moisture, but the effects were not seen in the current study.

The pH affects the acidity or alkanity of the juice. However, pH value was not directly related to titratable acidity. It is dependable on the concentration of free H^+ and buffering capacity of the extracted juice. Ascorbic acid is abundantly present in all plant cells and has many biological functions. As an antioxidant, vitamin C prevents browning of tissue, which is an oxidation reaction. Ascobic acid have been widely reported to increase under an organic cropping system, as was practiced for the current study. Again, it was not affected by the organic residues used for mulching the leaf lettuce crop (Kader, 2002).

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Conclusions

There were no significant main and interaction effects of allelopathic mulch residues and rates of mulch residue on postharvest quality characteristics of the leaf lettuce. This finding indicates that cucumber and sunflower mulches could be used safely for leaf lettuce production without affecting its quality. However, at this stage of the experiment, conclusions for the effect of mulches on the leaf lettuce quality might be too early to make, as the experiment was carried out only for 49 days (i.e. one crop cycle of leaf lettuce production).

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Effect of Thermal Processing on Bioactive Compounds, Antioxidant Activity and Microbial Properties of Chokanan Mango (*Mangifera indica* L.) Juice

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Introduction

Chokanan mango *(Mangifera indica L.)* is one of the most popular cultivar grown in Malaysia for local and export market. According to Spreer et al. (2009), there is a large stock of Chokanan mango every year as it has three harvests in May, June and August. This is due to its ability to yield off-season flowering without applying chemicals for initiation. Hence, this characteristic allows the fruit to be processed into products including juice, nectars, puree, pickles and canned slices that are globally accepted.

Several studies have reported bioactive compounds in mango which are good antioxidants and have been linked to the prevention of cardiovascular diseases and cancer (Block et al., 1992; Liu, 2003). Therefore, consumption of mango juice could provide substantial dietary source for the humans.

The popularity of natural fruit juices has urged studies on juice processing. Commercial thermal processing is the most preferred method to extend the shelf life of juices. The concern of the juice industry in thermal pasteurization is to inactivate microorganisms and enzymes which cause undesirable changes to the juice during storage. However, there has been reports that show loss of quality in thermally treated juices such as orange (Cortes et al., 2008), strawberry (Aguilo-Aguayo et al., 2009), and watermelon (Zhang et al., 2011).

The purpose of this study was to evaluate the impact of thermal treatments (mild and high heat pasteurization) on bioactive compounds (ascorbic acid and carotenoids) and antioxidant properties such as polyphenol content, radical scavenging activity, reducing power and total antioxidant capacity of Chokanan mango juice. In addition, the effect of thermal treatments on the microbial count (aerobic plate count, coliform, yeast and mould) of Chokanan mango juice was also studied.

Materials and Methods

Plant Material and Extraction of Juice

Mature green Chokanan mango were harvested at 13 weeks after anthesis from a mango farm in Tobiar (Kedah, Malaysia) which is located about 428 kilometres from the Postharvest Biotechnology Laboratory, University of Malaya. The fruits were left to ripen naturally at room temperature (25 ± 1 °C). Ripe pulp were macerated using a domestic juice extractor (Philips Juice Extractor HR 2820, Holland), and then centrifuged (Beckman J2-MI Centrifuge, California) at 12000 rpm for 10 minutes at 4 °C. The supernatant was filtered and stored in sterile glass bottles prior to deployment into experiment.

Thermal Treatment

Freshly squeezed mango juice stored in glass bottles were pasteurized in a covered water bath (Memmert, Germany) at 90 ± 1 °C for 30 seconds (mild heat pasteurization) and 90 ± 1 °C for 60 seconds (high heat pasteurization). After the thermal treatment, the juice samples were immediately cooled by immersing in an ice-water bath for 5 minutes.

The following terms were used to describe the different treatments in this study: Control (freshly squeezed or no treatment); MP (mild heat pasteurization) and HP (high heat pasteurization). All treatments and analysis were carried out in triplicates.

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Bioactive Compounds (Ascorbic Acid and Carotenoid Content)

The ascorbic acid content in samples was determined based on the 2,6-dichlorophenol-indophenol (DCPIP) visual titration method (Ranganna, 1977).
The carotenoid extraction was performed according to Lee et al. (2001), while the total carotenoid content was determined using β-carotene as a reference (Scott, 2001).

Antioxidant Activity

Total polyphenol content of juice samples were determined using Folin-Ciocalteu assay modified to a microscale (Bae and Suh, 2007) and results were reported as milligrams of gallic acid equivalent (GAE) per 100 ml juice extract.
The DPPH radical scavenging assay is based on the method described by Oyaizu (1986) and Bae and Suh (2007), and results were reported as micrograms of ascorbic acid equivalent (AAE) per ml juice extract.

Reducing power assay was measured with a spectrophotometric method by Oyaizu (1986) and results were reported as micrograms of ascorbic acid equivalent (AAE) per ml juice extract.
Total antioxidant capacity was evaluated by the phosphomolybdenum method according to Prieto et al. (1999) and results were reported as micrograms of ascorbic acid equivalent (AAE) per ml juice extract.

Microbial Inactivation Analysis

The microbial count of juice samples were determined using Petrifilm plates (3M Center, MN, USA) for aerobic bacteria, coliforms, yeast and mould. Diluted sample (1 ml) was placed on Petrifilm plates. The aerobic plate count, coliform count and yeast and mould count in samples were expressed as log colony-forming units (CFU) per ml of juice.

Statistical Analysis

The data obtained were subjected to statistical analysis using SPSS 19.0 software (SPSS Inc., IBM). In this study, data was represented as mean values ± standard deviation (SD). The significant differences between mean values of juice samples were determined by analysis of variance (one way-ANOVA) using Tukey's HSD (Honestly Significant Difference) test at a significance level of $p < 0.05$.

Results and Discussion

Bioactive Compounds (Ascorbic Acid and Carotenoid Content)

The results of the effect of thermal treatments on ascorbic acid and carotenoid content in juice extracts are shown in Figures 1 and 2. In this study, significant decrease in the ascorbic acid (Vitamin C) content was observed in juice processed by both thermal treatments when compared to control. HP sample recorded the highest degradation (65%) of ascorbic acid when compared to the control. The loss of ascorbic acid might be due to its heat-sensitive characteristic.

Thermal treated juice exhibited significant decrease in carotenoids ($p<0.05$), where MP sample showed 56.13 µg/100 ml and HP sample showed 48.92 µg/100 ml of carotenoid content, respectively, in comparison with control (82.03 µg/100 ml). In a previous study conducted by Lee and Coates (2003), it was observed that thermal treatment induces geometric isomerization of carotenoids, resulting in significant loss of carotenoids in orange juice. In addition, heat causes instability of the conjugated double bond system of carotenoids, resulting in oxidation (Rodríguez-Amaya, 1997).

Antioxidant Activity

The results of the effect of thermal treatments on antioxidant activity in juice extracts are shown in Table 1. The Folin-Ciocalteu method is based on the detection of phenolic compounds by reduction of reagent, which contains tungsten and molybdenum oxides. In this study, a significant reduction in extractability of polyphenols was observed in heat treated juices (Figure 2), being lowest for HP sample (37.8% reduction).

The DPPH radical scavenging assay measures the hydrogen donating capacity of the antioxidant to the stable free radical DPPH, which forms diphenylpicrylhydrazine (Shon et al., 2003). In this study, juice samples subjected to both thermal treatments showed no significant changes in percentage of DPPH inhibition.

The reductive ability of a compound (Fe^{3+} to Fe^{2+} transformation) may serve as an important indicator of its potential antioxidant activity. The presence of antioxidants in the sample extract may cause the reduction of the ferricyanide complex to ferrocyanide complex in this assay (Rama Prabha and Vasantha, 2011). With regard to reducing power, thermal treatments showed non-significant decrease when compared to the control. The reducing power was 355.00 µg AAE/ml and 345.00 µg AAE/ml in MP and HP samples, respectively compared to control (360.71 µg AAE/ml).

Total antioxidant capacity is based on the reduction of Mo (VI)–Mo (V). The presence of antioxidants in the sample extract may cause formation of a green phosphate/Mo (V) complex at acidic solution (Prieto et al., 1999). In this study, juice samples subjected to both thermal treatments showed no significant changes in total antioxidant capacity.

Microbial Inactivation Analysis

In this study, juice samples after thermal treatment showed a significant reduction of microbial count (Figure 3). The aerobic bacteria, coliform, and yeast and mould counts in freshly squeezed Chokanan mango juice were 2.74 log CFU/ml, 0.99 log CFU/ml, 2.12 log CFU/ml, respectively. Thermal treatment regardless of mild or high heat pasteurisation inactivated 100% aerobic bacteria, coliform and yeast and mould. The results obtained are in agreement with thermally pasteurised blended orange and carrot juice, where 100% reduction of microbial load was observed (Rivas et al., 2006)

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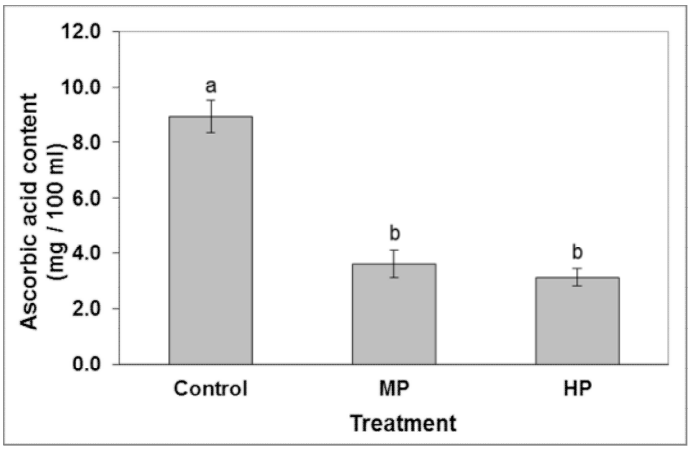

Figure 1. Effects of thermal treatments on ascorbic acid content of Chokanan mango juice. Means followed by different letters (a-b) are significantly different ($p<0.05$).

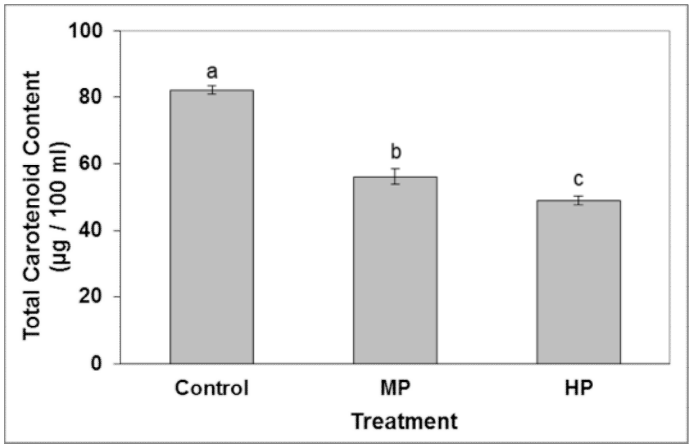

Figure 2. Effects of thermal treatments on total carotenoid content of Chokanan mango juice. Means followed by different letters (a-c) are significantly different ($p<0.05$).

Table 1. Effects of thermal treatments on antioxidant activity of Chokanan mango juice extract.

	Total polyphenol content (mg GAE/100 ml)	DPPH radical scavenging activity (µg AAE/ml)	Reducing power assay (µg AAE/ml)	Total antioxidant capacity (µg AAE/ml)
Control	97.8 ± 1.8 [a]	8.3 ± 0.2 [a]	360.7 ± 3.2 [a]	1022.2 ± 2.8 [a]
MP	82.9 ± 1.6 [b]	8.0 ± 0.2 [a]	356.0 ± 2.5 [a]	1017.5 ± 3.5 [a]
HP	60.9 ± 1.4 [c]	8.1 ± 0.1 [a]	354.0 ± 1.6 [a]	1019.7 ± 2.2 [a]

Means followed by different letters in the same column (a-c) are significantly different ($p<0.05$).

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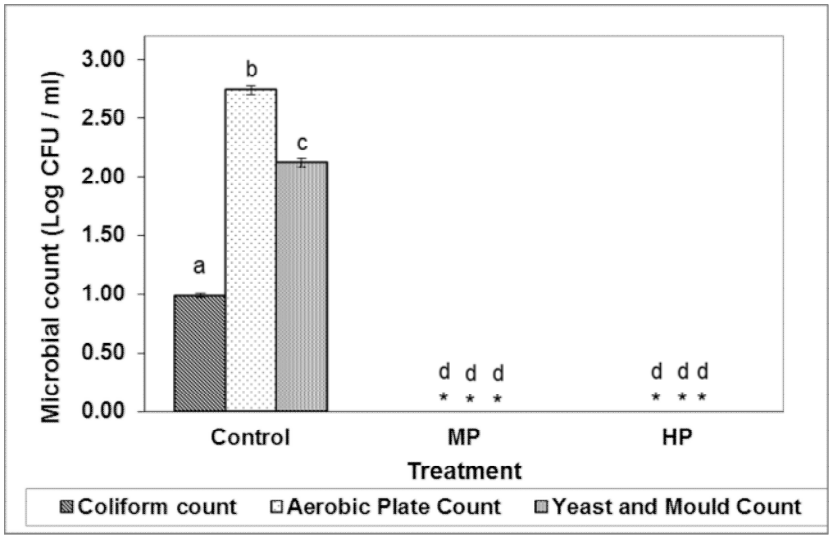

Figure 3. Effects of thermal treatments on microbial count of Chokanan mango juice. Means followed by different letters (a-d) are significantly different ($p<0.05$). *Not detected.

Conclusions

The juice samples subjected to high heat pasteurization exhibit greater loss in ascorbic acid, carotenoid content, and phenolic compounds, when compared to mild heat pasteurization. Thermal treatment regardless of mild or high heat pasteurization retained the antioxidant activity and reduced the microbial loads to below detection limits. The results obtained showed that although thermal treatment completely inactivated microbial growth in the juice, significant loss in quality was observed. Therefore, process optimisation of thermal processing should be considered to minimise the effect on the quality of Chokanan mango juice for the satisfaction of consumers.

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Submicron Dispersios of Chitosan a Green Technology: Against *Colletotrichum gloeosporioides* through Scanning Electron Microscopy (SEM)

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Introduction

Colletotrichum is the ubiquitous fungal specie that is responsible for anthracnose in dragon fruits (Masyahit et al. 2009). Anthracnose can affect the developing fruits in the fields and also damage mature fruits during storage. The farmers lost almost 90% of their normal production that equals to 1.125mt/ha/month worth about RM 3,375/ha/month due to this fungal disease. In year 2008, a total loss of RM 11.2 million has been reported only in Malaysia due to this disease (Cheah and Zulkarnain, 2008).
Currently, synthetic fungicides are used for the control of this disease (Hoa, 2008). However, rising public awareness about the toxicological effects of fungicide to human health, as well as the environmental impact such as poisoning of soil necessitate the development of non-toxic biofungicide.

Chitosan can be one of the potent antifungal agent which is obtained from crab and shrimp shells. Anti-microbial activities of chitosan to control pre and postharvest diseases of various fruits and vegetables were well reported (Ali et al., 2010). However, an alternative of using the conventional form of chitosan against *C. gloeosporioides*, colloidal carriers such as nanoemulsions holding chitosan will have the intense potential. Nanoemulsions are highly stable, so that they have been applied commercially in various agrochemical industries. These nanoemulsions have higher encapsulation efficiency so that active components can be encapsulated within the droplet size, which trim down the practice of chemical degradation of the active compound.

Thus, the objective of this study was to evaluate the effect of chitosan loaded nanoemulsions on inhibition of mycelial growth, and to investigate the alteration of fungal structure through SEM studies.

Materials and Methods

Isolation and Inoculum Preparation of C. Gloeosporioides

Infected dragon fruits were used for the isolation of *C. gloeosporioides* and inoculums were maintained on potato dextrose agar (PDA) plates for further studies.

Preparation of Submicron Dispersions of Chitosan

Different concentrations of chitosan (0.5, 1.0, 1.5, and 2.0%) mixed with emulsifiers (Brij 56, Span 20) and then subjected to ultra-sonication using an ultrasound bath to obtain 600 nm submicron dispersion of chitosan.

Antifungal Assay of Submicron Dispersion of Chitosan

Antifungal assay of submicron dispersion encapsulated with chitosan was performed based on inhibition in radial mycelial growth of *C. gloeosporioides* on PDA medium using the poison food technique. Alteration in spores structure were observed by using scanning electron microscope (FEI Quonta 400 FE-SEM). All the experiments were conducted in completely randomized design (CRD) having four replications of twenty Petri

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plats for each treatment. Results were analyzed by analysis of variance (ANOVA) for the data using SAS 9.1 software and the means were separated according to Least Significant Difference test at ($P < 0.05$).

Results and Discussion

With a change in the chitosan concentration, a significant ($P < 0.05$) change in the sonication time to which the sample should be subjected to was observed; and the time of sonication also changed for obtaining different droplet sizes (Table 1). To obtain smaller droplet size, longer was the time period with which the sonication was applied. It is proven previously that the time of sonication required to prepare nanoemulsions varies with the type and concentration of the material (McClements, 2005). The increase the sonication time, the lower would be the viscosity of the solution (Table 1). As it is already proved that ultrasonication is the input of energy and temperature of the solution increased due to energy input, thus reduces the viscosity (McClements, 2005).

Table 1. Time of sonication and viscosity of different droplet sizes at various concentrations of chitosan

Chitosan conc. (%)	**Time of sonication (min)**	**Viscosity (cp)**
0.5%	13 ± 1.0 d	24.4 ± 2.6 d
1.0%	19 ± 1.5 c	48.4 ± 4.7 c
1.5%	22 ± 1.0 b	428.6 ± 9.5 b
2.0%	24 ± 2.0 a	756.3 ± 12.3 a

Results were expressed as the mean ± SEM (n=10). Means with different letters are significantly different using LSD test ($P < 0.05$).

The result of radial mycelial growth showed no inhibition in control plates while the chitosan loaded nanoemulsions showed a remarkable inhibition at all the concentrations. The highest inhibition was observed at 2.0% chitosan with 600 nm droplet size (81.07%) which is non-significantly different with 1.5% (80.86%) and 1.0% (79.57%) at 600 nm droplet (Figure 1). In a recent study by Ali et al. (2010), it was observed that chitosan markedly inhibited the mycelial growth of *C. gloeosporioides*. However, in the present study, chitosan in the form of nanoemulsions added to growth media showed the greatest effect against *C. gloeosporioides* the causal agent of anthracnose in dragon fruits.

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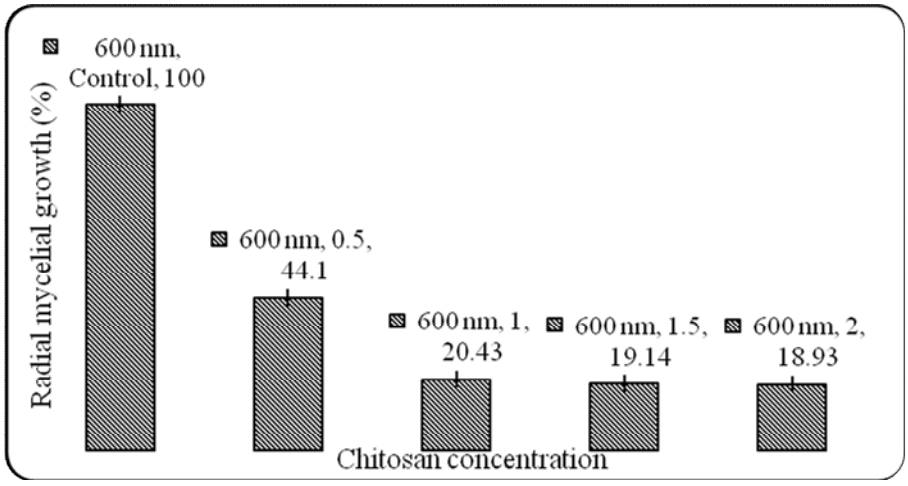

Figure 1. Effect of 600 nm on radial mycelial growth of *C. gloeosporioides* isolated from dragon fruits after 10 days of incubation. The vertical bar represents the standard error of means for four replicates.

The results obtained through SEM showed a remarkable difference between control mycelium and the mycelium treated with 600 nm droplet at 1.0% chitosan concentration (Fig. 2).
Scanning electron microscopic studies of the fungus tested indicates that the conidial germination inhibition, hyphal agglomeration and abnormal shapes of conidia in response to chitosan loaded nanoemulsions might be due to marked cellular changes. Similar results were obtained in a recent study by Al-Hetar et al. (2010), where they found marked cellular changes in the morphology *F. oxysporum* f. sp. *Cubense* after using chitosan. The changes in the morphology of the various other pathogenic microorganisms have also been reported when grown on chitosan (El Ghaouth et al. 1997).

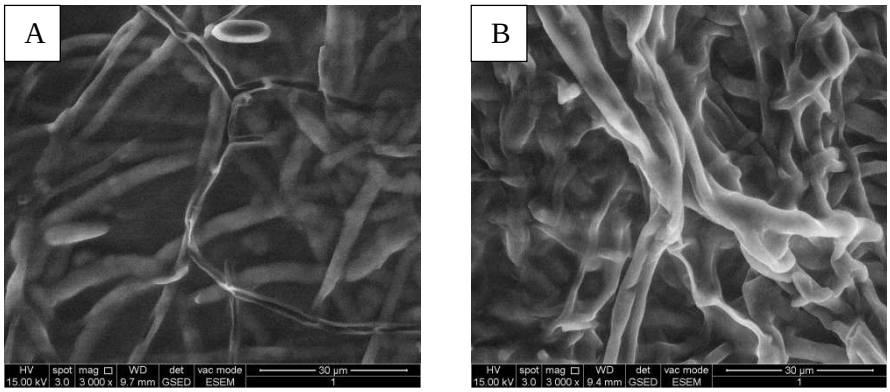

Figure 2. (A). Normal hyphae (B). hyphal agglomeration

Conclusion
It is concluded from this experiment that chitosan in the form of submicron dispersions act more effectively against C. gloeosporioides and 600 nm is an accurate size to penetrate inside the fungus and disrupt its structure.

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Application of Propolis as a Green Technology for Maintaining the Quality of Bell Pepper

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Introduction

An estimate of 10 -50% of yield of marketable bell pepper within Malaysia is lost as a result of bell pepper anthracnose (Nik et al., 1998). Bell pepper anthracnose is caused by *Colletorichum capsici*, which was commonly controlled by chemical fungicides (Roberts et al., 2001).

Several harmful properties of chemical fungicides have been identified, which negatively affect the environment and consumers and provide a selection pressure for resistant and pathogenic fungi. Thus, there has been a move from chemical fungicides towards natural products. Propolis is a resinous beehive product which has been used in folk medicine since 300 BC and is attributed with properties that enable the prevention of diseases such as diabetes, heart disease and cancer (Banskota et al., 2001). Studies have demonstrated that this natural product possesses a range of biological activity that include antioxidant, antibiotic, anti-inflammatory (Ghisalberti, 1979; Scazzochio et al., 2006). Propolis is rich in flavonoids and phenolic compounds, and according to Marcucci (1995) and Bankova et al. (2000) more than 300 biologically active constituents have been identified.

The majority of research that has been carried out on the effect of propolis on plant pathogens has been confined to *in vitro* studies. Thus, the aim of this study was to utilise this natural product as a coating on bell pepper and test its ability to maintain the postharvest quality of bell pepper. The ability of the coating to control anthracnose development will be assessed, along with the effect of the coating on physical quality parameters of the fruit.

Materials and Methods

Microorganisms, Fruit and Treatments

Pure cultures of *Colletrotrichum capsici* were obtained by isolation from an infected bell pepper tissue and maintained on Potato Dextrose Agar (PDA) plates. Spore suspension was prepared by flooding the sporulating cultures with distilled water, and the concentration was determined using a haemocytometer and diluted to 1 x 10^5 spores/ml.

Mature green bell peppers were purchased from a commercial market in Semenyih. They were selected for their uniformity in size, shape and colour, for being free of damage and infection. The fruits were washed with 0.05% sodium hypochlorite, rinsed with distilled water and air-dried at room temperature.

The treatments were selected from initial screening studies as 1, 5 and 10% propolis solutions, and two controls were used (water and 70% ethanol). The preparation of the propolis solutions was carried out from stock propolis that was purchased from Semenyih. The powdered propolis was mixed with 95% propolis, agitated on an orbital shaker and filtered with Whatmann Grade No. 1 paper. A Rotary Evaporator (Buchi Rotavapor R-200, Switzerland) set at 40°C and 1 bar was used to evaporate the filtrate to dryness, which was then completely dried in an oven at 60^0C. Following that, 70% ethanol was added to a weighed sample to create a 10% solution, which was filtered again and diluted to 1, 5 and 10% solutions. After dipping in the respective treatments, the fruits were stored at 13 ^{0}C and 70 – 80% relative humidity for 28 days.

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Disease Incidence

Anthracnose disease incidence was visually evaluated on a weekly basis for fruits that were dipped in the spore suspension before treated with their respective coatings (water control, 70% ethanol control and 1, 5 and 10% propolis solutions). The results were then expressed as percentage of fruits infected.

Determination of Quality Parameters

Weight loss, colour and firmness were assessed on a weekly basis as indicators of the postharvest quality of the fruits. The fruits were weighed weekly and percentage weight loss was calculated. Colour was determined using a Miniscan XE Plus 24 colorimeter (Virginia, USA) based on the CIELAB (L*a*b*) system. The firmness property of the fruit was determined using Instron Universal Testing Machine Single Column Model (Instron, USA), that was equipped with a 6.00 mm plunger tip. The penetration force was set at speed of 10mm/min and the maximum force (N) required for penetration of the fruit at the equator of the fruit was recorded.

Statistical Analysis

The experiment was performed using a Completely Randomized Design (CRD) with four replicates per treatment. Data were analysed by one way analysis of variance (ANOVA) and mean separations was performed by Least significant difference (LSD) test ($P>0.05$). All statistical analyses were performed with GENSTAT software.

Results and Discussion

Effect of Propolis Coating on Disease Incidence

A comparison between the two controls and the propolis treated fruits (Figure 1) indicated that the propolis treatment significantly reduced anthracnose disease incidence ($P>0.05$). All concentrations of the propolis coating were found to completely inhibit anthracnose development on bell pepper (0% DI). Nonetheless, the disease symptoms observed on the infected fruits were mere small lesions and by the end of the storage period there was no completely rotten fruit.

The prevention of fungal decay incidence on the treated fruits is in agreement with Candir et al. (2009) and Ozdemir et al. (2010) who used similar concentrations of propolis. With regards to the observed disease symptoms, they were confined to small lesions even on the most diseased fruits. There was no report of completely rotten fruits at the end of the period. This might be a result of the fruits being stored at cold conditions, which is the recommended temperature for commercial storage, while *C.capsici* is more aggressive at 27^{o}C.

Effect of Propolis Coating on Quality Parameters

It can be observed in Figure 2A that the weight loss increased continuously for all treatments ($P>0.05$). The weight loss associated with the 10% treatment was significantly lower than the other treatments ($P>0.05$). Although the ethanol control showed the highest weight loss, the water control, 1% and 5% treatments showed no significant difference from each other ($P<0.05$). The firmness property of the fruits did not significantly change during the storage period for all treatments ($P<0.05$) (Figure 2B). The L* value increased continuously throughout the storage period, while the a* and b* value decreased. Although, initially higher L* values were observed for the propolis treatments, there was no significant difference between all treatments for all three values ($P<0.05$) (Figure 3A).

Propolis plays an important role in moisture retention in beehives (Ghisalberti, 1979). Therefore, the application of propolis as a coating was hypothesized to reduce water loss from the fruits due to increased water retention. However, the results indicate that concentrations of 10% or higher of propolis are required for

the coating to provide efficient moisture retention. With regards to firmness, although no significant difference was observed between the different treatments and over the storage period, the authors observed that the fruits became softer and more wrinkled. This was also observed as an increase in the time required for the cylindrical probe to penetrate the fruits' surface. Similar difficulties were reported by Ball (1997) who observed that the maximum penetration force was recorded at a compression of 6 mm for the fresh fruits, while the same penetration force was recorded for the older fruits although at 12 mm penetration. The authors observed higher L* values for the coated fruits, with the value steadily increasing as the concentration of the propolis coating increased. This might be accounted for by the actual colour of the EEP coating, yielding a lighter fruit colour at higher concentrations.

Conclusions

Although the application of propolis coating did not have a significant effect on the quality parameters of the bell pepper, it significantly inhibited anthracnose development on the fruits. The health improving properties attributed to propolis are numerous, thus by incorporating this natural medicinal product into postharvest technologies, numerous economic and health benefits can be reaped.

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Figures

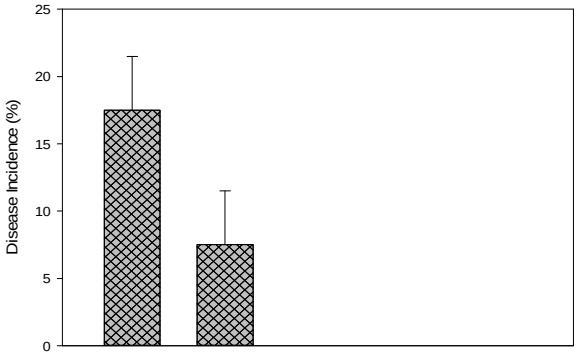

Figure 1. The effects of different concentration of ethanolic extracted propolis on anthracnose disease incidence on inoculated bell peppers (10^0C, 90% RH) after 28 days. Results are the means ± SE

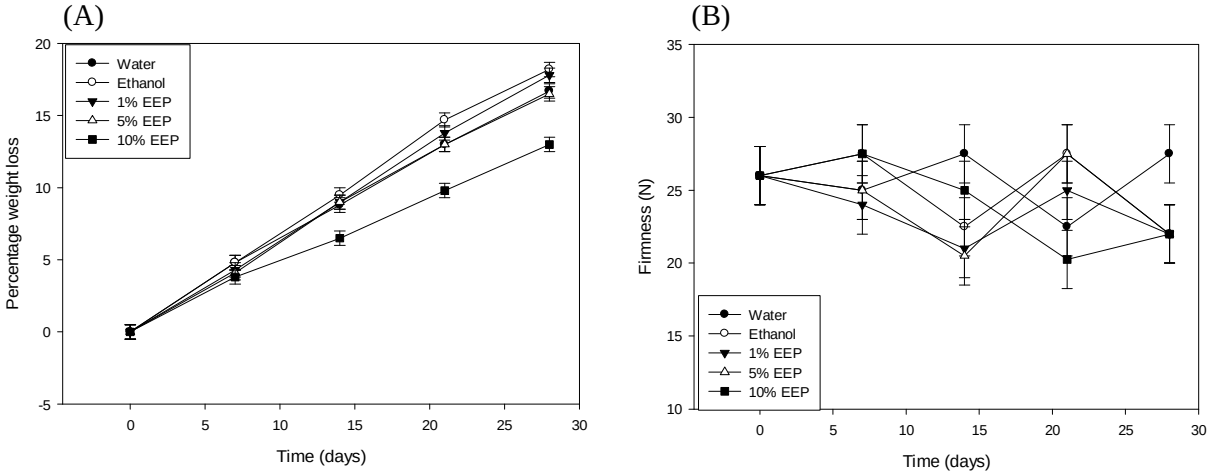

Figure 2. The effects of different concentration of ethanolic extracted propolis (EEP) on (A) percentage weight loss and (B) firmness of bell peppers (10^0C, 90% RH) after 28 days. Results are the means ± SE

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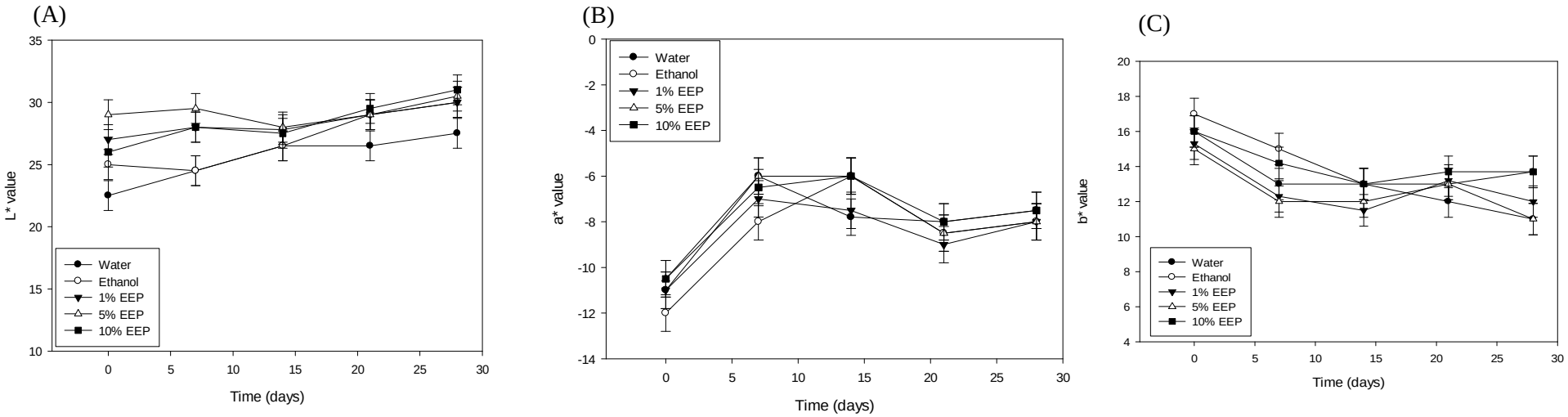

Figure 3. The effects of different concentration of ethanolic extracted propolis (EEP) on colour (A) L* value (B) a* value and (C) b* value of bell peppers (10^0C, 90% RH) after 28 days. Results are the means ± SE

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CHAPTER 3

ECO-PHYSIOLOGY AND STRESS BIOLOGY

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Relationship of Chlorophyll and Flavonoids Content in *Labisia pumila* Benth. under Nitrogen and Potassium Fertilization

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Introduction

There are three varieties of *Labisia pumila* found in Malaysia; *L. pumila* var. *alata*, *L. pumila* var. *pumila* and *L. pumila* var. *lanceolata* which are different in terms of their physical, chemical and biological activity. This herb is known as queen of plants and its extract prepared by boiling the roots, leaves or whole plant traditionally is taken orally to accelerate labor, shrink the uterus, improve the menstrual cycle, and for weight loss (Jamal et al., 2003; Ibrahim et al., 2011). The extracts of *L. pumila* was reported to have antioxidant activity as providing significant protection to human dermal fibroblasts and from cell damage caused by UV radiation due to the presence of secondary metabolites. Some components discovered in water extracts are flavonoids, ascorbic acid, beta-carotene, anthocyanin and phenolic compounds (Norhaiza et al., 2009). These secondary metabolites are assumed to be responsible for the pharmacological and medicinal properties found in the herbs.

Nitrogen availability is strongly related to the concentration of secondary metabolites where plant growing in nitrogen-poor condition tends to contain more bioactive compounds than plants growing in a nitrogen-rich environment according to the carbon/nutrient balance hypothesis (Ibrahim et al., 2011). Potassium is vital for osmoregulation and stimulating photosynthesis process (Mohamed and Aly, 2008). In condition of low potassium availability in soil more potassium fertilizer is possible to increase flavonoids accumulation in plant, but in high potassium availability is to reduce and there is not a tradeoff between the growth and accumulation of secondary metabolites in plant (Su et al., 2010).

Despite of its high demand due to the medicinal value, there is a scarcity of the plant sources and materials. The objective of this study was to examine the effects of different nitrogen and potassium levels on chlorophyll, flavonoids content in two varieties of *L. pumila*; var *alata* and *pumila*. Relationships among the parameters were also determined to characterize their cross involvement.

Materials and Methods

Experimental Location, Plant Materials and Treatments

Experiment was carried out in growth houses at Field 2, Faculty of Agriculture Glasshouse Complex, Universiti Putra Malaysia. Three-month-old of *L. pumila* seedlings (var. *alata* and var. *pumila*) were left for a month to acclimatize in a nursery. After a month, seedlings were fertilized with three rates of nitrogen (urea; 0, 90, 180 kg/ha)(Table 1) and potassium (MOP; 0, 90, 180 kg/ha)(Table 2). Seedlings were planted in soilless medium containing 10:1 (v/v) ratio of cocopeat and chicken dung in 20 cm diameter polyethylene bags. Day and night temperatures in the greenhouse were maintained at 27-30 ^{O}C and 20-23 ^{O}C respectively and relative humidity around 50-60%. All the seedlings were irrigated 3 times per day for 5 minutes each time. The experiment design of this experiment was split-split plot with nitrogen levels being the main plot, potassium levels as the sub-plot and varieties as sub-sub plot, replicated three times.

Table 1. Different levels of nitrogen fertilization during the experiment

Nitrogen (kg N/ha)	Total nitrogen fertilizers per plant (g)
0	0
90	0.1
180	0.2

Table 2. Different levels of potassium fertilization during the experiment

Potassium (kg K/ha)	Total potassium fertilizers per plant (g)
0	0
90	0.07
180	0.16

Nitrogen source used was urea (46% N) and potassium source was MOP (muriate of potash, 60% K). Every treatment receives TSP (triple super phosphate, 46% P) at standard rates of 180 kg N ha^{-1}.

Chrolophyll Content

Total chlorophyll content was measured by using fresh weight basis. Before the destructive harvest each seedling was analyzed for the leaf chlorophyll relative reading (SPAD Meter 502, Minolta Inc., USA). The leaves of *L. pumila* were selected for analysis and total leaf chlorophyll content was analyzed. The relative SPAD value was recorded (three points/leaf) and the same leaves sampled for chlorophyll content determination was conducted followed modified method based on Ibrahim and Jaafar, 2011.

Total Flavonoids Quantification

Total flavonoids compound was measured by the aluminum chloride colorimetric assay based on Ibrahim et al. (2011). Total flavonoid compound of extracts were expressed as mg quercetin equivalent/g dry weight (DW).

Results and Discussion

For chlorophyll content, there are no varietal different in all levels of fertilization ($p \leq 0.05$). The leaf chlorophyll content was found to be the highest at 90 kg N/ha (15.61 mg/g fresh weight) and decreased when nitrogen level increased to 180 kg N/ha (13.59 mg/g fresh weight) (Figure 1). This is due to the effect that higher concentration of nitrogen will limit the uptake of other essential macro (potassium) and micronutrients by the plant (Vijay et al., 2009). Therefore, increasing the concentration of nitrogen beyond the optimum level will not further increase the plant growth and development (Hussain et al., 2006).

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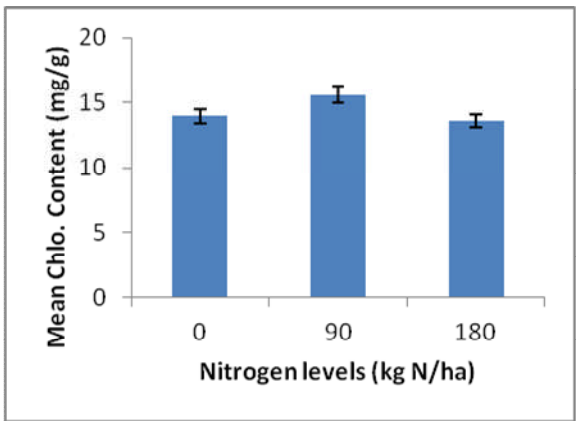

Figure 1. Chlorophyll content (mean ± SE) of *Labisia pumila* under different levels of nitrogen fertilization

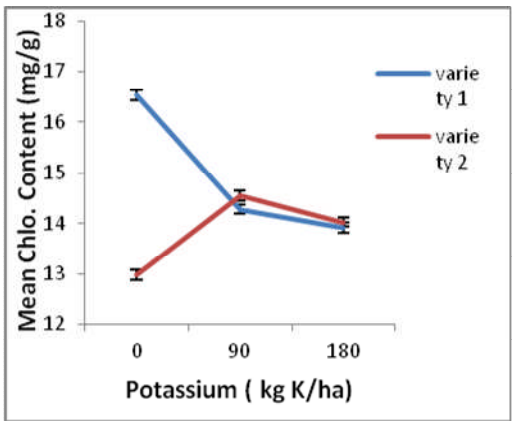

Figure 2. Interaction effects of potassium and varieties of *Labisia pumila* in chlorophyll content (mean±SE)

The interaction between potassium and varieties was shown in Figure 2. Variety *alata* (variety 1) has the highest rate of chlorophyll content at 0 kg K/ha and the chlorophyll content decreased with the increased of K fertilization. For variety *pumila* (variety 2), leaf chlorophyll content increased from 0 kg N/ha and has the higher chlorophyll content at 90 kg N/ha but decreased when increased to 180 kg N/ha. Variation between varieties may due to the different in sizes and age of var *alata* and var *pumila* Studies presented that with optimum concentration of potassium resulted in lower stomatal conductance, transpiration rate and higher chlorophyll content which are important for cell function and photosynthesis (Xu et al., 2011). However, high potassium content will also reduce the water absorption of plants which will decrease the rate of plant growth.

Production of flavonoids was found to be the highest at 0 kg N/ha (1.086 mg quercetin /g dry weight) and 180 kg K/h**a** (1.033 mg quercetin /g dry weight). There is varietal different in the flavonoids production where variety *pumila* has higher flavonoids content than variety *alata*. There are interaction effects between the N, K and varieties. For variety *alata*, production of flavonoids is highest with 180 kg N/ha and 90 kg K/ha. Production of flavonoids in *alata* has no increment even the K fertilization increased if the rate of N was 0 kg N/ha. Variety *pumila* has higher flavonoids content at 0 kg N/ha and 180 kg K/ha (Figure 3). The increased of flavonoids content under limited nitrogen supply had been reported in previous study by Nguyen et al., 2011 and Ibrahim et al., 2011 based on the Carbon Nutrients Balance (CNB) theory. Low nitrogen supply will increased the phenylalanine availability due to restriction of protein under nitrogen deficiency. Contrary, potassium deficiency will decrease the flavonoids content in many medicinal plants due to limitation or reduced activities of enzymes in the process of flavonoids synthesis (Liu et al., 2010).

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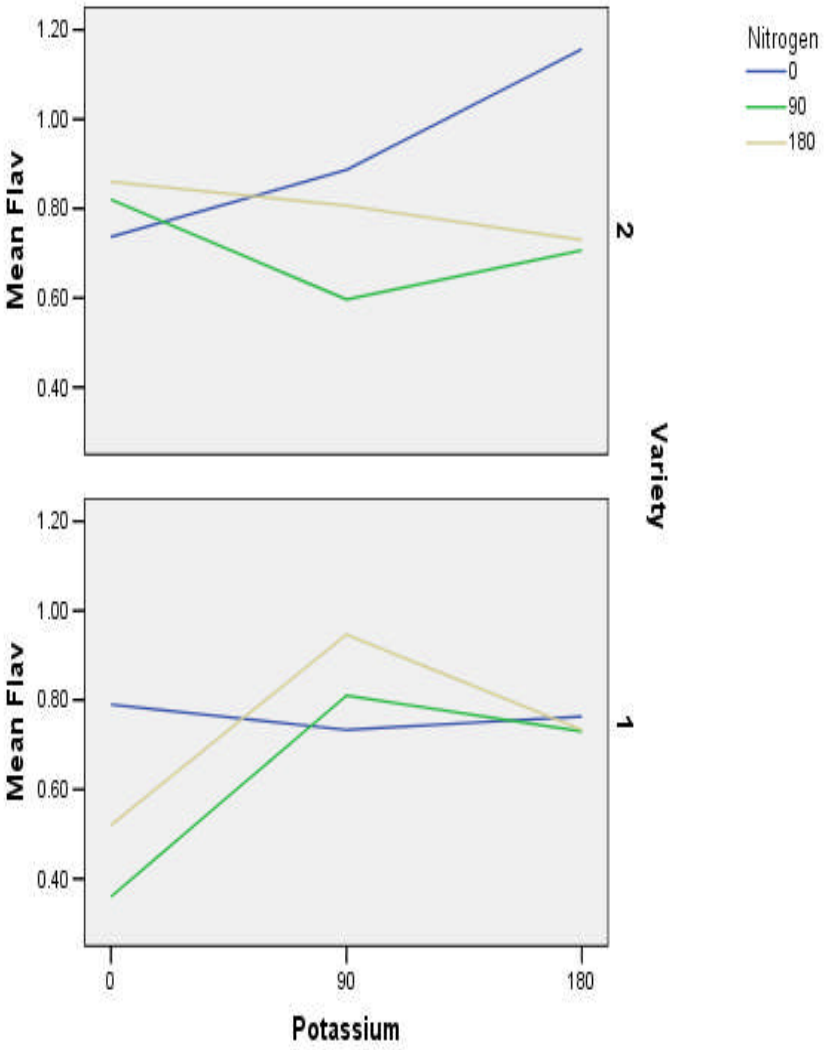

Figure 3. Flavonoids content of two varieties of *Labisia pumila* (1) *alata*, (2) *pumila* under different levels of potassium and nitrogen fertilization.

The increased production of flavonoids was found to be negatively correlated with the chlorophyll content (Figure 4). The production of flavonoids increased with low leaf chlorophyll content. Low nitrogen fertilization will reduced the chlorophyll content of leaf and in another words increased the flavonoids production. Low availability of nitrogen limits the growth of the plants, therefore plant allocates the extra carbon that cannot be used for growth to the production of carbon based secondary metabolites (CBSM).

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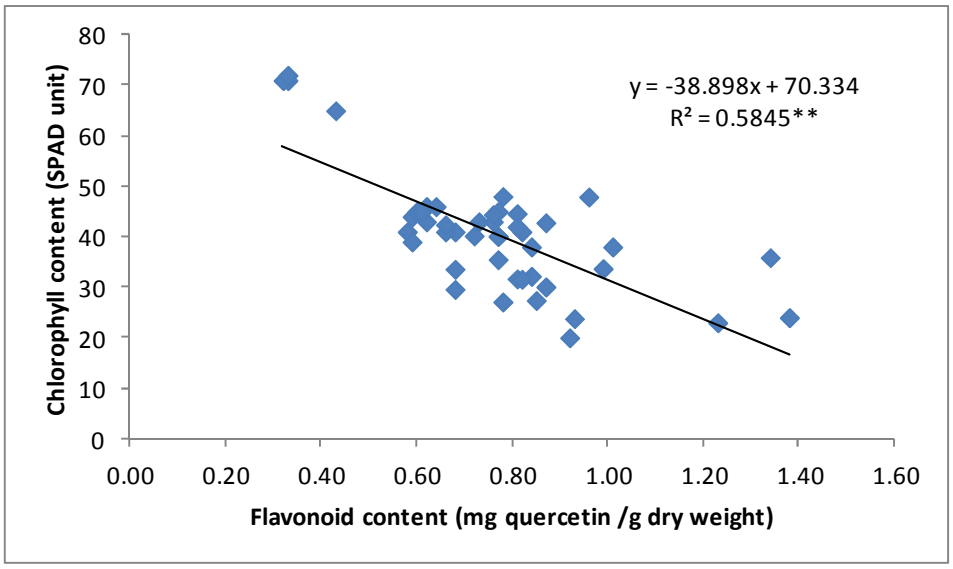

Figure 4. Relationship of chlorophyll and flavonoids content in *Labisia pumila* under different levels of N and K fertilization

Conclusions

Flavonoids content in *L.pumila* is negatively correlated with the chlorophyll content while the flavonoids content reduced when there are high content of chlorophyll in plants based on the Carbon Nutrients Balance theory.

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Combining Ability for Physiological Traits in Wheat under Drought Stress

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Introduction

One of the most complex designs that has been used extensively for the genetic analysis of quantitative characters such as drought is the diallel cross (Desalegn et al., 2001). The diallel cross designs are frequently used in plant breeding research to obtain information about genetic properties of parental lines or estimates of general and specific combining abilities and heritability. The approaches of Griffing (1956b) and Hayman (1954) are statistically similar in their analyses of variance,the Griffing's (1956a, b) general and specific combining abilities are mathematically identical to Hayman's (1954) additive and dominance components. They differ, however, in the genetic assumptions, information and interpretation. Therefore, this study was conducted to estimate the combining ability of selected lines and cultivars for tolerance to drought using physiological traits as indirect indicators.

