

# DETERMINING THE OPTIMAL CONCENTRATION OF MANNOSE AS AN EFFECTIVE SELECTION AGENT FOR TRANSFORMED OIL PALM CELLS USING THE PHOSPHOMANNOSE ISOMERASE (*pmi*) GENE AS A POSITIVE SELECTABLE MARKER

BOHARI BAHARIAH\*; GHULAM KADIR AHMAD PARVEEZ\* and NORZULAANI KHALID\*\*

## ABSTRACT

*The elimination of antibiotic or herbicide resistance gene usage in genetically modified plants is being encouraged due to public concern. In response to this, alternative selection systems for the recovery of transgenic oil palm were developed using positive selectable markers. To establish a selection system that utilises the phosphomannose isomerase (*pmi*) gene for oil palm transformation, we first determined the optimal mannose concentration for selecting the transformed cells. Non-transformed embryogenic calli were cultured on media containing various combinations and concentrations of mannose and a usable source of carbon, i.e. sucrose, ranging in content from 0 to 30 g litre<sup>-1</sup>. Sucrose is often used as a carbon source in plant tissue culture media. The embryogenic calli were subcultured onto similar fresh media every four weeks, and growth was recorded monthly up to five months. From the 10 combinations of mannose and sucrose evaluated, mannose:sucrose at 30:0 g litre<sup>-1</sup> was shown to be the most effective for selection because at this concentration the least plant growth was demonstrated for non-transformed embryogenic calli. We will thereafter use this particular concentration of mannose to select for oil palm embryogenic calli transformed with the *pmi* gene using biolistic bombardment.*

**Keywords:** phosphomannose isomerase, mannose, oil palm, embryogenic callus, selection agent.

**Date received:** 25 August 2010; **Sent for revision:** 21 September 2010; **Received in final form:** 10 February 2011; **Accepted:** 2 February 2012.

## INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq., Palmae, 2n=32) is the most important commodity crop in Malaysia. The mature palm is a single-stemmed and perennial monocotyledon with a long generation period of about seven to 10 years. Oil palm is the most efficient oil-producing plant species, requiring only 0.25 ha to produce 1 t of oil. By comparison,

soyabean, sunflower and rapeseed need 2.15, 1.50 and 0.75 ha, respectively. The rapid increase in area planted with oil palm in Malaysia, from 300 000 ha in 1970 to 4.49 million hectares in 2008 (Mohd Basri, 2009) indicates the economic importance of this plantation crop and the growing world demand for palm oil. However, the oil palm industry faces challenges such as the increased demand over supply due to the increasing world population, limiting arable land for future expansion, and competition from other oil-producing crops that are far more advanced in the application of genetic manipulations (Parveez, 1998). Due to the long regeneration time, narrow gene pool and open-pollinating behaviour of oil palm (Rajanaidu and Jalani, 1995), genetic engineering is expected to

\* Malaysian Palm Oil Board,  
P. O. Box 10620, 50720 Kuala Lumpur, Malaysia.  
E-mail: parveez@mpob.gov.my

\*\* Institute of Biological Sciences, Faculty of Science,  
Universiti Malaya, Lembah Pantai,  
50603 Kuala Lumpur, Malaysia.

overcome these challenges. It has been estimated that four to five years are required to produce useful putative transgenic plantlets from the initial date of explant culture (Parveez, 2000).

The successful regeneration of transgenic plants, including those of oil palm, has relied largely on the use of negative selectable marker genes. The negative selectable marker genes used in oil palm transformation are genes that confer resistance to the antibiotic hygromycin, *hygromycin phosphotransferase (hpt)*, and the gene that confers resistance to the herbicide Basta, *phosphinothricin acetyltransferase (bar)* (Parveez, 2000; Abdullah *et al.*, 2005). However, for improving the efficiency of oil palm genetic transformation and in eliminating the use of negative selection, a new concept for selection of transgenic plant cells called positive selection needs to be evaluated. Such a system could use carbohydrates such as mannose (Joersbo *et al.*, 1998; 1999), xylose (Haldrup *et al.*, 1998a, b) or deoxyglucose (Kunze *et al.*, 2001). Carbohydrates have been shown to be more efficient as selection agents for potato, tomato, tobacco and sugar beet transformation as compared to methods based on antibiotic selection. Most of these markers have been developed using model plants such as tobacco and *Arabidopsis*.