Materials and Methods

Twenty eight F_3 hybrids and their eight parents were sown in plastic pots filled with a soil mixture (soil:organic matter:sand in a ratio of 1:1:1) in Experiment Farm of University Putra Malaysia. Four seeds were sown in each pot. The pots were irrigated after 75% depletion of the soil water. Each pot was filled with 3 kg of air-dried soil and soil field capacity was calculated on the soil dry weight basis. SPAD with chlorophyll meter, stomatal conductance was measured using an IRGA (Infra-Red Gas Analyzer, LCA-4, Analytical Development Corporation, UK), membrane stability index The leaf discs (100 mg) were thoroughly washed in running tap water followed by a wash in double distilled water thereafter the discs were heated in 10 ml of double distilled water at 40o C for 30 minutes. Then, their electrical conductivity (C1) was recorded by EC (Electrical Conductivity) meter. Subsequently, the same samples were placed in a boiling water bath (100o C) for 10 minutes and their electrical conductivity was also recorded (C2). The MSI was calculated as: Membrane stability index (MSI) = [1 - (C1/ C2)] × 100. and relative water content was measured The leaf material was weighed (2 leaves) to determine its fresh weight and then placed in distilled water at 4°C for 19h and its turgid weight was recorded. Finally, the samples were dried in an oven at 65-70°C for 48 hours and their dry weights were recorded. RWC was calculated as:

RWC= [(fresh weight- dry weight)/ (turgid weight- dry weight)] x100.

Results and Discussion

Analysis of variance showed significant differences among parents and hybrids for physiological traits, indicating the different responses of genotypes to water stress condition and possible selection of drought tolerant genotypes under water deficit. Mean squares of GCA (general combining ability) and SCA (specific combining ability) for all the traits indicating the importance of additive and dominance genetic effects and variance of GCA for all traits except relative water content were more than variance of SCA and this indicates more additive control than non-additive effects for traits Chamran, Hamoon and Veery with significant negative and significant GCA effects were good combiners for stomatal conductance under drought stress. The best general combination with positive

and significant effects was Irena/Babax//Pastor and S-78-11 for membrane stability index, Irena/Babax//Pastor and S-78-11 for relative water content and cultivar Chamran for chlorophyll content.

Conclusions

The line Chamran had a high negative GCA value for stomatal conductance and this line is a good performance under drought stress. Cultivar Irena/Babax/Pastor had high positive value for cell membrane stability index and this line had a high GCA value for relative water content. Cultivar Chamran had the best performance for GCA effect among genotypes for chlorophyll content. Therefore, these lines and cultivars were recommend to put into the breeding program, chlorophyll content based on inbreeding depression values showed was impressed more than other trait in promising generation, and performance of these cultivars and lines can be used in crossing program.

Table1. Analysis of variance (mean squares) for combining ability in F_3 generation

Source of variation	DF	Stomatal conductance	Membrane stability index	Relative water content	Chlorophyll content
Replication	2	124.25	34.29	0.45	25.83
GCA	7	32258.4**	93.44**	146.95**	295.8**
SCA	28	7000.2**	87.51**	157.12**	28.23**
Error	70	133.1	22.59	4.28	9.09

**Significant at 1% level

Table 2. Values of general combining ability (GCA)

Line/Cultivar	Stomatal conductance	Membrane stability index	Relative water content	Clorophyll content
Irena/Babax//Pastor	63.8**	4.79**	1.72**	0.32
S-78-11	19.4**	1.85	1.72**	0.49
Tajan	35.3**	-0.82	-3.28**	-2.3**
Chamran	-54.7**	-2.15	5.33**	8.8**
Hamoon	-31.3**	-0.71	-2.33**	-4.4**
Moghan3	-28.1**	-1.60	-1.33**	-3.2**
Veery/Nacozari	-35.1**	-0.10	-2.17**	0.10
Hirmand	30**	-1.26	0.33	0.26
SE (gi)	3.41	1.40	0.61	0.88

** Significant at 1% level

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Table 3. Values of specific combining ability (SCA)

Hybrids	Stomatal conductance	Membrane stability index	Relative water content	Chlorophyll content
Irena×S-78-11	51.49**	-8.8**	-8.73**	3.09
Irena×Tajan	-30.7**	-0.82	1.60	0.25
Irena×Chamran	-61**	1.52	0.99	1.03
Irena×Hamoon	86.9**	5.07	-7	3.03
Irena×Moghan3	-47.5**	8.29*	6.3**	0.81
Irena×Veery	-23.9**	-2.87	11.8**	-3.19
Irena×Hirmand	24.8*	-2.37	-5**	-5.02
S-78-11×Tajan	-38.9**	-1.87	2.94	2.09
S-78-11×Chamran	11.7	-2.54	-5.01**	-0.13
S-78-11×Hamoon	23.3*	7.68*	6.33**	-3.13
S-78-11×Moghan3	-25.1*	-3.43	13.3**	1.64
S-78-11×Veery	14.1	6.47*	-6.5**	-1.02
S-78-11×Hirmand	-36.7**	2.57	-2.3	-2.52
Tajan×Chamran	-30.1**	2.13	-4.6*	1.37
Tajan×Hamoon	18.4	-2.98	-1.67	-2.63
Tajan×Moghan3	-3.1	-3.76	-3	-4.86
Tajan×Veery	35.2**	3.74	-3.8*	1.81
Tajan×Hirmand	49**	3.57	8.6**	1.98
Chamran×Hamoon	12.8	-6.32	4.7*	2.81
Chamran×Moghan3	4.7	7.24*	0.7	-1.41
Chamran×Veery	26.6*	-5.60	-5.4**	-2.08
Chamran×Hirmand	35.1**	3.57	8.7**	-1.58
Hamoon×Moghan3	-22.6*	-3.87	-5.29**	-3.41
Hamoon×Veery	-30.06**	-1.37	6.21**	2.25
Hamoon×Hirmand	-88.9**	1.79	-3.2	1.09
Moghan3×Veery	27.44**	2.18	-3.7*	1.70
Moghan3×Hirmand	66.2**	-6.65*	-8.2**	5.53*
Veery×Hirmand	-49.5**	-2.48	1.5	0.53
SE.sij	10.46	3.30	1.87	2.7

*and ** Significant at 5% and 1% statistical levels

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Seed Quality of Christmas Palm (*Veitchia merrillii*) as Affected by Desiccation

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Introduction

Malaysia is one of the countries with the largest number of palm genus in the world with 34 genera and 220 species (Whitmore, 1985). Among the widely used palm species for horticulture purpose in Malaysia is *Veitchia merrillii* (Christmas Palm) (Ismail, 1993). The only feasible manner in propagating this palm is by using seeds as this monopodial palm does not produce offshoots and the seeds are produced in large quantities (Wagner, 1982). Often, there is a need to store seeds prior to use. An important consideration in seed storage is to preserve quality and minimize loss of viability and vigor. Thus, understanding the storage behaviour of Christmas Palm seed is crucial. One of the important factors in ensuring the viability of seeds upon storage is the initial seed quality (Cromarty et al,. 1990). Seeds of high initial viability are much more resistant to unfavourable storage environmental conditions than seeds with low viability. Najib (2010) reported that overripe seeds of the Christmas Palm have the highest vigor compared to other stages of maturity thus is the best stage for storage. In order to store the seeds, they need to be desiccated to a low moisture content to avoid high metabolic rate which can affect the seed health and viability during storage. However, seeds will deteriorate if the moisture content is too low and might not be viable or cannot germinate after being desiccated due to desiccation injury. Therefore, the objective of this study is to determine the ability of Christmas Palm to tolerate desiccation using both quantitative and qualitative tests.

Materials and Methods

Determination of Moisture Content and Desiccation of Seeds

Fruits harvested at overripe stage were processed, depericarped and the initial (fresh) moisture content were determined using the low constant temperature oven method (ISTA, 1996). To ensure even and efficient drying, the seeds were placed on a modified culture rack with wire mesh and desiccated inside the Laminar Air Flow for duration of 48, 96 and 144 hours.

Germination and Seedling Evaluation

Some of the desiccated seeds were subjected to germination test using sand media. After 4 weeks of germination, the germination percentage was recorded and the seedlings were evaluated and ranked based on the morphological differences as in Figure 1.

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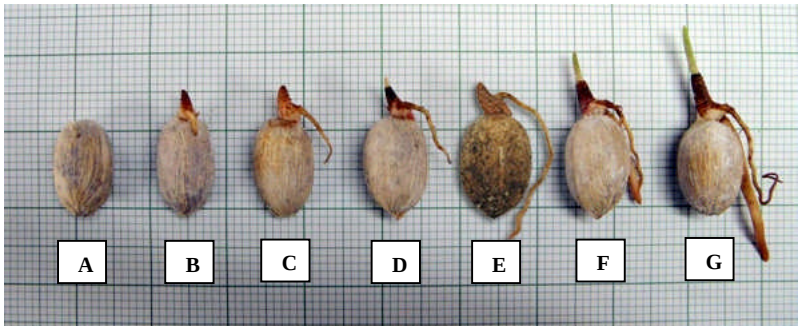

Figure 1. Categorization of Christmas Palm seedling evaluation

Tetrazolium Test on Embryo

For the remaining desiccated seeds, the embryos were excised, submerged in 0.1% tetrazolium solution for 2 hours with temperature of 35 ˚C (ISTA, 2003). The stained embryos were evaluated and ranked based on staining pattern as in Figure 2.

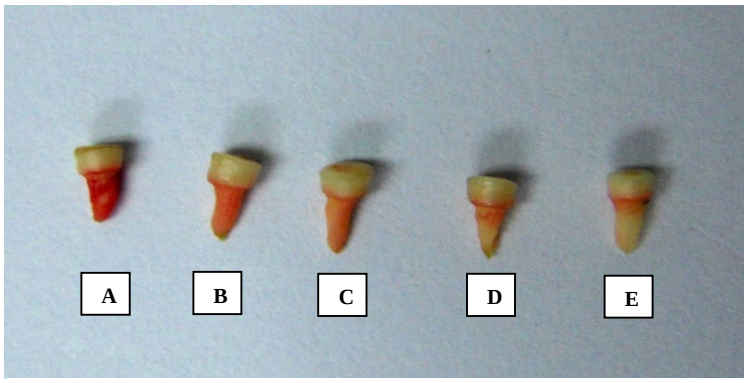

Figure 2. Categorization of Christmas Palm embryo staining intensity evaluation

The experiments were arranged in Completely Randomized Design (CRD) with four treatments of desiccation period. Germination test and tetrazolium test were carried out in 4 replicates of 20 seeds each and 4 replicates of 10 embryos each respectively. The data obtained was subjected to the Analysis of Variance (ANOVA) and differences among treatments means were compared using Least Significant Design (LSD).

Results and Discussion

Effects of Desiccation Treatment on Seed Moisture Content

The results showed that Christmas Palm at overripe stage had an initial moisture content of 48.4%. When the seeds were subjected to desiccation for a continuous period of 48, 96 and 144 hours, the moisture content decreased to 42.4%, 40.4% and 39.4% respectively (Figure 3).

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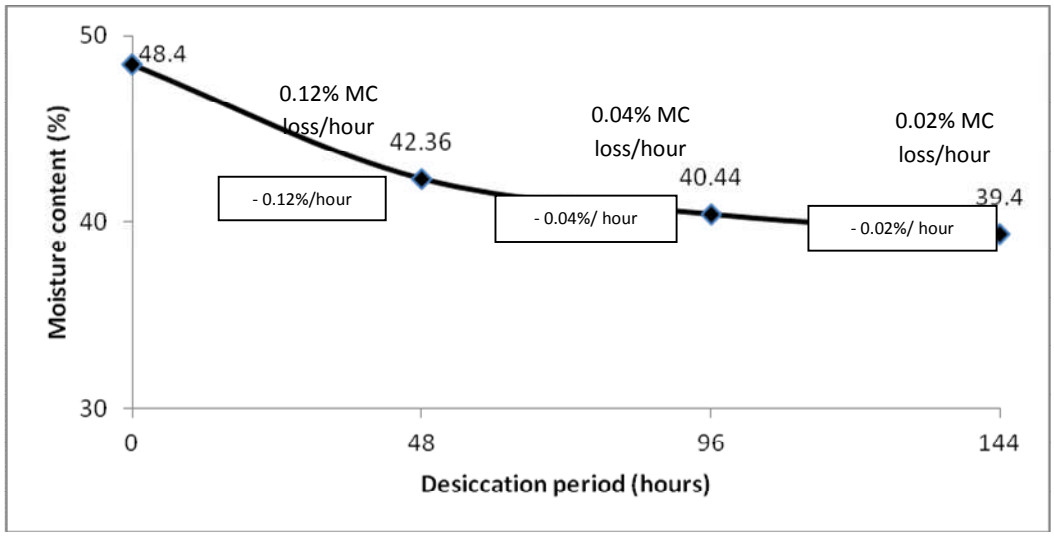

Figure 3. Moisture content of Christmas Palm after desiccation of 48, 96 and 144 hours

The rate of desiccation for Christmas Palm was highest during the first 48 hours which declined at a rate of 0.12% moisture content per hour. However, for desiccation up to 96 and 144 hours, the rate decreased tremendously to only 0.04 and 0.02% moisture content per hour respectively. This pattern of loss in moisture content can be related to the water chemistry whereby the rapid loss is due to loss in free water while the slower rate is attribute to lose in either loosely bound or tightly bound water. From the pattern of declining in moisture content from Figure 3, it will be very hard to further desiccate the seed as the moisture content will hardly decline.

Effects of Desiccation Treatments on Seed Germination

Results of germination revealed that germination percentage for all treatments were above 95% with no significant difference in all treatments (Table 1). Desiccation has not reached threshold limit that may affect viability of Christmas Palm seeds.

Table 1. Germination percentage as affected by period of desiccation. (Means with the same letter are not significantly different

Desiccation (Hour)	Germination (%)
0	100[a]
48	95[a]
96	100[a]
144	100[a]

Effects of Desiccation Treatments on Seed Vigor

Even though there was no significant difference in terms of germination percentage between the treatments, there were differences in vigor between the treatments when the seedlings were categorized according to their morphological differences. Table 2 shows that the vigor of desiccated seeds was lower. For seeds desiccated 48 hours, most of the seeds which germinated ranked into category F (71.25%) and G (23.75%). As for seeds desiccated for 96 hours, less seedlings fell into category G (22.5%) and more were categorized F (75%). When the seeds were desiccated for 144 hours, only 2.5% seedlings were categorized F, none belonged to score G and most

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of the seeds were scored C and D. This means that the seed lost its vigor when were desiccated due to seed deterioration and when the seed is deteriorating, it takes a longer time to complete germination process.

Table 2. Percentage of Christmas Palm seedling according to various morphological categories for four different desiccation periods

Score	Percentage of seedling in different rank			
	0 hour	48 hours	96 hours	144 hours
A	3.8	5	0	0
B	3.8	0	0	6.3
C	0	0	0	41.3
D	0	0	1.3	41.3
E	0	0	1.3	8.8
F	40	71.3	75	2.5
G	52.5	23.8	22.5	0

Effects of Desiccation Treatments on Seed Viability – Tetrazolium Test

Results from the Tetrazolium test showed that 57.5% of the embryos from seeds desiccated for 48 hours were scored B and the rest were scored A and C (Table 3). As desiccation period increases, more embryos were scored D and E and none were scored B. This corresponded to the result of the germination test which showed that as desiccation period increases, the seeds started to lose its vigor since the staining intensity reduced and portions of embryos that were stained are less.

Table 3. Percentage of Christmas Palm embryo according to various staining pattern for four different desiccation period based on tetrazolium chloride test

Score	Percentage of embryo in different rank			
	0 hour	48 hours	96 hours	144 hours
A	42.5	12.5	2.5	0
B	57.5	57.5	0	0
C	0	30	15	5
D	0	0	47.5	32.5
E	0	0	35	57.5

Tetrazolium test for Christmas Palm embryo appears to be a very sensitive test which can be interpreted as loss of vigor rather than loss of viability. However, further studies is required on tetrazolium staining intensity of Christmas Palm embryo as the loss in staining intensity appears to be rather drastic considering that all 5 irrespective categories (A – E) had germination percentage of above 95%. Besides that, further studies on histology and biochemical content of the embryo need to be conducted in order to determine the factor that influence staining pattern of Christmas Palm embryo since even the embryo from non-desiccated seeds, the blunt end was not stained. The region that was stained was the region from the middle of the embryo until the sharp end (Figure 4).

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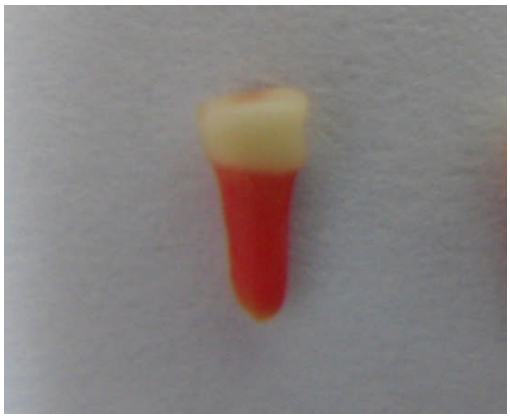

Figure 4. Staining pattern of embryo from non-desiccated seed of Christmas Palm tested with tetrazolium

Conclusions

Desiccation rate of Christmas Palm appears to be very drastic for the first 48 hours and desiccation beyond 144 hours would be hard considering the rate has nearly reached plateau. Desiccation for 48, 96 and 144 hours in *Veitchia merrillii* seeds did not affect the viability of the seeds but affect the vigor of the seed as seed desiccated longer will have lower vigor due to deterioration.

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Physiological Adaptation of *Jatropha curcas* to Water Deficit

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Introduction

Jatropha curcas L. is a drought resistant large shrub or small tree, belonging to the genus *Euphorbiaceae* which in previous years has the requisite potential of providing a promising and commercially viable alternative to diesel oil (Dehgan and Webster, 1979). The plant can be grown over a wide range of arid or semi-arid climate conditions (Patil, 2004). Naturally, hot and humid environment is preferred for the emergence of its seeds. In India, low water requirement has always been one of the major reasons for the selection of *J. curcas* and hence its suitability to dry and arid lands (Jongschaap et al., 2007).

In many plant species, stomatal closure has been suggested as the main regulatory mechanism of photosynthesis under water stress (Farquhar and Sharkey, 1982). During evolution, many tree species have developed various mechanisms to enhance their drought adaptation, including plant structure modification and increased of water use efficiency (WUE). It is also important to note the role played by the plant hormone abscisic acid (ABA) which reduce stomata opening and vegetative growth in a response appropriate to the reduction in available water (Liang et al., 1997). The structural and physiological adaptations associated with drought tolerance of plants are numerous and diverse and appear to be more important for plant survival than for high growth rates (Dewar and McMurtrie, 1996). It is well known that drought has profound effects on growth, yield, and plant quality (Hale and Orcutt, 1987). For instance, other plant parts including fruit may not enlarge because rapidly transpiring leaves create lowered water potentials in the xylem, which may result in water actually leaving the fruits. In plant physiology, water deficit can cause a decrease in photosynthesis rate either by a direct effect on photosynthesis capacity of the mesophyll or by carbon dioxide limitation resulting from stomatal closure (Tezara et al., 1999; and Lawlor, 2002).

Previously there is little knowledge on how water deficit affects the survival, growth and yield performances of *J. curcas* particularly in Malaysia. Furthermore, the documentation on the plant responses and physiological adaptation to water deficit for survival is still lacking, which needs immediate attention if cultivation of *J. curcas* was to be undertaken seriously in dry arid environment or during drier period of the season. Therefore, this study was conducted with the following objectives: (i) to investigate the effect of water deficit on selected *J. curcas* fruit characteristics, (ii) to investigate the impact of water deficit on the gas exchange characteristics of *J. curcas*, and (iii) to examine the role of ABA and stomata conductance in regulating transpiration during event of water deficit.

Materials and Methods

The experiment was a field study and conducted in a 20 m x 29 m plot near Universiti Malaysia Sarawak (UNIMAS) in Kota Samarahan, Sarawak, Malaysia. The type of soil at planting site was the *Triboh* series which has a low to moderate permeability status, typical of a sandy clay loam (Soil Survey Staff, 1992). The test crop in this study was *J. curcas*. The seeds were germinated in a sand bed at a germination rate of 80%. The seedlings were then transplanted into polythene bags with a mixture of soil, sand, and peat (7:3:2). Uniform seedlings (with

a height of 1 m) were selected and transplanted at the planting site. The study was conducted in the month of August 2009.

Experimental Design and Treatments

The experiment was arranged in a completely randomized design (CRD) with four treatments replicated six times. Treatments were: (i) control (W0) – plants exposed to soil water potential (SWP) at field capacity $\geq$ -0.03 MPa, (ii) mild water stress (W1) – plants exposed to SWP between -0.30 and -0.10 MPa, (iii) moderate water stress (W2) – plants exposed to SWP between -1.0 and -0.8 MPa, and (iv) extreme water stress (W3) – plants were grown at soil permanent wilt point $\leq$ -1.50 MPa. Different water stressed levels were established within the experimental plots based on a similar study done by International Resource Group (2006) and Jongschaap et al. (2007). In the study, water-proof canvas sheets (0.2 mm thick) with a surface area dimension of 4 m x 8 m covered the soil surface to prevent rain water from entering into the respective treatments. Treated plants were compartmentalized by establishing small drains surrounding it to ensure the desired SWP. To prevent lateral movement of water in the soil, aluminium metal sheets were inserted into the soil to a depth of 1.5 m surrounding the treatments. Soil water potential was monitored by using soil moisture sensor equipment (W.E.T. Sensor, Eijkelkamp, Netherlands).

Fruit Size Seed Mass

To compare and determine fruit size from each treatment, measurement of the fruit diameter was obtained. The maximum diameter of the fruit was measured using a vernier calliper in millimetre up to two decimal places.

Approximately 30 fully mature seeds were drawn randomly from each treatment to determine the expected yield. This was done by measuring the oven dry weight of the seeds. Seeds were oven dried for 24 hours at a temperature of 60^{o}C. The dry weight of 30 seeds from each treatment will then be used and expressed as mass of whole seeds (Arndt et al., 2001).

Gas Exchange Measurement

Gas exchange measurement was determined according to the method by DiCristina and Germino (2006), carried out on young fully expanded leaves with the same orientation and the same layer in the crown (middle bottom). Measurements of net photosynthesis on an area basis (*A*, µmol CO_2 $m^{-2}s^{-1}$), leaf stomatal conductance (*g*s, mol H_2O $m^{-2}s^{-1}$), and transpiration rate (*E*, mmol H_2O $m^{-2}s^{-1}$) of five different leaves per treatment were monitored using a LICOR 6400 XT (Lincoln, Nebraska, USA) infrared gas analyzer (IRGA). Light intensity (Photosynthetically active radiation, PAR) within the sampling chamber was set as close to outside PAR at 1500 µmol $m^{-2}s^{-1}$. The CO_2 flow into the chamber was maintained at a concentration of 400 µmol mol^{-1}. The humidity flow into the chamber was fixed at 500 µmol s^{-1}. To assess the trade-off between CO_2 uptake and water loss, instantaneous water-use efficiency (WUE) was calculated as ratio between photosynthetic rate and transpiration rate (*A*/*E*, µmol CO_2/µmol H_2O). Statistical assessment was done on gas exchange parameters at between 1100 to 1200 h, which was presumed to be the diurnal period when photosynthetic rates would be maximal.

Foliar ABA Concentration

Based on a modification from the method by Kozukue et al. (1984), leaves were collected and wrapped with aluminium foil, immediately dipped in liquid nitrogen and kept at -20^{o}C in the freezer.

Dried leaves (10 g) were grounded in cold MeOH-water combined. The extract was filtered and the solid materials were again grounded with cold MeOH-water and filtered. The two filtrates were combined, and the

MeOH was removed using the rotor evaporator. The crude extracts were then analyzed using Thin Layer Chromatography (TLC) and Column Chromatography (CC) to identify the fractions obtained.

The compound identification of ABA was then performed by the co-injection method. Volume of 20 µl of authentic ABA (5 mg per 2.5 mL MeOH) with a concentration of 2.0 mg/L was prepared and the solution was injected into the Lichrospher 100 RP-18 E column. Comparison of retention times of the peaks in authentic hormone and in the purified leaf extracts were obtained by the High Performance Liquid Chromatography (HPLC). Quantification of ABA was accomplished by comparing their peak areas in samples to the peak area of the standard.

Quantification of ABA (ng/g) was calculated using the formula:

$$\text{ABA concentration (ng/g)} = \left[\frac{\text{Conc. of standard from calibration curve (mg/L)}}{\text{Peak area sample}}\right] \times \left(\frac{1000000 \text{ ng}}{\text{Peak area of standard from calibration curve}}\right) \quad (1000 \text{ g})$$

Statistical Analysis

Data were analyzed using one way analysis of variance (ANOVA) with the SPSS software (version 15). The Tukey's Honest Significance Difference (HSD) Test, at $\alpha = 0.05$ level of significance was done to compare the means and to determine whether there were any differences in between treatments. The relationship of *E – gs* and foliar ABA concentration – *gs* were correlated using regression of best fit.

Results and Discussion

Fruit Size and Seed Mass

Table 1 shows that W0 had a 90% bigger fruit diameter than W2 could be partly due to the impact of drought to water-stressed plants. At the end of the study, there was no substantial difference detected among treatment W0, W1, and W3 (Table 1). The result suggests that at soil water potential of -0.8 MPa, the size of fruits in plants grown under moderate water stress conditions were significantly affected by drought. This was the case for plants grown under W2 whereby fruit cell division is most active immediately after flowering, so water stress at this stage significantly reduces fruit size at harvest since it is to some extent dependant on the number of cells in the fruit (Goodwin and Tatura, 2002). However, the extreme water-stressed plants (W3) exhibited bigger fruit size than the moderate water-stressed plants (W2). The finding concurs with that of Goodwin and Tatura (2002) in their work with grapes. They reported that the physiological reaction to water stress will affect the growth and development of the fruit depending on the timing and level of water stress which may increase the plant's adaptive capability to drought. That being the case, plants subjected to extreme water stress (W3) treatments may be affected by the chemical breakdown or formation of important acids at fruit maturity (Liang et al., 1997) which may cause a slight increase in fruit diameter.

The effects of different water regimes on *J. curcas* seeds mass is shown in Table 1. The well watered plants significantly ($p < 0.05$) produced higher seeds mass than those under water stressed. Seeds mass was increased slightly by 3 % in the well watered plants. Like any other plant, *J. curcas* requires water from the soil for various physiological and biochemical processes to produce functional carbohydrates through photosynthesis (Jongschaap et al., 2007). Similar study by Patil (2004) reported that the fairly hot and humid environment with rain was preferred and most beneficial for the growth and development of *J. curcas* fruit in India.

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Table 1. Fruit diameter and seed weight of *J. curcas* under different watering regimes

Treatments	Fruit diameter (cm)	Seed weight (g)
W0	3.60 ± 0.49^{a}	32.03 ± 0.57^{a}
W1	3.38 ± 0.23^{b}	31.01 ± 0.84^{b}
W2	3.02 ± 0.66^{c}	30.01 ± 0.29^{b}
W3	3.28 ± 0.91^{b}	30.01 ± 0.84^{b}

Note: Figures with same letter superscript within columns are not statistically different using Tukey's at $P > 0.05$ probability level. (Mean$\pm$SD, n = 6; seed weight n = 30).

Gas Exchange and Foliar ABA Physiological Response

Photosynthesis rate was reduced by 51 % as the soil water potential decreased from field capacity to $\leq$ 1.5 MPa indicating that water stress depressed photosynthesis of *J. curcas* plants substantially (Table 2). In a similar study by Cornic and Massacci (1996), they reported that the decreased can be attributed to the direct inhibition of biochemical processes through ionic, osmotic or other conditions which were induced by loss of cellular water. Some other factor that contributed to the decreased in *A* might be the limited CO_2 diffusion into the intercellular spaces of the leaf as a consequence of reduced stomatal conductance (Lawlor, 2002). Table 2 shows that transpiration rates were lower for plants subjected to extreme water-stressed condition declining by 52% of the control value. Result for leaf transpiration rates (*E*) rate concurs to that of Ashizawa et al. (2003) in which they concluded that *E* rate was progressively reduced under conditions of decreasing soil moisture. Table 2 shows no significant difference in WUE between treatment W0 and W3. There is a possibility that W3 plants might already be less susceptible to drought during that time. This observation was similar to that of Arndt et al. (2001) which can be attributed to the postponement of the damaging effects caused by water deficit in trees through stomatal control, thereby optimizing carbon gain during drought. As water deficit increased, carbon was remobilized from leaves and preferentially redistributed to stems and roots. This finding was coherent to a study done by Niklas and O'Rourke (1987) whereby they discovered that the reserve of water stored in the succulent trunk of *Euphorbia trigona* might presumably buffered its susceptibility to water loss.

Table 2. Gas exchange properties and foliar ABA concentration of *J. curcas* under different watering regimes

Trt.	Photosynthesis (*A*) (μmol CO_2 $m^{-2}s^{1}$)	Transpiration (*E*) (mol H_2O m^{-2} s^{-1})	Stomatal Conductance (*gs*) (mol H_2O m^{-2} s^{-1})	Water Use Efficiency (WUE)	Foliar ABA Concentration (ngg^{-1})
W0	20.27 ± 0.22^{a}	3.44 ± 0.03^{a}	0.14 ± 0.23^{a}	5.89 ± 0.10^{a}	523.00 ± 3.35^{a}
W1	15.47 ± 0.26^{b}	2.35 ± 0.01^{b}	0.13 ± 0.23^{b}	6.60 ± 0.13^{b}	546.00 ± 3.58^{b}
W2	11.32 ± 0.14^{c}	2.17 ± 0.06^{c}	0.12 ± 0.33^{c}	5.23 ± 0.15^{c}	885.33 ± 3.01^{c}
W3	9.90 ± 0.07^{d}	1.65 ± 0.03^{d}	0.10 ± 0.11^{d}	5.99 ± 0.12^{a}	2437.33 ± 4.59^{c}

Note: Figures with same letter superscript within columns are not statistically different using Tukey's at $P > 0.05$ probability level (Mean$\pm$ SD, n = 6).

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It can be said that the reduced leaf stomatal conductance (*gs*) that affected both *A* and *E* was triggered by the plant hormone ABA which played a major role during events of water stress. There were significant ($p < 0.05$) differences among all treatments in *gs* as affected by water stress (Table 2). At the end of the experiment, *gs* of W3 was reduced by 29 % of the control as the soil water potential decreased from field capacity $\leq$ -1.5 MPa. The result was parallel to that of Schulze and Hall (1982) where tree species from the deserts and temperate regions closed its stomata as the soil water potential decreased rapidly. The ABA levels from each leaves extract are shown in Table 2, expressed as ng per g fresh weight. The result showed that the levels of ABA in fraction W3 was substantially higher than those of W2, W1, and W0. Taylor (1991) carried out similar experiment and found that ABA accumulation in plants in response to water deficit is thought to act as a signal for the initiation of various adaptation processes. As a response to water stress, the production of ABA increased thus directly inhibiting stomatal openings which led to lower transpiration and photosynthesis rates.

Figure 1 shows the relationship between transpiration rate (*E*) and leaf stomatal conductance (*gs*). The strong relationship between *E* and *gs* regardless of treatments shows a polynomial cubic regression line of zero intercept with $r^2 = 0.83$ indicating that higher *gs* increased *E* in plants. Previous report by Lecoeur et al. (1995) concluded that stomata impose a critical control over water loss and exchange of gases between the atmosphere and leaf cells hence limits transpiration of plants growing in dry soils, avoids leaf water potential becoming too negative and xylem embolism developing (Kramer, 1987; Ryan et al., 1994; Kessler, 2008).

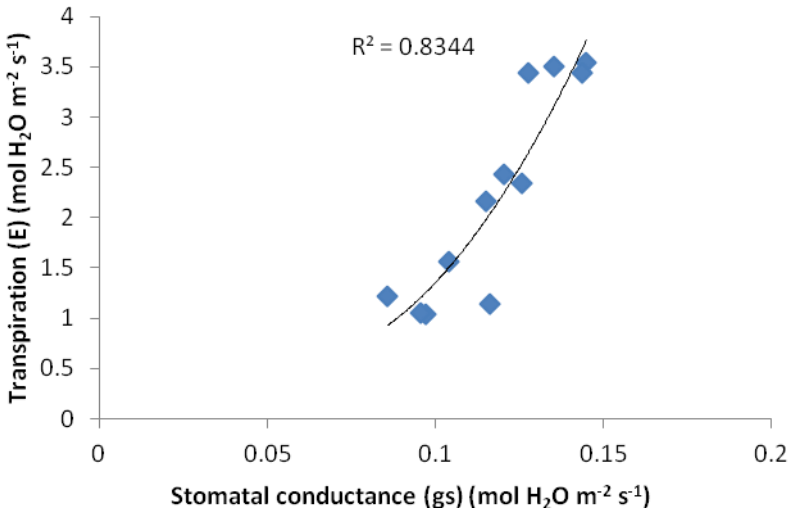

Figure 1. Relationship between transpiration and stomatal conductance in *J. curcas* subjected to water deficit. Values are means$\pm$SE of six leaves taken from different plants per treatment. The regression line (continuous) is shown. The values of the determination coefficient are included.

Similarly, the relationship between leaf stomatal conductance and foliar ABA concentration of *J. curcas* exposed to different levels of water stress were highly correlated, $r^2 = 0.85$ (Figure 2). The relationship between the two regardless of treatments was best described by a polynomial cubic regression line of zero intercept which explains a value of around 85% of the variation in leaf stomatal conductance. This consequence showed close relations between the two in which leaf stomatal conductance increased with decreasing foliar ABA concentration. Zainudin and Awang (2004) reported that when plants underwent stress, it stimulates the production of ABA in roots which travels via the xylem to the leaf where it decreases stomatal conductance.

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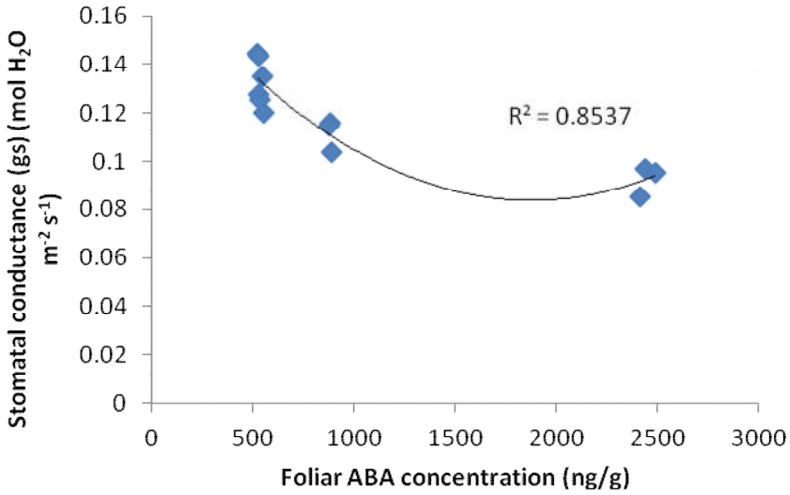

Figure 2. Relationship between stomatal conductance and foliar ABA concentration in *J. curcas* subjected to water deficit. Values are means±SE of six leaves taken from different plants per treatment. The regression line (continuous) is shown. The values of the determination coefficient are included.

Conclusions

The well-watered plants responded better in terms of its fruit characteristics and other selected physiological properties than that of the water-stressed plants. On the other hand, plants under water deficit managed to effectively control its growth and survival especially when water supply is limited through various physiological mechanisms. In well-watered (W0) plants, the high availability of soil water at field capacity (-0.03 MPa) affected the fruit characteristics of *Jatropha curcas* considerably by showing comparatively larger fruits and more mass in seeds. Photosynthesis rates (*A*), stomatal conductance (*gs*), and transpiration rates (*E*) of the well-watered plants were significantly higher than the water-stressed plants. Nevertheless, the WUE for W0 and W3 showed insignificant difference concluding that plants exposed to longer drought period managed to adapt through carbon gain optimization process. Furthermore, it was found that foliar ABA concentration and transpiration rate (*E*) was significantly correlated to stomatal conductance. It can be said that due to water stress, the increased of foliar ABA responded by helping to mediate stomatal conductance and thus the transpiration and photosynthetic rate of the leaf. The structural and physiological adaptations associated with drought tolerance of plants are numerous and diverse and appear to be more important for plant survival than for high growth rates.

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CHAPTER 4

DEVELOPMENTAL PHYSIOLOGY AND ASSIMILATION PRODUCTION

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Performance of Lowland Cauliflower (*Brassica oleraceae* var. *botrytis*) Grown Using Fertigation in Soilless and Soil-based Systems under Protective Structure

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Introduction

Cauliflower is one of the popular vegetables consumed by Malaysians. It is a temperate vegetable and is usually grown in the cool highland areas of the Cameron Highlands. Presently, very little cauliflower is cultivated. Domestic needs are met through imports worth around RM 30 million annually. There is thus a need to increase local cultivation of cauliflower to reduce imports. Due to limited land in the highlands, large scale production should now be in the lowlands. However, cultivation of cauliflower in the tropical lowland conditions requires some innovative methods to ensure high yield and quality produce. Growing under protective structures (rainshelter) using fertigation is one of the techniques. A study was conducted with the following objectives: (a) To determine the growth and yield of lowland cauliflower grown using the fertigation technique in soilless and soil-based systems under protective structure; (b) To determine suitable growth media in the cultivation of lowland cauliflower using fertigation in the soilless system; and (c) To assess the effectiveness of the fertigation technique on lowland cauliflower grown in the soil-based system.

Materials and Methods

The study was conducted under a tunnel-shaped rainshelter with side netting structure at MARDI Station, Serdang, Selangor located on a mineral soil. Cauliflower var. White Shot was used. Treatments consisted of the following: 1 – fertigation in soilless system with cocopeat; 2 – fertigation in soilless system with burnt rice husk (BRH); 3 – fertigation in soilless system with cocopeat + burnt rice husk; 4 – fertigation in soil-based system with compost; 5 – fertigation in soil-based system with processed chicken manure; and 6 – soil-based system with conventional fertilizer. Parameters taken were plant canopy diameter, head weight, head diameter, and yield in ton/ha. Analysis of growth media and the soil were carried out before the end of experiment, and leaf analysis done after harvest.

Results and Discussion

Plant Canopy and Head Diameter

There were no differences in plant canopy among plants grown under fertigation in soilless and soil-based systems, but plant canopy was least in plants grown on the soil with conventional fertilizer application (Table 1), indicating better plant growth in fertigation systems. Cauliflower from plants in cocopeat with fertigation and soil-based system with conventional fertilizer had the lowest head diameter. But overall, fertigation treatments had larger cauliflower heads (14.1 – 17.0 cm) compared to the soil-based system with conventional fertilizer.

Head Weight and Yield

Fertigation in soil-based systems produced cauliflower with higher head weight (582.3 – 589.3) than heads from plants grown under fertigation in the soilless system, with head weight of 452.0 – 463.3 g (Table 2). Plants in soil-based system with conventional fertilizer produced the least head weight. The highest yield was also from plants in soil-based system with fertigation (11.17 – 11.26 t/ha), followed by plants under fertigation in soilless system (8.68 – 8.90 t/ha). The soil-based system with conventional fertilizer produced the lowest yield.

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Table 1. Effects of treatments on plant canopy and head diameter of cauliflower

Treatment	Plant canopy (cm)	Head diameter (cm)
T1 – Fertigation in soilless system with cocopeat	65.1 a	11.8 b
T2 – Fertigation in soilless system with BRH	67.0 a	15.1 a
T3 – Fertigation in soilless system with cocopeat + BRH	65.7 a	15.1 a
T4 –Fertigation in soil-based system with compost	66.2 a	14.1 a
T5 – Fertigation in soil-based system with processed chicken manure	63.1 a	17.0 a
T6 – Soil-based system with conventional fertilizer	48.6 b	12.1 b

Values in a column with the same letter are not significantly different at p <0.05 according to the DMRT
w.a.t. = weeks after transplanting
BRH = burnt rice husk

Table 2. Effects of treatments on head weight and yield of cauliflower

Treatment	Head weight (g)	Yield (t/ha)
T1 – Fertigation in soilless system with cocopeat	452.0 b	8.68 b
T2 – Fertigation in soilless system with BRH	452.3 b	8.69 b
T3 – Fertigation in soilless system with cocopeat + BRH	463.3 b	8.90 b
T4 –Fertigation in soil-based system with compost	589.3 a	11.26 a
T5 – Fertigation in soil-based system with processed chicken manure	582.3 a	11.17 a
T6 – Soil-based system with conventional fertilizer	365.5 c	7.02 c

Values in a column with the same letter are not significantly different at p <0.05 according to the DMRT

Growth Media and Soil Analysis

Soils under fertigation had higher N, P and K levels than the fertigated growth media of cocopeat, burnt rice husk and cocopeat + burnt rice husk (Table 3), while Ca levels are generally lower in soilless systems. High Mg levels were recorded in cocopeat and soils with fertigation. It was also found that soil-based systems with fertigation had higher Fe and Mn concentrations (Table 4). In general, fertigation in soilless and soil-based systems improved soil nutrient levels compared to the soil-based system with conventional fertilizer. The higher nutrient levels in the fertigated soil and growth media had resulted in higher yields from the plants.

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Table 3. Growth media and soil analysis (N, P, K, Ca and Mg)

Treatment	N (%)	P (ppm)	K (%)	Ca (%)	Mg (%)
T1 – Fertigation in soilless system with cocopeat	0.17 d	987 b	0.23 d	1.92 ab	0.25 c
T2 – Fertigation in soilless system with BRH	0.18 d	643 bc	0.31 d	0.46 c	1.07 ab
T3 – Fertigation in soilless system with cocopeat + BRH	0.32 c	929 b	0.52 c	1.08 cb	0.29 c
T4 –Fertigation in soil-based system with compost	0.97 a	1387 a	1.15 a	1.91 ab	0.92 b
T5 – Fertigation in soil-based system with processed chicken manure	0.73 b	1567 a	0.96 b	3.03 a	1.55 a
T6 –Soil-based system with conventional fertilizer	0.13 d	518 c	0.17 d	2.14 ab	0.18 c

Values in a column with the same letter are not significantly different at p <0.05 according to the DMRT

Table 4. Growth media and soil analysis (B, Fe and Mn)

Treatment	B (ppm)	Fe (%)	Mn (ppm)
T1 – Fertigation in soilless system with cocopeat	83 ab	0.18 d	106 b
T2 – Fertigation in soilless system with BRH	86 a	0.10 d	82 b
T3 – Fertigation in soilless system with cocopeat + BRH	84 ab	0.16 d	66 b
T4 –Fertigation in soil-based system with compost	81 ab	1.63 a	179 a
T5 – Fertigation in soil-based system with processed chicken manure	84 ab	1.49 b	146 a
T6 –Soil-based system with conventional fertilizer	72 b	0.57 c	78 b

Values in a column with the same letter are not significantly different at p <0.05 according to the DMRT

Foliar Nutrient Content

Plants on the soil with conventional fertilizers had lower leaf N, P and K contents (Table 5). Leaf Ca content was also lower in soil-based system with conventional fertilizer compared to plants under fertigation in soilless system with BRH, fertigation in soil-based system with compost and fertigation in soil-based system with processed chicken manure. In terms of micronutrients, leaf B and Fe were lowest in soil-based system with conventional fertilizer (Table 6). Leaf Mn was higher in soilless systems. The leaf contents of all of these nutrients in plants under fertigation in soilless and soil-based systems are above the critical levels are above the critical concentrations as cited by Scaife and Turner (1983).

In general, higher yields from crops grown in soilless and soil-based systems with fertigation compared to those in soil-based system with conventional fertilizer are attributed to more nutrient availability in the growth media and the fertigated soil, and higher leaf nutrient contents.

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Table 5. Macronutrient contents of cauliflower leaves

Treatment	N (%)	P (%)	K (%)	Ca (%)	Mg (%)
T1 – Fertigation in soilless system with cocopeat	5.26 a	0.68 a	5.83 a	2.98 bcd	0.63 b
T2 – Fertigation in soilless system with BRH	4.91 a	0.65 a	6.04 a	2.90 cd	0.83 a
T3 – Fertigation in soilless system with cocopeat + BRH	5.14 a	0.63 a	6.36 a	3.18bc	0.73 a
T4 –Fertigation in soil-based system with compost	5.53 a	0.69 a	6.15 a	3.80 a	0.60 b
T5 – Fertigation in soil-based system with processed chicken manure	5.21 a	0.69 a	6.13 a	3.54 ab	0.62 b
T6 – Soil-based system with conventional fertilizer	3.56 b	0.43 b	3.71 b	2.50 d	0.52 b

Values in a column with the same letter are not significantly different at $p < 0.05$ according to the DMRT

Table 6. Micronutrient contents of cabbage leaves

Treatment	B (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
T1 – Fertigation in soilless system with cocopeat	42.40 c	145.21 c	39.44 b	54.95 ab
T2 – Fertigation in soilless system with BRH	53.24 a	178.12 a	80.01 a	44.25 b
T3 – Fertigation in soilless system with cocopeat + BRH	44.18 bc	148.22 cb	70.05 a	48.09 ab
T4 –Fertigation in soil-based system with compost	47.65 b	177.58 a	39.98 b	47.54 ab
T5 – Fertigation in soil-based system with processed chicken manure	46.90 bc	172.40 ab	45.05 b	59.33 a
T6 – Soil-based system with conventional fertilizer	25.10 d	109.22 d	39.18 b	49.51 ab

Values in a column with the same letter are not significantly different at $p < 0.05$ according to the DMRT

Conclusions

Cauliflower, a temperate vegetable, can be successfully grown in the tropical lowland conditions using the fertigation technique under protective structures. For cultivation of lowland cauliflower using fertigation in the soilless system, suitable growth media is cocopeat or burnt rice husk. Fertigation is also effective in the soil-based system. The crop grown on raised soil-beds with fertigation gives a higher yield than that grown using fertigation in the soilless system.

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Effect of Different Media Use on Rooting and Growth of Eksotika II Papaya Cuttings (*Carica papaya L.*)

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Introduction

Eksotika II papaya is considered as a well-renown papaya hybrid which has been released by Malaysia Agriculture Research and Development Institute (MARDI) in 1991. Apparently, the hermaphrodite fruit is elongated in shape has created a greater demand than rounded female fruits for export markets since two decades ago (Chong et. al, 2008). Currently, most of the papaya growers in Malaysia used seeds in their cultivation process. However, the production of hermaphrodite fruits from seedling was reduced, due to low production of hermaphrodite trees. This may be due to sex segregation incidence that occurs inside the seed with the ratio estimated to be 1 hermaphrodite: 1 female (Chan, 2004).

One approach to solve this problem is to produce 100% hermaphrodite of selected traits through vegetative propagation such as microcutting. Microcutting technique is the process whereas the pieces of the plant will be cut and placed or sown in a suitable environment, so they can grow into a whole new plant (Awang et. al, 2009). The rooting of cuttings is influenced by quite a few aspects. Whilst, the planting material like media for cuttings is one of the main factors that should be concerned (Dumitrascu et. al, 2003). The selective media used has an ability to be as one of the regulator to initiate callus formation resulting in the root development of the cuttings (Ab Kahar, Personal communication, 2010). Furthermore, most of the propagators choose to prepare the rooting substrate by mixing the components. This is to achieve the suitable air flow and drainage that conditioned the media to be moist but not water logged during roots initiation and development (Bilderback, 1995). Thus, this study was conducted to find an attempted within discussing about the suitable media that can be used to promote rooting and growth of Eksotika II papaya cuttings. The objective of this study is, to select the promising media for rooting Eksotika II cuttings and also, to observe the growth of Eksotika II papaya cuttings on the different media use.

Materials and Methods

This study was performed at the nursery, MARDI Station, Bukit Tangga, Kedah. The experiment was carried out in Complete Randomized Design (CRD) consisting of four replicates for each media treatment. Seed of Eksotika II papaya was obtained and germinated in the planting media. The uniform seedlings were selected and each of the selected seedlings was cut at the main stem. The cuttings were sown in the different treatment of media. All the cuttings were placed in the microcutting box. Data recording the percentage (%) of the rooted cuttings were taken at day 30 microcutting technique started. While, the data for leaves number, height, stem diameter and biomass of the rooted cuttings were taken at day 40 microcutting technique started. The treatments were:-

Control – Peat moss 100%
T1 – Peat moss: Sand (1:1)
T2 – Layering media, with 10% of sand as a top layer + Peat moss : Sand (1:1)
T3 – Peat moss: Perlite: Vermiculite (1:1:1)
T4 – Peat moss: Soil (1:1)

The data were analysed using Statistical Analysis System (SAS)

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Results and Discussion

Results showed that, for the rooting percentages parameter, there were significantly differences between T3 compared with the other treatments respectively (Table 1). The T1 and T2 recorded 100% rooting. It was followed by Control and T4 with 83% rooting. No rooting was observed in T3 might be due to their physical properties of the media which has coarse-textured. Therefore, the bulk density and the water holding capacity in the media combination are low and the substrate did not facilitate holding of water to supply moisture to the cuttings. The moisture content appears to be properties of a major concern for propagation (Bilderback, 1995). Plate 1 showed T3 cuttings that were rotten and dry. The symptoms initiated from the basal part of the cuttings. The other treatment showed different trend of root development after 40 days of rooting. Leaves number of rooted cuttings (Table 1), it is found that T4 is significantly different compared with the other treatments. It might be due to the texture of substrate is not in an appropriate condition to support the growth of the rooted cuttings. The combination between peat moss and soil was considered as a fine-textured substrate compared with other treatments. Finally, the water flow was reduced and caused the media become too moisten. The too moisten condition caused the air space between the fine-textured substrates particles were replaced by water. Oxygen diffused much slowly in water filled pores than in open pores (Wever, 1995). The T1 and T2 are not significantly different between the treatments itself, but were found significantly different and higher compared to Control and T4 (Table 1). Sand is one of the particle that was integrated in these two treatments (T1 and T2). The sand particle was coarse-texture resulting in improved drainage system and also provide good aeration for the growth of cuttings (Abo-Rezq et. al, 2009). Robbins and Evans (2003) clarified that using of sand and peat moss substrates were beneficial in aspect of good physical properties for the growing medium.

Table 1. Effect of different media use on rooting and growth of Eksotika II papaya cuttings (*Carica papaya L.*)

Trt. No.	Treatments	ɔoting percentages (%)	Leaves number	Heights (cm)	Fresh weight (g)
Control	Peat moss 100%	83.33[a]	4.0833[a]	19.16[a]	4.444[b]
T1	Peat moss: Sand (1:1)	100[a]	4.5833[a]	21.16[a]	7.6195[a]
T2	Sand Layer + Peat moss: Sand (1:1)	100[a]	4.5833[a]	23.54[a]	7.3565[a]
T3	Peat moss: Perlite: Vermiculite (1:1:1)	0[b]	0[c]	0[c]	0[c]
T4	Peat moss: Soil (1:1)	83.33[a]	2.9167[b]	10.16[b]	4.8951[b]

Means followed by the different letter within the same column are significantly different at $p<0.05$ according to the Duncan Multiple Range Test (DMRT)

Plate 1. The rooting performance on different treatment of media

Conclusions

Based on the overall results from the different parameters studied namely the rooting percentage (%), leaf number, height (cm) and biomass of the rooted cuttings, we can conclude that, the combination between peat moss and sand can be adopted or recommended as a viable growing medium for microcutting technique on Eksotika II papaya cuttings. The combination of the substrate will promote the root initiation and enhancing the growth performances of Eksotika II papaya cuttings.

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In Vitro Germination of Oil Palm Synthetic Seeds

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Introduction

An artificial or synthetic seed (SS) is defined as a somatic embryo (SE)/tissues located inside a coating resembling a zygotic seed. The coating may serve as a synthetic endosperm consisting of carbon sources, nutrients, growth hormones, anti- microbial agents, etc. The synthetic coat must be non-damaging to the embryo, protect the embryo from mechanical damage during handling and allow germination to occur. The coating and embryo seed may be hydrated (resemble a recalcitrant seed) or desiccated (resemble true seed).

The application of SS was proven useful in many crops such as carrot (Timbert et al., 1996), *Carica papaya* (Castilo et al., 1998), *Palonia elongata* (Ipekci and Gozukirmizi, 2003), Citrus reticulate (Antonietta, 2007), rice (Roy and Mandal, 2008), ginger (Sundararaj et al., 2010) and cauliflower (Rihan et al., 2011). However, there are still problems hindering the routine production of large numbers of SS which can be planted as true seeds. Selection of good cultures, coating materials, hardening and germination process need to be optimised for production or propagation purposes.
The objective of the study is to develop a synthetic coating material for various tissues of oil palm.

Materials and Methods

Plant materials

Embryogenic aggregates, zygotic embryos and *in vitro* shoot apices were used for encapsulation (Figure 1).

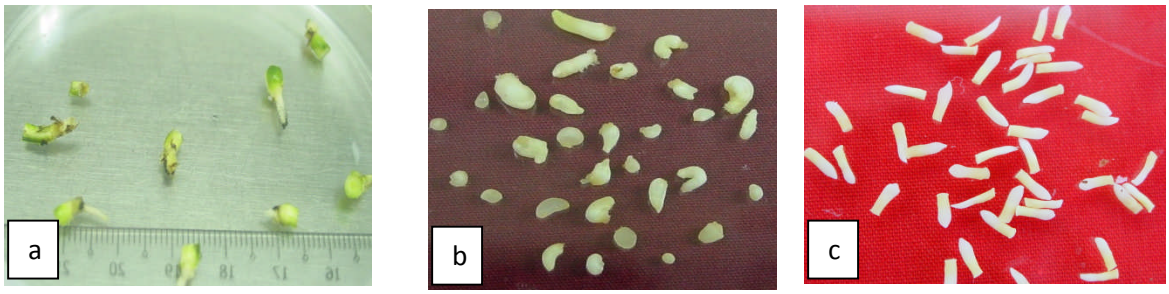

Figure 1. Selection of tissues for encapsulation.
a. zygotic embryos
b. Embryogenic aggregates
c. Shoot apices

Media for Encapsulation

a. Sodium alginate media

Sodium alginate solution (3%) was prepared in liquid MS supplemented with 3% sucrose and 0.0168g/l naphthaleneacetic acid (NAA) and autoclaved at 121^{o}C under 105 kPa pressure for 15 min.

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b. Calcium chloride solution

0.1 M $CaCl_2$ was prepared in liquid MS supplemented with 3% sucrose and autoclaved at 121^{o}C under 105 kPa pressures for 15 min.

Encapsulation Process

Zygotic embryos, embryogenic aggregates and shoot apices were mixed in the sodium alginate media for a few seconds, picked up and placed in a sterile aqueous solution of calcium chloride with occasional agitation. Calcium alginate beads encapsulating the tissues were formed within 15 – 30 min. Beads were recovered by decanting the $CaCl_2$ solution, then washed with sterile distilled water and surfaced dried with sterile blotting paper. Beads were then cultured onto MS medium.

Results and Discussion

Zygotic Embryos

The germination rate of encapsulated embryos was less than 50%.The germination rate appeared to depend on the quality of the seeds **(**Figure 2).

Figure 2. Germination and conversion of encapsulated zygotic embryos

Embryogenic Aggregates

About 90% of encapsulated embryogenic aggregates of selected clones produced shoots when cultured onto solid MS media (Figure 3)

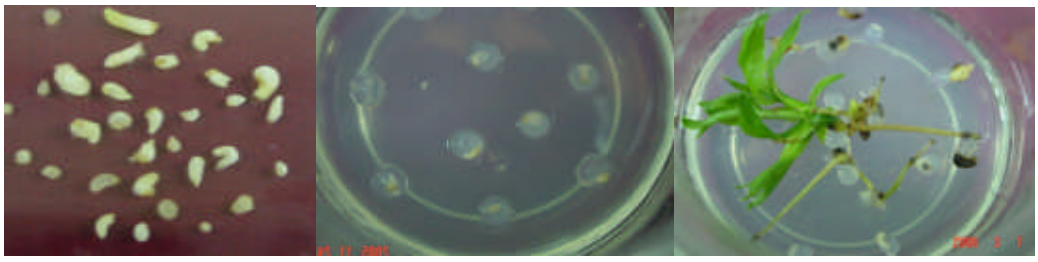

Figure 3. Germination and conversion of encapsulated embryogenic aggregates.

Shoot Apices

All encapsulated shoot apices from selected clones produced shoots when cultured onto solid MS media (Figure 4)

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Figure 4. Germination and conversion of encapsulated shoot apices.

Conclusions

Comparatively, shoots apices were observed to be the best tissue for synthetic seed production of oil palm *in vitro* cultures. They can be easily germinated *in vitro* and further developed into single plantlets. The development of oil palm synthetic seeds offers a convenient and practical means for long distance delivery of oil palm *in vitro* cultures. The same approach was proven useful in synthetic seeds of Valeriana wallichin (Mathur et al., 1989) and *Solanum tuberosum* (Nyende et al., 2003).

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Tropical Plants: Attractions for Tourism and Environmental Education

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Introduction

Plants are valuable assets in maintaining the sustainability of life. Not only for the economic benefits, plants in our environment are functional in improving air quality, protecting water, saving energy and providing aesthetic benefits. As the world is changing towards sustainable management of natural resources, plant functions are even more appreciated today. Plants have now become a subject of interest by many as indicated in the surge of popularity of tree planting activities around the globe, where Malaysia is not an exception. Tourists of today also demand for special learning experiences. Thus, there is a great potential of utilizing plants as attractions in educational tourism.

FRIM campus in Kepong is a well known nature recreation site and its greeneries are very much appreciated by visitors. The annual visitation to FRIM has reached about 300,000 visitors (Noor Azlin et al. 2010). To ensure that there is always a reason for people to come and revisit, seasonal attractions need to be highlighted. As a tropical forest, green is the dominant colour of the FRIM campus. However, certain species give special characters to the site. Not only the ornamental plants, forest plants or timber species too yield very attractive leaf shoots, flowers and fruits. Animals especially birds, give the campus a more colourful picture. These attractions will also become a potential tourism product that is seen to boost tourism industry for the country.

Many countries have successfully marketed their plant based tourism products. The Japanese Tourism Organization, for example, promotes the country's seasonal plant attraction through "Japan's Four Seasons and Flowers" which consists of cherry blossom tree in East Japan from late April to late May and in West Japan from late March to late April. Japan also promotes the autumn leaves of Hokkaido from mid-September to early October and the plum tree of Ibaraki during late February to mid-March. A tourism promotion called the "Various Flower Attraction" highlighting species such as lavender, peach tree, azaleas and hydrangea, at different places and flowering seasons is also promoted by Japan (Japanese National Tourism Organization 2012).

Another example of plant related tourism is in Kargil, India where apricot blossoms became a new tourist attraction. In May, the entire countryside are decorated with fragrant white apricot blossoms while August, the ripening fruit lends it an orange hue that interests visitors to the sites (Ladakh Tourism, 2012).

With the data from the phenological observations, FRIM started to publish a column called "Colours of FRIM" on its official website (Colours of FRIM, 2012).

Materials and Methods

The "Colours of FRIM" initiative utilizes the data from the phenological observations of plants in FRIM. Not only for visitors, through the online publication, dates of flushing, flowering or fruiting of various plants in FRIM were recorded and can be compared to similar plants at other places. The rigorous program of observing the phenological events started in January 2012 and the articles are published on FRIM's main web site since March 2012.

FRIM also features the colourful phenological articles on its facebook page which have recently showed marked increase in positive comments and "like" acknowledgements. The articles featured in "Colours of FRIM" have been extracted by newspapers as well, further disseminating information on Malaysian tropical plants to

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Malaysian readers. The *Diospyros terengganuensis* or Terengganu ebony that was flushing red young shoots, the *Pterocapus indicus* or angsana that flowers and fruits profusely and the *Lepisanthes alata* or Terengganu cherries with delectable looking fruits, were some of the examples featured in the column. The articles also indicate the location of these attractive species to make it easier for visitors. Maps have also been produced for selected species. Pictures used to highlight the plants are as in the example of *Pterocarpus indicus* in Figure 1.

Figure 1. The published article on the flowering and fruiting of *Pterocarpus indicus* attracts tourists to FRIMs grounds.

Phenology is a study of periodic biological events, such as bud break, flushing, flowering and fruit development, closely regulated by climatic and seasonal changes (Cautín and Agustí, 2005). Thus, relationships of phenological patterns to weather conditions will also be analysed that can help us understand the effects of microclimatic changes.

Results and Discussion

Some of the species featured in “Colours of FRIM” include the *Diospyros terengganuensis* or Terengganu ebony that was flushing red young shoots, the *Pterocapus indicus* or angsana that flowers and fruits profusely and the *Lepisanthes alata* or Terengganu cherries with delectable looking fruit that invites many birds to feast on the trees. All species highlighted in the column are accompanied with beautiful coloured pictures.

From the results of weekly observations, the record of the phenological events is as indicated in Table 1.

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Table 1. Phenological observation on selected species on FRIM main campus from January to June 2012

Species	**Months and Weeks in 2012**																							
	Jan				**Feb**				**March**				**April**				**May**				**June**			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Tabebuia ochracea	*	*																						*
Alphonsea maingayi	*	*	*	*	+	+	+	+																*
Elateriospermum tapos		/	/	/	/																			
Amesiodendron chinense		/	/	/													*	*						
Saraca cauliflora				*	*	*																		
Firmiana malayana				*	*	*	*	*																
Amherstia nobilis						*	*																	
Shorea agamii					*	*	*	*																
Diospyros terengganuensis							/	/	/	/														
Eurycoma longifolia										+	+	+	*	*										
Pterocarpus indicus										*	*	*	*	+	+	+	+	+	+	+				
Phyllanthus emblica																		+	+					
Dracontomelon dao																			+	+				
Tabebuia pentaphylla																				*				
Prainea limpato var. *longipedunculata*																								

Note: "*" indicates flushing, "/" flowering and "+" fruiting.

Conclusions

Many realize that plants are valuable assets in maintaining the sustainability of life. Not only for economic sustainability, plants in our environment are functional in improving air quality, protecting water, saving energy and providing aesthetic benefits. Thus, plant observation is a highly potential nature-based educational activity and rare plants would attract many visitors to a site, contributing to a sustainable tourism industry.

Another great potential for plant based tourism attraction is the medicinal gardens where visitors can learn about plants used is traditional medicines. Medicinal use of plants has always been one of the popular subjects used in

environmental interpretation. Medicinal gardens are getting popular as it can be a venue for outdoor education within parks or schools, which do not require very much space. In FRIM, an ethnobotanical garden established to showcase these plants is increasingly gaining popularity for educational visits. An example of a feature article on flowering and fruiting tongkat Ali can be found in FRIM (2012).

The knowledge on plants is a source of wealth that can be shared with tourists and extended to the public. The showiness of the plants during different phonological stages is one way to attract visitors and readers. As a significant contributor to Malaysian economy, the tourism industry can be boosted by utilizing information on tropical plant resources in terms of their attractiveness, medicinal or traditional uses as well as for their educational conservation status information.

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The Effect of Plant Density on the Erosion Rate of Slope Soil

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Introduction

Erosion is one of the major environmental problems especially in countries with high rainfall intensity like Malaysia. Each year, erosion and landslide tragedies cause many property damages and fatalities. They were claimed to be caused by increased in soil moisture that consequently contribute to the reduction of soil shear strength, thus, decreasing slope safety (Akmal, 2006). The activities of the natural processes accompanied with bad human practices have put the stress to the slope environment. In addition, the permanent loss of vegetation cover will increase the water yield and risen up the level of water table (Greenway, 1987), thus, exacerbated the runoff activities as well as contribute to severe slope soil degradation.

Recently, establishment of vegetative elements has become an alternative way instead of engineering approach for slope stabilizing against erosion as well as minimizing the occurrence of landslides. In addition, Andrés and Jorba (2000) and Tormo et al. (2006) reported that the creation of bare and steep surfaces will increase the need of vegetation to provide stabilization. While Morgan (2007) in his research stated that the main role of vegetation cover is to protect soil by intercepting rainfall and reducing runoff flow velocity. However, not all species are suitable due to harsh characteristics of the slope soil such as acidity, low fertility and water holding capacity. As the erosion rate was also found to be subjected to type of plant coverage and coverage percentage (Petrone and Preti, 2010), this study is aimed to determine the erosion rate of slope soil grown by the species selected, namely *Melastoma malabathricum* at different plant density. This study also will deduce the relationships between plant density, soil saturation level as well as the erosion rate. It is anticipated that the potentials of *M. malabathricum* at several plant densities in this study will produce a viable approach to improving the information on the soil protection, especially on slope.

Materials and Methods

Plant Material

Seeds of *Melastoma malabathricum* were germinated in the glasshouse (temperature 25-32^{o}C, ranging PAR 300-2000 µE m^{-2} s^{-1} and relative humidity 60-90%), Institute of Biological Sciences, Faculty of Science, University of Malaya. After reaching the average height of 1 m, seedlings of *M.malabathricum* were transferred to the slope.

Experimental Set Up

Twelve experimental plots of 5.5 m x 5.5 m each with 0.5 m buffer zones were set up on the slope chosen (temperature 25-32^{o}C, PAR ranging 400-2000 mE m^{-2} s^{-1} and relative humidity of 60-85%) at the Guthrie Corridor Expressway, Selangor. Five erosion boxes (modified Gerlach-type microplots, Bochet et al., 2006) with the size of 0.5 m x 0.5 m were installed in each plot of four treatments; one seedling (Treatment A); two seedlings (Treatment B); and three seedlings (Treatment C) per box, and bare soil surfaces as the control (Treatment D) in three replications in Completely Randomized Design (CRD).

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Plant Transplanting

The transplanting of *Melastoma malabathricum* was conducted using a Microclimate Plant Propagation Technique (Yushayati and Barakbah, 1996) with a modified soil depth (Normaniza, 2004). Each seedling was transplanted into a hole which was cored using a soil coring machine (Eijelkamp Agrisearch Equipment, Model Cobra, The Netherlands) at 0.6 m of soil depth supplemented with NPK fertilizers, sphagnum moss and rock-phosphate (15 g/hole) were applied at the beginning of treatment.

Data Measurements: Erosion Rate, Physiological Data [Photosynthetic Rate, Transpiration Rate and Leaf Area Index (LAI)] and Soil Water Content

The erosion rate was measured by weighing the eroded soils in the PVC container that placed below the erosion boxes and was manually calculated and determined (g/m^2). Photosynthetic rates, stomatal conductance, as well as transpiration rates were measured using Portable Photosynthesis System (LICOR 6400XT, USA) in an open system mode. The Leaf Area Index (LAI) values were measured using PAR/LAI Ceptometer (AccuPAR LP-80, Decagon Devices, Inc.). The soil water content (SWC) was also determined. Cylindrical soil cores (11 cm in diameter; 45 cm of soil depth) were sampled using a soil coring machine (Eijelkamp Agrisearch Equipment, Model Cobra,The Netherlands)

Results and Discussion

Figure 1 showed that erosion rate decreased with increasing plant density. The treatment with three seedlings (Treatment C) showed lowest erosion rate throughout the observation of six months, implying that increased in plant coverage contributed to increase surface roughness by stems and foliage which slowed down velocity of runoff. Thus, this will provide resistance against soil movements and water erosion (Gray and Sotir,1996). The result was in line with Ayed (2008) who found that soil erosion was lower at the area with high vegetation density. While, the highest erosion rate recorded on the bare soil (Treatment D) which showed that erosion rate was much attributed to rainfall, where the raindrops directly hit the soil without interception.

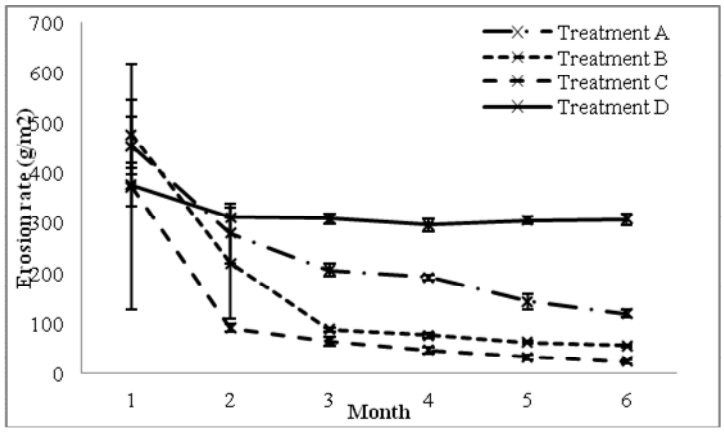

Figure 1. The erosion rate of four treatments; Treatment A, Treatment B, Treatment C, and Treatment D during six months of observation at the selected slope.

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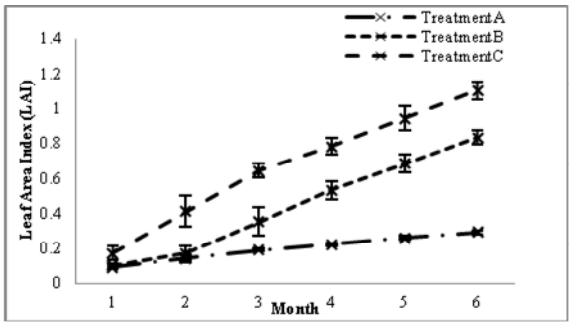

Figure 2. LAI of Treatments A, B, and C during six months of observation.

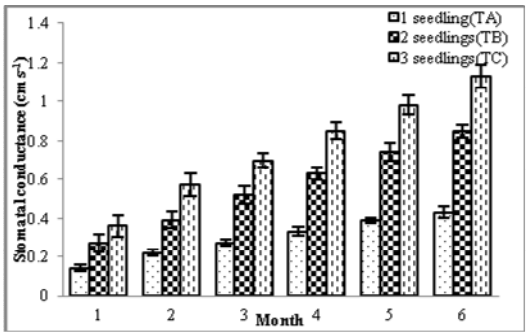

Figure 3. Stomatal conductance of Treatments A, B, and C during six months of observation.

Value of Leaf Area Index (LAI) in Treatment C appeared to be the higher than Treatments B and A. The results implied that seedlings of Treatment C had high number of leaves which increased the interception. This will reduce soil detachment on slope especially during intense rainfall (Bui et.al., 1992; Foody, 2002). In addition, the higher values of stomatal conductance contributed to increase both photosynthetic rate and LAI, exhibiting a positive plant growth performance. This promising plant growth will contribute to increase ecosystem restoration leading to acceleration of natural succession process (Normaniza & Barakbah, 2011). Apart from that, the stomatal conductance of Treatment C showed the highest values compared to the other treatments (Figure 3), indicated that the density treatment gave more positive effects on the growth of the species studied. During the maximum light intensity, the stomata opening are highest, carbon dioxide will diffused into the leaf as well as water vapor will extracted out from the leaf through the process of transpiration which in turn increased the water absorption from the soil. This will eventually lead to reduce soil water saturation.

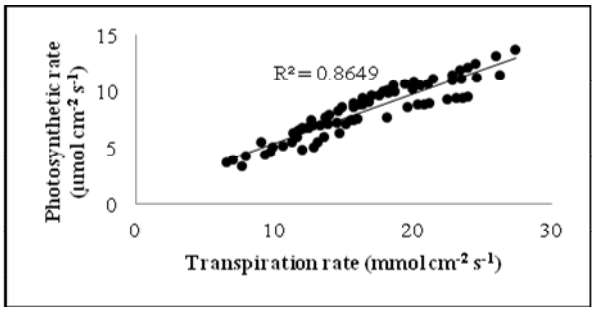

Figure 4. Relationship between photosynthetic rate and transpiration rate.

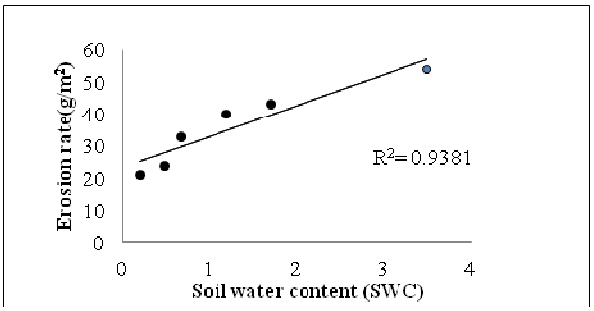

Figure 5. Relationship between soil water content (SWC) and erosion rate

From the results obtained, some correlations were observed in this study. The photosynthetic rates at all treatments were positively correlated with transpiration rate (Figure 5). This implied that increased leaf area coverage contributed to more stomatal opening in order to allow water extraction and CO_2 exchanges, resulting in a higher transpiration and photosynthetic rates. Furthermore, the erosion rate showed a strong positive trend with soil water content (Figure 6). This indicates that as the soil water content increases, the possibility for the soil to be eroded is higher. In terms of soil water content (SWC), increasing amount of water in the soil refers to low amount of water extracted through evapo-transpiration process. This resulted in oversaturated of the soil and tendency for the soil particles deformation to be higher and thus, lead to the slope failure. Nevertheless, the role of

vegetation cover is crucial in governing the erosion, hence, the higher the vegetation density, the better the water extraction, the more stable is the slope.

Conclusions

Within six months, monthly results of erosion rate decreased with increasing plant density. The treatment with the highest plant density (Treatment C) resulted with lowest erosion rate. In terms of LAI and stomatal conductance, Treatment C showed the highest values, followed by Treatment B, A, and D. In a conclusion, higher density of *M. malabathricum* contributed in reduced soil runoff by intercepting rainfall, thus, enhance the slope stability.

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The Limiting Factor for Commercial Vase Life of Cut *Acacia holosericea* Foliage Stem by Dye Tracking Method

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Introduction

Acacia holosericea is a potential cut foliage stem, animal fodder (Vercoe, 1987) and land rehabilitation (Langkamp and Dalling, 1983). Various species of *Acacias* gained interest in horticultural industries worldwide including EU, Japan and USA (Horlock et al., 2000; Ratnayake and Joyce, 2010). The species survival in semi-arid region of Australia is specially determined by its simple perforation plate that is relatively low resistance to water flow and demand for greater hydraulic efficiency (Damunupola et al. 2011). The internal flow of water is measured as xylem hydraulic conductivity (K_h) normalized for the length of the transport path and reciprocal of hydraulic resistance (K_r) (van Ieperen, 2007).

The study was carried out to confirm xylem physiological occlusion as a wounding response is a major cause of vase life termination of *A. holosericea* (Ratnayake et al. 2011; Celikel et al. 2011). The termination of vase life for cut flowers and foliage is coincident with the development of occlusions that reduce xylem flow (Faragher and Mayak, 1984). Blockages in xylem conduits can be by bacteria proliferation at the stem end (Liu et al. 2012; Ratnayake et al. 2011), tyloses (Cochard and Tyree, 1990) and/or by gel formation (Sun et al., 2008; Che Husin et al., 2012) and include the deposition of phenolic compounds (Olien and Bukovac, 1982).

Dye movement mimicking water flow in the stem of *A. holosericea* was investigated by using Safranin O as it is permanently bound to lignin (Ruzin, 1999; Larson et al., 1994; Zimmermann, 1983). Also tested was the hypothesis that the increase of cationic charge (K^+) with 10 mM potassium chloride (KCl) would lead to shrinking of the poly-electrolyte pectin, lignin and/or hemicellulose in pit membranes thus improve K_h (Aasamaa and Sober, 2010; Nardini et al., 2011; Zwieniecki et al., 2001). By using dye, an estimate distance travelled through conductive conduits (stained) versus non-conductive conduits (unstained) was distinguishable and quantifiable. The non-conductive conduits usually represent occluded vessels. Thus, staining observations could localise occlusions triggered by pre-harvest wounding at the specific times and distances from cutting sites.

Materials and Methods

Acacia holosericea stems were collected in November 2010 from 1 year old trees grown at The University of Queensland's Gatton campus. The stems were harvested at 0800 h, stood in a pale of de-ionised water (DIW) and delivered to the laboratory within 10 min in air-conditioned car. The stems were re-cut below DIW surface to *ca.* 80 cm in Experiment 1 and to *ca.* 42 cm in Experiment 2. The assessments were in the controlled environment room of 22 to 27 °C, 70 to 90% relative humidity (RH) and *ca*. 40 µmol m^{-2} s^{-1} irradiation at 12 h daily light period.

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Experiment 1

The experiment was conducted with 12 experimental units in three replicates. The *ca*. 80 cm long cut foliage stems were stood in 250 mL DIW in 300 mL capacity vases. One mL of 0.04% Safranin O was added to the vase solution individually in a new vase containing fresh DIW at serial 1 day intervals for 4 days of the vase period. The dye infusion was for 24 h before dye tracks were observed by dissection and been photographed.

Experiment 2

The experiment consisted of 16 experimental units with two single stem replicates for each of two treatments as follows, 40 mL of DIW (Treatment 1) and 10 mM KCl (Treatment 2). As the controls, two stems from each treatment were stood into DIW and left unstained with the dye. The remaining stems were shifted into 50 mL 0.04% Safranin O dye solution at 1 h intervals for 1 h infusion throughout 4 h evaluation period. The infused cut foliage stems were then transferred into new vases with fresh DIW and immediately measured for dye tracks. The stems in both experiments were transverse sectioned (TS) and analysed under light microscope (LM) (Olympus BH-2) and dissecting microscopy attached to a digital camera (Canon PowerShot G5, Japan).

Results and Discussions

Dye tracks were observed in stems TS (Figure 1) under dissecting microscope. Dye tracks in *ca.* 80 cm stem lengths in Experiment 1 was reduced across time. The stop points for dye movement from below the shoot tip of the *ca.* 80 cm cut stems in Experiment 1 were in a range of at 23 to 26 cm distal (70.8% of full stem length) on day 1, at 16 to 23 cm distal (73.9% of full stem length) on day 2, at 54 to 61 cm distal (30.3% of full stem length) on day 3, and at 49 to 61 cm distal (35.0% of full stem length) on day 4 (Figure 1).

However, no reduced length of dye tracks in *ca.* 42 cm stem lengths in Experiment 2 was observed over 0 to 4 h after cutting (Table 1). The decreased proportion of dye containing vessels in the stem TS with increased distance from end cut (stem position) was significant ($P<0.05$) (Table 1). The use of 10 mM KCl neither significantly ($P>0.05$) increased the length of the dye tracks nor the proportion of dye conducting vessels (Table 1). Thus, there were no measured effects of the cation K^+ on water (dye) uptake in this species.

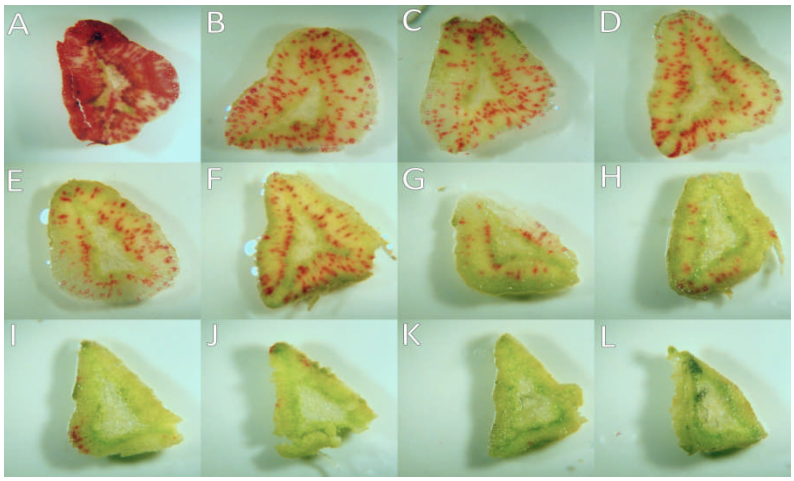

Figure 1. Transverse sections of *A. holosericea* stem show the xylem water uptake using Safranin O dye at day 1 in Experiment 1. The distribution of dye was observed at specific stems length measured from the meristem apex at, (A) 87 cm; (B) 82 cm; (C) 77 cm; (D) 72 cm; (E) 67 cm; (F) 62 cm; (G) 57 cm; (H) 52 cm; (I) 47 cm; (J) 42 cm; (K) 37 cm; and (L) 35 cm.

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The xylem cells around conductive vessels were stained red. In contrast those around non-conductive vessels did not stain, suggesting that gel occlusion/s in supply vessels had blocked dye movement to the particular position in the cut stem. The obstruction dye uptake could explain reduced water uptake by the cut *A. holosericea* foliage stems during the vase life. Occlusions were evident at the stem cut ends and 10 to 15 cm more distal from the cut ends when stem TSs were examined at higher magnification by LM (Figure 2). Gels were colourless and transparent at 10 to 15 cm distal from the basal stem end cut within 4 h after cutting

(Figure 2i-ii). When dehydrated, the early form gels structures could be broken, but regained their form in the presence of water (Figure 2iii-iv).

Table 1. Mean value of the proportion (%) of dye tracking conduits and the distance of dye conduction along *ca*. 42 cm cut *A. holosericea* foliage stems held in deionised water (DIW) (T1) and 10 mM KCl (T2) within 4 h in vases and infused for 1 h with 0.04% Safranin O dye in Experiment 2. The measurements were made in stem positions at stem end cut (0 cm) followed by every 5 cm distal until up to 25 cm more distal from the stem cut end.

		Mean % dye tracking conduits ± se	Mean dye tracking lengths (cm) ± se
Treatment	DIW	62.8 ± 4.24	30.4 ± 1.54
	10 mM KCl	65.4 ± 4.22	30.0 ± 2.61
F-test probability		0.3026	0.7650
$LSD_{0.05\ (n = 10)}$		-	-
Time (h)	0	73.3 ± 6.09^{a}	31.4 ± 1.35
	1	57.7 ± 6.33^{ab}	31.9 ± 1.30
	2	61.5 ± 7.17^{ab}	29.3 ± 1.79
	3	52.2 ± 7.51^{b}	27.6 ± 0.80
	4	75.9 ± 4.75^{a}	30.9 ± 1.59
F-test probability		<.0001	0.2731
$LSD_{0.05\ (n = 4)}$		21.1	-
Stem position (cm)	0	100.0 ± 0.00^{a}	-
	5	86.1 ± 2.44^{b}	-
	10	76.2 ± 3.33^{c}	-
	15	60.1 ± 5.41^{d}	-
	20	38.3 ± 5.19^{e}	-
	25	23.9 ± 5.70^{f}	-
F-test probability		<.0001	
$LSD_{0.05\ (n = 4)}$		8.44	

Values followed by different superscript letters are significantly different (P<0.05) by the LSD test.

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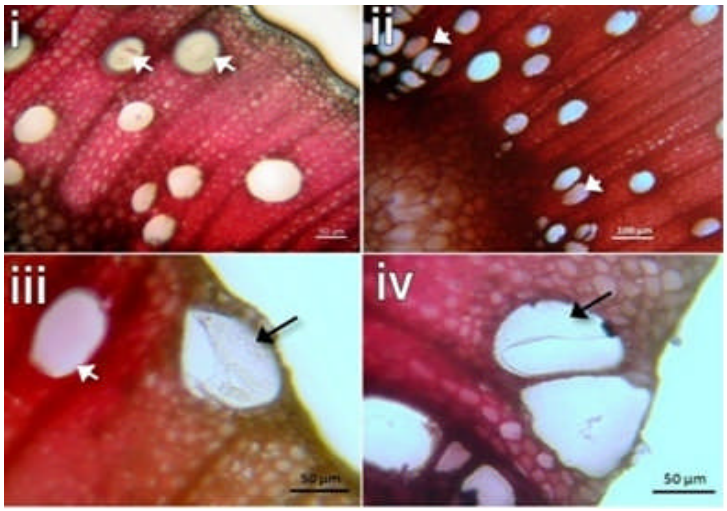

Figure 2. Occlusions by gels within stems at (i and ii) 10 cm at 4 h and (iii and iv) 15 cm 0 h for stems in DIW (control treatment) in Experiment 2. White arrow head - gels at their earlier formation stage were colourless and transparent. Black arrow-dehydrated newly forms gels.

Conclusions

Two factors determining K_h in *A. holosericea* stems were vessel characteristics and the occlusion by gels. The reduced proportion of dye tracking conduits in the stem position above 35 cm distal from the basal end cut indicates for higher deposition of gels occlusions in the xylem vessels situated near to the injured area. Induced occlusion soon after cutting obstructs dye movement coincidence with reduced water uptake of cut foliage stem. Gel physiological plugging which also develops over time appears to be a major factor contributing to vase life termination of this species.

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CHAPTER 5

POSTHARVEST TECHNOLOGY AND QUALITY CONTROL

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Development of a Non-Radioactive Screening Protocol of Various Genetic Background of Oil Palm

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Introduction

Simple sequence repeats (SSRs) which consist of a variable number of tandem repeats of a simple motif sequences are very useful markers for a number of plant species (Semagn et al., 2006). The main advantages of SSRs are that they are abundant, reproducible, co-dominant, widely distributed in crop genomes and require a small quantity of DNA for PCR to detect polymorphisms (Chua et al., 2006). A routine protocol for SSR analysis has been established for oil palm (Billotte et al., 2005; Rajinder et al., 2007; Norziha et al., 2008; Ngoot-Chin et al., 2010). The protocols listed above mainly use radiochemicals to detect the SSR amplicons. Radioactive labels need licensing and there are many safety issues associated with radioactivity. Capillary electrophoresis (CE) is available as alternative to radioactive screening using SSR (Seng et al., 2011). However, these visualizations require rather expensive or hazardous chemicals which utilize highly sophisticated instruments and are more time consuming (Gupta et al., 2010). Simpler and cost-effective SSR analysis methods will be very useful for routine screening of samples by researchers. The method in this study does not require radioactivity for detection, nor does it require a large capital investment. The method described relies on the use of standard horizontal electrophoresis sets and Super Fine Resolution agarose (SFR) gels. It focuses on identifying suitable SSR primers for routine analysis of oil palm samples of different genetic background.

Materials and Methods

A total of 20 accessions of various oil palm samples were used (Table 1). Young fresh spear leaves were ground to powder in liquid nitrogen and the DNA was extracted using a protocol described by Doyle and Doyle (1990). The samples were then kept in -80 °C freezer until needed. The purity of the samples was measured using a Genesys 20 spectrophotometer and later diluted to 25 ng/µL using Tris-EDTA (TE) buffer before using it for PCR. Twenty-nine pairs of forward and reverse primers were also diluted to 5 µM and 1 µM consecutively and stored before use.

The PCR reaction was subsequently carried out in a final volume of 10 µL consisting of 1 µL of 10X PCR buffer, 1 mM dNTPs, 5 µM and 1 µM unlabelled forward and reverse primers, 0.5 U Taq DNA polymerase and 50 ng template DNA. PCR was done using Perkin Elmer 9600 thermocycler as follows: denaturation at 95 °C for 3 min; 35 cycles of 95 °C for 3 s, 52 to 58 °C for 3 s (depending on the primers as shown in Table 2) and 72 °C for 3 s; and a final extension at 72 °C for 5 min. The PCR reaction was stopped by the addition of 3 µL bromophenol blue dye into each microtube.

Successful PCR was confirmed by 4% SFR agarose (Amresco, USA) gel electrophoresis. Horizontal electrophoresis systems (Horizon model 20-25) were used to separate the amplicons using 1X Tris-acetate ethylene diamine tetraacetic acid (TAE) buffer. For the PCR products, 6 to 9µL DNA mixtures were loaded. Gels were run at constant voltage (140V for 3 hours) until the PCR amplicons were resolved. The cut gel was then stained using 20µg ethidium bromide on Heidolph Unimax platform shaker for 30min and rinsed with water. The gels were placed on a transilluminating ultraviolet light source and images were collected by a digital camera. Only clear fragments were scored and used in the data analysis. In analysing the genotype data, PopGene software version 1.32 was utilized (Yeh and Boyle, 1997). Associations were determined by visual evaluations of SSR

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band patterns in pools and can be validated by evaluations of other linked SSR markers. The software was also used in calculating the genetic distances of all the 20 samples.

Table1. List of oil palm samples used for screening SSR primers

No.	Sample	Background	No.	Sample	Background
1	T128-PK540/318	selfIng of palm T128	11	ML161	pisifera
2	T128-PK540/319	selfIng of palm T128	12	ENL 48	dura
3	T128-PK540/320	selfIng of palm T128	13	P2/1	tenera
4	T128-PK540/337	selfIng of palm T128	14	P2/2	tenera
5	T128	Nigeria palm T128	15	P2/10	tenera
6	UP1026	oleifera	16	P2/11	tenera
7	4-2-39/79	OxG hybrid	17	EPA1109	tenera
8	4-2-39/80	OxG hybrid	18	EPA3206	tenera
9	4-2-39/147	OxG hybrid	19	EPA2708	tenera
10	4-2-39/154	OxG hybrid	20	EPA3605	tenera

Table 2. List of primers used for screening various oil palm samples. The annealing temperature (TA) during PCR denaturation differs for each sample

No.	Primer name	TA(^{o}C)	SSR Motif	No.	Primer name	TA(^{o}C)	SSR Motif
1	sMo00222	57	(CT)8	16	sMo00208	58	(TC)10
2	mEgCIR2422	52	(GA)16	17	sMg00087	58	(AG)19AA(AG)6
3	mEgCIR3775	52	(GA)18	18	sMo00269	57	(GCC)7
4	mEgCIR3825	52	(GA)21	19	sMg00235	58	(GA)15
5	sMo00121	54	(TC)24	20	mEgCIR3649	52	(GA)15
6	sMo00166	57	(AT)8	21	mEgCIR0425	58	(CCG)9
7	mEgCIR2188	52	(GA)17	22	mEgCIR0795	56	(CA)6(GA)20
8	mEgCIR3402	52	(GA)19	23	sMg00025	52	(TC)11
9	sMo00071	56	(AG)22	24	sMg00239	54	(AT)25
10	sMg00197	56	(AG)15	25	sEg00154	57	(CAG)5
11	mEgCIR3813	52	(GA)19	26	mEgCIR0580	52	(GA)10
12	sEg00203	58	(CT)7	27	sMo00270	57	(TTC)6
13	mEgCIR0878	52	(GA)22	28	mEgCIR0905	52	(GT)14ctca(GA)11
14	mEgCIR3362	52	(GA)19	29	sEg00213	57	(GCT)5
15	sMg00198	56	(AG)14				

Results and Discussion

Electrophoresis protocol was initially done using 0.5X TAE buffer but took about 5to 6 hours before the PCR products were resolved. Later, the buffer was replaced with 1X TAE and the electrophoresis time was reduced to less than 3 hours. The voltage was also increased from 120V to 140V gradually using the same set of samples.

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The electrophoresis revealed products with 140V having the best allele separation. However, if the voltage was increased to 160V, no band was observed. This is due to the increase in temperature when the voltage was increased which degraded the DNA (Burger et al., 1999). In this study, both 3% and 4% SFR agarose gels were tested as well as the 4% metaphor agarose. The type and concentration of agarose however did not show notable difference in the banding patterns. Many other studies utilized either 3% (Akamatsu et al., 2007; Sipahi, 2011) or 4% SFR agarose (Chen et al., 2007; Perumal et al., 2008). Nevertheless, 4% SFR agarose were preferred in the study due to the ease in handling the gel during staining procedures. Example of polymorphic bands is shown in Figure 1.

Apart from visualizing samples, we also used PopGene to analyze the band profile (Yeh and Boyle, 1997). Twenty-nine primers were screened and twenty-eight were found to be polymorphic. Nei's (1972) genetic distances revealed divergence within and among samples ranging from 0.057 to 1.066. The highest distance among samples was observed between UP1026 and EPA2708 (1.066) and lowest between EPA3206 and EPA2708 (0.057). Samples from the same family clustered together. The UPGMA clustering of the 20 samples is illustrated in Figure 2 and it showed genetic diversity associated with the types of sample analyzed.

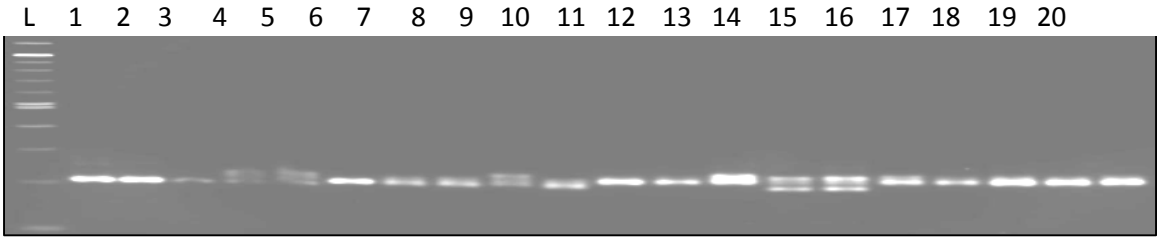

Figure 1. Bands showing polymorphism from different samples using primer sMo00222

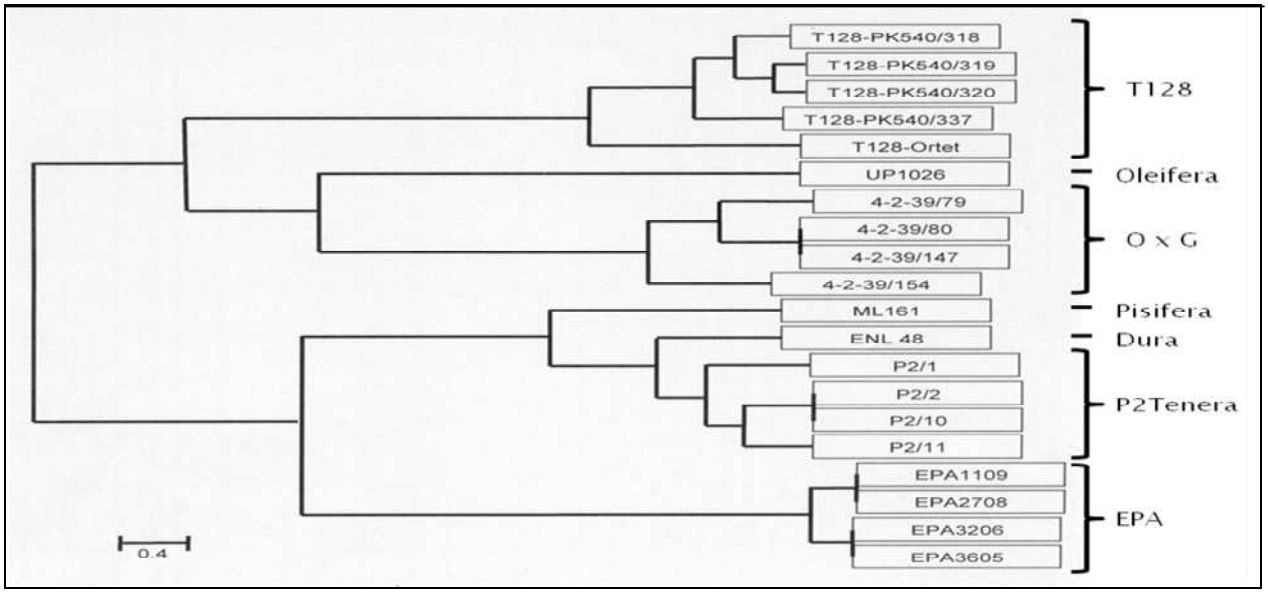

Figure 2. UPGMA clustering of 20 samples of different genetic background based on Nei's (1972) genetic distances

Conclusions

This study has demonstrated the application of non-radioactive protocol for screening of SSR using SFR agarose. The SSR was found to be suitable in characterizing the oil palm samples obtained from different genetic background. The results from this study also demonstrated the effectiveness of the SSR primers for fingerprinting different samples of the oil palm, *Elaeis guineensis, E. oleifera* or its hybrid.

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In Vitro Micropropagation of Temu Hitam

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Introduction

Curcuma aeruginosa Roxb. H or Temu hitam is used in traditional medicine to treat various ailments. This plant had been shown to exhibit antioxidant (Angel et al., 2012), antibacterial and antifungal (Tengku Kamezeri et al., 2012) activities. It can be used for food preservation, pharmaceutical treatment and natural therapies (Tengku Kamezeri et al., 2012).

However, propagation of this plant is very low through underground rhizome. Hence, tissue culture can be utilized to overcome this problem for a sustainable raw material supply in manufacturing *C. aeruginosa* products. The *in vitro* culture techniques can be used as the alternative for the superior planting material and as continuous provision of plantlet stocks for large scale field cultivation. We therefore investigated the suitable regeneration of *C. aeruginosa.* This protocol can be used for clonal propagation of this species.