Mannose, a hexose sugar, has been known for a number of years to inhibit the growth of various plant species (Malca *et al.*, 1967). Mannose and mannose derivatives are common constituents of living cells, and are key components of intermediary metabolism. Positive selection of transgenic plant cells using mannose has several advantages in plant transformation. First, the transformation frequencies obtained by positive selection appear to be higher than those obtained with antibiotic or herbicide selection (Haldrup *et al.*, 1998a, b; Joersbo *et al.*, 1998). Second, it allows the elimination of the use of antibiotic or herbicide resistance marker genes which cause widespread public concern because of inadequate knowledge of the agents' impact on the environment and on human health (Ferber, 1999). Third, positive selection does not cause any risk to animals, humans or the environment (Haldrup *et al.*, 1998a, b; Joersbo *et al.*, 1998; Negrotto *et al.*, 2000; Wang *et al.*, 2000). Other advantages include, but are not limited to, the solubility of this hexose in plant culture media, absorbance by plant cells without toxic effects, and being cheap and easily available (Aragão and Brasileiro, 2002). However, mannose will prevent cell growth and development when it is phosphorylated by hexokinase to mannose-6-phosphate. Accumulation of mannose-6-phosphate inhibits growth of the non-transgenic cells due to starvation with respect to intracellular phosphate and ATP (Sheu-Hwa *et al.*, 1975). Cells transformed with the selectable marker gene (the *pmi* gene derived from *E. coli*), acquire the ability to

convert mannose-6-phosphate to the metabolizable fructose-6-phosphate, thus enabling these cells to survive on mannose-containing media (Joersbo *et al.*, 1998).

The success of the genetic transformation process is monitored through the following three steps: establishing proof of DNA integration, protein expression and transmission of the transgene into its progenies. In practice, during genetic transformation, the foreign gene is transferred into the target tissue, which contains thousands of cells; of these, only few cells will become transgenic or will have the transgene stably integrated into its genome. It is very important to isolate only the transformed cells from the majority of untransformed cells by using a selection agent. The concentration of the selection agent needs to be carefully determined to avoid either being too low which causes undesirable 'escapes' or regeneration of chimeric plants, or too high which decreases the chance of capturing transformants with low to moderate levels of selective gene expression (Parveez *et al.*, 2007). The ability of plants to metabolise mannose is species-dependent (Malca *et al.*, 1967; Wang *et al.*, 2000). Plant species that are extremely sensitive to mannose and have been successfully transformed using mannose as the selective agent include maize (Wright *et al.*, 2001), wheat (Reed *et al.*, 2001), durum wheat (Gadaleta *et al.*, 2004), pearl millet (O' Kennedy *et al.*, 2004), bentgrass (Fu *et al.*, 2005), rice (Zai-Song *et al.*, 2006), onion (Aswath *et al.*, 2006), sugar-cane (Jain *et al.*, 2007) and sorghum (Songul *et al.*, 2009).

Based on the advantages of positive selectable marker genes demonstrated in other crops, a research project to develop transgenic oil palm based on a similar selectable marker gene was initiated. In this report, we elaborate on the work to determine the optimal concentration of mannose required for the eventual effective selection of stably transformed oil palm embryogenic calli carrying the *pmi* gene.

## MATERIALS AND METHODS

### Plant Materials and Culture Conditions

Leaflets from unopened (-6) fronds were aseptically transferred onto solid callus initiation media [MS salts (Murashige and Skoog, 1962) + Y<sub>3</sub> vitamins (Eeuwans, 1976) + 0.1 g litre<sup>-1</sup> myo-inositol and L-glutamin + 3% sucrose + 5 × 10<sup>-5</sup> M 2, 4-D + 0.25% activated charcoal + 0.7% agar], and incubated at 28°C in the dark. Any callus formed was subcultured every four weeks onto the same medium until embryogenic calli were formed.