Materials and Methods

Rhizomes were cleaned with detergent and tap water thoroughly to remove soil particles. Healthy sprouted buds were cut, and washed thoroughly with detergent and tap water before surface sterilized with Clorox. They were immersed in 95% ethanol for 30 s and then surfaced sterilized with 20% Clorox® for 15 min, and rinsed three times with sterile distilled water. They were again surface sterilized a second time with 5% Clorox® and rinsed again three times with sterile distilled water. The sterilized buds were cultured on MSO medium for 5 days prior to inoculation on MS medium containing various concentrations of 6-benzylaminopurine (BAP) (1.0 -10 mg/L).

Results and Discussion

Sprouted buds from rhizome were used as initial explants for the establishment of *in vitro* culture system of *C. aeruginosa.* The explants were cultured on Murashige and Skoog medium (MS) supplemented with 1 to 10 mg/L BAP to determine the best concentration for production of multiple shoots.

Initially one shoot per explant emerged after 5 to 8 days of inoculation and gradually the number of shoot buds per explant increased depending on BAP concentration. After 6 weeks, results showed that 100% of the explants produced shoot on induction medium. An average of one to three shoots were formed from each explant. MS supplemented with 5 mg/L BAP induced the most number of shoots, of three shoots per explant (Figure 1). Explants cultured on MS medium without any growth regulator differentiated into single shoot per explant.

These results showed that BAP was effective in the induction of multiple shoot. The stimulating effect of BA on multiple shoot formation has been reported earlier for eight wild species of *Curcuma* (Tyagi et al., 2004). BAP is considered as the most useful cytokinin in bud breaking in plants and has been widely used in plant micropropagation (Safdari and Kazemitabar, 2010; Alam et al., 2010). BAP promotes cell division, cell proliferation and shoot elongation. Nasirujjaman et al. (2005) stated that herbaceous plants such as *C. mangga* are highly responsive to treatment of BAP as it is able to induce auxiliary buds and produce robust, well-formed shoots suitable for further shoot proliferation.

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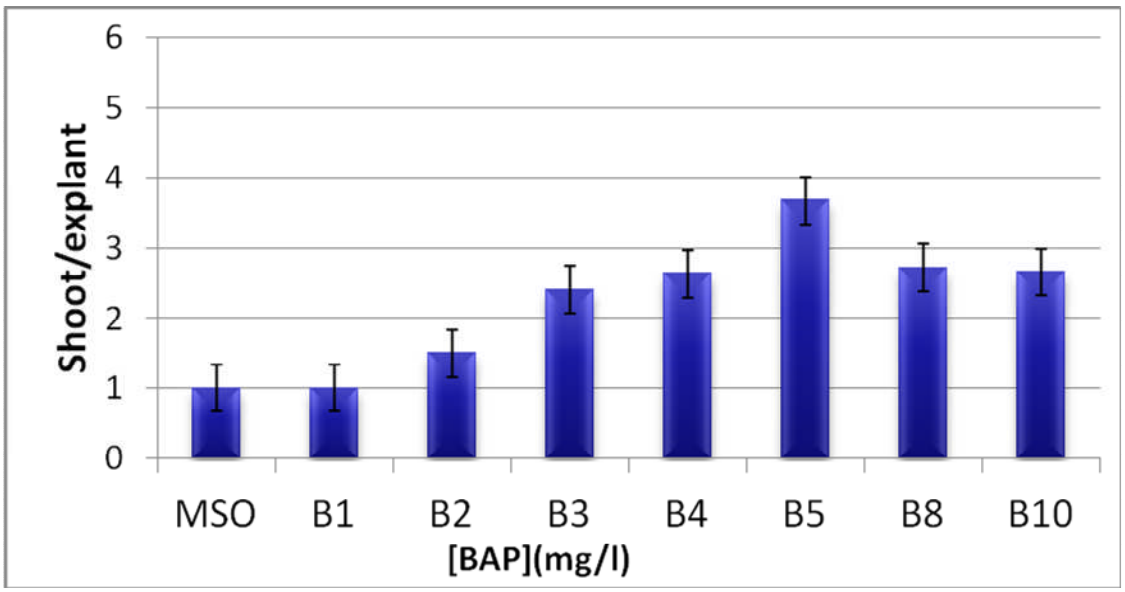

Figure 1. Effect of different concentrations of BAP on shoot induction of *C. aeruginosa*

However, on lowering the concentration of BA from their optimum concentration the number of shoots per explant was reduced. In general, the number of adventitious shoot buds per explant increased to a certain concentration and declined with the increase or decrease in concentration of cytokinin beyond their optimal level. Reduction in shoot number at concentrations higher or lower than optimal level has also been reported for several medicinal plants (Haw and Keng, 2003; Hiregoudar et al., 2006; Alatar et al., 2012). Higher concentrations of BAP not only reduced the number of shoots formed but also resulted in stunted growth of the shoots.

The regenerated shoots were excised and placed on MS medium without any growth regulator. Shoot elongation was simultaneously observed along with root induction. Rooted plantlets were removed from agar, washed thoroughly and placed in a mixture of sterilized vermiculite and sterilized soil (1:1) before being acclimatized in greenhouse.

Conclusions

Here, we report a protocol for clonal multiplication of *C. aeruginosa* using rhizome bud explants. Optimum adventitious shoot bud induction occurred at 5 mg/L BA with an average of three shoot buds per explant. This protocol can be suitably exploited for mass multiplication on a large scale for commercial purposes.

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Nucleotide Sequences Analysis of Coat Protein Gene of Cucumber Mosaic Virus from Three Different Plants

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Introduction

Agriculture was listed in NKEA of Malaysia and it is 3rd engine of growth in term of GDP after Manufacturing Industries & Service sectors. Almost 8.44 million hectares or 25% of land area devoted to agriculture. In Malaysia, CMV has been reported to be present in many important economic crops (Mohamad Roff and Anang, 1989) and is considered as one of the most virulence viruses infecting the horticultural crops with potential cause of virus epidemics in. Historically, CMV was first described in detail in 1916 on cucumber (Doolittle S.P, 1916) and other cucurbits, but is now known to occur worldwide in both temperate and tropical climates. CMV is present naturally in weeds and transmitted primarily by aphids. Infection of CMV to various vegetables, fruits and ornamental plants, results in economically losses in all parts of the world. Since the disease expresses a vast array of symptoms, hence it is difficult to detect its presence by judging the symptoms appeared. CMV is a pathogenic plant virus in the genus Cucumovirus, family Bromoviridae. It has the reputation of having the widest host range of any known plant virus including monocots and dicots, herbaceous plants, shrubs, and trees (Roossinck, 2002). Palukaitis et al. (2003) reported that CMV has a very large host range, which is estimated to be over 1000 species in 85 families. This research will give further information related to the entire length of the CMV coat protein gene and its relationship of the CMV isolate with other virus DNA sequences data available in website by molecular approach.

Materials and Methods

The CMV isolate were propagated on 3 different plants which are chilli, cucumber and tobacco plants at cotyledon stage and grown in a glasshouse. The healthy and infected CMV leaf from different plants were grounded separately using sterile mortar and pestle. Total RNAs were obtained from both healthy and infected CMV leaves from those plants were extracted with conventional phenol- chloroform method. The RT-PCR was carried out using the primer set CMV AY545, slightly modified from Madhubala et al. (2005). The primer pair was targeted at CMV coat protein gene with expected yielding product of approximately 650 bp in size. The purified specific amplification product was sent for commercial sequencing to confirm the amplicons sequence and primers specificity. Multiple alignment analysis of sequencing results between forward and reverse primers was carried out using BioEdit software package and Basic Local Alignment Search Tool (BLAST) for verification of amplified sequences in database.

Results and Discussion

Fragments of approximately ~650 base pairs were successfully amplified from the total RNAs extracted from each infected CMV leaves samples from chilli, cucumber and tobacco using RT-PCR technique with the designed primer set CMV AY545 (Figure 1). The optimum annealing temperature of the primers was found to be at 60 ^{o}C via gradient RT-PCR reaction. The purified specific amplification products were sent for commercial sequencing to confirm the amplicons sequence and primers specificity. Figure 2 shown a total number of 644 nucleotides for chilli, 619 nucleotides for cucumber and 601 nucleotides for tobacco were successfully sequence. The BLAST results from NCBI website were shown in figure 3. The homology of amplified products were 99% similar to CMV coat protein gene isolated from cucumber leaves from Thailand with accession no. FN552545 and there was

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another isolate from Thailand also give 99% homolog with the amplified product. The isolate was extracted from pepper leaf tissues with accession no. EF608461. The method was successfully used to detect as low as 0.01ng/µl RNA.

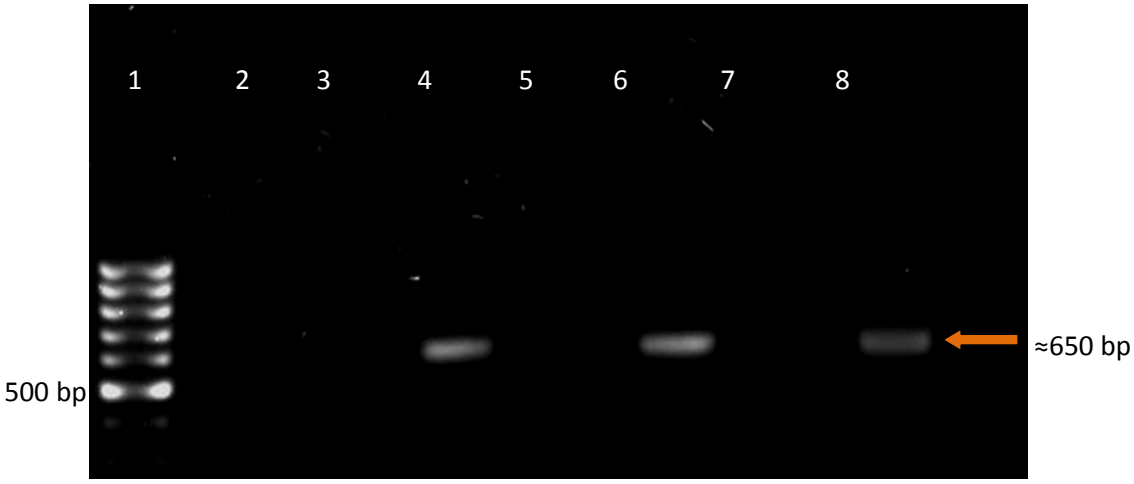

Figure 1. Gel diagram of RT-PCR amplification using primer set CMV AY545 with total RNA extracted from chilli, cucumber and tobacco leaves.
Lane 1: 100bp DNA Ladder
Lane 2: No-template control
Lane 3: Healthy chilli
Lane 4: Infected CMV chilli leaves
Lane 5: Healthy tobacco leaves
Lane 6: Infected CMV tobacco leaves
Lane 7: Healthy cucumber leaves
Lane 8: Infected CMV cucumber leaves

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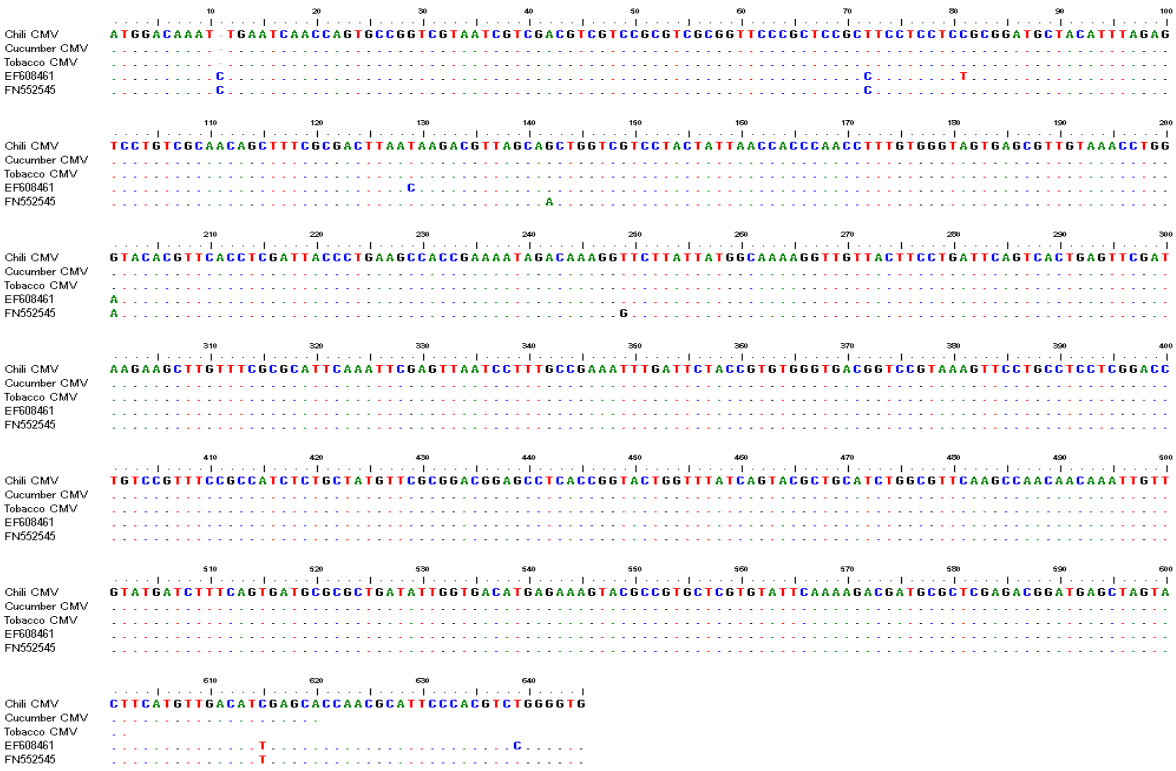

Figure 2. Nucleotide sequences comparison between CMV isolate from chilli, cucumber and tobacco with nucleotide sequence of CMV deposited in gene bank (accession number EF608461 and FN552545).

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
FN552545.1	Cucumber mosaic virus CP gene for coat protein, genomic RNA, isolat	1138	1138	100%	0.0	99%
EF608461.1	Cucumber mosaic virus isolate KPS 10 coat protein (CP) gene, comple	1133	1133	100%	0.0	99%
FN552542.1	Cucumber mosaic virus CP gene for coat protein, genomic RNA, isolat	1127	1127	100%	0.0	98%
FM999062.1	Cucumber mosaic virus CP gene for coat protein, isolate HC-56, geno	1127	1127	100%	0.0	98%
FM999063.1	Cucumber mosaic virus CP gene for coat protein, isolate HC-53, geno	1122	1122	100%	0.0	98%
FN552546.1	Cucumber mosaic virus CP gene for coat protein, genomic RNA, isolat	1110	1110	100%	0.0	98%
AY560556.1	Cucumber mosaic virus isolate SG15 coat protein gene, complete cds	1110	1110	100%	0.0	98%
AJ810259.1	Cucumber mosaic Virus cp gene for coat protein, genomic RNA, isolat	1110	1110	100%	0.0	98%
AY560555.1	Cucumber mosaic virus isolate 30RS coat protein gene, complete cds	1105	1105	100%	0.0	98%
FM999065.1	Cucumber mosaic virus CP gene for coat protein, isolate CRI, genomic	1099	1099	100%	0.0	98%
EU726631.1	Cucumber mosaic virus isolate Serdang-Malaysia coat protein gene, c	1094	1094	100%	0.0	97%
EU310928.1	Cucumber mosaic virus isolate CIMAP-India C18 coat protein gene, c	1094	1094	100%	0.0	97%
EF178298.1	Cucumber mosaic virus isolate Lucknow-Gmt segment RNA3, complete	1094	1094	100%	0.0	97%
DQ910858.1	Cucumber mosaic virus isolate Lucknow coat protein gene, complete	1094	1094	100%	0.0	97%
JF798578.1	Cucumber mosaic virus isolate Petunia-Lucknow coat protein gene, c	1088	1088	100%	0.0	97%
EF593025.1	Cucumber mosaic virus isolate Rauvolfia segment RNA3, complete sec	1088	1088	100%	0.0	97%
DQ914877.1	Cucumber mosaic virus from Rauvolfia serpentina coat protein gene,	1088	1088	100%	0.0	97%
JF682239.1	Cucumber mosaic virus isolate Lucknow coat protein gene, complete	1083	1083	100%	0.0	97%
GU906293.1	Cucumber mosaic virus isolate Kanpur (U.P.) coat protein gene, comp	1077	1077	100%	0.0	97%
FJ168035.1	Cucumber mosaic virus isolate CMV-Ch-Ada coat protein (CP) gene,	1077	1077	100%	0.0	97%
DQ640743.1	Cucumber mosaic virus strain CMV-Maharastra coat protein (CP) gen	1077	1077	100%	0.0	97%
AF350450.1	Cucumber mosaic virus coat protein gene, complete cds	1077	1077	100%	0.0	97%
HM348786.1	Cucumber mosaic virus isolate chil coat protein gene, complete cds	1072	1072	100%	0.0	97%
EF593026.1	Cucumber mosaic virus isolate Jatropha segment RNA3, complete seq	1072	1072	100%	0.0	97%
EF153739.1	Cucumber mosaic virus isolate Jatropha coat protein gene, complete	1072	1072	100%	0.0	97%
AM158321.1	Cucumber mosaic virus CP gene for coat protein, genomic RNA, isolat	1066	1066	100%	0.0	97%
X89652.1	Cucumber Mosaic Virus (CMV) RNA for capsid protein gene	1066	1066	100%	0.0	97%
EU429567.1	Cucumber mosaic virus isolate BT coat protein (CP) gene, complete c	1062	1062	99%	0.0	97%

Figure 3. The BLAST result shown that homology of the amplified product were 99% similar to CMV coat protein gene deposited in gene bank database.

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Conclusions

The primer set CMV AY545 managed to amplify coat protein gene of CMV from three different plants which are chilli, cucumber and tobacco leaves. The coat protein gene nucleotide from chili, cucumber and tobacco samples were successfully sequences.

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Effects of Coapplication of Red Gypsum and EFB Compost on Zn Content in Sweet Corn (*Zea mays L.*)

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Introduction

Red gypsum (RG) is a waste material or by-product from the industrial production of titanium dioxide (TiO_2). Usually, RG is disposed off outside the titanium dioxide plant or left as stacks. Land application is one of the disposed options of this by product. RG might be suitable for use in agriculture, because of its very high calcium (Ca) and sulfur (s) contents. Also, study on this RG indicates that it can be a good soil amendment. It could loosen and aerates hard packed or heavy soil through a chemical reaction. The high Ca content of RG can be used to improve the fertility status of wide range of soils (Fauziah et. al., 1996). In order to alleviate subsoil Ca deficiency of Ultisol and Oxisols, GML has to be applied together with gypsum. A by product may not be ideal by itself for land application, thus to improve the effectiveness of RG, co-application of RG with organic material is recommended.

Empty Fruit Bunch (EFB) compost also can be used as soil amendment. EFB is a major product produced in oil palm plantations which need to be managed. Every 25 metric tons of full fruit bunch yield 16 metric tons of EFB. An increase in soil organic matter and nutrient availability after compost application has been observed by many. The use of compost on strongly acidic soils not only improves soil productivity, but it also a way of recycling waste materials and protecting the environment from pollution. The high content of stabilized organic matter and the presence of nutrients will guarantee agronomic advantages. Used of compost also makes the reuse of organic waste possible. Hence, through coapplication of red gypsum and EFB compost, hopefully more benefits can be obtained in improving the soil physico-chemical characteristics and also enhance plant's growth.

So far, there has been no study conducted on co-application of RG and EFB compost. Thus this study was conducted with the aim of investigating corn growth performance at different rates of RG and EFB compost application.

Materials and Methods

The pot experiment was carried out at the glasshouse unit of the Faculty of Agriculture, UPM. Sweet corn (*Zea mays* L.) was used as the test crop. Sixteen polybags were arranged in a randomized complete block design (RCBD). Air dried surface soil (0-15 cm depth) from Kuala Brang series were mixed with RG + compost. The experiments consist of 4 treatments and 4 replications. Treatments of RG + EFB compost were based on the 1:2 ratio vol/vol basis. Rate of RG that used was; 25 t/ha, 50 t/ha, 100 t/ha and 200 t/ha. Rate of EFB compost used will be 2 times the volume of RG. Polybags were filled with 20 kg of air dried soil and were applied with basic fertilizers (180 kg N as urea, 150 kg P_2O_5 ha^{-1} as triple super phosphate and 75 kg K_2O ha^{-1} as muriate of potash). Three plants were grown in per polybag. This plant was planted for 70 days and it was harvested after that. The fresh weight was recorded before it was dried in the oven at the temperature of 65^0C. The dry matter was weighed and ground and sieved at 1-cm size. The prepared foliar samples then were subjected to undergo elemental analysis of N (Kjeldahl method), P, K, Ca, Mg, Fe, Zn (dry ashing method) using auto-analyzer and Pelkin Elmer 5100 atomic adsorption spectrophotometer.

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Results and Discussion

Soil, RG and EFB compost Properties

Table 1. Basic Properties of soil, RG and EFB compost

Properties	*Kuala Brang Series Soil*	*Red Gypsum*	*EFB Compost*
pH (H_2O)	4.12	7.72	7.25
Total C (%)	0.95	0.358*	28**
Cation exchange capacity ($cmol_c/kg$)	8.25	n.d	n.d
Exc. potassium (%)	0.55	0.016	2.5
Exc. calcium (%)	0.36	13	3.2
Exc. magnesium (%)	0.08	0.384	2.00
Total zinc ($mgkg^{-1}$)	0.018	167*	90.7**
Total ferum ($mgkg^{-1}$)	0.25	3.02*	1.2**

Note *Data from Fauziah et al. (2011)
** Data from Baharuddin et al. (2009)

Effect of Red Gypsum and Compost on Plant Dry Biomass

The plant dry biomass was shown in Figure 1. There is no significant difference in plant dry biomass up to 200t/ha of RG compared to the lowest rate used. Plant dry biomass was highest by addition of 200t/ha RG. However, addition of 50t/ha resulted in significantly lower plant dry biomass compared to other treatments. The highest RG content probably increased soil pH and resulted in reduced Al toxicity problem. Also the highest rate used high amount of EFB compost which probably supply more nutrient to the plant.

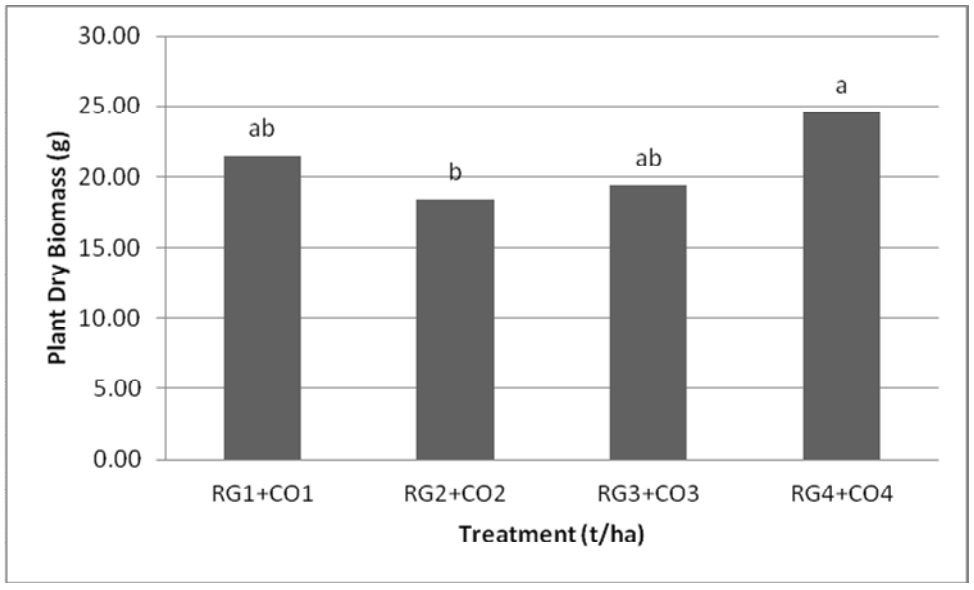

Figure 1. Mean of plant dry biomass with the different rate of red gypsum and EFB compost application (25, 50,100 and 200 t/ha) of red gypsum (bar value in column with different letters are significantly different ($P \leq 0.05$) based on LSD test).

Effect of Different Rates Red gypsum and Compost Application on the Macronutrients Content in Plant Tissue

Table 2. The Macronutrients Status in Foliar of different rates of RG and compost treatments

Treatment	Rate (t/ha)	N (%)	P (%)	K (%)	Ca (%)	Mg (%)
1	25	2.71a	0.27a	3.81b	0.08b	0.02b
2	50	2.72a	0.23a	4.39a	0.15ab	0.02b
3	100	2.71a	0.27a	3.52b	0.29ab	0.08ab
4	200	2.77a	0.26a	3.81b	0.39a	0.12a
NSR*		3.50-5.00	0.30-0.50	2.5-4.00	0.30-0.70	0.15-0.45

* Nutrient Sufficiency Range for corn. (Means with different letters are significantly different (P≤0.05) based on LSD test).

The macronutrients status of plant was compared with the nutrient sufficiency range of corn to determine the effect of RG and EFB compost application on plant nutrient. As shown in table2, the N, P and Mg percentage are lower than NSR value with the exception of potassium. For Ca, it shows an increase percentage with the addition of RG and EFB compost to the soil. The first two rates shows a percentage value lower than NSR; however the highest two rate shows an adequate percentage of Ca in plant. The Ca content increased with the application of RG on the soil due to the rich content of Ca in the RG. There was no significant difference between treatments for N and P percentage, K at the rate of 50t/ha shows significant difference compared to other treatments.

Effect of Red gypsum and EFB Compost on Zn Concentration in Plant Tissue

Concentrations of Zn in foliar tissues were significantly (P<0.05) decreased with the application of RG and EFB compost to the soil with the exception of treatment 2.This indicates that by adding the large amount of RG mixture with EFB compost, Zn content in plant tissues can be reduced. This result probably indicates that RG can be suitable agent for Zn in soil. A study done by Fauziah (2011) found that increasing the RG amendment rates clearly reduced the Zn concentrations in soil, thus RG has the potential to fix Zn in soil system and make it less phytoavailable. Comparison of Zn concentration with NSR shows that Zn concentration in plant was sufficient with the increasing rate of RG and compost application, with the exception of highest rate (T4). Concentration in T4 was much lower than NSR; means high application of RG rate may cause Zn deficiency in corn plant.

Table 3. Zinc concentration in the plant tissue

Mg/kg	T1	T2	T3	T4	NSR* ($mgkg^{-1}$)
Zn ($mgkg^{-1}$)	83.75a	75.65a	39.20b	9.63c	20-60

*Nutrient Sufficiency Range for corn. Means with different letters are significantly different (P≤0.05) based on LSD test.

Conclusions

The co-application of RG and EFB compost at rate up to 100t/ha could be used as amendment towards the growth of the plant. The used of RG and compost at rate of 200t/ha gives the best result on the corn growth, however the application of RG at very high rate can result in Zn deficiency for corn and thus need to be monitored.

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Isolation and Identification of Bacteria from Infected Banana Samples on TZC Medium using 16S rRNA Gene-cloning Method

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Introduction

In Malaysia, banana remains the second most important commercial fruit crop. In 2009, banana covers about 11% of the total fruit production areas with an estimated production value of RM 452.4 million (Husain and William, 2011). The production area is expected to expand due to increase demand for banana locally and globally as well as the listing of banana as one of the six fruit crops for development in the newly implemented Economic Transformation Programme (ETP). One of the most important and a major constraint to the production of bananas are pests and diseases. In Malaysia, Panama and Moko diseases post a significant threat to the banana industry, where yield loss can be up to 100% (Husain and William, 2011). Since mid-2012, banana farms in several states have been infected by a wilt disease causing yellowing and wilting of the older leaves, which become necrotic and collapse. When cut, fruits and trunks of affected banana plants showed brown discoloration and the latter also exude bacterial ooze, symptoms synonym to Moko but not found in Panama Disease (Husai and William, 2011).

Moko disease in banana is caused by the bacteria *Ralstonia solanacearum* race 2 and was first identified in Malaysia in 2007 (Husain and William, 2011). The most commonly used method worldwide for detection and identification of *R. solanacearum* has been isolation on tetrazolium chloride (TZC) medium; yet, for highly specific and sensitive detection and identification of the pathogenic bacteria, assays using PCR are usually utilized (Pradhanang et al., 2000). It is possible to identify bacteria to the genus or species level based on the amplification of the 16S rRNA gene sequence as ribosomal subunit exists universally among bacteria and includes regions with species specific variability (Vandamme et al., 1996).

In an effort to isolate and identify the causative agent of the current outbreak affecting the banana industry, samples were collected from an infected banana farm in Sabak Bernam that showed symptoms of Moko disease. The samples were processed where the aim of this study was to isolate bacteria from the samples on TZC medium and to identify bacteria with similar colony morphology as *R. solanacearum* (based on literature) using 16S rRNA gene-cloning method.

Materials and Methods

Sample Collection, Sample Processing and Bacterial Isolation

Samples from different parts of infected bananas plants such as the banana trunk, leaves, fruits, ooze from the trunk and soils were collected and processed. The samples except for the ooze and soils were first cut into smaller species before soaked in 10 ml sterile distilled water in 50 ml conical tubes. The samples were then shake in an incubator shaker at 28 ^{O}C for 30 minutes and serially diluted up to 10^{-5}. Each dilution was then spread on TZC agar. After incubation at 28 ^{O}C for 48 hours, plates containing between 30-300 colonies were examined. Single colonies suspected as *R. solanacearum* based on their colony morphology were selected and purified by streaking them onto fresh TZC agar for further identification.

Bacterial DNA Extraction

Pure cultures of the selected bacteria were grown at 28 ^{O}C for 48 hours in liquid nutrient broth. Extraction of the bacterial genomic DNA was then performed using the Gen Elute Bacterial Genomic DNA Extraction Kit (Sigma-Aldrich, USA) following the manufacturer's protocol.

PCR Amplification of 16S Rrna Gene

The 16S rRNA gene of the bacteria was amplified by polymerase chain reaction using the universal primers F8 (5'-AGAGTTTGATCMTGGCTC-3') and rP2 (5'-ACGGCTACCTTGTTACGACTT-3') in a 25 ul reaction volume using thermostable DyNAzyme^{TM} EXT DNA polymerase (Finnizymes, Finland) in a PTC-200 thermal cycler (MJ Research, USA). Cycling conditions were as follows: initial denaturation for 3 min at 95 ^{O}C, followed by 30 cycles of 30 sec at 94 ^{O}C, 45 sec at 55 ^{O}C and 2 min at 72 ^{O}C; and final extension at 72 ^{O}C for 10 min. The 16S rRNA gene product generated was subjected to gel electrophoresis in 1% agarose and visualized under UV transillumination after staining with ethidium bromide.

Cloning and Sequencing of 16S rRNA Gene

For cloning, the PCR product was recovered from the gel using QIA quick gel extraction kit (QIAGEN, Germany), ligated into TOPO plasmid vector using the TOPO TA Cloning Kit (Invitrogen, USA) and transformed into chemically competent Top 10 *E. coli* cells. Recombinant plasmids DNA were isolated using QIAprep Spin miniprep kit (QIAGEN, Germany) and digested with *EcoRI* restriction enzyme to determine the presence of the cloned insert in the vector. Positive clones were selected and sent for DNA Sequencing service (Research Biolabs Technologies, Singapore).

16S rRNA Gene Sequence Analysis

The forward and reverse sequences obtained were aligned using the BioEdit software. The determined 16S rRNA gene sequences were subjected to BLAST analysis against the non-redundant nucleotide sequence collection at the National Center for Biotechnology Information (NCBI) database for bacterial identification. Neighbor-joining method (NJ) was used to construct a phylogenetic tree using the MEGA5 program.

Results and Discussion

The banana industry in Malaysia is currently facing an unidentified outbreak, resulting in yield lost and rendering areas unsuitable for banana production. Symptoms of the outbreak include yellowing and wilting of the oldest leaves and discoloring of internal fruit and vascular tissues. These symptoms are similar to the Moko disease in banana. Samples were collected from infected banana farms in Sabak Bernam to isolate and identify the causative agent of the outbreak, presumptive *R. solanacearum*.

The samples were processed and spread plates were done on Kelman's tetrazolium chloride (TZC) medium, a medium which is commonly used to isolate *R. solaneacearum* if suspected, due to the relatively low cost, simplicity of use and consistency of results between different laboratories (Pradhanang et al., 2000). Typical virulent *R. solanacearum* colonies on TZC medium are fluidal and irregular with red centre and whitish periphery characteristics whereas avirulent mutant colonies are butyrous, deep-red often with a bluish border (Kelman, 1954). Similarity to avirulent *R. solanacearum* colony morphology are also taking into consideration as the bacteria is easily mutated once cultured (Kelman, 1954). After 48 hours, three distinct bacterial colonies, B7.2, B15 and HR2.2, were suspected as *R. solanacearum* based on colony morphology in which B15 colony matches virulent *R. solanacearum* whereas B7.2 and HR2.2 were inferred as avirulent (Figure 1). The bacteria were then purified and further identified using the 16S ribosomal RNA sequence based identification method. A PCR

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product, which was approximately 1500 bp in size, was successfully amplified using the universal primer F8/rP2. After BLAST analysis of the 1.5 kb 16S rRNA gene sequence and construction of a phylogenetic tree, the isolated bacteria, B7.2, B15 and HR2.2 were identified as *Methylobacterium* spp., *Enterobacter* spp., and *Staphylococcus* spp respectively; none were identified as *R. solanacearum* (Figure 2). Both *Enterobacter* and *Methylobacterium* are commonly found in soils and plant surfaces while *Staphylococcus* is a small component of the soil microbial flora (Miller and Keane, 1997; Madhiyan et al., 2007; Madigan and Martinko, 2005).

TZC, the medium that was used to isolate the bacteria, is used as a redox indicator in culture medium for differentiating bacteria, which is colorless in the oxidized form. As bacteria with suitable reducing system grow in the presence of TZC, the dye is absorbed into the bacterial cells where it is reduced to the insoluble red-colored pigment formazan (MacFaddin, 1972). *R. solanacearum* and *Methylobacterium* are aerobe while

Enterobacter and *Staphylococcus* are facultative anaerobe; therefore, in the presence of oxygen, the bacteria are able to incorporate and reduce TTC to a red colored formazan dye. *Enterobacter* spp. is the only motile bacteria among the three that were isolated. As motility occurs, small to very large regions of color can be observed around the area of inoculation, which explains the similarity of *Enterobacter* spp. colony morphology to virulent *R. solanacearum*, where colonies are fluidal and irregular, as the latter is also motile.

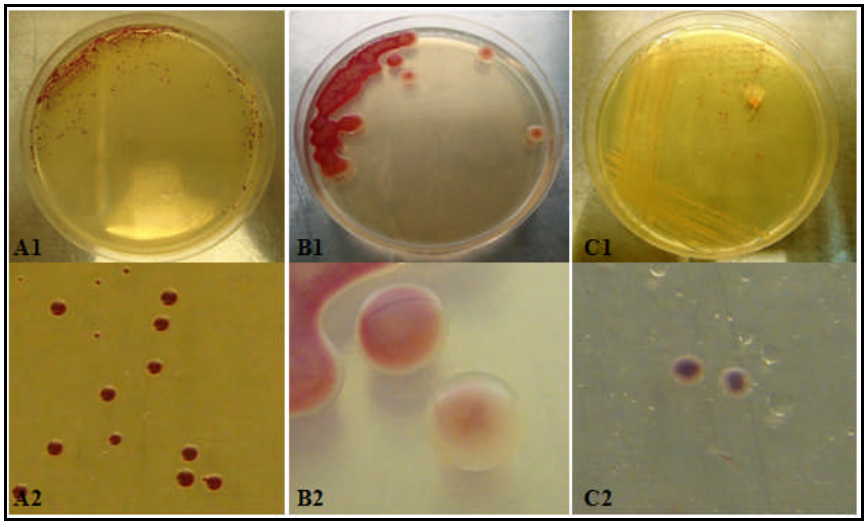

Figure 1. The isolate B7.2, B15, and HR2.2 on TZC medium ([A1,A2], [B1,B2], and [C1,C2] respectively).

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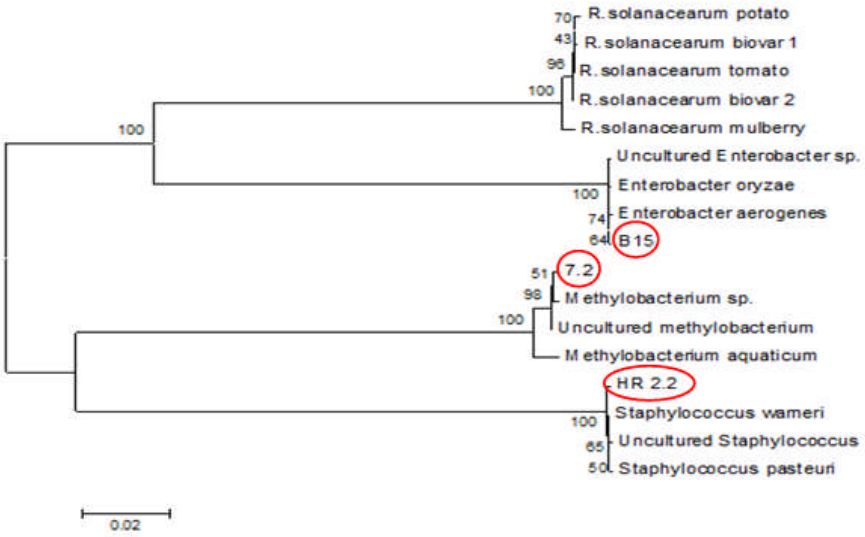

Figure 2. Neighbor-joining phylogram based on 16S rRNA gene sequences showing the position of isolate B7.2, B15 and HR2.2.

Identification of bacteria based on phenotypic methods such as culture on selective media present some inherent setbacks. First, there is a possibility of substantial amount of variability among strains belonging to the same species and in addition, corresponding databases may not yet include newly described species. Furthermore, certain tests may rely on subjective interpretation that varies among individuals (Mignard and Flandrois, 2006). Identification based on the 16S rRNA gene sequence is of interest as ribosomal subunit exists universally among bacteria and includes regions with species specific variability, making it possible to identify bacteria to the genus or species level by comparison with databases in the public domain (Vandamme et al.,1996). The 16S rRNA identification method was useful in showing that not all fluidal colonies with reddish centers obtained from isolation plates are *R. solanacearum* as reported in literatures and may result in false-positive diagnosis. Phenotypic identification should be confirmed with genotypic identification such as the 16S rRNA gene-cloning method.

Conclusions

In conclusion, three bacteria, B7.2, B15, and HR2.2, which were isolated from infected banana samples showing Moko disease symptoms and showed similarity to the colony morphology of *R. solaneacearum* on TZC medium, were identified as *Methylobacterium* spp., *Enterobacter* spp., and *Staphylococcus* spp. respectively using the 16S rRNA gene-cloning method. Therefore, to avoid false positive diagnosis, genotypic identification such as the 16S rRNA gene-cloning method should be used to confirm phenotypic identification.

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Isolation and Purification of Mitochondria DNA from Oil Palm (*Elaeis guineensis* Jacq.)

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Introduction

Mitochondria are the main site for synthesis of ATP that provides energy required by the cell. Mitochondria are currently viewed as an integrated endosymbionts, originating from a large group of eubacteria having their own genome with essential genes (Gray 1992). A great interest in mitochondria DNA (mtDNA) of higher plants has occurred in the past as investigators have documented the range of complexities in mitochondrial genomes, studied cytoplasmic male sterility in various plant systems, undertaken molecular mapping of mitochondrial genomes, isolated mitochondrial structural and non-structural genes and documented many molecular polymorphs by mtDNA restriction fragment profiles. Various researches have been conducted regarding the use of mitochondrial as an indicator of productivity in wheat, maize and oil palm (Sage and Hobson, 1973; McDaniel and Sarkissian, 1968; Kouame &and Noiret, 1981).

The ability to isolate intact mitochondria from plant tissue is a key approach in the study of the genome, proteome, and metabolic function of plant mitochondrion. To study the function of the organellar genome in the oil palm, efficient preparation of pure mtDNA that is intact and free of nuclear DNA contamination is required. Plant mitochondria have been conventionally purified from total cell extracts of storage organs, etiolated tissues, photosynthetic shoot or leaf samples or cell cultures by a combination of differential centrifugation and sucrose, PVP and/or Percoll gradients (Douce et al., 1977; Neuburger et al., 1982; Day et al., 1985) while elimination of nuclear DNA contamination or purification of organellar DNA is accomplished by cesium chloride (CsCl) gradient. Herein, we report the isolation of free nuclear-mitochondria DNA to study its involvement in clonal abnormality as well as its relationship with regards to yield and productivity.

Material and Methods

Plant Materials

Normal (P255/0436; 67) and abnormal (P255/0436; 328) leaves of *E. guineensis* were obtained from Teluk Intan, Perak.

Isolation of Mitochondria from Plant Tissue

Mitochondria isolation was carried out using Binder and Grohmann (1996) with modifications. Leave samples (100 g) were kept in the cold room for 72 hours and cut into small pieces prior to extraction. Leaves were homogenized for 5 sec in a Waring blender at 4 °C. The filtered extract was further purified *via* differential centrifugation. The pellet was treated with 300 ug/ml DNase I to remove the remaining nuclear and chloroplast-derived DNA. The pellet was then thoroughly dispersed with a fine paintbrush and homogenized using a hand homogenizer for 5 s, 2 times before being loaded onto a freshly prepared Percoll step gradient. The Percoll gradient composed of, from bottom to top, 1.8 ml 45% Percoll, 3.6 ml 28% Percoll, and 3.6 ml of 14% Percoll. Each Percoll solution containing 400 mM mannitol, 20 mM Tricine pH 7.2, 1mM EGTA. The gradients were centrifuged for 48 minutes at 19,700 rpm (SW41Ti rotor, Beckman, USA). Mitochondria were removed from the

28/45% interface and subjected to repeated dilution and centrifugation to get rid of the Percoll before finally storing it at -80 °C until used.

Isolation of Mitochondria DNA (Mtdna)

Mitochondria DNA isolation was carried out according to Mourad, G. (1998) with minor modifications. Mitochondria were lysed for an hour at 37°C with lysis buffer (5% sodium sarcosinate, 50 mM Tris-HCl pH 8, 25 mM EDTA) and 10mg/ml proteinase K was added. Final purification of mtDNA was performed by isopycnic centrifugation on CsCl density gradients. Solid CsCl in a ratio of 1g/ml of final suspension and 10mg/ml ethidium bromide (EtBr) were added, mixed and ultracentrifuged at 41,000 rpm for 24 hours at 19°C. MtDNA band was visualized using a long wavelength UV hand-held lamp and the band was collected by a syringe needle. EtBr was extracted four times using isopropanol equilibrated with NaCl and water. mtDNA was dialyzed against 2 liters of LTE buffer for a period of 48 hours and the dialyzed samples were precipitated with 0.1V of 3 M sodium acetate (pH 5.0) and 2V of ethanol. The pellet was dissolved in LTE.

Mitochondria DNA Characterization

Digestion was carried out by restriction enzyme to verify the purity of mtDNA samples. 0.75µg mtDNA was digested with *EcoR*V, electrophoresed on an 0.9% agarose gel and visualized under UV light.

Results and Discussion

The isolation of mitochondria from plants are particularly challenging as they tend to contain phenolic compound, oxidation products and polysaccharides that co-purify with organelle DNA, fragility of mitochondrial and contamination with broken chloroplast and thylakoid membranes. In this study, mitochondria isolation of normal and abnormal palm leaves was carried out according to Binder and Grohmann (1996) with modifications. Using this method, mitochondria were successfully obtained from these samples. The mitochondria were successfully isolated using the buffer with EDTA or EGTA, bovine serum albumin (BSA), and sulfhydryl reagent such as 2-mercaptoethanol to overcome the acidity, phenolic compounds and oxidation products that lead to the rapid inactivation of mitochondria. In order to obtain a maximum yield of intact mitochondria, a minimum grinding procedure using Waring blender was carried out. To minimize the effects of vacuoles on plant mitochondria, a low ratio of tissue to grinding buffer was performed.

Differential centrifugation is a key feature of mitochondria isolation to separate the bulk of nuclei, plastids and cellular debris from the mitochondrial fraction, which differs in particle size or density. Normally, DNase is used to remove the remaining external DNA of the mitochondria. The effectiveness of this DNase step requires penetration of the DNase into nonintact contaminating plastids and nuclear debris and sufficient mitochondrial integrity to prevent the enzyme from entering the organelles. Mitochondria are then further separated from the remaining plastids and nuclear debris by gradient centrifugation. Percoll density gradients medium was used for mitochondria purification (Figure 1). According to Lang and Burger (1997), the usage of Percoll allows separation of mitochondria and thylakoid membranes from green tissues. The purified mitochondria were successfully obtained by percoll density gradient method.

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Figure 1. Isolation of intact organelles from oil palm leaves. Three-step Percoll gradient for the purification mitochondria made from 1.8 ml of 45% Percoll, 3.6 ml of 28% Percoll and 3.6 ml of 14% Percoll solution, from bottom to top. This gradient was ultracentrifuged at 19,700 rpm in swing out rotor for 48 min. Plastid are concentrated in the 14 to 28% interphase (a), mitochondria in the 28 to 45% interphase (m).

The isolated mitochondria were then purified according to Mourad (1998). The purified mtDNA of normal and abnormal leaves resulted the yield of 8.4 µg and 10 µg DNA respectively. Electrophoresis gel agarose analyses showed that an intact band of mtDNA for both normal and abnormal leaves were successfully isolated (Figure 2).

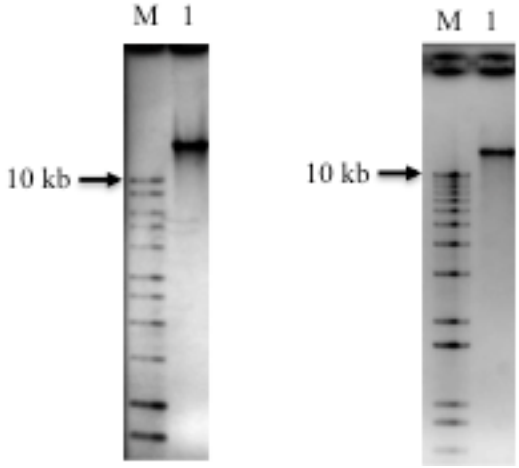

Figure 2. Agarose gel electrophoresis (0.9%) of mtDNA from (a) Normal palm leaves (b) abnormal palm leaves. Lane M: 1 kb DNA ladder; lane 1: mtDNA

The intact band of the purified mtDNA was verified by restriction enzyme analysis. Restriction patterns of most orgDNA consist of well-separated bands (with little background smear) adding up to sizes of several to more than 100 kbp (Lang andBurger, 1997). Gel electrophoresis analysis showed that mtDNA for normal and abnormal palm leaves were successfully digested with restriction enzyme (*EcoR*V) (Figure 3).

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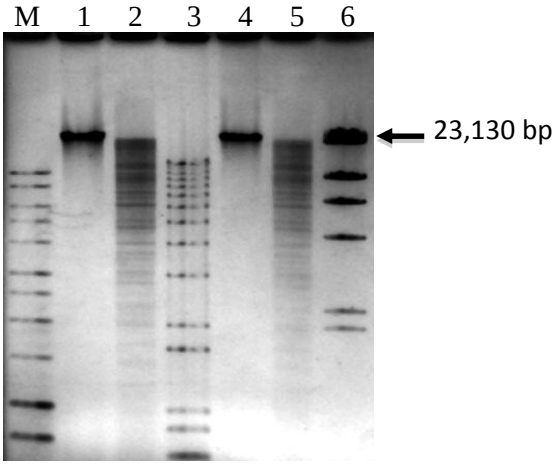

Figure 3. Restriction digestion pattern of mitochondrial DNA (0.75µg DNA) from normal and abnormal palms (200g fresh spear leaves) resolved on 0.9% agarose gel. Lane M: 1 kb DNA ladder; lane 1: undigested mitochondrial DNA (normal palms); lane 2: mtDNA (normal palms) digested with *EcoR*V (Promega); lane 3: 12 kb Plus DNA ladder; lane 4: undigested mitochondrial DNA (abnormal palms); lane 5: mtDNA (abnormal palms) digested with *EcoR*V (Promega); lane 6: λ *Hind*III DNA ladder.

Conclusions

MtDNA from normal and abnormal oil palm leaves were successfully isolated using the combined protocols of Binder and Grohmann (1996) and Mourad (1998). The isolated mitochondrion is currently being used to generate the organellar genome information in order to study the nuclear-mitochondrial genetic interactions.

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Effect of Plant Growth Regulators on Callus Induction of *Polygonum minus*

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Introduction

Polygonum minus also known as 'kesum' by the locals is well known for its health benefits. It has been used in traditional medicine for ages treating digestive and dandruff problems. Famous for its pharmacological and anti-inflammatory properties, this aromatic plant also lowers high cholesterol and blood pressure. Callus induction of *P. minus* is an important aspect to look into because with certain optimization of culture conditions, secondary metabolite production in callus culture can be achieved. Kesum is a potential source for secondary metabolite production, as it contains high levels of antioxidant properties (Suhailah et al., 2012).

Secondary plant products, also commonly known as secondary metabolites are probably important source of active pharmaceuticals serving as antibiotic, antifungal and antiviral treatments (Bourgaud et al., 2001). They are now widely used in pharmaceutical, cosmetic, and nutraceutical industries focusing valuable compounds. Today, 75% of the world's population relies on plants for traditional medicine. In the US, 25% of the pharmaceuticals used are based on plant-derived chemicals (Farnsworth, 1985: Payne et al., 1991; Ramachandra and Ravishankar, 2002) and over 60% of the anticancer drugs and 75% of drugs for infectious diseases currently used originated from natural sources (Newman et al., 2003; Cragg and Newman, 2009).

This high demand for secondary metabolites has put pressure in finding new sources. However, production of secondary metabolites through conventional method of planting and harvesting is not efficient as it requires huge land clearance, high cost and is labour intensive. In addition, usage of pesticides as some plants are very susceptible to pathogens is not environmental friendly and does not support green economy. Thus, as a preliminary work, callus induction of *P. minus* is an important aspect to look into as an alternative way of secondary metabolite production.

Materials and Methods

Previously established *in vitro* grown *P. minus* cultures from Ulu Yam population were used as a source of explants. Sucrose was added at the concentration of 30 g/l and 3.2 g/l gelrite was used as a solidifying agent in all the treatments. The pH of the medium was adjusted to 5.7-5.8 with 1 N HCL and 1 N NaOH before autoclaving at 121 °C for 20 minutes. *In vitro* grown leaves were excised at 1.0 cm x 1.5 cm each and transferred aseptically onto MS medium with different concentrations and combinations of plant growth regulators (PGR): (a) 2,4-dichlorophenoxyacetic acid acid (2,4-D) (1,2,3 mg/L) and 6- benzylaminopurine (BAP) (0.5, 1.0 mg/L); 2,4-D (1,2,3 mg/L) and kinetin (Kin) (0.1, 0.5 mg/L); and picloram (Pic) (1,2,3 mg/L) and Kin (0.1,0.5 mg/L). MS medium supplemented with 2mg/L of 2,4-D and 4mg/L α-naphthalene acetic acid (NAA) under dark condition (Faizan, 2011) was also tested for comparison of optimal treatment for callus induction. There were 10 samples for each treatment and each treatment was replicated 4 times. The cultures were incubated in a culture room of 16:8 photoperiod (irradiance of 22.26 μmol m^{-2} s^{-1}) and temperature 25 ± 1 °C. Observation was done weekly and the percentage of callus induction and callusing response were noted. Every two weeks, all samples were sub-cultured onto the same treatment medium. All data obtained were then analyzed with Statistical Analysis Software (SAS, USA) version 9.3. Duncan Multiple Range Test (DMRT) was used to compare the treatments tested.

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Results and Discussion

Plant growth regulators largely govern growth and morphogenesis of plant tissue under *in vitro* conditions. It was found that types and concentrations of PGR have a significant effect on the frequency of callus induction and callusing response. To study the combined effect of auxins and cytokinins on callus induction, two levels (0.1 and 0.5 mg/L) of Kin were tested in combination with 2,4-D and Pic at 1, 2 and 3 mg/L. BAP (0.5, 1.0 mg/L) was also tested in combination with 1, 2 and 3 mg/L 2,4-D.

Frequency of callus induction was observed based on the initiation of good quality calli, which were yellow to light yellow in color, dry and friable in appearance. Taking morphological criteria of the initiated calli into consideration, the highest frequency of callus induction, 72.5% was achieved on MS medium supplemented with 2mg/L and 3mg/L 2,4-D with 0.1mg/L Kin (Figure 1a). However, medium supplemented with 2mg/L 2,4-D in combination of 0.1mg/L Kin gave higher frequency of vigorous callusing (10.0%) compared to medium supplemented with 3mg/L 2,4-D and 0.1mg/L Kin (2.5%), although they were found not to be significantly different (Table 1). Higher frequency of vigorous callusing samples is important because callus induction with huge amount of callus initiation gives an indication of healthier and better proliferating callus line compared to samples, which induces little/small amount of callus.

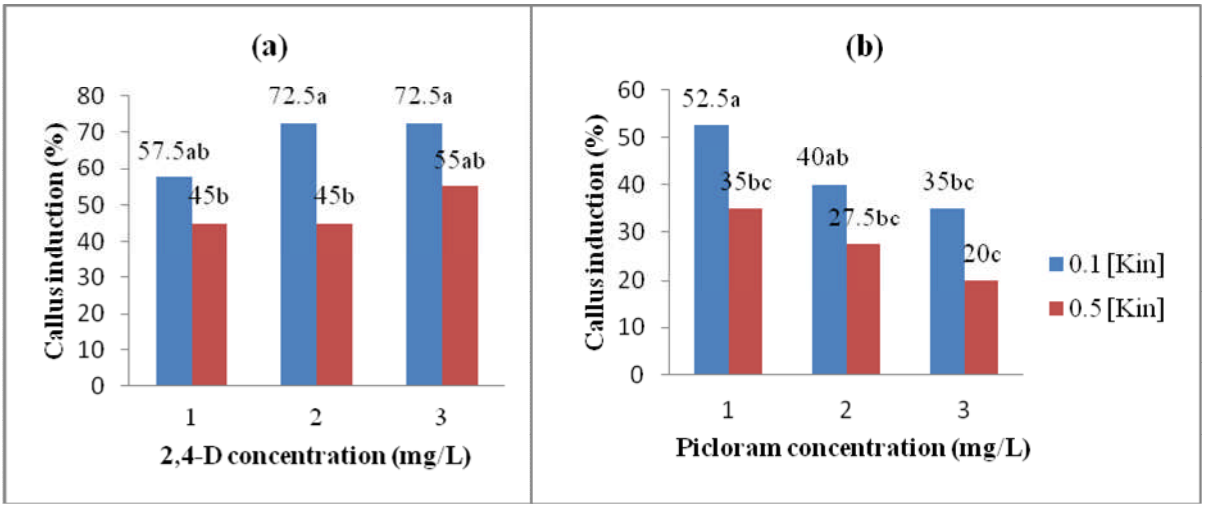

Figure 1. Effect of plant growth regulators (PGRs) on callus induction frequency (%) of kesum leaf segments after 10 weeks of culture: (a) 2,4-D and Kin (b) Pic and Kin. Means were calculated from four replications, with 10 leaf samples per replication. Means within the graph followed by the same letter are not significantly different using Duncan's multiple range test at the 0.05 level or less (≤ 0.05).

Within the PGR combination of Pic and Kin, the highest frequency of vigorous callusing response was observed on medium supplemented with 0.1mg/L Kin regardless of the concentration of Pic in combination (Table 2). All three concentrations (1, 2 and 3 mg/L) of Pic observed to give 7.5% of vigorous callusing rate although the highest frequency of callus induction was observed on medium supplemented with 1mg/L Pic and 0.1mg/L Kin (52.5%). However, when 1mg/L Pic supplemented in combination with 0.5mg/L Kin, the callus induction frequency was significantly different (35.0%) (Figure 1b). This could be due to high ratio of auxin to cytokinin (1:10) in 1mg/L Pic and 0.1mg/L Kin treatment, which favors induction of callus of compared to lower ratio of auxin to cytokinin (1:2) in 1mg/L Pic and 0.5mg/L Kin treatment. Rafique et al. (2011) also suggested that high auxin or cytokinin ratio is usually used for initiation of the embryogenic callus, while a low ratio is used for the regeneration of plantlets. PGR combination of 2,4-D and Kin was also reported in immature inflorescence culture

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of kodo millet (*Paspalum scrobiculatum)* where medium supplemented with lower and higher levels of Pic in combination with Kin was reported to develop organogenic callus with shoot buds and direct somatic embryo formation respectively (Kaur and Kothari, 2004).

Table 1. Effect of combination 2,4-D and kinetin on callusing response of callus derived from leaf segments of kesum after 10 weeks of culture.

[2,4-D] mg/L	[Kinetin] mg/L	Callusing Response		
		+++	++	+
1	0.1	5.0[a]	10.0[a]	42.5[ab]
1	0.5	2.5[a]	20.0[a]	22.5[b]
2	0.1	10.0[a]	10.0[a]	52.5[a]
2	0.5	0.0[a]	7.5[a]	37.5[ab]
3	0.1	2.5[a]	15.0[a]	55.0[a]
3	0.5	5.0[a]	10.0[a]	40.0[ab]

Callusing response of the explants inducing callus was expressed as follows:
+, slight callusing; ++, less callusing; +++, vigorous callusing.
Values are means of four replications, with 10 leaf samples per replication. Means within the column followed by the same letter are not significantly different using Duncan's multiple range test at the 0.05 level or less (≤ 0.05).

Table 2. Effect of combination picloram and kinetin on callusing response of callus derived from leaf segments of kesum after 10 weeks of culture.

[Picloram] mg/L	[Kinetin] mg/L	Callusing Response		
		+++	++	+
1	0.1	7.5[a]	10.0[ab]	35.0[a]
1	0.5	2.5[a]	2.5[b]	30.0[ab]
2	0.1	7.5[a]	17.5[a]	15.0[c]
2	0.5	0.0[a]	7.5[ab]	20.0[bc]
3	0.1	7.5[a]	15.0[a]	12.5[c]
3	0.5	0.0[a]	5.0[ab]	15.0[c]

Callusing response of the explants inducing callus was expressed as follows:
+, slight callusing; ++, less callusing; +++, vigorous callusing.
Values are means of four replications, with 10 leaf samples per replication. Means within the column followed by the same letter are not significantly different using Duncan's multiple range test at the 0.05 level or less (≤ 0.05).

After 10 weeks of culturing, explants on optimal medium reported by Faizan (2011) (2mg/L 2,4-D and 4mg/L NAA) under dark condition were only observed with early phase of callus induction activities. They had big, soft callus-like structure suggesting more time period required for formation of friable callus. Therefore, the medium and culture condition reported by Faizan (2011) is not favorable for callus induction since callus with desired morphology was not achieved in this medium compared to other treatments tested in this study (after 10 weeks).

Other than that, all treatments under combination of 2,4-D and BAP showed only preliminary callus induction activities, failing to produce callus as desired**.** Our observations on kesum callus induction of this PGR

combination is different compared to as been reported in *Acacia raddiana*, where addition of BAP in culture media already containing 2,4-D improved callogenesis rates (Sané et al., 2000; Sané et al., 2012).

Conclusions

In conclusion, an important factor for callus induction of kesum has been optimized throughout this study. This study has shown that combinations of different types and concentrations of auxins and cytokinins gave various callus induction frequencies and callusing responses in kesum. 2mg/l 2,4-D and 0.1mg/l Kin were found to give the highest callus induction frequency and callusing response with desired callus morphology thus, is reported as the best plant growth regulator combination and concentration for kesum callus induction from leaf segments. Further studies on optimization of other culture conditions are required, in order to establish a standardized protocol for callus induction and proliferation of *P. minus* in a shorter time period and also as an alternative way for secondary metabolite production.

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Antibody Optimization for the Development of Lateral Flow Strip for Cucumber Mosaic Virus Detection Using Surface Plasmon Resonance Technique

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Introduction

Cucumber mosaic virus (CMV), a recognised species under family Bromoviridae and genus Cucumovirus, is economically one of the most important viral pathogen of crop plants with worldwide distribution (Roossinck et al. 1999). CMV, transmitted by several species of aphid, is known to infect more than 1000 species of plants in 365 genera of 85 families (Gallitelli, 2000). This common plant virus caused yellow mottling, distorted leaves and stunted growth in a wide range of garden plants, not just cucumbers. CMV was also found to be the most common viruses infecting and damaging chili in Malaysia which results in the losses of yield ranges from 10%-15% if infection came in late and may reach up to 60% if plants were infected at early stage. As a result, production of chili is insufficient to meet the local consumption and consequently, an approximately RM30 million worth of chili is imported annually.

Several sensitive methods are available for the detection of CMV. Serological based tests, such as lateral flow immunoassay (LFA) can possibly be applied as a method for CMV detection in plants. LFA is a simple device used to detect the presence/absence of analytes including antigen and antibodies. This device is very sensitive, detecting viruses up to 90-97% accuracy and has been the method of choice for the detection and assay of various plant viruses because it is sufficiently sensitive for most applications (Salomone et al. 2004). This technology has several advantages over traditional immunoassays, such as its simplicity of procedure, rapid operation, quick results, low cost, and no requirements for special skills or expensive equipment.

As with other immunoassays, the sensitivity of an LFA is influenced by the affinity of the antibodies used in the test. In optimal physiological conditions, the affinity constant of an antibody has a value that cannot be improved upon and is ultimately responsible for the sensitivity of the test (Davies, 1994). The affinity constant can be affected by temperature, pH, and buffer constituents. As it is not usually possible to change the concentration of antigen, therefore, the optimal working concentration (dilution) of each individual antibody must be determined for each application and set of experimental conditions. Hence, to develop a sensitive LFA, the best available antibody and the best combination of materials should be utilized. Previously, we have successfully purified polyclonal antibodies from rabbit injected with CMV. Surface Plasmon Resonance (SPR) profiles for the purified antibody interaction between CMV infected leaves and healthy leaves revealed that the rabbit polyclonal antibody was able to detect the presence of CMV in the samples. Thus, the aim of this work was to report on the initial work to optimize the antibody concentration in the development of lateral flow strip for CMV detection via the SPR technique using gold chip sensor.

Materials and Methods

CMV Isolate

The same isolate of CMV was used throughout the experiments. The virus was propagated in tobacco plants, grown in a greenhouse at 20-25^{o}C. CMV was purified as previously described by Mossop et al. (1976). Virus concentration was estimated by measuring the absorbance with a spectrophotometer at 260nm.

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CMV Antibody

Male rabbit (3 months old) was immunized with purified CMV antigen. Blood samples were taken for antibody purification using a two step procedure involving ammonium sulphate precipitation and column chromatography using protein A. The purified antibodies used in this experiment were serially diluted in phosphate buffered saline (PBS).

Surface Plasmon Resonance Assay

Gold chip sensor was coated with mercapto-undecanoic acid overnight (11-MUA). Each chip was inserted into an Autolab ESPRIT SPR system. CMV coat protein 10µg/mL was directly flowed on the sensor surface which was already immobilized with different antibody concentrations (20, 15, 10 and 5µg/mL). Analyses were performed using the Autolab ESPRIT with pre-programmed parameters. Data were analyzed using Autolab ESPRIT Kinetic Evaluation version 5.0. Samples were analyzed in triplicates.

Results and Discussion

Compared to other technologies, SPR biosensors have several advantages such as real time monitoring, label free detection and small sample volumes (McDonnell, 2001). Until now, most studies using the SPR assay have detected antigens or pathogens such as *Cowpea mosaic virus* and *Tobacco mosaic virus* (Dubs et al. 1992). However, using the SPR system to detect antibodies has been rare except in studies of tumor antigens (Campagnolo, 2004). The SPR-based assay was used for the first time to determine the prime antibody concentrations against CMV in rabbit serum.

In the present paper, we used antigen to bind with antibody immobilized on the sensor chip. The binding of antigen to antibody leads to the formation of large couplers that could cause a large shift of the resonant wavelength. The sensitivity of the assay can be optimized with adjusting the concentration of the antigen and antibody preparations. Compared to other methods of sensitivity enhancement, this method is directed at optimizing the antibody and antigen preparations used as analytes rather than by optimizing the biosensor surface. This greatly simplifies the assay operation. From the four antibody concentrations tested, 20µg/mL gave the highest response (m^0) for the antigen-antibody binding signal. This was followed by concentrations of 15µg/mL, 10µg/mL and 5µg/mL (Figure 1).

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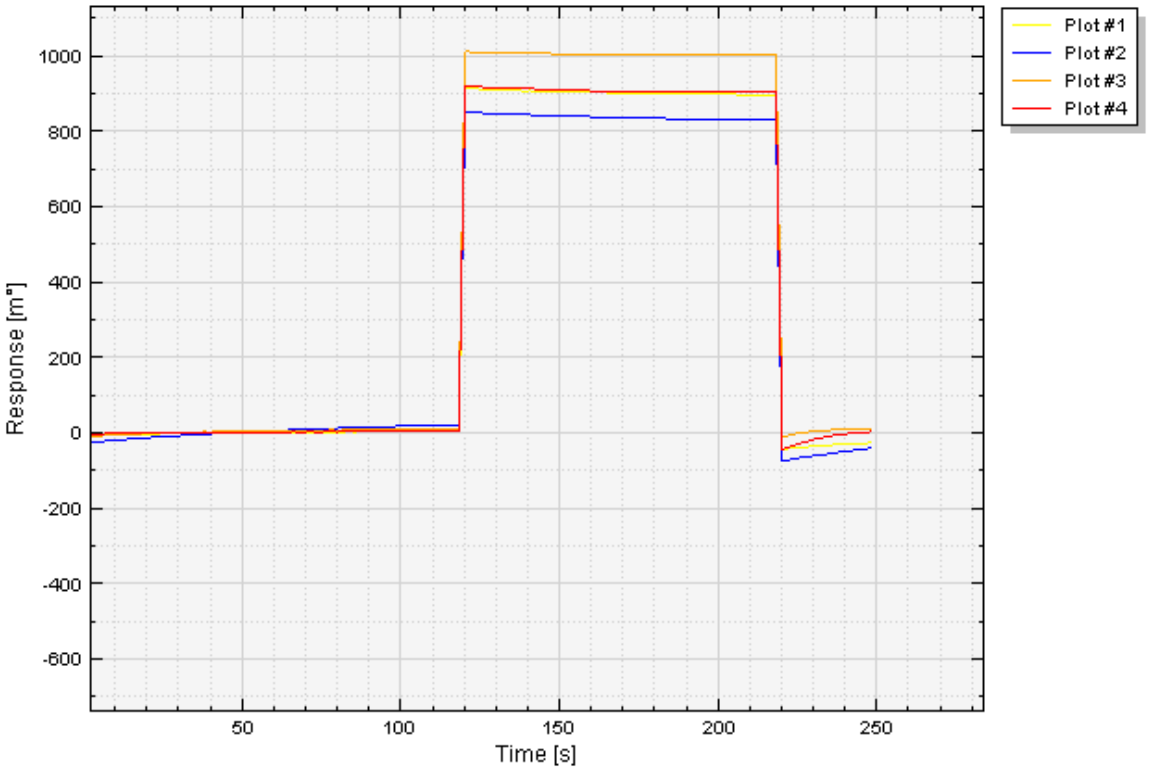

Plot#1: 10µg/mL
Plot#2: 5µg/mL
Plot#3: 20µg/mL
Plot#4: 15µg/mL

Figure 1. SPR profile for different antibody concentrations for the detection of between CMV.

The accurate determination of antibody-antigen binding activities is crucial for optimizing antibody based bioassays. These findings would be used as a basis to determine the initial antibody concentration for antibody titer determination using ELISA technique.

Conclusions

The results demonstrated the best concentration of CMV antibody was 20µg/mL. Similarly, the least optimum concentration of CMV antibody was 5µg/mL. At the same time, this indicates that the quality of CMV antibody is favourable as it is capable of giving antibody-antigen binding signal at very low concentrations. This can be said as an essential discovery as to develop a reliable plant pathogen diagnostic method requires high sensitivity and specificity of the antibody.

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Micropropagation and the Optimization for the Multiplication of Tissue Cultured Pineapple Plantlets

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Introduction

Pineapple *(Ananas comosus L.)* is one of the most economically important tropical fruits cultivated in most tropical and in some mild climate regions. The major challenge is that there is an acute shortage of pineapple planting material to meet the high demand from the market. Traditionally, pineapple is propagated via slips, suckers or crowns but the rate of production is slow and may not meet the market requirements. Thus, *in vitro* multiplication would be a useful alternative for mass production of this crop. *In vitro* micropropagation of pineapple plantlets has many advantages over conventional methods of vegetative propagation in which this technique allows an efficient and rapid increase of selected elite pineapple varieties.

This paper studies the use of plant hormone, carbon sources, organic additives and amino acids to increase the multiplication rate in pineapple tissue culture.

Materials and Methods

Plant Material

Pineapple suckers bought from Malaysian Agriculture and Research Institute (MARDI) were used as explants. Leaves were removed and the axillary buds were then excised into a cube of approximately 5 mm^3.The explants were put under running tap water for an hour and immersed in 95% (v/v) ethanol for a minute. Surface sterilization was carried out twice with 70% Clorox for 20 minutes and rinsed thrice with sterile distilled water. Sterilized explants were cultured and subcultured every month on solidified MS medium (Murashige and Skoog, 1962) supplemented with 5 mg/L BAP and 30 g/L sucrose to produce multiple shoots as stock plants.

Optimization for Multiplication

Healthy pineapple shoots from the stock explants with height around 2.5 to 3.5 cm were used. The shoots with their leaves trimmed were subcultured into the same MS media containing different cytokinins (BAP, KIN, TDZ and ZEA) at 5mg/L, carbon sources (sucrose, glucose, sorbitol, mannitol and galactose) at 30 g/L, organic additives (banana homogenate, pineapple homogenate and coconut water) at 3% (v/v) amino acids (glutamine, alanine, phenylalanine, asparagine and arginine) at 50 mg/L. The efficiency of the propagation was determined by the number of shoots and leaves produced after four weeks of culture.

Results and Discussion

Shoots Proliferation

The results obtained showed that BAP, ZEA and KIN at 5mg/L were able to promote shoot proliferation in which ZEA produced a slightly higher total number of shoots per culture (3) compared to BAP (2) and KIN (1) whereas TDZ proved to be ineffective. This result was in agreement with Danso et al. (2008) whereby the proliferation of

MD-2 plantlets produced significantly more pineapple plantlets in a low concentration of BAP (5.0 mg/L) liquid than in solid media (7.5 mg/L). It is important that there is no significant difference between the effect of BAP and ZEA since Zeatin is an expensive cytokinin and relatively unstable. From the different carbon sources used, the control (household sugar, sucrose) gave the same proliferation rate (2.67 shoots per culture) as laboratory grade glucose and galactose while mannitol showed adverse effect where the leaves turned pale yellow. Several researchers reported that various organic additives have been used in plant tissue culture to promote the growth of plants including coconut water, banana pulp, potato homogenate and juice, honey, date palm syrup, corn extract, papaya extract and also beef extract (Islam et al., 2003; Murdad et al., 2010). The finding of this study indicated that all the organic additives used (banana homogenate, pineapple homogenate and coconut water) were not effective in stimulating the shoot formation though the highest number of shoot showed in coconut water treatment. This result was in agreement with Nasib et al. (2008) which stated that coconut water was found to be able to increase the number of shoots, length and nodes of kiwi fruit. Further result showed that shoot proliferation was induced by the addition of arginine (3) and phenylalanine (2) at 50mg/L and this stimulation may be attributed to the role of these amino acids as exogenous nitrogen sources to the growth of the plantlets.

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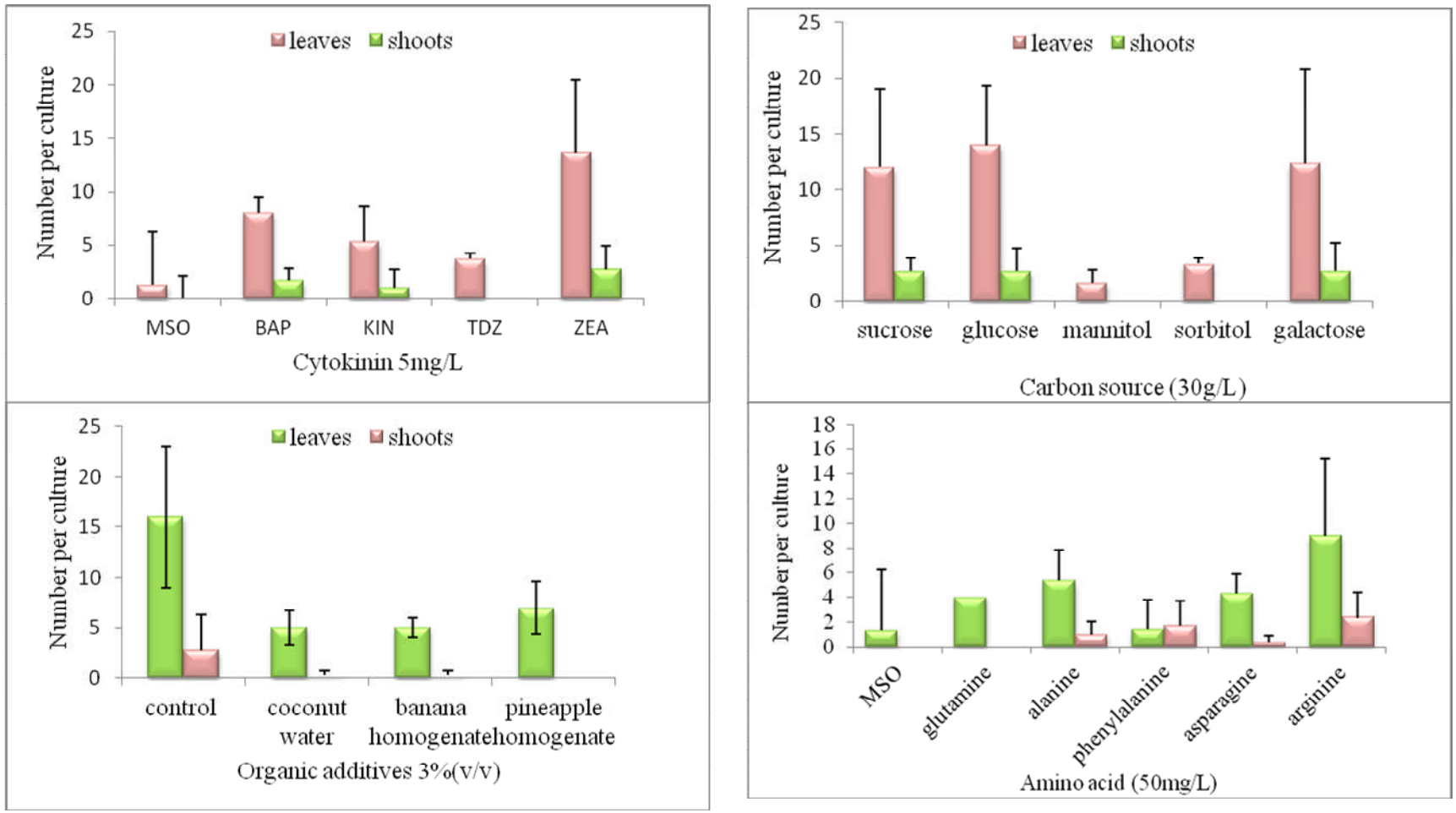

Figure 1. Proliferation of Maspine pineapple after four weeks of culture on solidified MS medium supplemented with different cytokinins, carbon sources, amino acids and organic additives. Values are mean ± standard deviation based on three replicates

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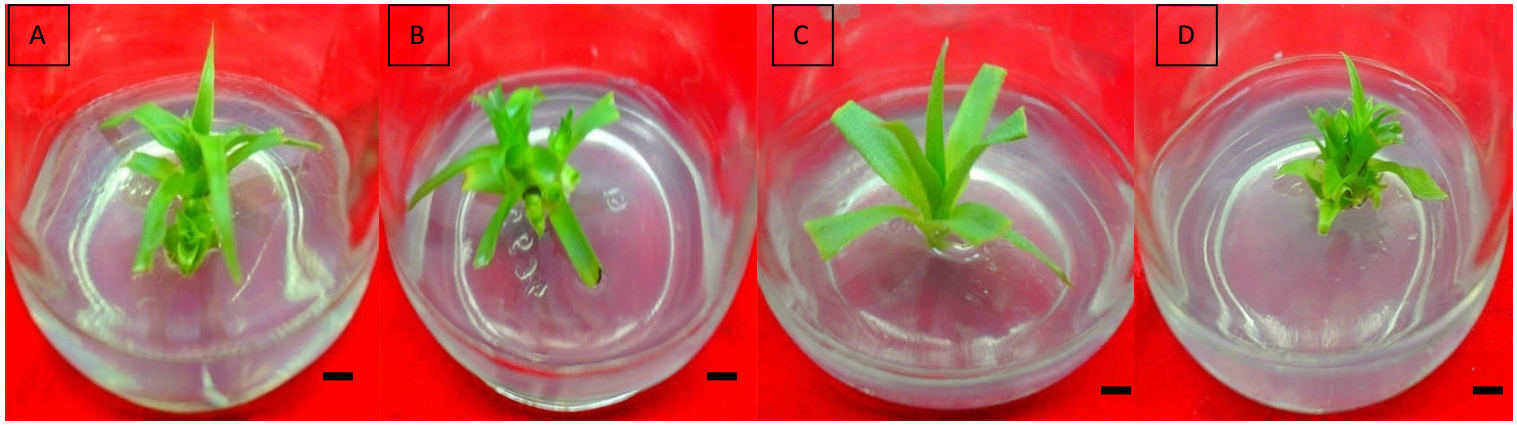

Figure 2. Maspine pineapple morphology after four weeks of culture on solidified MS medium supplemented with (A) BAP (control) (B) Kinetin (C) TDZ and (D) ZEA at 5 mg/L (Bar = 1 cm)

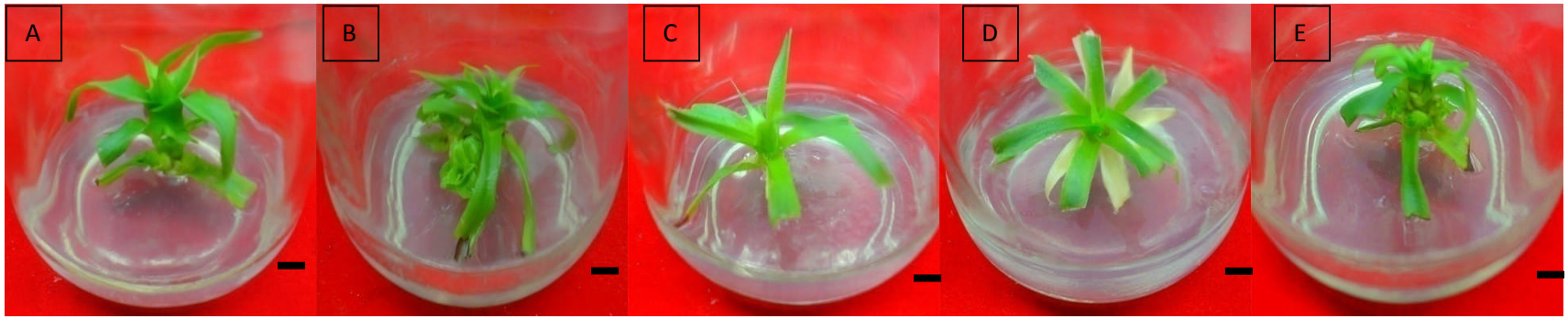

Figure 3. Maspine pineapple morphology after four weeks of culture on solidified medium supplemented with (A) sucrose (control) (B) glucose (C) sorbitol (D) mannitol and (E) galactose at 30 g/L(Bar = 1 cm)

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Conclusions

The results obtained for the shoots proliferation of Maspine pineapple showed that the best cytokinin was Zeatin but considering the cost, the usage of BAP is acceptable. The suitable carbon sources were found to be sucrose, glucose and galactose, and it is suggested that the use of sucrose is more practical because glucose and galactose are more expensive. Arginine and coconut water can be added into the medium to stimulate the proliferation of pineapple culture.

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Discovery of Single Nucleotide Polymorphism (SNP) Markers in Papaya for Improvement of Economically Trait

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Introduction

Papaya *(Carica papaya L*.) belongs to *Caricacea* family and has a genome size of 372 Mbp with nine pairs of chromosome (Paull et al., 2010). In Malaysia, papaya is among the major fruit crop to be exported. Hence a greater understanding and finding to improve papaya desirable traits is needed to increase the production of papaya with high quality. The genomic studies on papaya have started in last few years with the first draft genome of papaya was sequenced on 2007 (Wei and Wing 2008). Prior to this study, no large scale single nucleotide polymorphism (SNP) database has yet been made available for this important crop, limiting the use of genotyping with these preferred markers.

Molecular markers are pieces of DNA that are known to be located near genes and inherited traits of interest (Duran et al., 2009). Advancement in high throughput sequencing and genotyping technology has made SNP as a preferred marker and important tool for marker trait of interest (Brautigum and Gowik 2010). Although SNPs are less polymorphic than Simple Sequence Repeat (SSR) due to bi-allelic nature but SNPs are amenable to high throughput automation using current genotyping technology platform. In modern agriculture, SNP has become a predominate marker for marker development of important traits, genetic diversity analysis, fingerprinting, cultivar identification and association with agronomic traits (Ahmad et al., 2011).

A good papaya cultivar is represented by certain traits such as sweetness, softness, fruit ripening and also resistance to disease. In this study, we conducted genome re-sequencing of three papaya varieties with the aim to discover 800 SNPs within desirable traits for papaya marker development. We reported on the discovery of a SNP from three papaya varieties (Solo, Eksotika 1 and Sekaki) using Illumina sequencing technology and bioinformatics. High throughput sequencing and bioinformatics has been a powerful tool to accelerate the functional genomics analysis and SNP marker development. Bioinformatics analysis of the sequencing data and characterization of SNPs will involve sequence mapping, SNPs discovery and SNPs selection.

Materials and Methods

Plant Materials and Growth Condition

Three papaya varieties (Solo, Eksotika 1 and Sekaki (ACC)) were planted at Horticulture Research Centre glass house. Leaves were harvested from 4 months old papaya seedlings and stored at -80°C until further use. DNA extracted from the papaya leaves were visualized by 0.8% agarose gel. Analysis of the DNA quality and quantity were also carried out using nanodrop spectrophotometer.

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Bioinformatic Analysis

About 51 000 papaya scaffolds are publicly available and were downloaded from *C.papaya* plant genome database (CpGDB) (http://www.plantgdb.org/CpGDB/) released on March 2011. The papaya scaffolds has generated four chromosomes of papaya and were used as a reference sequence to mapping against papaya raw reads.

Using bioinformatics approach, we have designed a SNP discovery workflow for high throughput SNP discovery from papaya genome re-sequencing (Figure 1). This workflow allows the large detection of SNPs by comparing with a reference sequence from the same or a different species. This pipeline is based on public software (BWA, SAMTOOLS, FASTX Toolkits and custom made perl script. All these tools have different functions in order to discover the SNP.

The quality of papaya raw reads was checked and trimmed using FASTQC and FASTX toolkits. The papaya raw reads were then aligned against the reference sequences using BWA, an aligner tool. SNPs were detected using SAMTools mpileup. The identified SNPs with flanking region were done using custom made perl script. The SNP selections were then performed to select useful SNPs marker for papaya marker development. SNPs were then specifically selected based on these six criteria namely depth alignment, phred-like score (Q> 100), SNP present in reads (DP > 10), excluded redundant SNPs position among three varieties, SNPs with BLAST result and excluded if two or more SNPs around 150bp length.

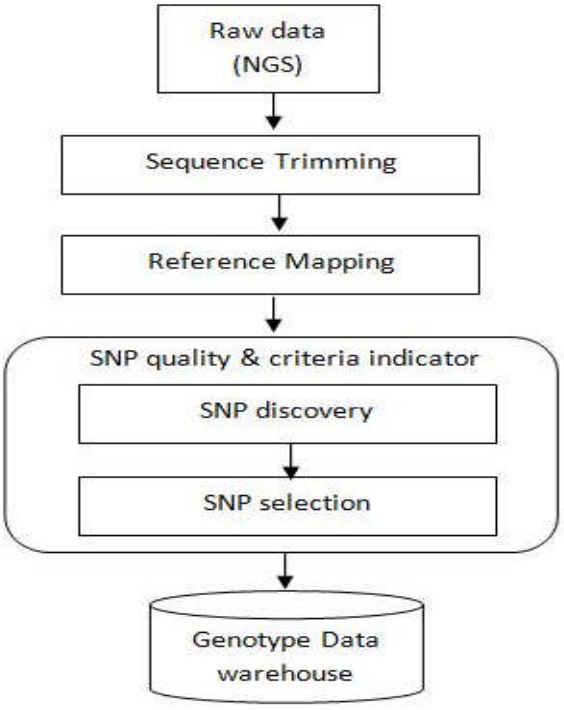

Figure 1. SNP discovery workflow of papaya genome re-sequencing

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Results and Discussion

Intact genomic DNA was obtained with the concentrations of DNAs between 500 to 2000 ng/µl with the ratio of A_{260}/A_{280} at 1.7 to 1.9. Three genomic libraries from these papaya varieties were sequenced in paired end reads (forward and reverse) using Illumina HiSeq sequencing technology. A total of 233,731,438 with 101 pair end read length has been generated from Solo, Eksotika1 and Sekaki. The quality of raw Illumina reads was examined and trimmed to ensure the good quality reads has produced by the sequencer provider. A sufficient sequencing coverage of papaya is important in order to obtain useful putative SNP discovery. The insert size and sequencing coverage parameters were 641bp and 36X for Solo, 590bp and 44X for Eksotika 1 while 603bp and 48X for Sekaki. Based on the sequencing coverage it is predicted to provide better mapping results when aligned against papaya reference sequences.

SNP discovery is a process of aligning homologous sequences from a variety of crops or breed. Scanning of the aligned sequences was carried out to locate bases that do not match the consensus of the alignment to produce a list of putative SNP markers. As the papaya scaffold and papaya genome annotation are publicly available, this has been advantagous to the researchers in order to predict whether a SNP falls within or near a gene of interest. It can be very useful in determining whether a particular SNP is likely to be responsible for a phenotype of interest.

Illumina paired-end short reads from Solo, Eksotika1 and Sekaki were aligned separately against the papaya scaffolds using Burrows-Wheeler Aligner (BWA) tool. SNP detection was performed using SAMTOOLS mpileup at default settings appropriate for diploid organism. We have set up the criteria for SNP selection in order to avoid SNPs that are false positives. This effort has identified a large quantity of SNPs in Solo, followed by Sekaki and Eksotika 1.

Comparison of these variation data has defined potential SNPs marker to be highly distributed in Solo compared to Sekaki and Eksotika 1. The different numbers of SNP between Solo and its progeny, Eksotika 1 and Sekaki is also quite high. These differences probably arise due to the high conservation of Solo against papaya reference sequences. The results also showed Sekaki has scored the highest number of reads that are successful aligned against the papaya reference sequences. This is due to the high sequencing coverage of Sekaki which is, 48x compared to Solo and Eksotika 1. A statistic summary of papaya mapping and SNP discovery is shown in Table 1. The non redundant and redundant SNP positions among Solo, Eksotika 1 and Sekaki was performed and showed on Venn diagram. The highest redundant on SNPs position was found to be between Sekaki and Solo (Figure 2).

Table 1. Papaya sequence mapping and SNP discovery statistics

Variety	Solo	Eksotika1	Sekaki
Total Reads	59,300,253	56,536, 110	60,568,735
Sequencing Coverage	46	44	48
Total Mapped Reads (%)	79.47	77.17	81.49
Total unmapped reads	12,175,884	12,904,529	11,213,712
Total SNP discovered	869,847	186,476	261,172
Total SNP filtered (based on criteria 1-4)	513,278	482	92,478

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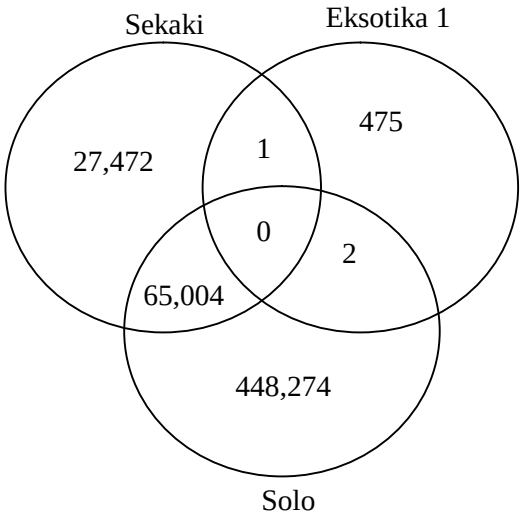

Figure 2. Overlap of papaya SNPs

Conclusions

The Illumina Hi-Seq 2000 sequencing technology has produced high quality sequencing coverage of three papaya varieties and represented high quality putative SNP. We report here discovery of potential papaya SNP, which can provide additional clues to the molecular basis of the remarkable traits in papaya with economic importance. The predicted SNPs could be located in candidate genes responsible for economically important traits in papaya. This can be achieved through the annotation with papaya gene information. Further SNP selection will be carried out by following on the other two criteria, namely SNPs with BLAST result and excluded if two or more SNPs around 150bp length, in order to obtain 800 potential SNPs marker. This will permit the development of physical mapping of papaya and papaya SNP linkage to functional genes for marking Quantitative Trait Loci (QTL) to be applied in papaya trait improvement.

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Development of *Arabidopsis thaliana* Lines Containing 2-Propenyl and 3-Butenyl Glucosinolates for Biological Testing

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Introduction

Glucosinolates, naturally occurring β-thioglucosides present in *Arabidopsis thaliana*, have been the subject of extensive studies in relation to understanding the role of these compounds in plant defence. When tissue damage occurs through the feeding behaviour of insects or by infection with a microbial pathogen, the integrity of the glucosinolate-myrosinase system breaks down and hydrolysis of glucosinolates takes place giving rise to a number of products depending on the presence or absence of certain proteins that work in conjunction with myrosinase (Kissen et al., 2009). A very important determinant of biological activity of the degradation products is the structure of the glucosinolate side chain. The structure of the side chain is highly variable and is often controlled by a set of four genetic loci (Parkin et al., 1994; Giamoustaris and Mithen, 1996; Kliebenstein et al., 2001a). In *A. thaliana*, these loci are *GSL-Elong*, *GSL-OX*, *GSL-ALK*, and *GSL-OH*. The *GSL-Elong* locus encodes a set of methylthioalkylmalate synthases (MAM) that control the variation in carbon chain length of the glucosinolate structure (Benderoth et al., 2006) leading to accumulation of three-carbon or four-carbon long glucosinolates (Mithen et al., 1995; Kliebenstein et al., 2001b; Kroymann et al., 2001). *GSL-OX* is controlled by a family of flavin monoxygenases that oxidize methylthioalkyl glucosinolates to their corresponding methylsulphinylalkyl derivatives (Hansen et al., 2007). The *GSL-ALK* locus controls the type and amount of glucosinolate biosynthesised depending upon the expression of two genes, *AOP2* and *AOP3*. If *AOP2* is expressed, the plant accumulates alkenyl glucosinolate from the methylsulphinylalkyl precursor glucosinolate, while *AOP3* determines the production of hydroxyalkyl glucosinolates (Kliebenstein et al., 2001b). The final member is the *GSL-OH* locus, which controls the oxidation of 3-butenyl glucosinolate to 2-hydroxybut-3-enyl glucosinolate (Hansen et al., 2008).

Plants with different glucosinolate hydrolysis product profiles show different biological activity further advancing our knowledge of the biochemistry of these compounds. For example, a major glucosinolate breakdown product, 4-methylsulfinylbutyl isothiocyanate found in *A. thaliana* Col-0, displays *in vitro* toxicity to assorted fungi and bacteria (Tierens et al., 2001). *In vivo* experiments with *A. thaliana gsm-1*, which lacks or has reduced amounts of aliphatic glucosinolates showed that the biotroph fungal pathogen *Psedumonas syringae* pv tomato DC3000 was unaffected *in vivo* by 4-methylsulfinylbutyl-isothiocyanate (Tierens et al., 2001). One study showed that the alkenyl glucosinolates are more deterrent than the non-alkenyl glucosinolates, with the 3 carbon 2-propenyl glucosinolate being more toxic than the 4 carbon 3-butenyl glucosinolate (Kliebenstein, 2004). Further, the alkenyl glucosinolate hydrolysis product, 2-propenyl isothiocyanate (2-propenyl-isothiocyanate) has been shown to be the most toxic of all glucosinolates tested against *Leptosphaeria maculans* (Mithen et al., 1986).

Metabolic engineering of secondary metabolites has been reported as the key goal of many research programs, but in most cases, is subject to trial and error (Morant et al., 2007). The selection of promoters that control transgene expression is crucial, depending on the species. In some plant species, copies of the same promoter may be introduced without any undesirable effects, while in other species this may be the opposite. Hence, crossing experiments were initiated to offer an alternative in developing *A. thaliana* lines containing alkenyl glucosinolate for biological experiments. This paper presents the development of *A. thaliana* plants with alkenyl glucosinolate

profiles in a Col-0 background. *Arabidopsis thaliana* Col-0 contains a 5-bp deletion in its AOP2 cDNA and genomic sequence generating frameshift mutation which resulted in a truncated protein and accumulated methylsulphinylalkyl glucosinolates. It was hypothesised that the introduction of *GS-ALK/AOP2* gene in Col-0 would result in AOP2 regaining functionality and the conversion of the precursor methylsulphinylalkyl glucosinolate to the alkenyl glucosinolate will be evident. Thus, by crossing a *A. thaliana* accession containing alkenyl glucosinolates with Col-0, it is possible to switch the plant from a methylsulphinylalkyl glucosinolate producer to one with alkenyl glucosinolates.

Materials and Methods

Development of the Crosses

The inflorescences of *A. thaliana* plants were selected and flowers which were too young were removed. The flowers of recipient plants were left to mature. Pollen from male plants was obtained by removing the stamen after which the stamen was used to brush the prepared stigma of the matured recipient flowers. The crosses were label accordingly and left to develop. The siliques were collected and sown to get new lines of plants for the next cross. All crosses were developed in the Col-0 background.

Selection by Glucosinolates Degradation Profile

The leaf tissues of *Arabidopsis* plants were crushed and extracted with dichloromethane (DCM). The extracts were spun for 5 min and the supernatant (DCM) was dried with sodium sulphate (anhydrous) and re-centrifuged for 2 min. The DCM extracts were concentrated to 200 µl and analysed on a GC–MS (Hewlett Packard 6890 GC linked to a 5973 MSD). Separation was carried out on a HP-5MS 5% Phenylmethylsiloxane (30 m × 250 mm) column in the split mode (20 : 1) injection temperature = 225 °C, column temperature = 50 °C for 5 min, 5 °C min^{-1} to 180 °C for 26 min and 10 °C min^{-1} to 280 °C for 20 min. All compounds were identified by their mass fragmentation patterns.

Plants Inoculation

Arabidopsis thaliana challenged with Pseudomonas syringae DC3000

Plants were grown for 5-6 weeks before inoculation. A cell suspension of *Pseudomonas syringae* DC3000 was prepared in 10 mM $MgCl_2$, adjusting the OD_{600} to 0.002 absorbance units. The bacterial suspension was inoculated using needleless syringes into small incisions on the adaxial epidermis of expanded leaves. Leaf disc samples from inoculated leaves were excised at the 3 day time point post-inoculation for observation of bacterial growth.

Arabidopsis thaliana challenged with Botrytis cinerea

The leaves from *A. thaliana* plant were placed into plastic humidity boxes. A final spore suspension of 2 x 10^5 spores/ml in 1/8th strength PDB was used for the inoculation. Two sets of four leaves each were inoculated and symptom development after 3 days inoculation was assessed by visual microscopy using stereomicroscope (Leica M80).

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Results and Discussion

Generation of A. thaliana Plants Expressing AOP2 with Alkenyl Glucosinolate Profile on Col-0 Background

Arabidopsis thaliana Col-0 and Ru-0 accessions which are isothiocyanate producing lines accumulate different glucosinolate hydrolysis products that vary in their side chain length and side chain structure, verifying the populations. Col-0 contained predominantly 4-methylsulfinylbutyl isothiocyanate and trace levels of 3-methylsulphinylpropyl isothiocyanate (Figure 1). Ru-0 accumulates 2-propenyl isothiocyanate (Figure 1). Accumulation of alkenyl glucosinolate hydrolysis product is a strong indication of the presence of a functional *AOP2* gene in the plant. Hence, this biochemical phenotypic selection method was used throughout the study to segregate between the phenotypes of the plants.

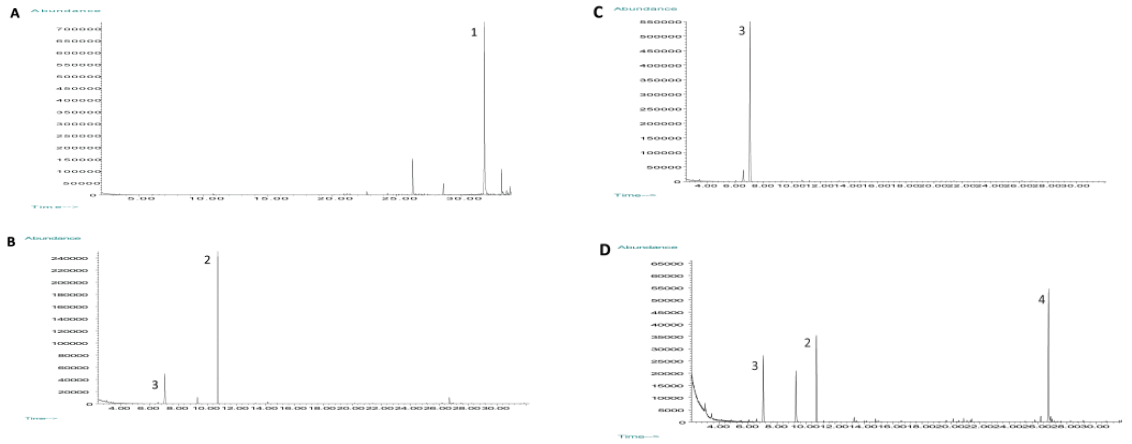

Figure 1. Representative GC chromatogram of volatiles released by endogenous myrosinase hydrolysing in fresh leaves of *A. thaliana* plants (A) Col-0 contains 4-methylsulfinylbutyl-isothiocyanate (peak 1); progenies expressing AOP2 with (B) 3-butenyl-isothiocyanate (peak 2), (C) progenies with 2-propenyl-isothiocyanate (peak 3) and (D) progenies with profile that contain a mix of 3-butenyl-isothiocyanate (peak 2), 2-propenyl-isothiocyanate (peak 3) and goitrin (peak 4).

Table 1. Mass spectral data of glucosinolate hydrolysis products

Hydrolysis product	*Retention time (min)*	*MS spectral data*
4-methylsulfinylbutyl isothiocyanate	31.80	163, 160, 72, 55
3-butenyl isothiocyanate	10.70	113(M^+), 85, 72, 55
2-propenyl isothiocyanate	5.0	99 (M^+), 72
Goitrin	28.0	129 (M^+),85, 68

Combination of functional and null alleles at GS-ELONG, GS-ALK and GS-OH explains the variation in glucosinolate profile of *A. thaliana* plants. The presence of following genes: *MAM1* and *MAM2* (*GSL-ELONG*) controls side chain elongation (Magrath et al., 1994); *AOP2* (GS-ALK) mediates the conversion of methylsulphinylalkyl to alkenyl glucosinolates (Mithen et al., 1995; Kliebenstein et al., 2001b) and GSL-OH regulates the hydroxylation of butenyl glucosinolate (Hansen et al., 2008).

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Arabidopsis thaliana Line Containing 2-Propenyl Glucosinolate and Pathogen (P. Syringae DC3000) and Fungal (B. cinerea) Interaction

After infiltration with a DC3000 bacterial inocula, the parent, Col-0 (lacking *AOP2*) which accumulates predominantly 4-methylsulfinylbutyl glucosinolates developed chlorotic and necrotic lesions on the leaves at 3 days after inoculation, meanwhile a small reduction in bacterial growth was observed in *A. thaliana* progenies containing 2-propenyl glucosinolates (Figure 2). For fungus infection, leaves of *A. thaliana* Col-0 (which lack *AOP2*) showed coalescing lesions within the inoculum droplet at 3 days after inoculation of *B. cinerea* (Figure 3) meanwhile leaves of *A. thaliana* progenies containing 2-propenyl glucosinolates showed intermediate size lesions as compared to Col-0. These observations suggest that 2-propenyl glucosinolates in *A. thaliana* progenies might restrict *P. syringae* and *B. cinerea* growth.

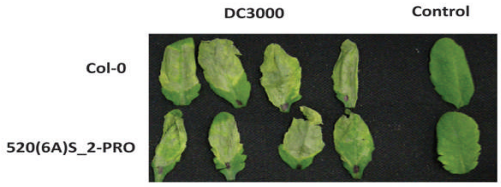

Figure 2. Alkenyl glucosinolate and pathogen interaction**.** Disease symptoms of *A. thaliana* leaves caused by DC3000 in Col-0 parent and 520(6A)S_2-PRO. *520(6A)S_2-PRO: Progenies containing 2-propenyl glucosinolates.

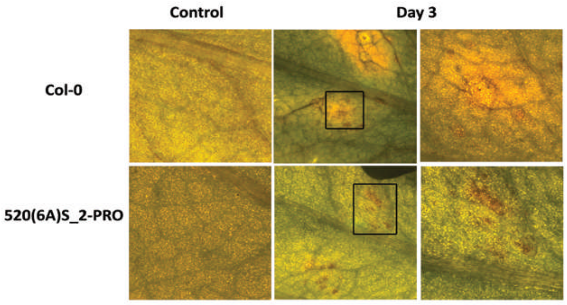

Figure 3. Resistance to *B. cinerea* in *A. thaliana* plants. Lesion development in *A. thaliana* accession Col-0 parent and 520(6A)S_2-PRO inoculated with *B. cinerea*. *520(6A)S_2-PRO: Progenies containing 2-propenyl glucosinolates.

We have revealed in our study that 2-propenyl-isothiocyanate seems to be more toxic to pathogen and fungus than the non-alkenyl 4-methylsulfinylbutyl-isothiocyanate (Figures 2 and 3). Previous studies reported that glucosinolates with the alkenyl side chains were more of a deterrent than non-alkenyl glucosinolates (Mithen et al., 1986; Kliebenstein, 2004). This suggests introduced functional GS-ALK AOP2 to Col-0 via natural selection produced *A. thaliana* plants with elevated level of toxicity.

Conclusions

Our strategy to produce *A. thaliana* lines with alkenyl glucosinolates with Col-0 background appears to be successful. These alkenyl isothiocyanate producing lines could offer the researcher a useful tool to study the relevance of specific alkenyl glucosinolates and their role in natural enemy attraction/deterrent.

This study presents that modifying the leaf glucosinolate content of *A. thaliana* by the crossing method influences pathogen/fungal interactions. By exploiting *A. thaliana* line with a different alkenyl glucosinolate profiles with the same (Col-0) background, we would also be able to study the effect of alkenyl isothiocyanates on the palatability of vegetative tissue and the significance in influencing the crop pest behaviour. Nevertheless, the newly generated *A. thaliana* lines can also be utilized for animal and human studies.

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Degradome Analysis of Phenylpropanoid Biosynthesis Pathway in *Polygonum minus*

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Introduction

To date, more than 60% of drugs produced are based on the active substances originated from plants (Newman and Cragg, 2012). The secondary metabolites of plants such as phenolic compounds are long known to promote better health. Thousands of different secondary metabolites can be classified into three major groups namely phenylpropanoids and their derivatives such as flavonoids, tannins, glycosides and lignins, terpenes and nitrogen-containing compounds like alkaloids (Korkina, 2007). Only a small portion of plants in the world have been studied and there is still a huge potential in the discovery of new beneficial active compounds.

Polygonum minus or kesum from Polygonaceae family is an aromatic plant which has been recognised by the Malaysian government in the Herbal Product Blueprint as an essential oil-producing crop (Wan Hassan, 2006). Apart from that, *P. minus* is shown to have antioxidant, antimicrobial and anticancer properties (Nanasombat and Teckchuen, 2009; Mackeen, et al., 1997; Nornisah et al., 2005). All the attractive medicinal and commercial values of *P. minus* justify it as a good model plant for further research. In this study, we are particularly interested in the regulation of the phenylpropanoid biosynthesis pathway as it comprises a complex series of branching biochemical reactions with many valuable plant secondary metabolites as the end-products.

Many of plant-derived phenolic compounds such as flavonoids and lignins are the secondary products of phenylpropanoid (PP) metabolism (Douglas, 1996). Secondary metabolites are produced to protect the sessile plants in surviving abiotic and biotic stresses. All these compounds are found to be beneficial to humans. Many plant responses trigger the activation of PP metabolism where the branch pathways lead to the synthesis of compounds having protective functions such as antimicrobial activity (Cos et al., 2002). Plant-derived PP and their derivatives are commonly used in food, perfume, wine and essential oil industries as well as contributing significantly to the medicinal aspect such as antioxidants, anticancer and wound healing agents (Dembitsky, 2005). Chemical synthesis of PP is expensive, thus, it is proposed that the yield in plants is increased through genetic engineering. Nowadays, small RNAs (sRNAs) are known to play an important role in post-transcriptional and translational regulation of gene expression (Khraiwesh et al., 2010). Therefore, this study is conducted with the aim of understanding how the sRNAs are involved in the regulation of biochemical pathways especially on phenylpropanoid biosynthesis pathway. With this knowledge, it is possible to manipulate the pathway to either increase the production of valuable metabolites or reduce the unwanted products such as lignin. For instance, low lignin content is desirable in pulp industry as it increases pulp yield and reduces processing cost which is more environmentally friendly and aligned with the green economy trend worldwide (Hu et al., 1999).

sRNAs have been shown to be involved in the regulation of gene expression in many cellular processes such as development, adaptive responses towards non-favourable environment stresses in most eukaryotes (Zhou et al., 2007). sRNAs also known as non-coding RNAs are usually made up of ~22 nucleotides in length, typically divided into two major classes which are microRNAs (miRNAs) and small interfering RNAs (siRNAs). In our study, we focused more on miRNAs. In plants, miRNAs regulate gene expression post-transcriptionally by direct cleavage or just repression of the translation of their target mRNAs (Bartel, 2004). Since *P. minus* showed good adaptation ability to adverse environment, thus it is hypothesised that sRNAs are involved in the regulation of secondary metabolite production in response to stress.

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In this study, degradome sequencing was carried out to study the trend of sRNA regulatory system in phenylpropanoid pathway. Since there is not much genomic information regarding *P. minus* in the database, degradome sequencing is one of the best option to detect cleaved sRNA targets without relying much on the predictions or overexpression (Addo-Quaye et al., 2008).

Materials and methods

Plant Materials

P. minus Huds plants originated from Ulu Yam were harvested from the plot in Universiti Kebangsaan Malaysia (UKM). The sample was washed thoroughly with distilled water, ground in liquid nitrogen and directly used for RNA isolation. The remaining sample was stored at -80 °C until use.

Total RNA and Poly(A) RNA Isolation

Conventional RNA extraction method was used with some modifications to remove high amounts of phenolic and carbohydrate compounds in *P. minus* plant sample (López-Gómez and Gómez-Lim, 1992). About 800 ng of total RNA was used for Poly(A) RNA isolation using PolyATtract mRNA isolation kit (Promega, USA). RNA integrity was assessed via gel electrophoresis and was then quantified using Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The RNA was stored at -80 ˚C until use.

Degradome Library Construction

The degradome library was constructed as previously described with some modifications (German et. al. 2009). Briefly, 5’ RNA adapter (5’ – GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA – 3’) from RLM-RACE kit (Ambion) was ligated to the cleavage products which have their 5’ monophosphate and poly(A) tail, omitting the enzymatic treatments. The ligated products were then reverse-transcribed using the oligo dT provided in the kit (5’ –GCGAGCACAGAATTAATACGACTCACTATAGGT12VN – 3’) with the suggested condition. PCR was carried out using 5’ RACE outer primer (5’ – GCTGATGGCGATGAATGAACACTG – 3’) and 3’ RACE outer primer (5’ – GCGAGCACAGAATTAATACGACT – 3’) included in the kit as forward and reverse primers with SuperTaq Plus DNA polymerase (Ambion, USA). The amplification was done for 35 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 3 min). The PCR products were checked for size by running on 1% agarose gel. The size range was about 300 to 2000 bp. The purified PCR products were subjected to 454 sequencing by Roche GS FLX sequencer at Malaysia Genome Institute (MGI).

Analyses of Degradome Data

The sequence output was trimmed using Seqclean and assembled using iAssembler pipeline with settings for 454 sequencing dataset. The assembled sequences were mapped with Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database.

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Results and Discussion

Eight enzymes (different colour-coded) involved in phenylpropanoid biosynthesis pathway were successfully mapped with the degradome sequences obtained (Figure 1). They are caffeate methyltransferase (EC 2.1.1.68), cinnamoyl-CoA reductase (EC 1.2.1.44), cinnamate hydroxylase (EC 1.14.13.11), 4-coumarate CoA ligase (EC 6.2.1.12), shikimate hydroxyl cinnamoyl transferase (EC 2.3.1.133), cinnamoyl-alcohol dehydrogenase (EC 1.1.1.195), peroxidase (EC 1.11.1.7) and caffeoyl-CoA methyltransferase (EC 2.1.1.104). The transcripts of these genes are likely to be cleaved by sRNAs. sRNAs serve as an elegant plant regulatory system where they can regulate the expression of a few genes simultaneously, which is more rapid and efficient compared to individual gene regulation. This is important especially for plants to adapt to the changing environment. Further work will be carried out to identify the possible sRNAs targeting the genes above. The sRNAs involved are able to be manipulated in future to increase the yield of desired compounds or vice versa.

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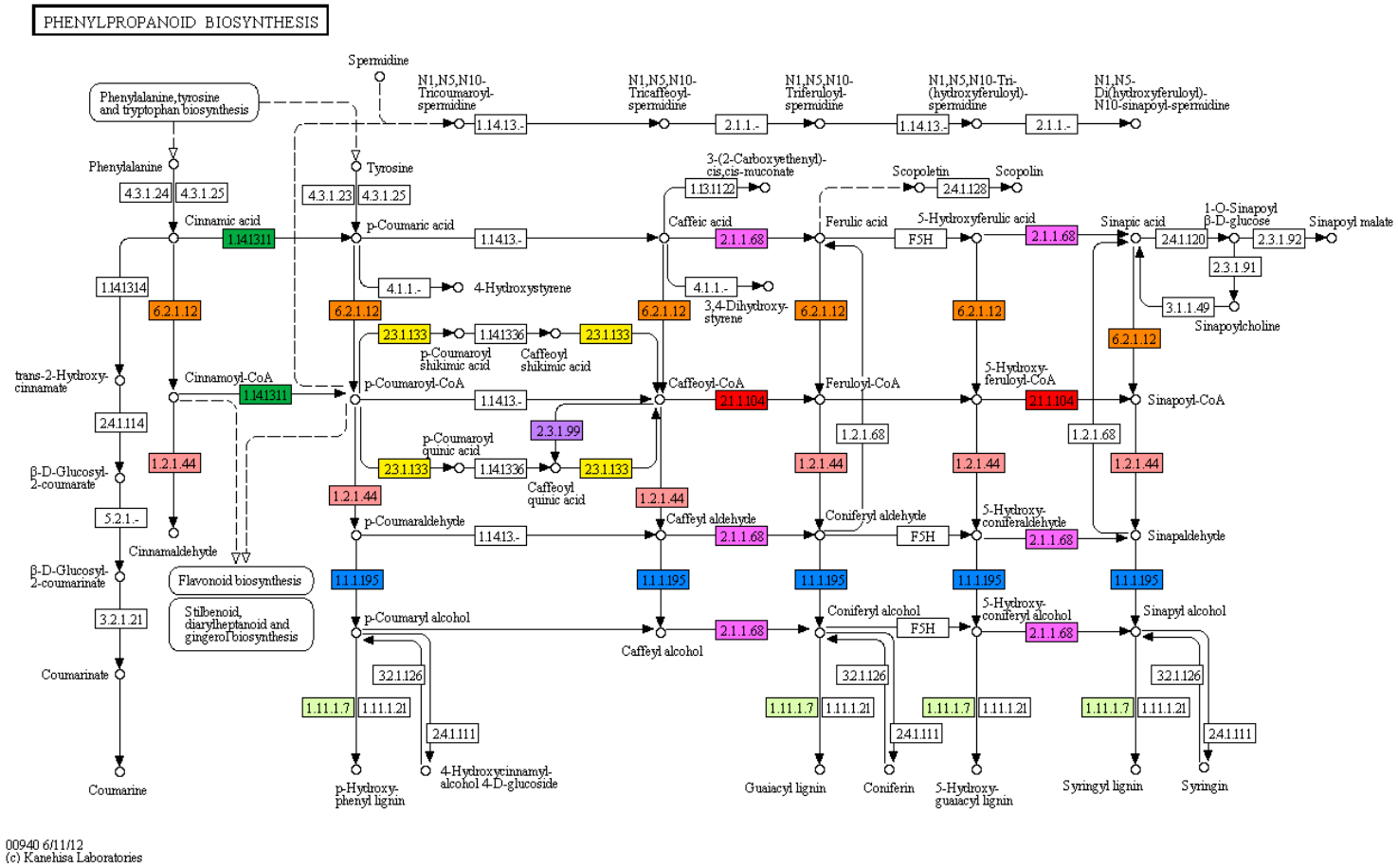

Figure 1. A schematic diagram showing the phenylpropanoid pathway map from KEGG. The colour-coded boxes indicate individual enzymes which match the degradome sequences in this study.

Conclusions

In conclusion, phenylpropanoid biosynthesis pathway is likely to be regulated by small RNA mechanism. The degradome sequences showed putative transcripts cleaved by sRNAs, supporting those reported previously (Addo-Quaye et. al. 2008). The degradome sequences will be aligned with the transcriptome sequences to obtain the sRNA sequences. Next, sRNAs will be verified by real-time reverse-transcription polymerase chain reaction and to confirm the identity of sRNAs. Expression and functional analysis will also be performed to study the role of these sRNAs in the phenylpropanoid biosynthesis pathway.

In future, functional RNAs could be manipulated to regulate the expression of several genes simultaneously. Hopefully, this could increase the yield of valuable natural secondary metabolites such as flavonoids, in place of relatively expensive synthetic plant secondary metabolites. In the long run, we can minimise the mass exploitation of lands for planting by applying genetic engineering.

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Molecular Characterization of Napier Grass (*Pennisetum purpureum* Schumach.) Varieties Using RAPD Markers

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Introduction

Napier grass (*Pennisetum purpureum)* occurs naturally throughout tropical Africa and particularly in East Africa. It is a tall, stout and deep-rooted perennial bunch grass well known for its high yielding capability and usage as forage for livestock (Woodard and Prine, 1991). Napier grass was first introduced in Malaysia in the 1920s and is currently the most popular fodder grass in dairy and feedlot production systems. Over the last two decades various Napier grass accessions have been introduced such as Taiwan Napier, Dwarf Napier, King Grass and Red Napier.

Napier grass has been the most promising and high yielding fodder (Anindo and Potter, 1994). A key for identifying the various Napier grass varieties using morphological and agronomic characters has been developed in Africa. In Ethiopia, ten Napier accessions were studied on their variation in growth, yield, chemical composition and *in vitro* dry matter digestibility. Four Napier grass accessions were categorized as high quality (Zewdu, 2005). In the morphological studies, variety Taiwan A-144 presented the highest dry matter production (Bach et al., 1995). In Ethiopia, 56 accessions were used to assess agronomic potential of Napier grass (Lowe et al., 2003). Studies have shown that molecular markers generated by polymeraze chain reaction (PCR) of randomly amplified DNA (RAPDS) have been extremely useful in differentiating different accessions of Napier grass (Smith et al., 1993; Daher et al., 2002; Lowe et al., 2003; Passo et al., 2005). In a trial involving eleven Napier grass accessions using RAPDs, it was found that there was enough genetic variation between the 11 cultivars to allow successful separation (Jamnadass, 1999). Random Amplified Polymorphic DNA (RAPDs) markers have a number of advantages over other molecular markers. It is rapid, low cost, requires small DNA and suitable for study on anonymous genome as no prior DNA sequence is required (Hadrys et al., 1992). The markers are also able to detect the polymorphism in any sequences in organism (Haymer and Mclnnis, 1994).

The varieties of Napier grass introduced in Malaysia from various sources recently are known by various common local names leading to some confusion as to the actual identity of the varieties. No systematic evaluation has been done on these accessions with regards to the identification and characterization of the plant genotype. Farmers need meaningful advice on Napier grass cultivars and a practical field key would be useful for their identification. Therefore, the objective of this study was to characterize the genetic variation of several Napier grass varieties grown in Malaysia.

Materials and Methods

The experiment was conducted on nine varieties of Napier grass replicated in five blocks in a randomized complete-block design. The varieties were King Grass, Common Napier, Red Napier, Taiwan Napier, Uganda, Indian Napier, Dwarf Napier, Dwarf 'Mott' and Australian Dwarf. Genetic studies were conducted using DNA extracted from young leaves for each variety using RAPD fragments.

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DNA was extracted using Geneall plant kit on 0.1 g samples of young leaf of Napier grass varieties. The quality and quantity of DNA were measured by NanoDrop 2000C (Thermo Scientific, USA).

A total 30 operon primers were screened and the primers that showed positive amplification and polymorphism were used in the genetic diversity determination of nine Napier grass varieties.

DNA amplification mix reaction that comprised 6.5 µL polymerase chain reactions (PCR) Mastermix ($MgCl_2$, dNTPs and Taq polymerase), 2.5 µL PCR Buffer, 9 µL dH_2O, 4 µL primer and 3 µL DNA. Reaction mixtures were placed in a DNA Thermal cycler programmed following these conditions of pre-denaturation at 94 ^oC for 3 min, 40 cycles of 1 min at 94 ^oC, 1 min at 39.6 ^oC for annealing and 2 min at 72 ^oC with a final extension of 10 min at 72 ^oC.

RAPD fragments were separated by electrophoresis in 1.5% (w/v) agarose. Agarose gel was prepared using 0.9g agar, 60 µL TBE and 2 µl ethidium bromide. The electrophoresis process was run using 80 volt. DNA banding patterns were visualized by UV transillumination and photographed.

Cluster analysis (UPGMA) has been used to group similar varieties and the genetic relationship was analyzed using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) as described by Rohlf (1990). A dendrogram was constructed based on Jaccard's similarity coefficient (Jaccard, 1908).

Results and Discussions

Thirty operon primers (Figures 1a and 1b) were used for Napier grass varieties for reproducibility of the amplified fragments. Out of 30, 8 primers were used for DNA profiling in this study. They were OPA 1, OPA 6, OPB 1, OPB 4, OPB 5, OPB 9, OPB 19 and OPB 20 (Table 1). All eight primers chosen showed high and clear polymorphic bands. These primers were used for the full study of nine varieties to quantify genetic distances and to distinguish the plant varieties.

The highest number of RAPD loci generated from the genetic analysis was OPA 6 primer with 16 and followed by OPB 5 with 14. OPB 4, OPB 9, OPB 19 and OPB 20 showed the same number of loci with 12 each. The lowest number of fragments was generated by the primer OPA 1 and OPB 1 (6 each). The genetic profile showed high polymorphism which ranged from 0.40 to 0.67 among the Napier grass which indicates that there are high genetic variations among the varieties.

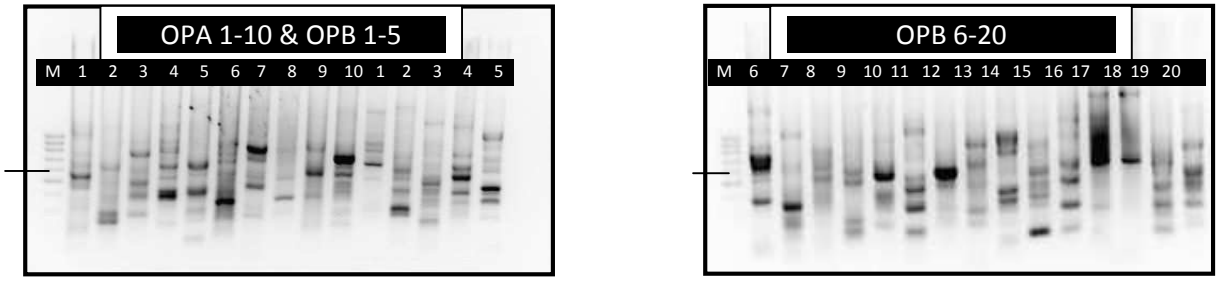

Figure 1. Screening of (a) 10 operon A primers and 5 operon B primers, and (b) 15 operon B primers on two varieties of Napier grass (Taiwan Napier and Uganda) showing positive amplification. M = 100 bp step ladder.

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Table 1. Sequence of RAPD primer used to screen for polymorphism in *P. purpureum* varieties from Operon Technologies Inc.

PRIMER	SEQUENCE (5' ⟶3')
OPA 1	CAGGCCCTTC
OPA 6	GGTCCCTGAC
OPB 1	GTTTCGCTCC
OPB 4	GGACTGGAGT
OPB 5	TGCGCCCTTC
OPB 9	TGGGGGACTC
OPB 19	ACCCCCGAAG
OPB 20	GGACCCTTAC

The data obtained from DNA amplification was used to generate a matrix of genetic distances (Table 2). The King Grass (1) and Dwarf 'Mott' (8) showed the lowest similarity index (0.40). Both of them varied considerably in agronomic performance, dry matter yield and nutritive quality too. Moreover these two varieties obviously were different in terms of plant height where the King Grass was from a taller varieties and Dwarf 'Mott' came from shorter varieties. These two varieties could be useful for hybridization since according to Xiao et al. (1996) hybrid vigor has a positive relation with genetic distance. The varieties with the highest similarity index of 0.70 were Taiwan Napier and Uganda, Indian Napier and Dwarf Napier and Taiwan Napier with Indian Napier.

The similarity coefficient indices were used as input data for cluster analysis using NTSYS-pc program and the resulting dendrogram is shown in Figure 2. Based on the dendrogram, the varieties have been grouped into three major clusters. Cluster 1 contained only one variety (King Grass). Second major cluster was formed by Common Napier and Red Napier. The third major cluster consisted of Taiwan Napier, Uganda, Indian Napier, Dwarf Napier, Dwarf 'Mott' and Australian Dwarf. The Taiwan Napier and Uganda varieties were grouped close together as well as Indian Napier and Dwarf Napier varieties which mean that the varieties are highly similar to each other.

Conclusions

As a conclusion, the genetic distances ranged from 0.40 to 0.67, indicating high genetic variability for the set of varieties. From that, considerable diversity existed among the Napier varieties, RAPD is quite efficient in bringing out this diversity at DNA level. Cluster analysis of nine varieties in this study formed three major groups. This study also identified highly diverse varieties where King Grass and Dwarf 'Mott' for use in hybridization program for Napier grass improvement.

The finding has provided information on the genetic variation and distance among nine varieties Napier grass grown in Malaysia. Farmers and growers can choose the two varieties with higher diversity as parents for hybridization and cultivation in order to get better varieties. The study was able to resolve the identity of available Napier varieties and to identify genetic characteristics of each variety. Finally, the genetic distance data obtained in this study might be useful in future work for a better understanding of the genetic variability of the species.

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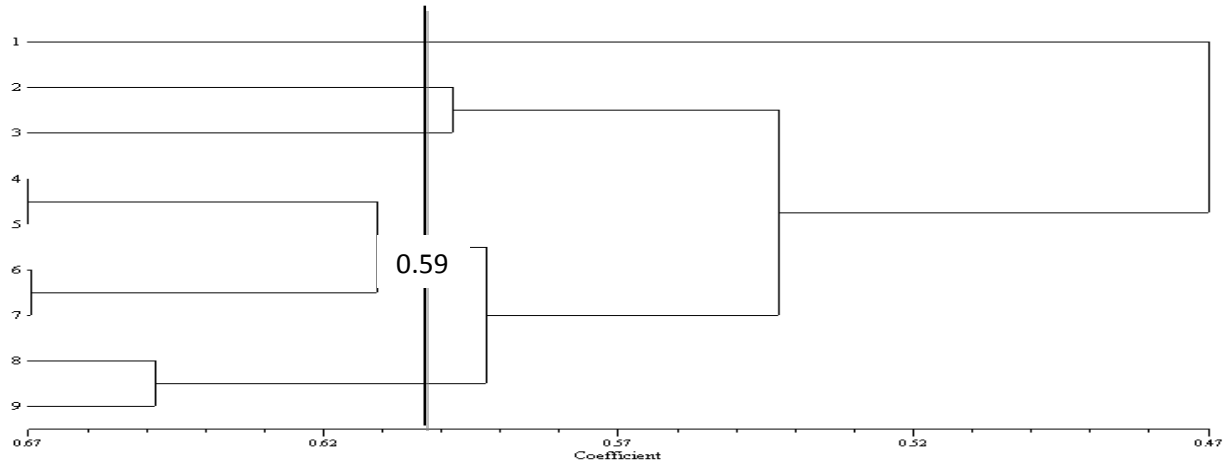
Table 2. Nei's (1979) genetic distance matrix of 9 varieties of Napier grass based on RAPD analysis. 1= King Grass; 2= Common Napier; 3= Red Napier; 4= Taiwan Napier; 5=Uganda; 6= Indian Napier; 7= Dwarf Napier; 8= Dwarf 'Mott'; 9= Australian Dwarf.

	1	2	3	4	5	6	7	8	9
1	1.00								
2	0.48	1.00							
3	0.49	0.60	1.00						
4	0.45	0.55	0.60	1.00					
5	0.56	0.50	0.55	0.67	1.00				
6	0.49	0.56	0.61	0.67	0.65	1.00			
7	0.46	0.53	0.54	0.56	0.57	0.67	1.00		
8	0.40	0.58	0.50	0.58	0.50	0.63	0.59	1.00	
9	0.42	0.52	0.47	0.62	0.60	0.64	0.60	0.65	1.00



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In Vitro Plant Regeneration via Somatic Embryogenesis from Zygotic Embryo Explants of Rubber (*Hevea brasiliensis* Muell. Arg).

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Introduction

The rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important industrial crop for natural rubber production. At present about 40 countries are devoted to rubber tree cultivation with a production of about 6.5 million tons of dry rubber each year. The world supply of natural rubber is barely keeping up with a global demand for 12 million tons of natural rubber in 2020. Rubber tree is not only an economic plant regenerating income to growers but also a big perennial plant which increases green space and oxygen to the atmosphere, conserves the environment and reduces deforestation and soil erosion. It can be considered as a plant factory for solar energy conversion and a carbon sink by virtue of the process of photosynthesis. It has the ability to absorb carbon dioxide 33.25 metric tons per hectare in a year and 979.37 metric tons per hectare in one life span (25 years) (Khun, 2006). As *Hevea* is mainly cultivated in regions of high photosynthetic productivity and has a capacity to fix 90 million tons of carbon per year, it would appear that a rubber plantation is almost as effective during photosynthesis as a virgin forest in consuming the products of fossil fuel burning, as well as producing life sustaining oxygen. Furthermore, natural rubber is natural plastic produced in clean process which saves energy by 7 to10 times, which is lower than synthetic rubber production. Hence rubber cultivation will be an advantage and a benefit for growers to earn extra from selling carbon credit. The rubber tree is propagated mostly by grafting. The interaction between rootstock and scion of grafted rubber tree affects growth, yield as well as the physiological and biochemical characteristics of rubber trees (Sobhana et al., 2001; Huang and Lin, 2003). Somatic embryogenesis is a rapid and efficient vegetative propagation method. Plant regeneration via somatic embryogenesis in *H. brasiliensis* has reported using several explants such as immature anther, (Wang et al., 1980), inner integument of seed (Carron and Enjalric,1985) and immature inflorescence (Sushamakumari et al., 2000). However, the technique is still not sufficient to be utilized in crop improvement programmes. Reliable somatic embryo formation is limited to only a few genotypes of *Hevea*. Embryogenic capacity is fugacious, and the rate of conversion of the embryos to planlet is very low. However, while only a few clones exhibited a high frequency plantlet induction (22%), many clones exhibited a very low embryoid and plantlet regeneration rate and some clones did not show any embryoid induction at all. This study was therefore carried out to develop a reliable regeneration system from zygotic embryos via somatic embryogenesis for Malaysian rubber clones.

Materials and Methods

Seeds were obtained from Malaysian Rubber Board of *Hevea* (clone RRIM 901) in September 2011. The endosperm along with embryo were sterilized with 15% Clorox containing one drop of Tween-20 for 15 min followed by thorough washing with sterile water. The endosperm was dissected aseptically and embryos were excised from the cotyledons. The excised embryos were inoculated into the primary callus induction medium. Three embryos were placed per culture containing 30 mL medium.

Callus Induction and Proliferation

Two basal media, MS (Murashige and Skoog, 1962) and WPM (Lloyd and McCown, 1980) supplemented with B5 vitamins and different phytohormones were used for callus induction from the zygotic embryo explants of *Hevea.* Various auxins namely 2, 4- dichlorophenoxyacetic acid (2,4-D),1-naphthalene acetic acid (NAA),

Picloram, and Dicamba at different concentrations alone or in combination with 6- benzylaminopurine (BAP) and Kinetin (KIN) were used. All media were solidified with 2.75 g/L gelrite. A total of 20 cultures were produced for each treatment. The cultures were incubated at 25 ± 2 ˚C in the dark. The frequency of callus formation was determined one month after culture initiation.

Embryogenic Callus Growth and Maintenance

The induced primary calli were subcultured for proliferation and optimization of the callus growth. MS medium with B5 vitamins, supplemented with 2,4-D, BAP and KIN in the presence of NAA were used in this experiment. Cultures were incubated at 25 ± 2 ˚c in the dark. The maximum diagonal length of callus, fresh and dry weight of callus and callus morphology were recorded after every week of culture to obtain the embryogenic callus and the best callus maintenance medium.

Embryo Induction, Maturation and Germination

Three weeks old proliferated calli were tested for embryo induction. The embryo induction medium comprised of modified MS medium with B5 vitamins, glutamine (100 mg/L) along with organic supplements of coconut water (10% v/v) and casein hydrolysate 200 mg/L and 5% (w/v) sucrose. The media was supplemented with reduced levels of auxins 2,4-D, and NAA along with different combinations of the phytohormones, BAP, KIN and GA3. Enlarged embryos having shoot apex were transferred for plant regeneration to hormone free MS medium and ½ MS medium. The media for embryo induction, maturation and germination were also supplemented with 0.2% (w/v) activated charcoal. Callus induction, embryo induction, maturation and germination of embryos were recorded based on visual observations. All experiments were repeated twice with three replications.

Results and Discussion

Between the two basal media tried, MS medium was found to be ideal for callus induction. Among the different auxins, 2,4-D and NAA successfully induced callusing but failed to proliferate further. The other two auxins, Picloram and Dicamba, showed very little response on callusing, when supplied individually (1 to 5 mg/L). In order to optimize callus growth and proliferation, two cytokinines, BAP and KIN, were used at different concentrations with 2,4-D and NAA. Among the different combinations of auxins and cytokinines, the combination of 2,4-D and BAP performed best. The maximum length of diagonal of callus, Fresh and Dry weight of callus per culture and callus morphology were recorded after three weeks of culture in MS medium supplemented with 2 mg/L 2,4-D, and 2 mg/L BAP in the presence of 0.5 mg/L NAA. Results are presented in Figure1and Figure 2. On further increase of BAP concentration reduction of callus growth was observed. Similar response was observed with increase in 2,4-D concentration (data not shown). The induced calli were then subcultured into the medium containing different cytokinins to obtain embryogenic callus. The yellow friable fast growing embryogenic callus was obtained in the MS medium supplemented with 2 mg/L 2,4-D and 2 mg/L KIN which further differentiated in proembryos after two weeks in the same medium. For embryo induction, the auxins were gradually reduced and finally removed from the medium and embryo induction was attempted with other growth regulators. Maximum embryo induction frequency and number of embryos were obtained in modified MS medium supplemented with BAP and GA3 (Figure 3).

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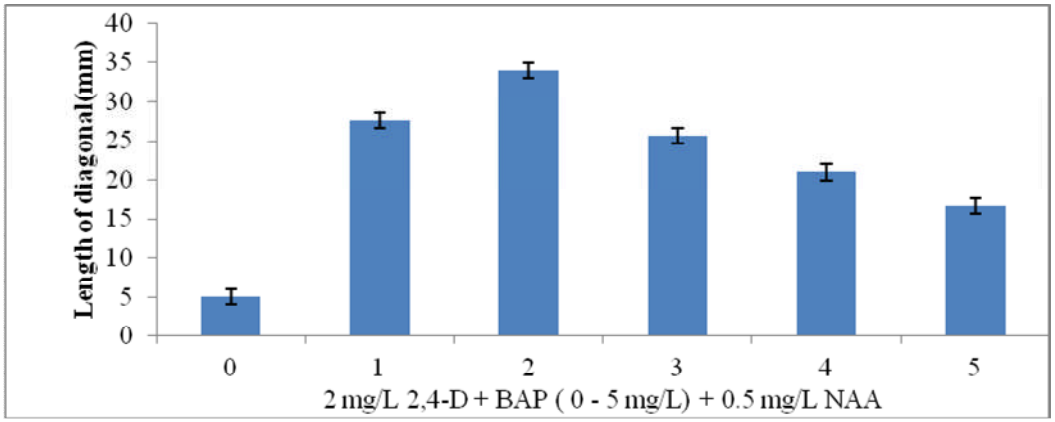

Figure 1. Effect of 2 mg/L 2, 4-D with different concentrations of BAP (0 – 5 mg/L) in the presence of 0.5 mg/L NAA in MS medium on callus growth after 3 weeks of culture

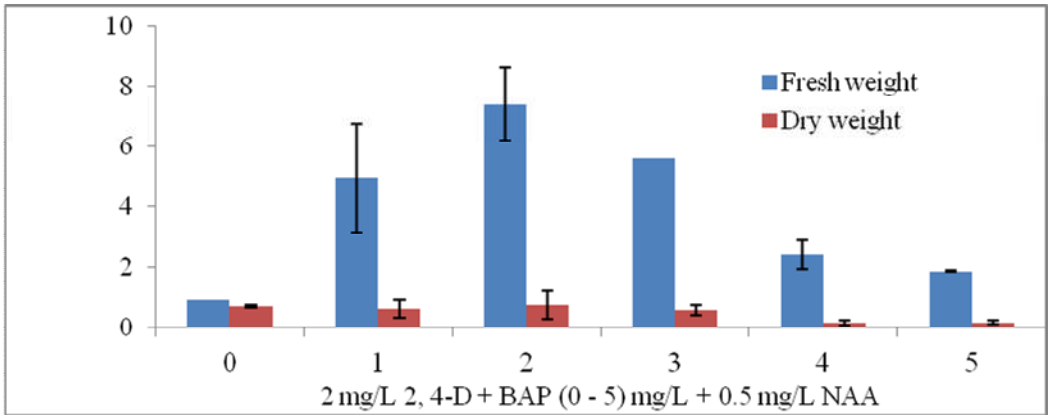

Figure 2. Effect of 2 mg/L 2, 4-D with different concentrations of BAP (0 – 5 mg/L) in the presence of 0.5 mg/L NAA in MS medium on FW and DW of callus growth after 3 weeks of culture

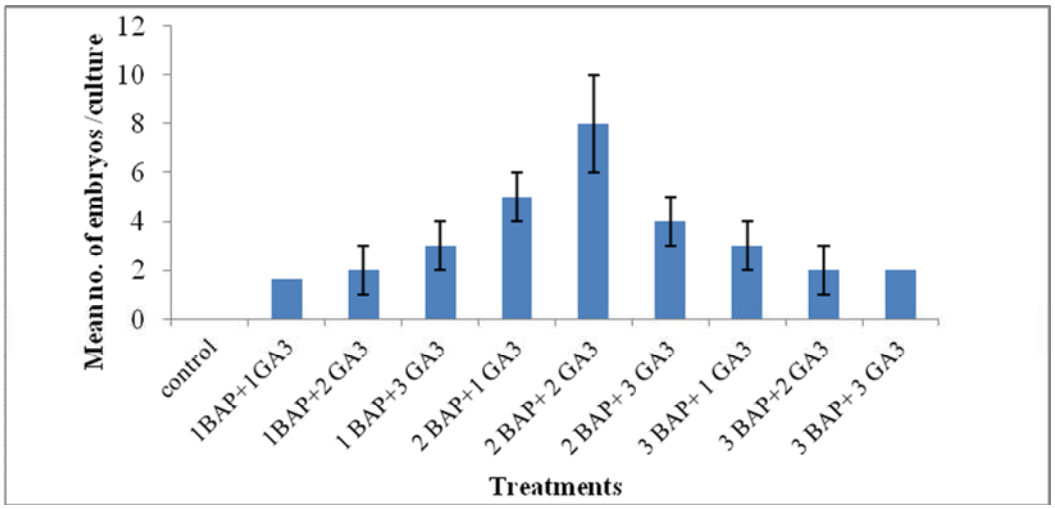

Figure 3. Effect of BAP and GA3 in modified MS medium on somatic embryo induction of *H. brasiliensis*

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Conclusions

A plant regeneration protocol via somatic embryogenesis was developed using zygotic embryo of *H. brasiliensis* (clone RRIM 901). MS and modified MS media supplemented with 2,4-D, NAA, BAP and GA3 as plant growth regulators were found to be ideal for embryogenesis. This protocol could be applied to several Malaysian rubber genotypes for production of large scale planting materials. The present regeneration system can also be used for developing transgenic plants either by *Agrobacterium* mediated gene transfer or by particle bombardment with desirable genes.

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Antimicrobial Screening of the Leaf, Stem and Root Extracts of Three Varieties of *Labisia pumila* Benth

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Introduction

Medicinal plants are now getting more attention in terms of medicinal and pharmacological uses. The medicinal value of these plants lies in the bioactive phytochemical constituents that produce definite physiological action in the human body (Akinmoladun et al., 2007). Medicinal plants are a critical link in the transition to a green economy – one that promotes sustainable development and also their chemical constituents have been used worldwide in folk medicine as an alternative treatment of various diseases (Castelucci et al., 2007). The use of plant extracts for medicinal purposes has long prevailed in different parts of the world (Gurib-Fakim, 2006) though it started enjoying great popularity in the 1990s (Cowan, 1999). According to Harvey (2000), natural products possess higher chemical novelty than chemically synthesized products and thus this fact has led to the search of active compounds in plants. *Labisia pumila,* locally known as Kacip Fatimah, is a forest-floor herbal plant has tremendous potential in the herbal industry (Ibrahim et al., 2010). It is one of the five herbal plants identified by the governments as one of the national key economic areas to be developed for commercial purposes. In Malaysia, it is an important and popular medicinal plant that has long been recognized and demanded for its properties value as female tonics and health products (Burkill, 1966). With recent discovery of estrogenic activity (Jaafar et al., 2008), demand for *L. pumila* is expected to soar. In an effort to provide scientific explanation for the use of *L. pumila* plant as an antimicrobial agent, the possible mechanisms of action of the extracts have to be elucidated. Therefore in this study, the antibacterial activity and antifungal properties of the crude extracts were investigated.

Materials and Methods

Sample Preparation

Seedlings of *Labisia pumila* varieties of *alata* and *pumila* were, respectively, collected from Kota Tinggi, Johor and raised under glasshouse for 18 months before use. Leaf, stem and root of three varieties were separated, frozen dried and kept for further analysis. Five grams of dried sample were mixed with 100 mL boiling water using a magnetic stirrer for 15 min. Then, the extract was filtered and evaporated using vacuum rotary evaporator. The crude extracts were then re-dissolved in 5 ml of respective solvents used in test analysis (Gulcin et al., 2004).

Antibacterial Assay

The antibacterial activity of leaf , stem and root of three varieties of *L. pumila* extracts were evaluated by paper disc diffusion (Boussaada et al., 2008) against four selected Gram-positive (*S. aureus, B. subtilis, B. cereus, M. letue*) and negative species (*E. coli, E. aeruginosa, K. pneumonie, P. auruginosa)*. Kanamycin at 1 mg/mL was used as reference control to evaluate susceptibility of tested strains.

Antifungal Activity Assay

The antifungal activity of the all extracts was carried out by the agar well diffusion assay against *Fusarium* sp., *Candida* sp. and *Mucor* (Quiroga et al., 2001). Amphotericin B (500 µg/well) was used as positive control.

Results and Discussion

The results of the antibacterial activity indicated significant difference ($p<0.05$) in inhibitory activity between leaf, stem and root in all three varieties of *L. pumila* Benth. These extracts exhibited moderate to appreciable antibacterial activities against four Gram-positive and four Gram-negative bacteria. In all three varieties, the leaf extract had higher activity compared to roots and stems extract. The *L. pumila* var. *pumila* had higher activity against Gram positive bacteria compared to var. *alata* and *lanceolata*, while *L. pumila* var. *alata* had higher activity against Gram negative bacteria compared with var. *pumila* and *lanceolata*. Meanwhile, the antifungal properties of the extracts exhibited weak to moderate inhibitory activities against three fungal species. Among different fungi tested, *Candida* sp. was found to be the most sensitive to all the extracts.

Conclusion

This study showed interesting results on the antimicrobial potential and antifungal properties of the three varieties of *Labisia pumila* Benth. Leaf was shown to posses highest antimicrobial and anti-fungal activity compare to root and stem. The antimicrobial activity of *Labisia pumila* could be attributed to various phytochemical constituents (flavonoid, phenolic and saponin compounds) present in the respective crude extracts. The purified components might have more potency with respect to inhibition of microbes.

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In Vitro Propagation, Rooting and Acclimatization of *Catharanthus roseus* (White Variety) – A Green Economy Conservation

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Introduction

Catharanthus roseus (L.) G Don which is also known as "Kemunting cina" in Malaysia and is widely used as an ornamental plant, belongs to the family Apocynaceae. The plant is also famous for its medicinal properties, particularly as anti-cancer, anti-diabetic, and antihypertensive remedies. In Malaysia, *C. roseus* has long been used in traditional medicine and one of the popular and potential medicinal plants for both cultivation and economy conservation. The aerial and underground organs of the plant are transcriptionally distinct. Ajmalicine is another major alkaloid from the roots. It is used in the treatment of hypertension and obstructive circulatory diseases.

In vitro techniques are very useful in ensuring sustainable, optimized sources of plant-derived natural products. However, *ex situ* cultivation should be preceded by proper evaluation of the plants for their ability to produce the required bioactive constituents before commencing cultivation or introducing the technology to potential growers. The ability of plants to produce certain bioactive substances is largely influenced by the physical and chemical environments in which they grow. One to low productivity and high production costs of these alkaloids by cultures of *C. roseus*, non-conventional methods have to be employed for better production. It is highly desirable to meet the large scale needs of the various industries and also to conserve the plants in *ex situ*. The present study is advancement from axillary and internodal explants of *C. roseus* (white variety) over the earlier protocol, because it describes the hormonal regulation, propagation, rooting and acclimatization of *C. roseus*. In addition, this study deals with the development of a rapid regeneration system, the quantification of secondary metabolites from axillary and internodal explants and the subsequent transplantation of the plantlets to natural environmental conditions.

Materials and Methods

Healthy, young axillary and internodal explant of *C. roseus* (white variety) were collected from field grown plants and maintained in the Department of Plant Science, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. First healthy and elite (2-3 months old) plants of *C. roseus* were trimmed and washed under running tap water for 5 min followed by a rinse with teepol solution for 2 min, 70% ethanol (v/v) for 1 min and 0.1% $HgCl_2$ for 5 min, the following explants were washed with sterile distilled water two or four times each, axillary bud were cultured on sterile MS medium (Murashige and Skoog, 1962). The nutrient culture medium consisted on MS salts and vitamins gelled with 0.8% (w/v) agar (Hi-media). Various growth regulators, such as 6-benzylaminopurine (BA, 1.0 – 5.0 mgl^{-1}), 6-Furfurylaminopurine (KN, 1.0 – 5.0 mgl^{-1}), Indole – 3 – acetic acid (IAA, 1.0 – 8.0 mgl^{-1}), Indole-3-butyric acid (IBA, 1.0 – 8.0 mgl^{-1}), α – naphthalene acetic acid (NAA, 1.0 – 5.0 mgl^{-1}) and 2,4-dicholorophenoxyacetic acid (2,4-D, 1.0 – 5.0 mgl^{-1}) were supplemented to MS medium either singly (or) in combination was supplemented with 3% (w/v) sucrose (Hi-media) as a carbon source. The pH of the medium was autoclaved at a pressure of 1.06 kg cm^{-2} at 121°C for 15 min. The sterilized explants were placed vertically on the MS basal medium containing 3% (w/v) sucrose, 0.8% (w/v) agar, with above mentioned growth regulators in different combinations and concentrations for the shoot initiation and proliferation. Multiple shoot plants (60-70

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mm) were kept in either quarter or half or full strength MS medium with IAA and IBA (0.1-3.0 mg/l) for *in vitro* rooting. Six-week old well rooted plantlets were removed from the culture tubes and washed to make it free of agar. The plantlets were transferred to plastic pots (5 cm diameter) containing red soil and vermiculite (1:3) and maintained at 25 ± 2ºC, 16 h day length (35-50 $mEM^{-2}S^{-1}$) and at 75 to 80% relative humidity. Then the grown plants were transplanted to earthenware pots (10 cm diameter) containing natural soil, kept under shade for 2 weeks and finally moved to the field. All the experiments were repeated thrice and used 30 replicates. The effect of different treatments was quantified as mean ± SE and the data were subjected to statistical analysis using Duncan's Multiple Range Test (DMRT) at 5% level significance (Gomez and Gomez, 1976).

Results and Discussion

Axillary node and shoot tip showed direct mode of regeneration and readily developed multiple shoots, whereas other explants (leaf, petiole, root and internode segments) produce only callus (data not shown). Auxiliary nodes initiation and multiplication failed to develop shoot buds in plant growth regulator free MS medium without growth regulators. Multiple shoot formation from auxiliary bud explants were observed in MS supplemented with BA and KN singly (or) in combination with IAA, IBA, NAA and 2,4-D. MS media supplemented with BA alone resulted in high bud sprouting frequency and shoot number with maximum percentage of response when compared to KN, after 45 days (Table 1).

Table 1. Effect of MS medium supplemented with cytokinins on multiple shoots and shoot length induction on *Catharanthus roseus*, after 45 days.

Plant growth regulators (mgl^{-1})	**Percentage of responses (%)**		**Multiple shoot numbers Mean ± S.E**		**Shoot length (cm) Mean ± S.E**	
	Shoot tip	**Axillary node**	**Shoot tip**	**Axillary node**	**Shoot tip**	**Axillary node**
BA - 1.0	40.7e	53.5e	6.1 ± 0.59d	5.5 ± 0.34e	2.1 ± 0.29e	1.8 ± 0.45e
2.0	59.5cd	67.4bc	6.4 ± 0.51bc	6.3 ± 0.21c	2.8 ± 0.26c	2.4 ± 0.19bc
3.0	73.4ab	72.5b	6.6 ± 0.48b	7.1 ± 0.40b	3.4 ± 0.17ab	2.8 ± 0.17b
4.0	**76.2a**	**80.0a**	**7.3 ± 0.40a**	**8.8 ± 0.32a**	**4.2 ± 0.21a**	**3.5 ± 0.22a**
5.0	65.0c	60.2d	5.9 ± 0.35e	6.2 ± 0.17cd	2.7 ± 0.14cd	2.1 ± 0.14d
KN - 1.0	62.1c	43.2e	5.8 ± 0.39cd	6.2 ± 0.32d	3.1 ± 0.16cd	3.2 ± 0.17cd
2.0	69.5ab	56.0bc	6.1 ± 0.43c	7.6 ± 0.18b	3.3 ±0.28ab	3.4 ± 0.32b
3.0	**72.0a**	**70.5a**	**6.8 ± 0.52a**	**8.1 ± 0.24a**	**3.6 ± 0.29a**	**3.8 ± 0.19a**
4.0	56.0d	62.0b	6.3 ± 0.34ab	6.5 ± 0.16bc	3.4 ± 0.21c	3.5 ± 0.14c
5.0	48.4e	55.3d	5.5 ± 0.43e	5.4 ± 0.19e	2.7 ± 0.17e	3.0 ± 0.10e

Values are mean of 10 replicates per treatment and repeated thrice. Values with same superscript are not significantly 5% probability level according to DMRT.

To enhance shoot multiplication, different auxins were combined with the optimized cytokinin concentration. Shoot number was the highest in the media containing NAA (4.0 mg/l) with BA (4.0 mg/l) for axillary explants of *C. roseus*, after 45 days (Figure 1 d -e). KN (3.0 mg/l) with IAA (3.0 mg/l) induced the maximum number of multiple shoots from shoot tip explants, after 45 days (Figures 1 a - c). Higher concentration of NAA (below 2.0 mg/l), IAA, IBA induced basal callus, sometimes root formation in IBA at cut ends and prevents multiple shoot induction. The shoots formed from explants of *C. roseus* showed stunted growth and phenolic exudation, yellowing of leaves. But combined BA and KN treatment showed better overall growth than individual treatments (Table 2). The effective concentration of NAA (4.0 mg/l) requirement for shoot bud induction was active to both

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the explants. Different media fortified separately with BA (4.0 mg/l) + KN (3.0 mg/l), [BA (4.0 mg/l + NAA(4.0 mg/l)], [KN (3.0 mg/l) + IAA (3.0 mg/l)], [BA (4.0 mg/l) + KN (3.0 mg/l] for shoot tip explants and BA (4.0 mg/l) + KN (3.0 mg/l), [BA (4.0 mg/l + NAA (4.0 mg/l)], [KN (3.0 mg/l) + NAA (4.0 mg/l)], [BA (4.0 mg/l) + KN (3.0 mg/l] for axillary node explants were examined to determine the optimum salt requirement for shoot initiation and shoot multiplication in *C. roseus*, after 45 days. The shoot buds sprouted slowly with limited development even if they were maintained for longer period in culture. The explants in culture showed phenolic exudation and yellowing of leaves. The highest frequency of response and shoot number was observed at node position 2, 3 and 4 found to be more responsive than from distal and proximal nodes. In *C. roseus*, development of shoots with larger internodes was observed on KN supplemented medium, the similar results were observed (Sabita and Sanghamitra, 1997). It shows that KN was necessary for the development of healthy normal shoots (Mondal et al., 1990). Combination of BA and KN in the culture medium prompted the multiple shoot induction and shoot sprouting frequency. Superiority of BA, KN combination has been formed for micropropagation of other woody perennials (Reuveni et al., 1990; Das et al., 1996; Komalavalli and Rao, 1997; Roy et al., 1998).

Table 2. Effect of MS medium supplemented with KN and auxins on multiple shoots and shoot length induction on *Catharanthus roseus*, after 45 days.

Plant growth regulators (mg/L)	**Percentage of response (%)**		**Multiple shoot number Mean ± S.E**		**Shoot length (cm) Mean ± S.E**	
	Shoot tip	Axillary node	Shoot tip	Axillary node	Shoot tip	Axillary node
KN + IAA						
3.0 + 1.0	30.2e	40.5e	4.3 ± 0.21bc	5.1 ± 0.18e	1.9 ± 0.12de	2.6 ± 0.20c
2.0	35.6c	59.8ab	5.0 ± 0.10b	6.4 ± 0.14c	2.6 ± 0.20b	3.0 ± 0.18ab
3.0	**46.2a**	**61.5a**	**5.4 ± 0.18a**	**7.2 ± 0.21a**	**3.2 ± 0.19a**	**3.2 ± 0.14a**
4.0	40.0b	54.2c	3.5 ± 0.14d	6.5 ± 0.14b	2.4 ± 0.14bc	2.4 ± 0.22cd
5.0	32.5cd	47.0d	2.8 ± 0.10e	5.8 ± 0.20cd	2.1 ± 0.19d	1.8 ± 0.16e
KN + IBA						
3.0 + 1.0	26.2e	32.8de	5.0 ± 0.16bc	5.8 ± 0.20e	2.5 ± 0.12d	1.8 ± 0.14de
2.0	29.0c	39.5d	5.4 ± 0.19b	6.6 ± 0.14cd	2.9 ± 0.22bc	2.4 ± 0.10c
3.0	**44.5b**	**60.4a**	**6.0 ± 0.22a**	**8.4 ± 0.19a**	**3.2 ± 0.16a**	**3.2 ± 0.16a**
4.0	54.4a	56.0ab	4.4 ± 0.24d	6.8 ± 0.10b	3.0 ± 0.10b	2.9 ± 0.19ab
5.0	42.5cd	53.2c	3.2 ± 0.10e	6.0 ± 0.18c	2.4 ± 0.14de	2.0 ± 0.10d
KN + NAA						
3.0 + 1.0	34.5e	34.2e	5.6 ± 0.12e	6.5 ± 0.14e	2.6 ± 0.14e	1.9 ± 0.10e
2.0	48.2cd	45.0cd	6.2 ± 0.10cd	7.1 ± 0.16cd	2.8 ± 0.20c	2.6 ± 0.14bc
3.0	54.0ab	56.4ab	7.4 ± 0.21b	9.0 ± 0.19a	3.0 ± 0.19b	3.2 ± 0.22b
4.0	**59.8a**	**62.8a**	**9.8 ± 0.19a**	**11.5 ± 022b**	**3.4 ± 0.22a**	**4.0 ± 0.16a**
5.0	49.3c	50.5c	6.8 ± 0.14c	7.3 ± 0.18c	2.1 ± 0.18cd	2.5 ± 0.12d
KN + 2,4-D						
3.0 + 1.0	32.0cd	35.4cd	4.9 ± 0.14e	5.8 ± 0.21e	2.1 ± 0.14de	2.0 ± 0.19e
2.0	44.2ab	46.8b	5.6 ± 0.10c	6.6 ± 0.14d	2.8 ± 0.22b	3.1 ± 0.20bc
3.0	**48.5a**	**49.5a**	**8.2 ± 0.14a**	9.0 ± **0.18b**	**3.2 ± 0.19a**	**3.5 ± 0.22a**
4.0	36.5c	38.0c	6.8 ± 0.22b	6.0 ± 0.10a	3.0 ± 0.18bc	3.2 ± 0.14b
5.0	30.0e	30.4e	5.0 ± 0.16cd	5.2 ± 0.14bc	2.3 ± 0.16d	2.6 ± 0.12d

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In our study among the various concentrations of BA involved (4.0 mg/l) BA with KN (3.0 mg/l) gave good survival percentage. Our results indicated that NAA (4.0 mg/l) concentration could modify positively the shoot induction response. Similar result was observed in *C. roseus*, MS medium supplemented with KN and BA each at 0.2 mg/l + IAA (0.1mg/l) (Adinpunya et al., 1997). IAA and IBA were used individually with full, half and quarter strength MS basal medium for rooting. In our study IBA was found to be more potent auxin for highest percentage of rooting. The highest root induction (82.5%) was observed on half-strength MS basal medium supplemented with auxins. The full strength MS medium significantly induced the basal callus, reduced root percentage and root length (Figure 2a-d). The maximum root induction was observed in half strength MS medium supplemented with IBA (4.0 mg/l) concentrations after 30 days. Similar results were observed in *Gymnema sylvestre* (Komalavalli and Rao, 2000) and *Eclipta alba* (Baskaran and Jeyabalan, 2005). Junaid et al., (2004) observed that matured green embryos of *C. roseus* were cultured on MS medium supplemented with optimized BAP (0.5 mg/l) produced shoots and roots.

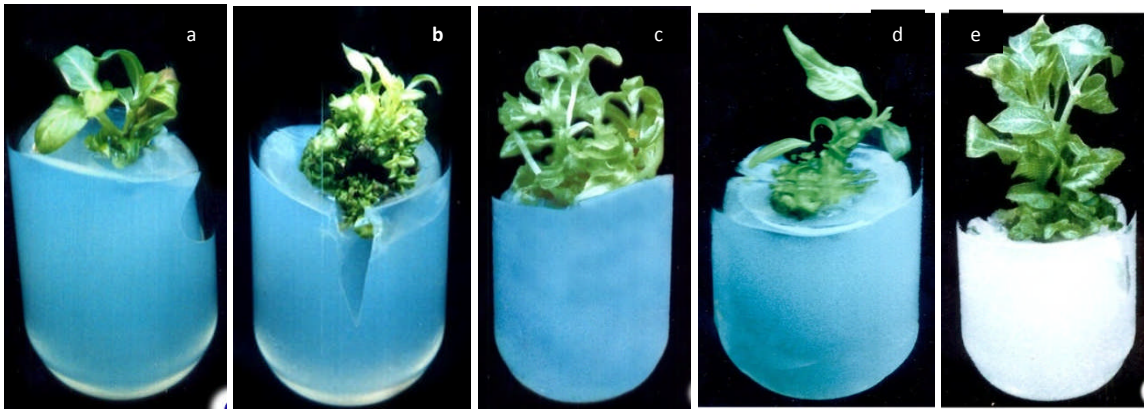

Figure 1. Multiple shoots from axillary and shoot tip explants of *C. roseus*

The protocol established in this study can be used for the efficient multiplication of *C. roseus.* MS basal medium in combination of BA (4.0 mg/l) and KN (3.0 mg/l) be influenced the better shoot induction and shoot multiplication. Half strength of MS basal medium with IBA (4.0 mg/l) effectively induced the number of roots per plant (Figure 2 (a-d). After 30 days, rooted plantlets were removed from culture tubes and washed with sterile distilled water to remove media, and they were then transferred to foam cups containing a mixture of autoclaved garden soil, sand and vermiculite (Keltech Energies Ltd., Bangalore, India) (1:1:1) respectively. The cups were covered with polyethylene bags to maintain high humidity. The hardened plants were maintained under controlled condition for 30 days and they were acclimatized in garden.

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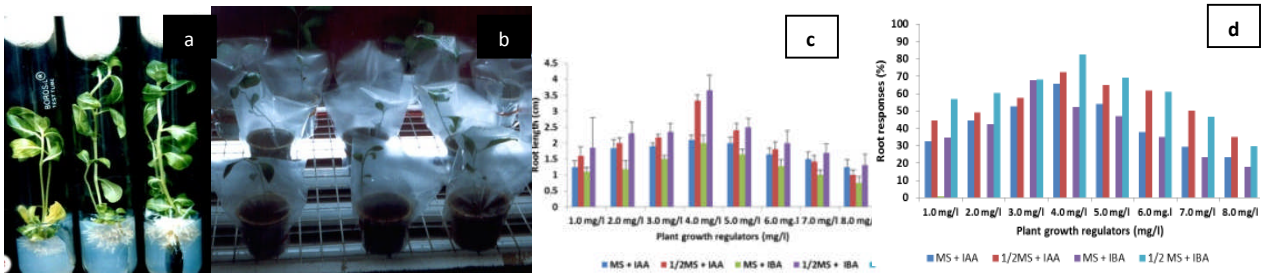

Figure 2. Rooting, acclimatization, root responses and root length of *C. roseus*

Conclusions

Successful plant regeneration was optimized from axillary and intermodal explants in the combination of BA and KN. The best multiple shoot induction on MS medium with BA (1.0 mg/l) and KN (2.0 mg/l) in the axillary nodel explants were optimum for plant regeneration. Our data on the effects of PGRs in *C. roseus* batch culture will be useful in enhancing the acculation of alkaloid compounds in such cells and can also be further extended to other plant culture models.

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Functional Characterization of UDP-Glucose Pyrophosphorylase cDNA Clone from *Eucheuma denticulatum*

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Introduction

UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9) represents an important enzyme for the biosynthesis of UDP-glucose which can be used in the synthesis of cell wall polysaccharides (Kleczkowski et al., 2004) and in production of the carbohydrate moiety (Flores-Diaz et al., 1997). UGPase carries out the reversible conversion of glucose-1-P and UTP to UDP-glucose and PPi. This enzyme was mainly found in eukaryotic and also prokaryotic organisms. In plant, several UGPase have been isolated and characterized, including *Hordeum vulgare* (Eimert et al., 1996), *Cucumis melo* (Dai et al., 2006) and *Oryza sativa* (Chen et al., 2007). The occurrence of UGPase enzyme in red algae was first demonstrated in *Porphyra perforata* (Su and Hassid, 1962). Recently, Lluisma and Ragan (1999) demonstrated the nuclear gene of UGPase from *Gracilaria gracilis*. Herein we report the isolation of cDNA clone, which encode UGPase from the marine red alga *E. denticulatum* and the functional analysis of this protein.

Materials and Methods

Plant Materials

E. denticulatum was purchased from Marine Borneo Research Institute, Universiti Malaysia Sabah via Lembaga Kemajuan Ikan Malaysia (LKIM) Sabah.

Isolation f UGPase Full-Length cDNA Clone

Total RNA from *E. denticulatum* was extracted using the method described by Lopez-Gomez and Gomez-Lim (1992). 5'-RACE technique were performed to obtain the 5' ends of the putative EdUGP. The 5' first strand cDNA was obtained and amplified using the SMART RACE cDNA Amplification Kit (Clontech). cDNA was sythesized from 1 µg total RNA isolated from *E. denticulatum* according to the manufacturer's protocol. This cDNA was used as the template for amplification of 5' cDNA fragments using the adapter-specific primer and the gene specific primers with the following PCR program: 40 cycles at 94 ^{o}C for 30 s, 68 ^{o}C for 30 s and 72 ^{o}C for 3 min. Full-length cDNA clones were constructed based on long-distance PCR (LD-PCR) strategy. A pairs of new primers were designed against the 5' and 3' untranslated region (UTR) of EdUGP cDNA. These primers and the first strand cDNA were utilized to obtain the full-length sequence by LD-PCR, using Advantage^{TM} 2 polymerase mix (Clontech). Sequencing was carried out using a PCR-based dideoxynucleotide terminator protocol and an ABI PRISM Automated DNA Analyzer (Applied Biosystems). Alignment of deduced amino acid sequences of EdUDP with other UGPase encoding sequences were obtained using the ClustalW program. A phylogenetic neighbour-joining analysis was conducted on deduced amino acid sequences using the Fitch-Margoliash algorithm (Fitch and Margoliash, 1967).

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Expression and Purification of UGPase

ORF of wild-type and mutant UGPase cDNA clones were subcloned into pQE-2 expression vector (Qiagen, USA). The resultant sequence-verified construct, respectively were used to transform into *Escherichia coli* M15 cells. The proteins were expressed in Luria Bertani (LB) medium containing 100 µg/ml ampisilin and 25 µg/ml kanamysin for 4 hours at 37°C for both wild-type and K491A mutant after induction with 1mM isopropyl-β-D-thiogalactoside (IPTG). The recombinant protein obtained was then purified by His-TRAP HP column using AKTA-purifier machine (GE Healthcare, USA). The purified protein was assayed according to Martz et. al (2002).

Results and Discussion

Cloning of UGPase full-length cDNA clone EdUDP was obtained by 5'-RACE and LD-PCR approaches. The full-length EdUGP cDNA sequence was found to comprise of 1509 bp ORF. Phylogenetic analysis using Neighbor-joining method showed that *E. denticulatum* UGPase was closely related to UGPase isolated from algae such as *Gracilaria lemaneiformis, G. gracilis* and other eukaryotic tissues. Multiple alignment analysis using ClustalW revealed that the deduced amino acid showed high identity with other plant species and the putative amino acid sequence of EdUGP contained five possible highly conserved Lys residues forming the active site of UGPase (Figure 1).

The wild type and mutant recombinant UGPase were over-expressed in *E. coli* pQE-2 and purified. The enzyme was a monomeric protein with apparent molecular weight of 55 kDa as determined by SDS-PAGE gel electrophoresis. The purified UGPase was kinetically characterized and the enzyme had optimum reaction conditions for pyrophosphorylase direction at pH 7.6 and 37 ºC, with a specific activity of 0.44 U/mg. Based on the site-directed mutagenesis study, we found that the point mutation on Lysine-491 (K491A) (Figure 2) resulted in 10% higher activity compared to wild-type (0.4774 U/mg).

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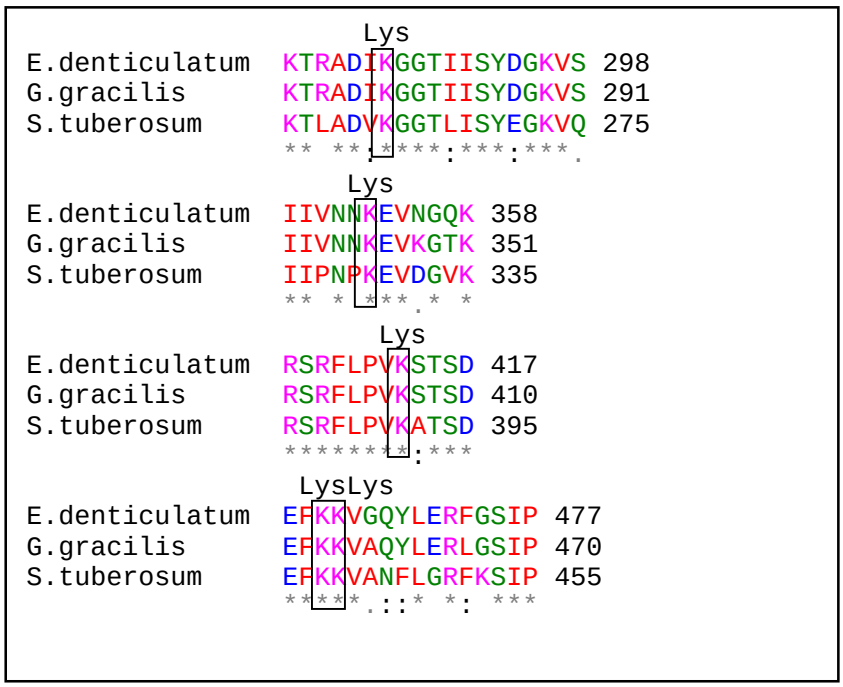

Figure 1. Amino acid alignment of EdUGP with other plant UGPase proteins using ClustalW. The sequence conservation around the Lys residues forming the active site of UGPase.

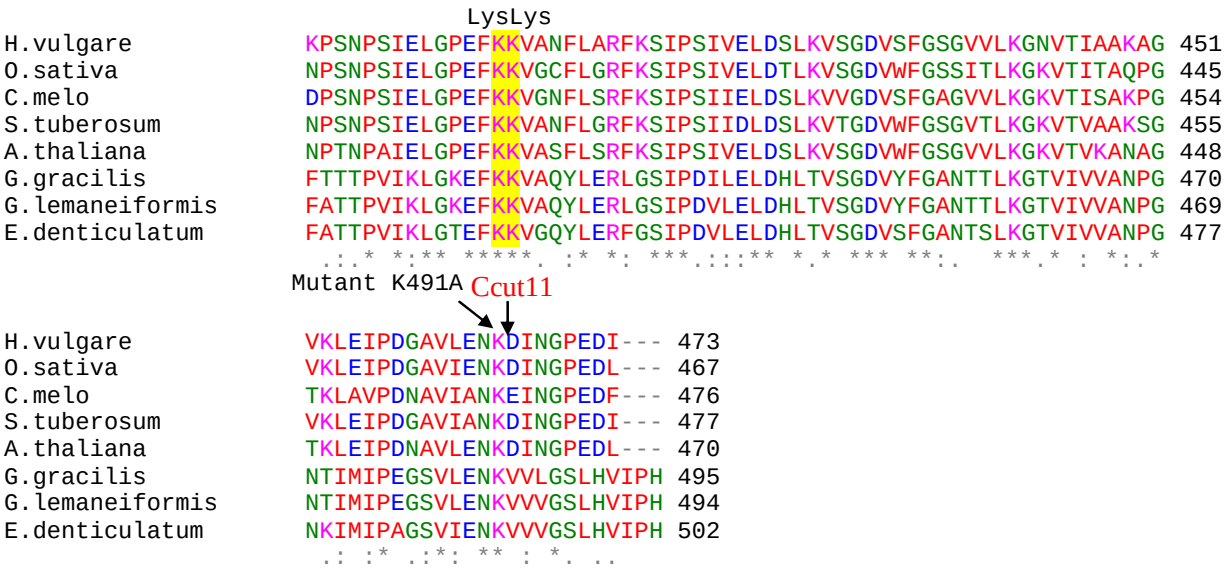

Figure 2. Amino acid alignment of EdUGP with other plant UGPase proteins using ClustalW showing the point mutation at Lys491 which is conserved in all plant UGPase gene sequences.

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Conclusions

A full-length cDNA clone encoding UGPase, EdUDP, with the size of 1.5 kb was successfully isolated. It shared high amino acid sequence identities with other plant UGPases and contains conserved Lys residues believed to play important roles for catalysis and substrate binding. Recombinant protein of wild-type and mutant UGPase were successfully expressed and assayed. Mutation of a conserved lysine residue at the C-terminus seemed to improve enzyme activity.

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Characterization of Protein Biomarker for Detection of Dieback Disease in Papaya

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Introduction

Papaya fruit is of considerable economic importance in Malaysia accounting for 21% of the global export market in 2004 (Chan and Baharuddin, 2010). However since 2005, papaya production in Malaysia faced a major threat from Papaya dieback disease which caused rapid decline in production. The disease affects all popular export varieties such as Eksotika, Solo and Sekaki and is characterized by leaf spot as well as greasy spot and water soaked lesions in all of the plant parts including leaves, fruits and stem. Later, dieback of the infected shoot occurs, leading to the destruction of the papaya trees (Noriha et al., 2011). In 2009, the disease has destroyed nearly one million trees or about 800 ha out of 2100 ha papaya growing areas. Total yield loss was estimated 200,000 tons, equivalent to USD 60 millions (Chan and Baharuddin, 2010).

The causal agent of papaya dieback disease was first reported as *Pantoea agglomerans* in 2005 (Mohamed and Wan Kelthom, 2005) and later as *Erwinia papayae* in 2008 (Maktar et al., 2008). Recently the disease was confirmed to be caused by *E. mallotivora* based on phenotypic observations, biochemical analysis and genetic studies (Noriha et al., 2011). Whole bacterial cells (i.e., *Erwinia*) and lipopolysaccharide (LPS), the major surface component of gram-negative bacteria specifically implicated in plant pathogenesis, are major antigens capable of initiating an immune response in various animal hosts. LPS is an outer membrane component composed of three distinct regions: O-specific oligosaccharide (consisting of long chains of repeating units), core oligosaccharide and lipid A (responsible for endotoxicity) (Yi and Hackett, 2000). There are different methods for extraction and purification of LPS including the use of butan-1-ol, petroleum ether, methanol-chloroform and phenol (Prescott et al., 2002). In the present study, we have employed a modified phenol-water extraction protocol accompanied with proteinase K digestion of bacterial proteins for extraction of LPS from *E. mallotivora* with high purity.

Materials and Methods

Bacterial Strains and Growth Conditions

E. mallotivora were grown in Luria Bertani (LB) agar at room temperature (24-25^{o}C) or 28-30^{o}C for 48 hours and then grown in broth medium at room temperature in incubator shaker for 48 hours. After centrifugation of culture media, sedimented bacteria were harvested and used for LPS extraction and purification.

LPS Extraction and Purification

LPS of *E. mallotivora* was purified by modified hot phenol-water extraction method. LPS was extracted following the method of Westpal and Jann (1965) with some modifications. In brief, 60 g of bacterial cells (10^8 colony-forming units/mL) were suspended in 350 mL distilled water at temperatures between 65 and 68 °C. In the next step, an equal volume of hot phenol (65-68°C) were added to the mixtures followed by vigorous shaking at 65 to 68° for 15 min. Suspensions were then cooled on ice, and centrifuged at 1800 g for 25 min. Phenol phases were re-extracted by distilled water at 65 to 68°C. The suspension was then cooled on ice and centrifuged at 3000 rpm using Beckman Coulter Centrifuge for 30 min. Extensive dialysis against distilled water at 4 °C was carried out at

the next level until the residual phenol in the aqueous phase was totally eliminated and opalescent solution obtained. The opalescent supernatant was then concentrated with polyethylene glycol. The extracts were stored at -80°C overnight to precipitate LPS. Crude LPS was then resuspended at 1mg/mL in distilled water. Extensive dialysis against distilled water at 4 °C was carried out in the next step until the residual phenol in the aqueous phases was totally eliminated. Final purified LPS product was lyophilized and stored at 4 °C.

Silver Staining

The purified LPS was solubilized in sample buffer to the desired concentration (1mg/mL), and boiled for 5 min. Each sample was separated in 16 µl/well in 15% SDS gel with 4% stacking gel under reducing conditions at 100 mA for 2 hr using mini-PROTEAN electrophoresis (BioRad, USA). Silver staining of the gels was performed according to the standard protocol.

LAL Assay

The potency of the purified LPS sample was evaluated using a quantitative, kinetic colorimetric Limulus Amebocyte Lysate assay (Lonza Kinetic-kQCL, USA). Briefly, a 0.1 mg/mL solution of the sample was diluted 100,000X and 1,000,000X in endotoxin-free water and assayed against Lonza's Control Standard Endotoxin (purified *E. coli* O55:B5); which is standardized against the United States Pharmacopoeia and European Pharmacopeia Reference Standard Endotoxin. The LAL assay was performed according to the manufacturer's instructions.

Results and Discussion

The result of silver staining after SDS-PAGE is shown in Figure 1. LPS purified by modified hot phenol-water extraction method showed high purity in comparison to crude as seen on silver staining. It was found to give typical lipopolysaccharide electrophoretic patterns with the ladder-like appearance due to the LPS-O-chain. Ladder-like bands are visible from approximately 35 to 60kDa in the crude and purified samples.

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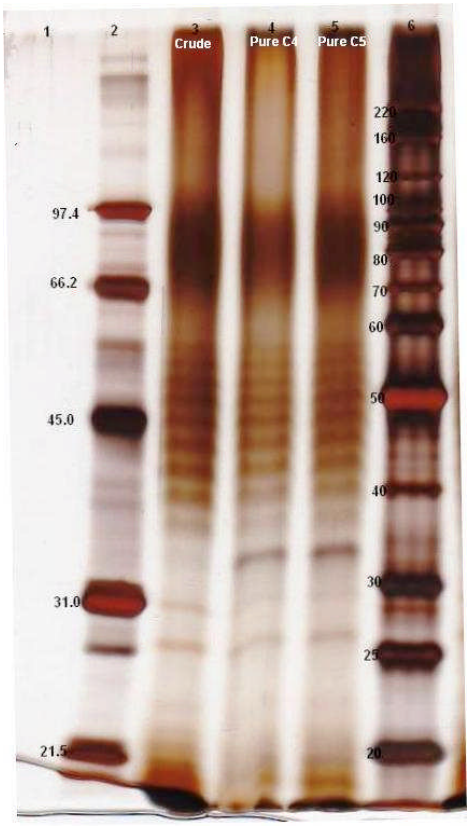

Figure 1. Silver staining of crude and purified LPS of *E. mallotivora*. Ladder pattern of LPS banding which is characteristic of smooth gram negative bacteria is seen. Lane 2, molecular weight marker; lane 3, crude *E. mallotivora*; lanes 4 and 5, purified LPS of *E. mallotivora*.

The results showed that the purified *E. mallotivora* LPS has a potency of 3.2 X 10^6 EU/mg. This showed that there is clear endotoxin reactivity contained in this preparation. However, the potency is 1 logs lower compared to the highly purified *E. coli* O55:B5 used as an international reference standard, which has a reported potency of between 2-50 EU/ng. This may be due to the purity of the preparation, or the inherent properties of endotoxin from these bacteria, as the potency of endotoxin of Gram-negative bacteria is known to vary across species. Lipopolysaccharides which is also known as lipoglycans, are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond; they are found in the outer membrane of Gram-negative bacteria, which act as endotoxins and elicit strong immune responses in animals. In this context, several attempts have been made to extract and purify LPS. The method introduced by Westpal and Jann (1965) is still the most frequently employed procedure for LPS extraction because of its high yield. Due to the presence of contaminating substances in the final purified LPS during extraction and purification process and their interfering effects in most downstream immunological and biological experiments, a practical approach for their elimination is necessary (Westpal and Jann, 1965). Purified LPSs were characterized by SDS-PAGE electrophoresis followed by silver staining. The results of silver staining clearly showed the ladder pattern of bands with multiple rungs characteristics of smooth type of gram negative bacteria due to the carbohydrate chain length variation of the O-antigen portion (Hendrick et al., 1983). LPS can be classified as either smooth or rough type based on the presence or absence of ladder-like structure due to lack of 'O' specific chain containing repeating units of oligopolysaccharides.

Although the purity of LPS is a good measure of the performance of purification system, functional activity of the final product is important as well. In conclusion, the protocol presented here could be employed for isolation of functionally active LPS with high purity from *E. mallotivora*.

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CHAPTER 6

PEST AND DISEASE MANAGEMENT

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A Potential of *Eurycoma longifolia* Jack. (Tongkat Ali) Leaves Extract: A Preliminary Study of Non-Chemical Insecticide Control on *Brassica campestris* sp. *parachinensis* (Green Mustard)

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Introduction

Planting green mustard vegetable (*Brassica campestris* sp. *parachinensis*) encounter a lot of disturbance especially insect *Plutella xylostella* (leaf worm) that attack green mustard plants starting from early planting until harvesting, and can cause 100% yield loss without pest control. Other insect pests that frequently damage the green mustard are soil worm (*Agrotis ipsilon*), snail (*Acanthina fulica*) and other kinds of leave bedbug (Henik, 2010).

Tongkat Ali or its botanical name *Eurycoma longifolia* belongs to Simaraubaceae family. Its leaves are compound pinnate, where the length of the leaves are between 5 to 10 cm and 1.5 to 4 cm wide spear eye shaped and tough (Chooi, 2004). This plant is used extensively in medical field and also becoming quite popular now as herbal plant (Goreja, 2004), but study on its potential as control pest insecticide is still lacking. Generally, *E. longifolia* plant is not attacked by any pest other than tiger worms that eat its leaves. *E. longifolia* resembles tobacco (*Nicotiana tabacum*) in terms of bitterness in taste. The tobacco leave extract proved to be usable as insecticide (Yuyun, 2002). This preliminary study was done to determine the effectiveness of the *E. longifolia* leaves extract as a potential non-chemical control pest insecticide on green mustard vegetable.

Materials and Methods

The study was conducted at the nursery, School of International Tropical Forestry, Universiti Malaysia Sabah (UMS). The green mustard vegetables aged three weeks were obtained from a vegetable farmer in Kinarut, Sabah. The vegetables were selected based on the size of the least damaged leaves and fertile leaves, and were planted in polybags. The *E. longifolia* leaves were obtained from Bukit UMS. Meanwhile, tobacco leaves and red chilli fruit (*Capsicum* sp.) were bought from Gaya Street market, Kota Kinabalu.

Method of preparing the extract were based on the method to extract tobacco leaves by Subiyakto (2005). The leaves were dried in an oven at 40^{o}C for a week before being crushed into medium size pieces amounting to 35 g and soaked in 1000 ml distilled water for a week. Filtered extracts were kept in enclosed jar. Five treatments were used in this study, namely, *E. longifolia* leaves extract (T1), tobacco leaves extract (T2), red chilli fruit extract (T3), insecticide Wesco Malathion (T4) and control (5).

The extracts and insecticide were sprayed three times a week by using Triger Pump at a rate of 2.5 ml per plant for the first week and 5 ml for the second week. As for the third and fourth week, the extracts were sprayed at a rate of 10 ml per plant due to plant size. The same spraying rates were applied to each of the replicates. The insect pests were identified and leaf area damaged by the pests were measured (in mm^2) everyday from the first week until the fourth week. At the fourth week, the green mustard vegetable reached the age of seven weeks which has achieved the maturity for the green mustard (Halimatul, 2008).

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The means of leaf area damaged were compared between the treatments by using One-Way Analysis Variance (ANOVA).

Results and Discussion

Five common insect pests that damaged the green mustard leaves were identified as caterpillar, grasshopper, beetle, bedbug and snail. Figure 1 shows some of the leaf damaged by the insect pests. The results show significant difference ($p<0.05$) between the treatments where *E. Longifolia* leaves extract recorded the least damaged area (1665 mm²), followed by tobacco leaves extract (2405 mm²), chilli fruits extract (3661 mm²), insecticide (4466 mm²) and control (9053 mm²) (Figure 2). Based on the previous study, tobacco leaves extract (Eka, 2006) and chilli extract (Henik, 2010) were suggested for control of insect pests. This study shows that *E. Longifolia* leaves extract performed better in controlling insect pest on the green mustard leaves as compared to both tobacco leaves and red chili extracts.

Figure 1. Some of the green mustard leaves damaged by the insect pests

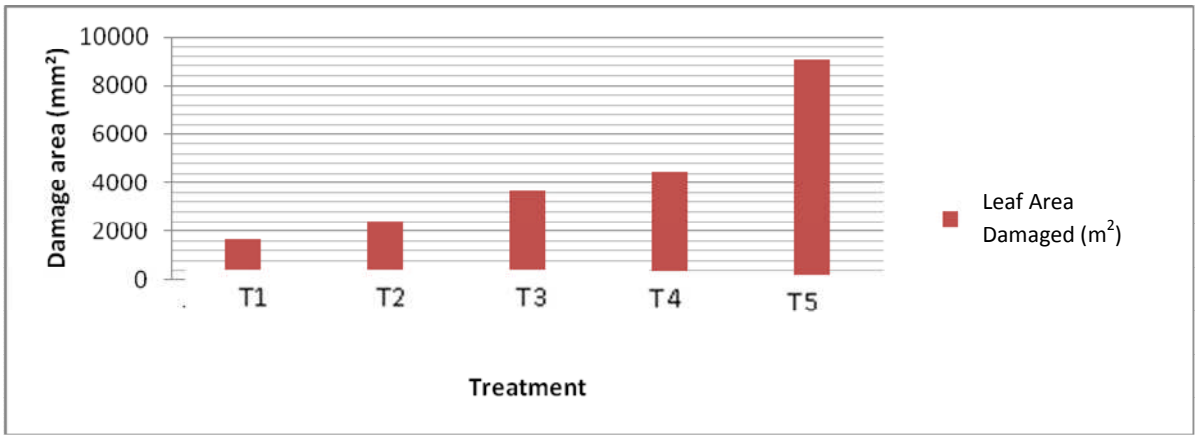

Figure 2. Leaf area damaged on green mustard by insect pests after spraying with differing pest control treatments.

Conclusions

The total damaged area of green mustard leaves were significantly difference among the treatments. *E. longifolia* leaves extract treatment shows the lowest total damaged area of green mustard leaves compared with the rest of the treatments. Based on this result, *E. longifolia* leaves extract has a potential to be used as an environmentally friendly insecticide on vegetables.

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Detection of S. Enteritidis in Artificially-Contaminated Raw Chicken Meat by Polymerase Chain Reaction

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Introduction

Salmonella enterica subspecies *enterica* serovar Enteritidis (*S.* Enteritidis) is the world-leading cause of salmonellosis and is often implicated in cases of human salmonellosis in Europe (Thorns, 2000), USA, China and England (D'Aoust and Maurer, 2007). *S.* Enteritidis is one of the most frequently isolated serotypes of *Salmonella* in poultry and poultry products from human food poisoning cases. In Malaysia, *S.* Enteritidis is among *Salmonella* serovar that was isolated from retail poultry samples (Rusul et al., 1996) with undercooked or raw eggs and poultry meat been a high risk for infection in humans (Gillespie et. al., 2005). The pathogen can cause mild to acute gastroenterititis in addition to other food poisoning symptom when infection occurred in human. Conventional culture methods for detection of *S.* Enteritidis in foods are laborious and time-consuming, taking 4-7 days to obtain results. Nucleic acid amplification technologies such as Polymerase Chain Reaction (PCR) have proved to provide rapid and sensitive tools for detection of bacterial pathogen. In this study, we reported the detection of *S.* Enteritidis by PCR using ENT primers targeting at 303bp of *sdfI* gene in artificially-contaminated raw chicken meat. Gene *sdfI* is a chromosomal region related to the invasiveness and infection of poultry and eggs. Thus, ENT primers targeting this gene fragment was used in this study and tested with some important *Salmonella* serovar to determine its sensitivity and specificity.

Materials and Methods

Bacterial Strains and Culture Conditions

Salmonella enterica subspecies *enterica* serovar Enteritidis isolated locally from chicken was used as a reference culture in this study. Culture from ATCC, *Salmonella* Typhimurium ATTC™ 53648, *Salmonella* Pullorum ATTC™ 10398 and *Salmonella* Gallinarum ATTC™ 9184 were used as reference serovar strains for primer specificity evaluation. All *Salmonella* serovar in this study were cultured overnight in TSB medium (Oxoid) at 37^{o}C, 150 rpm. For artificial inoculation of samples with *S.* Enteritidis, ten-fold serial dilutions of the overnight *S.* Enteritidis culture were prepared in TSB. To determine the number of cells for inoculation, 100 □l of 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} serial dilution culture were spread-plated on tryptone soy agar (TSA, Oxoid) in triplicate and incubated at 37^{o}C for 24 h.

Inoculation of Raw Chicken Meat Samples

Chicken meat breasts were purchased from local supermarket. Ten grams of each meat sample was autoclaved, transferred in a sterile 400 mL stomacher bag and inoculated with 100 µL of each *S.* Enteritidis dilutions. An uninoculated negative control was prepared by seeding 100 µL of sterile buffered peptone water into corresponding sample.

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Enrichment and Crude DNA Extraction

Following inoculation, each meat sample was homogenized at 230 rpm using stomacher (Stomacher®400 Circulator, Seward) for 1 min in 90 ml of sterile buffered peptone water (BPW, Oxoid). Each homogenate was transferred to a sterile 250-mL Erlenmeyer flask incubated at 37°C for 24 h. Crude DNA extraction was performed on each homogenate and each strain of pure culture. A 1mL portion of each broth culture was centrifuged at 15,000xg for 4 min. The pellet was resuspended in 500µL sterile distilled water and vortexed vigorously. The cell suspension was boiled for 10 min, immediately chilled on ice for 10 min and centrifuged again at 15,000xg for 4 min. The supernatant containing crude DNA was transferred into a new tube and 5 uL was used as DNA template in PCR.

Primers and PCR Amplification

Primers, ENT-F and ENT-R (Alvarez et al., 2004) for PCR was chosen to be used in this study after evaluation using *in silico* PCR program (insilico.ehu.es) against all genome database of *Salmonella* genus and by BLAST program (ncbi.nlm.nih.gov). Primers sequence used in this study is shown in Table 1. A reaction volume of 25 µL of PCR mixture using 1X PCR master mix (containing 2 mM $MgCl_2$, 0.025U/µL *Taq* DNA polymerase and 0.2 mM of each dNTP), 0.5 µM of each ENT primer, 5 µl of crude DNA extract, and nuclease-free water adjusted to a total volume of 25 µl. PCR reaction was performed out in a thermocycler (DNA Dyad, BioRad). The thermocyler was programmed by preheated 2 min at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 57°C, 30 s at 72°C and final extension for 4 min at 72°C. A 5µL of PCR product was analysed by electrophoresis on 2% agarose gel and stained with ethidium bromide to visualize the amplicons under UV light.

Table 1. Primers used in this study.

Primer name	**Sequences (5'- 3')**	**Gene**	**PCR product (bp)**
ENT-F	TGTGTTTTATCTGATGCAAGAGG	*sdfI*	304
ENT-R	GAACTACGTTCGTTCTTCTGG		

Results and Discussion

The concentration of *S.* Enteritidis for artificial inoculation in raw chicken samples was determined by plate count method. Plate 10^{-6} dilution was selected for plate counting since colonies grown on the plate ranging between 25-250 colonies which are recommended as countable range according to US Food and Drug Administration Bacterial Analytical Manual (BAM). The overnight *S.* Enteritidis stock culture was determined to be 9.4 X 10^8 cfu/mL. The stock culture was used for the serial dilution preparation prior to inoculation to raw chicken meat samples.

In silico PCR results showed that the primers amplified PCR product with *S.* Enteritidis only by generating a theoretical product of 303bp (lane 6) when tested with all 27 strains of *Salmonella* genome database (Figure 1). In addition, BLAST result showed that both ENT-F and ENT-R primers have 100% sequence similarity to *S. enterica* subsp. *enterica* serovar Enteritidis accession no. AM933172.1, AF370707.1 and AF370716.1, suggested that this primer set is specific to the targeted *S.* Enteritidis bacteria. The PCR procedure used in this study successfully amplified DNA of 303bp with crude DNA from *S.* Enteritidis, whereas no amplification for the other three *Salmonella* serovar, *S.* Typhimurium, *S.* Pollarum and *S.* Gallinarum crude DNA (Figure 2). The PCR results demonstrated that the product against the ENT primers was only present in *S.* Enteritidis serovar which confirmed the specificity of the ENT primers against *S.* enteritidis serovar. While the PCR sensitivity limit of the artificially-contaminated raw chicken meat samples in BPW was determined at 94 cfu/mL of inoculated *S.* Enteritidis concentration (Figure 3).

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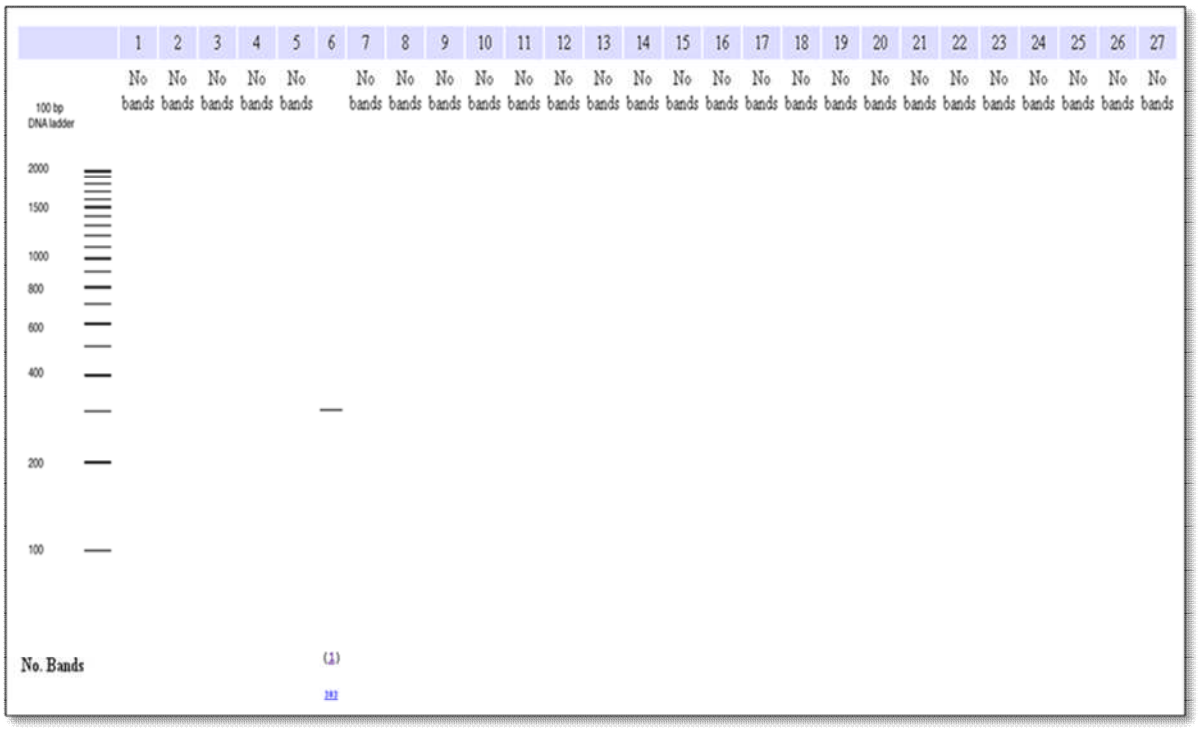

Figure 1. *In silico* PCR result of ENT primers against 27 strains of *Salmonella sp.*

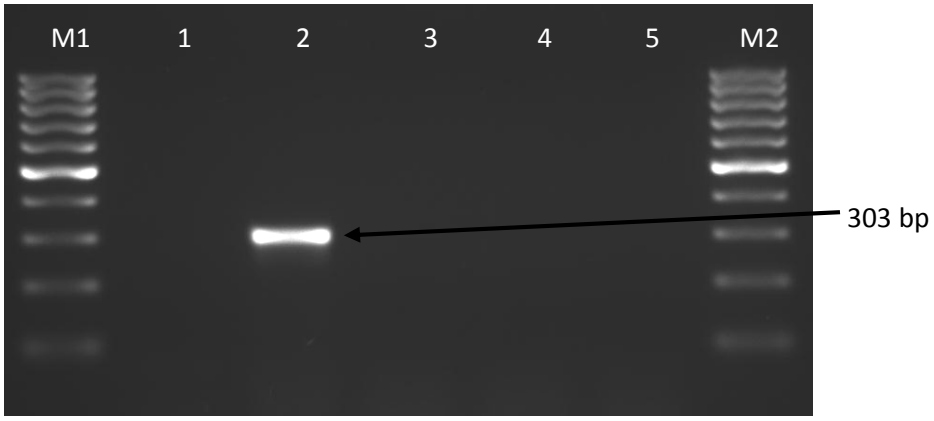

Figure 2. Specificity of PCR with ENT primers using crude DNA from selected *Salmonella* serovars.
Lane M1, M2: 100bp DNA ladder (Fermentas)
Lane 1: Negative control
Lane 2: *S.* Enteritidis
Lane 3: *S.* Typhimurium
Lane 4: *S.* Pollarum
Lane5: *S.* Gallinarum

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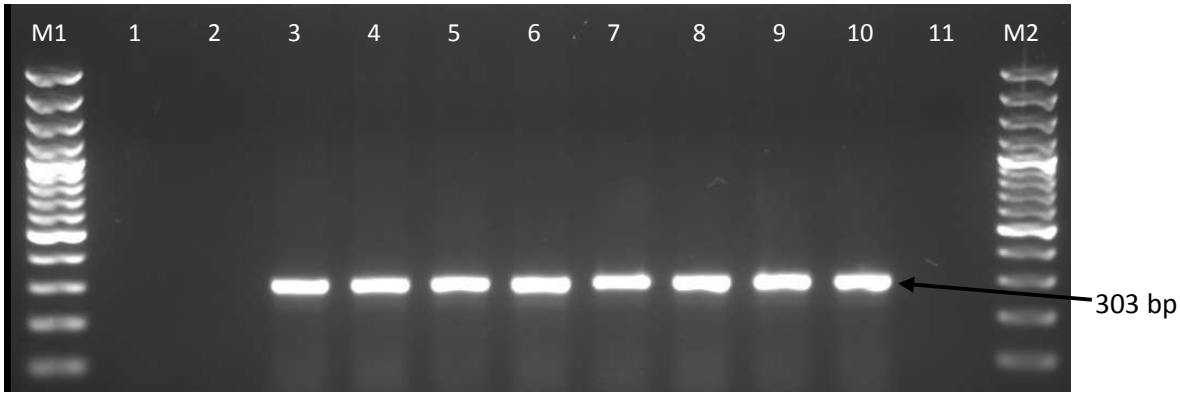

Figure 3. Sensitivity of PCR for *S.* Enteritidis detection in enrichment cultures of raw chicken meat samples inoculated with various concentrations of *S.* Enteritidis.
Lane M1 & M2: 100bp DNA ladder plus (Fermentas)
Lane 1 : Negative control (no DNA template)
Lane 2 : Negative control (spiked with sterile BPW)
Lane 3 : Raw chicken meat samples inoculated with 9.4 X 10^8 cfu/mL *S.* Enteritidis culture
Lane 4 : Raw chicken meat samples inoculated with 9.4 X 10^7 cfu/mL *S.* Enteritidis culture
Lane 5 : Raw chicken meat samples inoculated with 9.4 X 10^6 cfu/mL *S.* Enteritidis culture
Lane 6 : Raw chicken meat samples inoculated with 9.4 X 10^5 cfu/mL *S.* Enteritidis culture
Lane 7 : Raw chicken meat samples inoculated with 9.4 X 10^4 cfu/mL *S.* Enteritidis culture
Lane 8 : Raw chicken meat samples inoculated with 9.4 X 10^3 cfu/mL *S.* Enteritidis culture
Lane 9 : Raw chicken meat samples inoculated with 9.4 X 10^2 cfu/mL *S.* Enteritidis culture
Lane 10: Raw chicken meat samples inoculated with 9.4 X 10^1 cfu/mL *S.* Enteritidis culture
Lane 11: Raw chicken meat samples inoculated with 0.94 cfu/mL *S.* Enteritidis culture

Conclusions

The ENT primers had successfully amplified the targeted fragment of *sdfI* gene yielded product sized 303bp. The primers were specific to *S.* Enteritidis and sensitive, which can detect as low as 94 cfu/mL of inoculated S. *Enteritidis* culture after overnight incubation.

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Evaluation of Primer Set Targeting *Inva* Gene of *Salmonella* sp. by *In Silico* PCR Simulation against Major Foodborne Pathogens

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Introduction

In silico Polymerase Chain Reaction (PCR) via computer simulation aims to provide an easy way to analyse and obtain the theoretical PCR results we may expect from DNA, by using up-to-date sequenced bacterial genomes based on genome database (Bikandi, J. et. al., 2004). PCR allows amplification of specific DNA sequences by the use of primers, DNA template and PCR reagents in an optimized cycling condition. *In silico* PCR technique predicts the theoretical PCR products based on the input primer sequence against the chosen bacterial genome to be tested. Here, we report the use of *in silico* PCR program available at http://insilico.ehu.es to analyse primer set for *invA* which had previously been published (Rahn. et. al., 1992) to demonstrate the usefulness of the program in obtaining theoretical PCR products. In this study, primers invA-F and invA-R targeting *Salmonella* invasion gene, *invA* was evaluated by *in-silico* PCR against prokaryotic genome of major foodborne pathogens. A total of 127 strains of bacterial genome sequences from *Salmonella* sp. (27), *Escherichia* sp. (59), *Listeria* sp. (26) and *Campylobacter* sp. (15) were used as bacterial genome templates in the PCR simulation analysis. *InvA* primers targeting this gene fragment were used in this study and tested in PCR with some important *Salmonella* serovar to determine its amplifiability and specificity.

Materials and Methods

In Silico PCR and BLAST Analysis

Primers, invA-F (5'-GTGAAATTATCGCCACGTTCGGGCAA-3') and invA-R (5'-TCATCGCACCGTCAAAGGAACC-3') for PCR was chosen to be used in this study. They were evaluated using *in silico* PCR program (http://insilico.ehu.es) against genome database of *Salmonella* sp., *Escherichia* sp., *Listeria* sp. and *Campylobacter* sp. by BLAST program (www.ncbi.nlm.nih.gov) to predict their specificity and product sequence and size.

Bacterial Strains and Culture Conditions

Bacterial cultures used in this study were obtained from ATCC. *S. typhimurium* ATTC™ 53648, *S. pullorum* ATTC™ 10398, *S. gallinarum* ATTC™ 9184 and local isolate *S. enteritidis*. They were used as reference serovars for primers specificity evaluation. All *Salmonella* serovars were cultured overnight in TSB medium (Oxoid, USA) at 37 ^{o}C, 150 rpm.

Crude DNA Extraction

Crude DNA extraction was performed on each strain of pure culture. A 1 mL portion of each broth culture was centrifuged at 15,000x g for 4 min. The pellet was resuspended in 500 µL sterile distilled water and vortexed vigorously. The cell suspension was boiled for 10 min, immediately chilled on ice for 10 min and centrifuged

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again at 15,000x g for 4 min. The supernatant containing crude DNA was transferred into a new tube and 5µL was used as DNA template in PCR.

PCR Amplification and Analysis of PCR Products

A reaction volume of 25 µL of PCR mixture using 1X PCR master mix (containing 2 mM $MgCl_2$, 0.025 U/µL *Taq* DNA polymerase and 0.2 mM of each dNTP), 0.5 µM of each *invA* primer, 5 µl of crude DNA extract, and nuclease-free water adjusted to a total volume of 25 µl. PCR reaction was performed in a thermocycler (DNA Dyad, BioRad, USA). The thermocyler was programmed by preheated at 95 ^{o}C for 2 min, followed by 30 cycles of 95 ^{o}C for 30 s, 57 ^{o}C for 30 s, 72 ^{o}C for 30 s and final extension at 72 ^{o}C for 4 min. A 5 µL of PCR product was analysed by electrophoresis on 2% agarose gel and stained with ethidium bromide to visualize the amplicons under UV light.

Results and Discussion

The *invA* primers amplified *in silico* a band of 285 bp PCR product with all strains of *Salmonella* sp. except *S. bongori* and *S. enterica* subsp. *arizonae* when the mismatch of the primers was set to zero (Figure 1). Mismatch is the recognition errors between primers and DNA template. Zero mismatch make theoretical experiment very stringent and primers were very specific to the intended template. However, when less stringent theoretical experiment was used by setting the mismatch to 1, an additional band of identical size (Figure 2, Lane 2) was amplified *in silico* from *S. enterica* subsp. *arizonae* which demonstrated that the *invA* primers were capable to amplify DNA form all *S. enterica* strains including the subspecies *arizonae*. In addition, BLAST result showed that both invA-F and invA-R primers have 100% sequence similarity to *Salmonella enterica* subsp. *enterica* which imply the primers ability to amplify all *S. enterica* strains which was in agreement with the *in silico* PCR results. No cross-reaction was obtained with other bacterial genomes which indicated that the primer set was specific to *S. enterica* only (Figures 3 to 5). PCR experiments using invA-F and invA-R that were carried out in the laboratory had successfully amplified the expected 285 bp products using DNA from *S. typhimurium*, *S. enteritidis*, *S. polarum* and *S. gallinarum* (Figure 6). Based on the *in silico* PCR and BLAST results, it is assumed that these primers are able to amplify PCR products from other *S. enterica* subsp. *enterica* serovars other than those tested in our study (Fach et al., 1999).

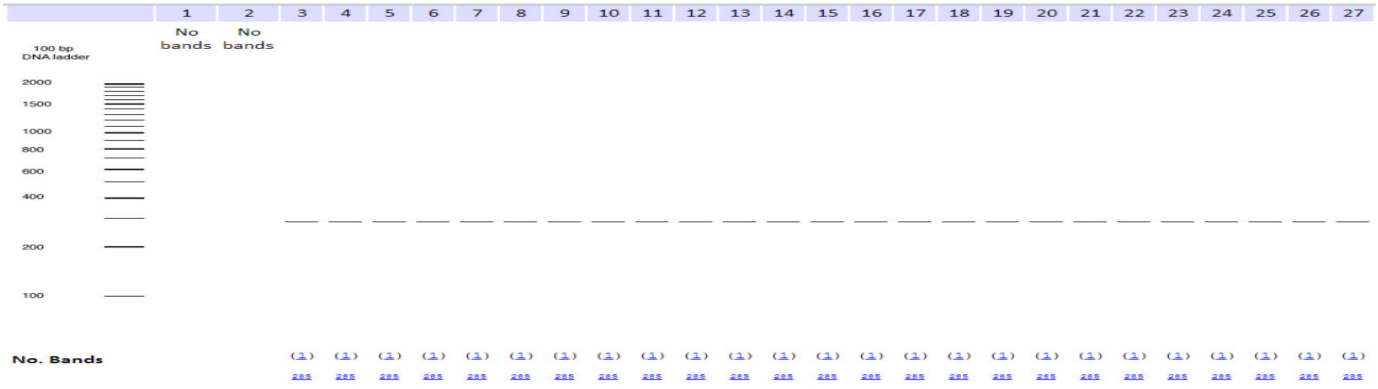

Figure 1. *In silico* PCR result of invA primers against all Salmonella serovar (Primer mismatch = 0)

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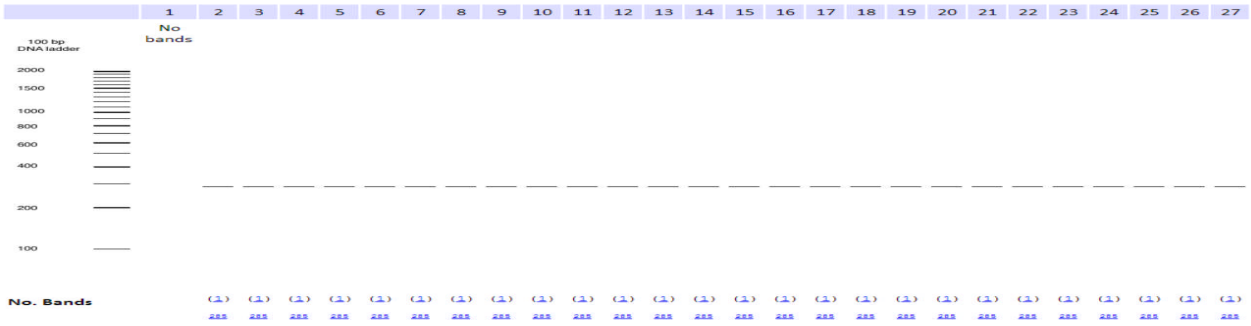

Figure 2. *In silico* PCR result of invA primers against all Salmonella serovar (Primer mismatch = 1)

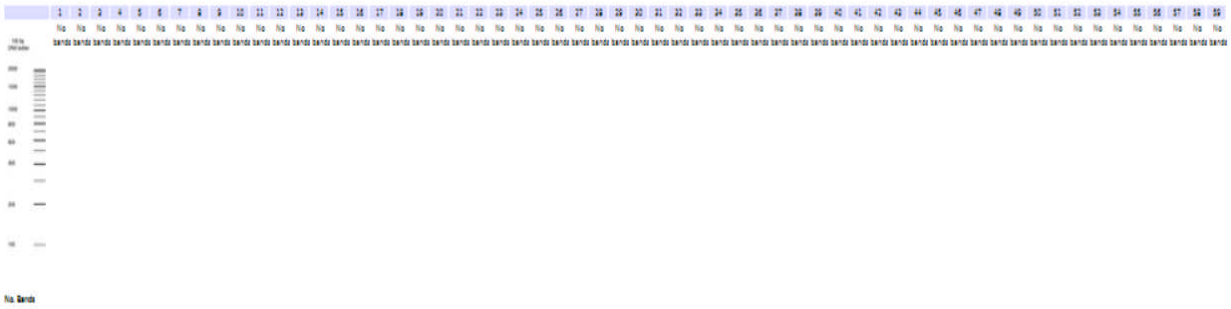

Figure 3. *In silico* PCR result of invA primers against *E. coli* (Primer mismatch = 0)

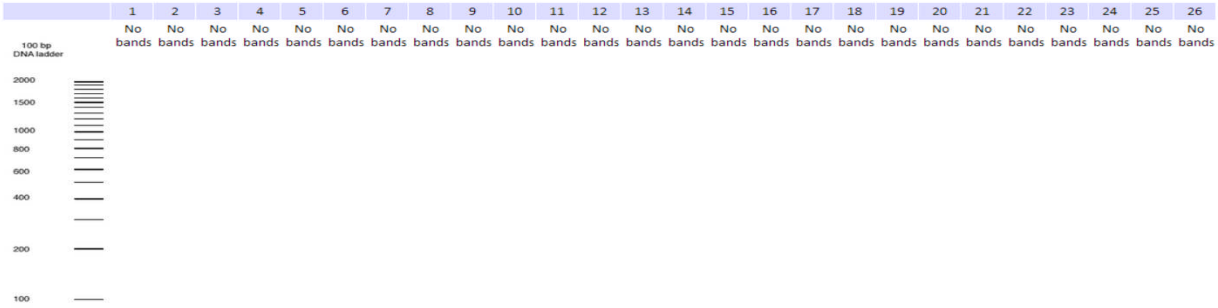

Figure 4. *In silico* PCR result of invA primers against *Listeria* sp. (Primer mismatch = 0)

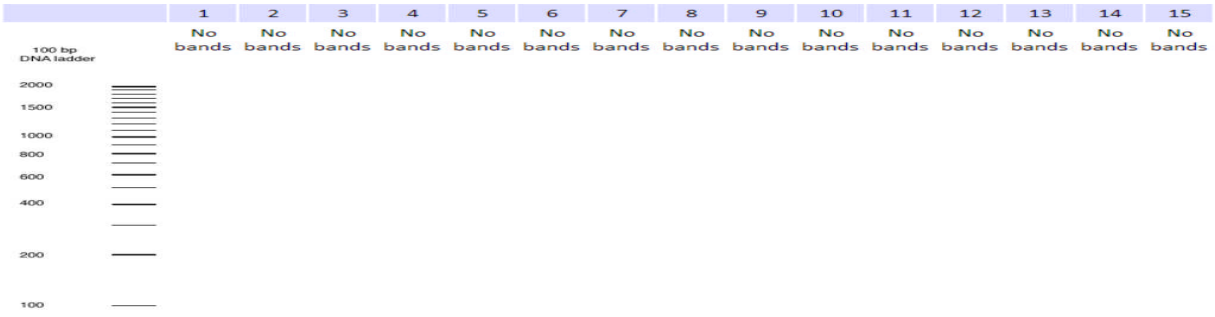

Figure 5. *In silico* PCR result of invA primers against *Campylobacter* sp. (Primer mismatch = 0)

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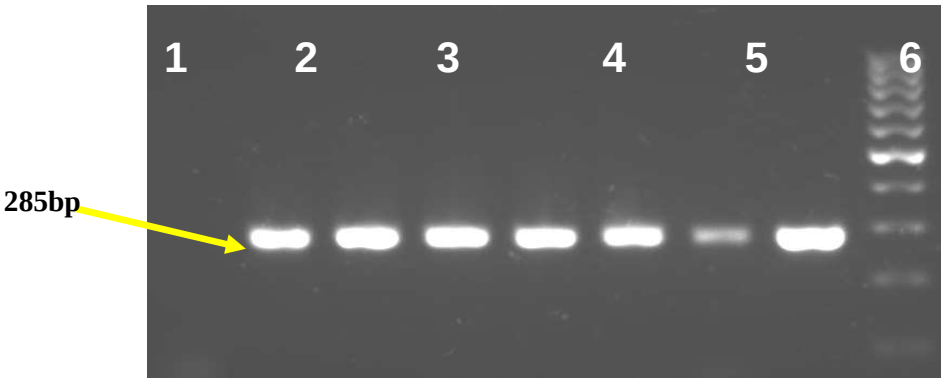

Figure 6. PCR with invA primers using crude DNA from selected *Salmonella* serovars
Lane 1: Negative control
Lane 2: *S. typhimurium*
Lane 3: *S. enteritidis*
Lane 4: *S. pollarum*
Lane 5: *S. gallinarum*,
Lanes 6 and 8: *S. typhimurium* (pure DNA)
Lane 7: Diluted *S. typhimurium* (pure DNA)
Lane M: 100 bp DNA ladder

Conclusions

In silico PCR is very useful to test primers specificity against targeted bacterial genome and able to predict the theoretical products size and sequence. Our study had showed that the *in silico* PCR have agreement with BLAST results as demonstrated by our evaluation of *invA* primers by *in silico* PCR against prokaryotic genome of major foodborne pathogens. The i*nvA* primers had successfully amplified the expected 258 bp *invA* gene fragment with some important *Salmonella* serovars which explained the usefulness of this technique. Nonetheless, further work need to be done to confirm the efficiency of the primers with other *S. enterica* serovars.

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Production of Polyclonal Antibody Against *Pyricularia oryzae* Using Germinating Conidia as an Antigen Preparation

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Introduction

Rice diseases are among the most significant limiting factors that affect rice production, causing annual yield losses conservatively estimated at 5% (Song and Goodman, 2001). More than 70 diseases caused by fungi, bacteria, viruses or nematodes have been reported on rice (Manandhar et al., 1998), among which rice blast is the most serious of disease affecting rice productivity (Song and Goodman, 2001).

Rice blast disease or Penyakit Karah, is caused by *Pyricularia oryzae* Cav. (previously known as *Magnoporthe grisea*). It is the most destructive of fungal diseases in rice crop and has caused significant yield losses worldwide including Malaysia with potential yield losses of more than 50 percent (Yang et al., 2012). According to the locations of the infected sites, rice blast is classified into seedling blast, leaf blast, rice node blast, neck blast and corn blast. The cycle of this fungal disease begins when a blast infects and produces a lesion on all parts of the shoot, as well as stem rot and panicle blight and ends when the fungus sporulates and releases new airborne spores (Ou, 1980). Rice blast disease moves from disease plants to healthy plants in nearby fields by airborne spores. A visual examination is not sufficient to discriminate between the pathogenic and non-pathogenic spores (Smiley et al., 1992). Presently, pathogenic spores can only be confirmed in a minimum of five to six days later by the development of lesion on the rice plants. By the time visible lesions have developed on the rice plants, it may be too late to eradicate rice blast by the application of fungicide resulting in the destruction of all or large portion of the entire crop (Smiley et al., 1992).

Nowadays, one of the most widely used methods of controlling rice blast is by spraying fungicide (Yang et al., 2012). However, intensive spraying requires an excessive amount of fungicide which increases cost, pollutes the environment and results in the development of multi-resistant fungal strains (LaMondia and Douglas, 1997). In some countries spore traps are used to predict rice blast epidemics. However the available traps are expensive and predictive data based on the number of spores trapped are applicable to the immediate area only.

Therefore, accurate and rapid identification of rice blast disease is essential for effective disease control. It enables more informed decisions to be made about cultivar choice and how and when fungicide can be used most effectively to control disease epidemics. Traditional approaches to rice blast diagnosis generally first involve the interpretation of visual symptoms (Stowell and Gelernter, 2001). This may be followed by laboratory identification, consisting of isolating the blast fungus from diseased tissue or trapped spores, culturing the fungus for spores and inoculating susceptible rice plants. But cultural isolation and identification is laborious, difficult and time-consuming (Ward et al., 2004). Other than that, several micro-detection based on molecular level such as polymerase chain reaction (PCR) and microscopy has also been developed. However, the major disadvantages of these methods are time-consuming and costly, requires professional operation and impossible to be applied in real-time detection (Yang et al., 2012).

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Recently, there has been a great progress in the development of very rapid diagnostic tests that are also simple. Therefore, the development of an inexpensive and accurate enzyme immunoassay screening kit for detecting *P. oryzae* in paddy field is highly desirable in deciding upon appropriate control for this fungus. In this work we have prepared germinating conidial suspension at concentration of 10^8 spores ml^{-1} as an antigen for immunization in rabbits for the production of polyclonal antibody.

Materials and Methods

Isolation and Culture of Fungi

The isolate of race 7.0 of *Pyricularia oryzae* was isolated at the MARDI Seberang Perai, Penang and identified by lesion reaction on a series of differential cultivars of rice. Oatmeal agar was used for culturing the fungal of the isolates. Mycelial colony on the oatmeal plate was cultured at 37 ^{0}C for 10 days. Large quantities of mass-produced conidia on oatmeal were collected by washing the colony surface on the oatmeal plate with 1 ml of ultra-pure water. Then this was filtered with cheesecloth.

Preparation of Germinating Conidia for Antigens

The spore suspension was centrifuged at 1000 g for 10 min and re-suspended about 1 x 10^8 conidia/ml in phosphate buffer saline (PBS). The suspension, used as immunogen was aliquot and stored at -20 ^{0}C until use.

Immunization Procedures

The spore suspension was prepared following the step above, and mixed with an equal volume of Freund's Complete Adjuvant and emulsified. The antigen-adjuvant mixture was injected into the New Zealand Rabbit. The first injection was followed by two booster injections at a three-week interval. For the second injection, spore suspensions were mixed with Incomplete Adjuvant. The third injection was done with equal volume mixture of suspension and PBS.

Serum Preparation

Blood sample was collected in tube which was exposed to room temperature for 5 min. Anti-serum was prepared by centrifugation at 3,000 rpm for 10 min and subjected to titration by ELISA.

Indirect ELISA Method for Detemination of Antibody Titer

Indirect ELISA was performed for screening of antisera. Aliquots of conidial suspension were dissolved in carbonate coating buffer (10^7 spore ml^{-1}), dispensed 100 µl per well, and kept for 2 hours at 37 ^{0}C . Thus the coated wells were washed four times with PBST and the uncoated well surface bloaked with 1% BSA at 37 ^{0}C overnight. After further washing for four times with PBST, well plate was incubated at 37 ^{0}C for 1 hour with 100 µl per well of antisera ranging from 1: 1000, 1: 4000, 1: 16000 and 1: 64000. Washing four times with PBST again and then incubating at 37 ^{0}C for 2 hours with secondary antibody were done. After final washing for four times, 100 µl substrate solution p-NPP was added per well and incubated for 15 min followed by OD reading in ELISA reader at 405 nm (Figure 1).

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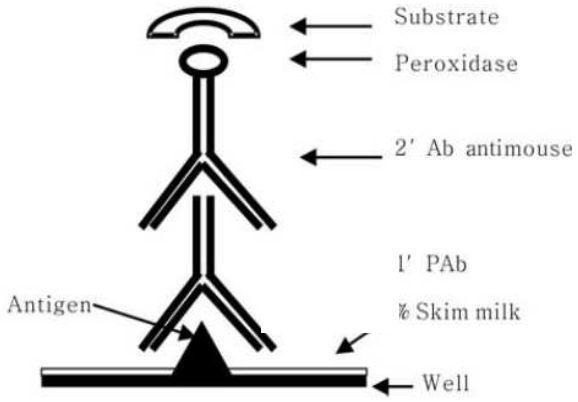

Figure 1. Scheme of indirect ELISA

Results and Discussion

In the present study, 10^8 conidia/ml has been used as an antigen for the production of polyclonal antibody against *P. oryzae*. After immunization, the titer of rabbit anti-sera against *P. oryzae* has been determined by indirect ELISA. Result in Figure 2 shows that the polyclonal antibody against *P.* was successfully developed by comparing the absorbance reading of the pre-immune, first bleed (1st bleed), second bleed (2nd bleed), third bleed (3rd bleed) and the negative control. Absorbance reading of the polyclonal antibody titer increased from the first to third bleed. Antibody production was the highest at the third bleed exceeded an absorbance of 3.0 at dilution 1:1000 while higher anti-serum dilutions resulted in weaker response which is at 1.0 at dilution 1:64,000.

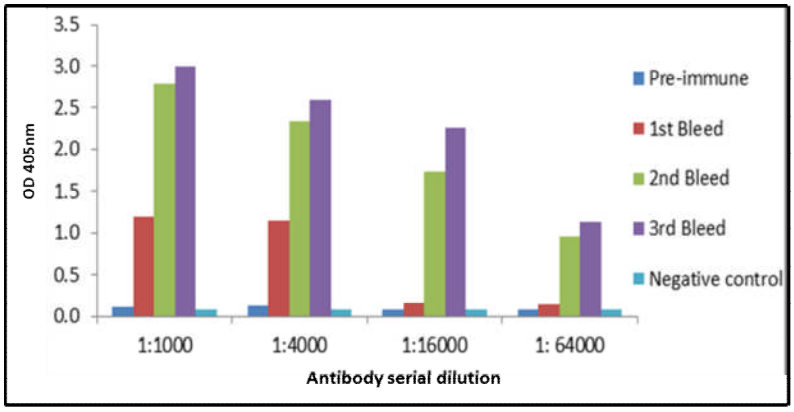

Figure 2. Titer of antibody against *Pyricularia oryzae*

An important aspect in raising polyclonal antibody against fungi for development of diagnostic probes is how to choose appropriate antigens (Xia et al., 1993). Theoretically, the choice of antigen is based on application of polyclonal antibody to specific fungal structures to be detected as well as on consideration of resulting specificity of the polyclonal antibody (Dewey et al., 1991). According to Xia et al., 1993, the immuno-gold labelling studies done by them shows that the epitope recognized by monoclonal antibody was found only in the cytoplasm of conidial cells but not in or on cell walls of conidia or in hyphae. Thus the choice of conidia as an antigen in this work is appropriate for production of polyclonal antibody.

The results confirm that the production of polyclonal antibody against *Pyricularia oryzae* using conidia as antigen is feasible by the titre results obtained. The titre of polyclonal antibody was determined by ELISA system by coating serial dilution of rabbit anti-sera. Absorbance value at 405 nm was 3.0 at 1: 1000 dilutions and was observable to the naked eye compared to control. This suggested that this polyclonal antibody could be used to develop early diagnostic kits.

Previous work by Xia et al., 1992 uses a monoclonal approach even though it is more specific. In other cases, it would be more useful to apply a polyclonal approach in the case of the detection of multiple pathogenic strain of the fungus.

Conclusions

In conclusion, polyclonal antibody is proven to be very sensitive and specific to the target pathogen, *Pyricularia oryzae*. Development of early detection kit for this fungal pathogen could save thousands of tonnes of rice lost yearly and the results obtained in this work is a promising start in realising this goal.

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Anthracnose-symptoms by Inoculation of *Colletotrichum capsici* and *Colletotrichum gloeosporioides* on Detached Ripen Chilli Fruit: Differences of Protein Patterns

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Introduction

Anthracnose disease is among the major plant diseases in chilli crop which caused by *Colletotrichum* spp. fungus. It is one of the most significant economic constraints to chilli production worldwide especially in tropical and sub-tropical regions, including Malaysia. Anthracnose disease can cause severe economic loss up to 50% which impair the crop productivity, fruit quality and marketability (Pakdeevaraporn et al., 2005). The disease symptoms are observed in three forms; (i) seedling blight or damping-off that is prevalent in the nursery, (ii) leaf spotting and die-back, which is initiated at different stages of growth; die-back infections starts from the growing point of secondary branches, gradually advancing downwards and inwards infecting the entire branch, and (iii) fruit spotting and rotting where mostly ripe fruits are infected. Anthracnose caused both pre- and post-harvest fruit decay. Therefore, fruit rot is a major significant impact which reduced crop yields. *Colletotrichum capsici* and *Colletotrichum gloeosporioides* have been reported to cause anthracnose disease in chilli crop in India, Indonesia, Korea and Thailand (Ratanacherdchai 2010). Yun et al. (2009) has reported these causal agents for anthracnose diseases in chilli for the first time in Sabah Borneo of Malaysia. C.*capsici* generally causes disease on ripe red fruits, while *C.gloeosporioides* produces disease on both green and ripe fruits. Its pathogenicity variability and approaches have leading to the disease management (Datar et al., 1995).

Plant responds to pathogen infection by producing a number of proteins believed to be important in protecting them from the deleterious effects of the pathogen. These proteins described as pathogenesis-related (PR) which is defined as any protein genetically coded by the host plant that is induced specifically in pathological or related situations following infection by a pathogen (Van Loon 1985). PR proteins are known to be selectively extractable in a buffer of low pH and are highly resistance to proteolytic degradation. The PR proteins have been classified into 14 families based on the amino acid sequences, serological relationship and/or enzymatic or biological activity. Many PR proteins purified from plants exhibit direct antifungal activity against a wide range of fungal pathogens . The activation of defense mechanisms in plants is considered to be consequent upon an initial recognition event in which the host plant detects molecular components of the pathogen, called elicitor. Several biotic elicitor including proteins, glycoproteins, peptides and lipids have been detected in germination culture fluid and cell walls of many phytopathogenic fungi (Kiba et al., 1999). These elicitor molecules bind to a receptors on the plasma membrane of plant cells and activate the signalling events required for the oncet of the defence responses. Knowledge of the mechanism of these pathogens infection and processes that trigger the host defence strategy would be informative towards developing rapid detection technologies for anthracnose disease, as well as supporting the sustainable green economy. Therefore, the aim of this study was to observe morphologically the pathogenicity effect of these both isolates on the susceptible chilli fruits and to identify any differences of proteins pattern extracted from the inoculated fruits, as preliminary study for investigate a role of defensive proteins in anthracnose chilli fruits.

Materials and Methods

Inoculation tests were done separately for each isolate of *C. capsici* and *C. gloeosporioides* on chilli wounded-surface by using plug inoculation method. Red chilli fruits which obtained from hypermarket were washed with sterile distilled water, swabbed with 70% ethanol (v/v) and dried on filter papers. Inocula were prepared by

culturing each isolate on PDA plate at room temperature for 10 days. Plugs (5 mm diameter) were cut from actively-sporulation areas near to colony periphery by using a sterilized cork borer and placed on the wounded-surface of chilli fruits. The inoculated fruits and controls (inoculated by placing only PDA plug) were transferred into humid plastic box and incubated for 25 days at room temperature. The developed lesions were observed and measured for each 5 days interval. After the incubation periods, the fruits were cut approximately 1cm size, followed by frozen with liquid nitrogen and stored at -20□C until used. For chilli protein extraction, samples were ground and homogenized in 0.1M phosphate buffer (EDTA, 2-mercaptoethanol) and centrifuged at 6000 rpm for 30 minutes. The protein extracts were precipitated with 60% ammonium sulphate and dialysed for overnight at 4□C. The electrophoretic of protein patterns were determined on 12% acrylamide SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) by Laemmli method and stained using silver staining method. The apparent molecular masses of proteins were determined by comparison with molecular mass standards (Promega).

Results and Discussion

Anthracnose disease was identified by small, brown, circular depressions and the fungus appeared as minute black acervulli on the surface of inoculated chilli fruits. In this study, the symptoms were observed and the lesion size was measured. The pathogens isolate were categorized based on the lesion size as described by Ratanacherdchai et al. (2010) as follow: 0-50 mm (low virulence, small lesion and no acervuli production 10 days after inoculation), 5.1-20 mm (moderate virulence, large lesion, tissue collapse and acervuli production 5 to 9 days after inoculation) and > 20 mm (high virulence, tissue collapse and acervuli production 1 to 4 days after inoculation). In this study, both *C. capsici* and *C. gloeosporioides* isolates showed to develop anthracnose lesions after 5 days of incubation after inoculated on the chilli wounded-surface. The observed symptoms were begun with water-soaked lesion, altered to dark and sunken lesion, and became circular rot with orange conidial masses. The measured lesion produced by *C.gloeosporioides* after 10 days of inoculation was 30mm, giving it more high virulence for disease incidence compared to *C.capsici,* which was only 15mm (Figure 1). These results were significantly correlated with their growth rate on PDA medium where *C. gloeosporioides* and *C.capsici* were achieved 11-12 mm and 5-6 mm per day of growth rates respectively. During the 25 days of incubation, the anthracnose lesions were fast gradually developed and achieved more than 20mm of lesion size towards rotted fruit.

The changed of protein profiles of inoculated chilli fruits during 25 days of incubation were analysed by SDS-PAGE. Figure 2 and 3 showed the accumulation of proteins from red chilli fruits as control was ranged between 10 to 100 kDa of molecular weight masses. Among them, the 35 kDa protein was predominant band and constitutively present at lower amount in control fruit but strongly induced once inoculated with *C. capsici* and *C. gloeosporioides* respectively. There were few protein bands which expressed differently in these inoculated chilli fruits. For *C. capsici* inoculated chilli fruits, the 20 kDa of protein band was present at lower amount in control fruit but it strongly induced after the pathogen inoculation particularly at 10 days of incubation, while the 10 kDa of protein band was gradually decreased onwards (Figure 2). However, 10 kDa-, 18 kDa- and 24 kDa-proteins from *C. gloeosporioides* inoculated fruit were strongly induced as compared to control, while the 25 kDa-protein was gradually decreased (Figure 3). Plants have evolved a defence mechanism by a group of cell wall proteins that called pathogenesis-related (PR) proteins which act as the first line of defence against fungal pathogen and induced along the infection. Shivashankar et al., (2010) also reported that a decline of polygalacturonase-inhibitor proteins towards fruit ripening in anthracnose susceptible varieties chilli has increased the susceptibility to fungal infection. Therefore, the strong and weak protein bands observed in this study might be involved in antifungal defence system, as they were induced during the infection periods.

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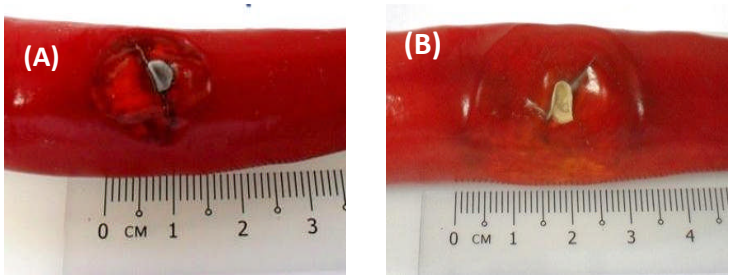

Figure 1. Anthracnose-symptom lesions produced by (A) *C. capsici* and (B) *C. gloeosporioides* at 10 days of incubation after artificial inoculation

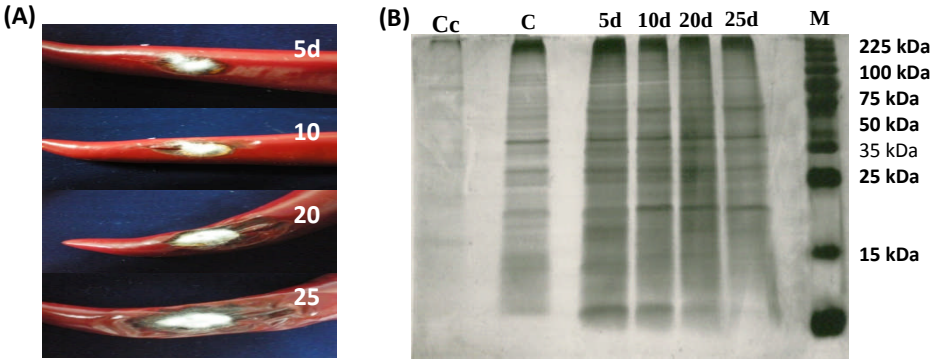

Figure 2. (A) Anthracnose symptom lesions developed during 25 days of incubation after inoculated with *C.capsici* (7 days growth) and (B) Electrophoretic pattern of extracted total proteins from *C. capsici*-inoculated detached ripen chilli fruit by using 12% acrylamide gel SDS-PAGE system; Cc (*C. capsici*); C (Control- non-inoculated fruit), 5d (5 days); 10d (10 days); 29d (20 days), 25d (25 days) and M (Promega broad range protein molecular weight marker)

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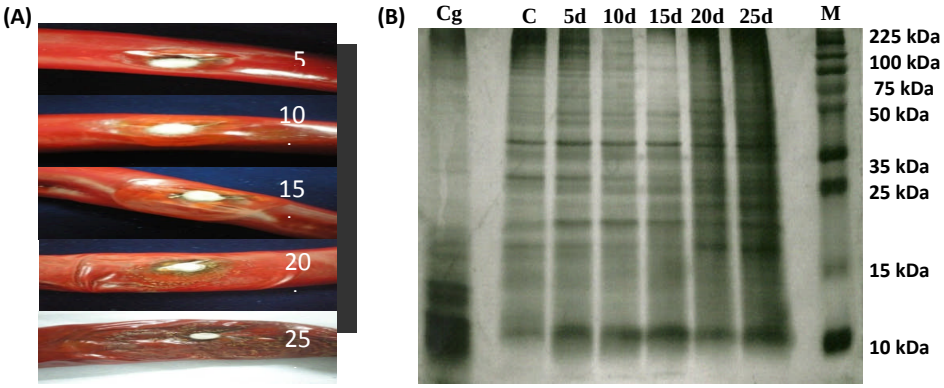

Figure 3. (A) Anthracnose symptom lesions developed during 25 days of incubation after inoculated with *C. gloeosporioides* (7 days growth) and (B) Electrophoretic pattern of extracted total proteins from *C. gloeosporioides*-inoculated detached ripen chilli fruit by using 12% acrylamide gel SDS-PAGE system; Cc (*C. gloeosporioides*); C (Control-non-inoculated fruit), 5d (5 days); 10d (10 days); 29d (20 days), 25d (25 days) and M (Promega broad range protein molecular weight marker)

Conclusions

C. capsici and *C. gloeosporioides* have shown the ability to develop anthracnose lesions on red chilli fruits after 5 days of artificial inoculation on fruit wounded-surface. The lesions were gradually developed during the incubation periods and caused fruit rot disease with damaged necrotic tissues. Differential proteins pattern with strongly and weakly induced during the pathogens infection might be involved in plant defence system. Therefore, knowledge of the mechanism of these pathogens infection and processes that trigger the host defence strategy would be informative towards developing rapid detection technologies for anthracnose disease, as well as supporting the sustainable green economy in crop productivity.

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Enterobacter spp. Found as the Dominant Bacteria in Infected Banana Farm at Sabak Bernam

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Introduction

Banana is among the important fruits cultivated in Malaysia. Present, banana is the second most important commercial fruit crops and listed as one of six fruits crops for development under the Entry Point Project of the National Key Economic Area for fruit production (Tengku et al., 2011). The area cultivated with fruits stand at 250 000 hectare and banana cover 27 500 hectare which constitute 11% of total fruits area in the country. The area is expected to expand in the future due to increasing local and foreign demand and newly implemented ETP by the government. In year 2009, Malaysia exported 19,934 metric ton of banana production value during the year is estimated to be RM452.4 million (Moktaruddin and Robert, 2011).

Disease is one of the most important factors that can significantly reduce the production of banana in Malaysia. Fusarium wilt caused by *Fusarium oxysporium* is one of the most devastating diseases contributing to low production and economic loss. The emerging of new bacterial disease which named Moko disease caused by *Ralstonia solanacearum*, has further dampened the banana industry. The disease was first observed to be attacking banana on 16th March 2007 in Pontian, Johore (Moktaruddin and Robert 2011). The yield loss can be up to 100%. Taking into consideration that 5 to 6 healthy bunches have to be destroyed per case of Moko infection, a production loss of 7.5 to 9 boxes (at 13 kilos per box) has to be expected for every mat infected with this disease. This adds up to thousands of kilos bananas and millions of RM that can be lost because of Moko infection.

Since banana is one of the export fruit and contribute in agricultural economy, it should be control from any diseases. In mid 2012, there have been reports from the villager that many of banana farms in Sabak Bernam, Selangor and Hilir Perak were infected with disease. In this study, we tried to find out the dominant microbe which might caused the banana disease from samples taken at Sabak Bernam.

Materials and Methods

Samples Collection

Samples were collected from infected banana farm at Sabak Bernam. Different parts of an infected banana tree (leaves, fruit, soil and banana trunk) were taken as samples for bacteria isolation and analysis. Healthy banana samples were also obtained from a villager.

Sample Processing and Bacterial Isolation

Samples were cut into small section and placed in tubes of 10 ml of sterile distilled water, shake for 30 minutes and spread on Kelman's tetrazolium chloride (TZC) medium. Spread plates were done until 10^{-5} dilution and incubate for 48 hours in room temperature. Colonies were observed for morphology.

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Genomic DNA Extraction and PCR Amplification of 16S rRNA Gene

Bacterial genomic DNA was isolated using Gen Elute Bacterial Genomic DNA Extraction Kit (Sigma-Aldrich, USA) according to protocol provided by the manufacturer. The universal primers ere used for PCR amplification of the 16S rRNA gene. PCR amplification was carried out in 25 ul reaction for 30 cycles using thermostable DyNAzyme^{TM} EXT DNA polymerase (Finnizymes, Finland) in PTC-200 thermal cycler (MJ Research, USA). PCR product was resolved using 1% of agarose gel and purified using QIA quick gel extraction kit (QIAGEN, Germany).

Cloning and Sequencing of 16S rRNA Gene

The purified PCR product as cloned directly into a vector using TOPO TA Cloning Kit (Invitrogen, USA). Plasmids DNA of recombinant clones were prepared using QIAprep Spin miniprep kit (QIAGEN, Germany). The plasmids were digested with *EcoRI* restriction enzyme to determine the presence of the cloned insert in the vector and sent for DNA sequencing service (Research Biolabs Technologies, Singapore). Nucleotide analysis sequence was perform using Bioinformatics tools at NCBI (http://www.ncbi.nlm.nih.gov/).

Results and Discussion

Villagers at Sabak Bernam reported that many of their banana farms were infected with disease. The banana plants showed sign and symptoms such as wilting and necrosis of the leaves, candle leaves turning black, discoloring and rotting of the banana fruits, and vascular tissue becomes discoloured in the middle and when cut and dip in water exudes bacterial ooze. Based on the sign and symptoms observed, the banana farms were likely infected with Moko disease which caused by bacteria *R. solanacearum*. Therefore different parts of an infected banana tree including leaves, fruit, soil and banana trunk were taken as samples for bacteria isolation and analysis. Healthy banana samples were also obtained from a villager for comparison.

Banana samples were spread on TZC media. After 48 hours incubation at room temperature, different colonies were observed on the spread plate. Full bacteria colonies were seen on infected samples and single colonies can only be seen on the dilution spread plate. Different observation was seen in healthy banana samples where bacterial colonies can be seen in undiluted samples and no bacteria were observed on the diluted samples.

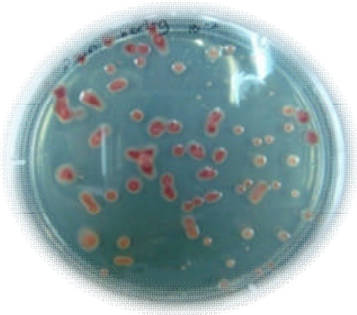

Figure 1. Single colonies on dilution spread plate

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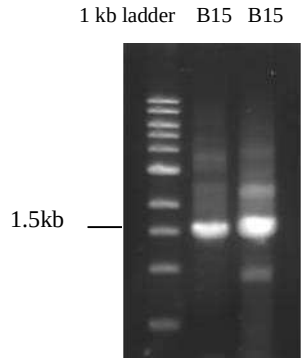

Figure 2. PCR amplification of 16S rRNA gene from dominant bacteria (B15).

From the morphological observation, a dominant bacteria, named as B15 was choosen to be identified using 16s rRNA method. PCR amplification of the 16S rRNA gene using the universal primers gave rise to the expected PCR product which was approximately 1500 bp in size (Figure 2). Surprisingly, blast result analysis showed that the 1500bp nucleotide sequences of the dominant bacteria (B15) which was found abundantly from the infected banana tree samples matched with the 16S rRNA gene of *Enterobacter* species listed in the gene Bank database with 99% homology. The result was different with our hypothesis where we suspect *R. solanacearum* as the dominant bacteria since the disease symptoms are more likely the same as Moko disease symptoms. Since *Enterobacter* species was found in high level in infected samples and in very low level in healthy samples, it might act as a dominant secondary bacterium that invades banana plant after infection by *R. solanacearum.* Several reports showed that *Enterobacter* spp. could cause bacterial wilt in worldwide crops such as rot of ginger rhizomes (Nishiijima et al., 2004), internal yellowing of papaya fruit (Keith et al., 2008) and mulberry wilt disease (Promsai et al., 2012). So far, there is no report stating that *Enterobacter* spp. could cause wilting disease in banana.

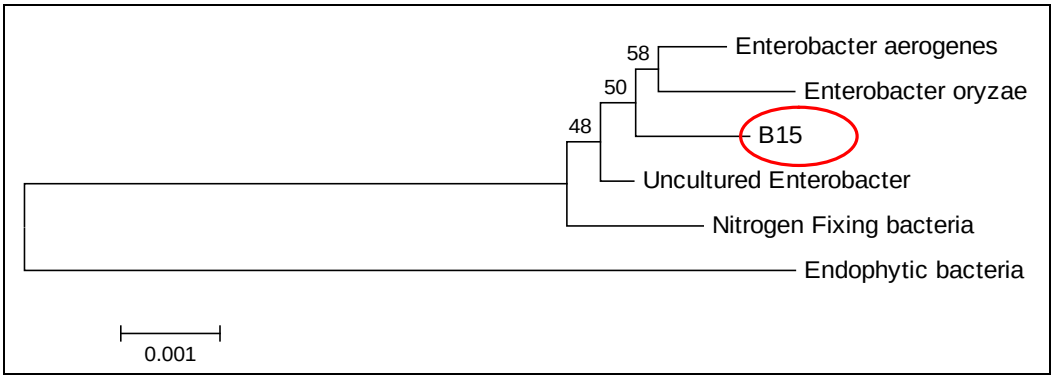

Figure 3. Phylogenetic tree analysis of 16S rRNA gene showed that the dominant bacteria (B15) was clustered together with *Enterobacter* group.

Conclusions

Enterobacter species were found abundantly in infected banana samples taken from Sabak Bernam. Eventhough the sign and symptoms of the infected banana plants were most likely the same as Moko disease symptoms, *Enterobacter* might act as a dominant secondary bacteria that invades banana plant after infection by *R. solanacearum* and therefore were found abundantly in severe infected banana plants.

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