Embryogenic calli were placed on solidified agar medium containing MS (Murashige and Skoog,

1962) macro and micronutrients supplemented with 1 mg litre<sup>-1</sup> naphthalene acetic acid (NAA) and 30 g litre<sup>-1</sup> sucrose. The medium was adjusted to pH 5.7 with NaOH or KOH, and autoclaved at 121°C for 15 min.

### Explant Sensitivity to the Selection Agent

Untransformed embryogenic calli were transferred onto embryogenic callus media containing various concentrations of mannose as the sole carbon source, or in combination with sucrose, in the following mixtures: 0:30, 5:25, 10:20, 15:15, 20:10, 25:5, 30:0, 10:30, 20:30 and 0:0 g litre<sup>-1</sup> of mannose:sucrose. This was to determine the ability of oil palm embryogenic calli to regenerate by metabolising mannose as a carbon source. The experiment was carried out by placing 0.5 g embryogenic calli on each plate (90-mm Petri dish) with five replicates per treatment. The embryogenic calli were transferred onto fresh media every four weeks, and their weights were recorded besides performing visual inspection. The above procedure was performed for five rounds of subculture.

### Determination of Mannose Concentration for Efficient Selection

The optimal concentration of mannose required for efficient selection against control tissues was determined by weighing the tissues prior to subculture and during every subculture. The percentages of proliferation for each treatment were calculated after five months of treatment on selection media. The proliferation percentage (%) was calculated as follows:

$$(A - B) / (C - D)$$

where A and B were the final and initial weights of the tissues/calli in the treated medium, respectively, C and D were the final and initial weights of tissues/calli in the control medium, respectively.

The proliferation percentage of the calli was used as a measure of mannose toxicity. The minimum mannose concentration that inhibited calli regeneration was determined using the formula modified from Dennehey *et al.* (1994). The growth rate of the untreated control embryogenic calli exposed to 0:30 g litre<sup>-1</sup> mannose:sucrose was used as a standard. The control was set at 100% proliferation. The means of five replicates were used in the final calculations.

### Regeneration of Plantlets

Embryogenic cultures were maintained on media containing MS macro and micronutrients and Y<sub>3</sub> vitamins supplemented with 100 mg litre<sup>-1</sup>

each of myo-inositol, L-glutamine, L-arginine and L-asparagine, 5 µM IBA, 0.7% agar and 30 g litre<sup>-1</sup> sucrose to form polyembryogenic cultures. The medium was adjusted to pH 5.7 with NaOH or KOH prior to autoclaving. Embryogenic calli were incubated at 28°C in the presence of light and were subcultured every 30 days onto fresh medium.

Small plantlets were produced from the polyembryogenic cultures on media containing MS macro and micronutrients and Y<sub>3</sub> vitamins supplemented with 100 mg litre<sup>-1</sup> each of myo-inositol, L-glutamine, L-arginine and L-asparagine, 0.1 µM NAA, 0.4% agar and 30 g litre<sup>-1</sup> sucrose. The medium was adjusted to pH 5.7 with NaOH or KOH prior to autoclaving. The polyembryogenic calli were incubated at 28°C in light until sufficient shoots were produced.

Root initiation from the small plantlets was promoted on media containing MS macro and micronutrients and Y<sub>3</sub> vitamins supplemented with 300 mg litre<sup>-1</sup> L-glutamine, 100 mg litre<sup>-1</sup> myo-inositol, 10 µM 2,4-D, 70 µM NAA, 0.15% activated charcoal and 60 g litre<sup>-1</sup> sucrose. The medium was adjusted to pH 5.7 with NaOH or KOH prior to autoclaving. The small plantlets were incubated at 28°C in light until roots were formed.

### Statistical Analysis

The statistical software SPSS version 8.0 was used to carry out ANOVA and Duncan's Multiple Range Test (DMRT), at the significance level of p ≤ 0.05.

## RESULTS AND DISCUSSION

### Explant Sensitivity to Mannose as the Selection Agent

The sensitivity of the oil palm embryogenic calli to mannose has to be determined before applying the selection systems for generating transgenic oil palm. It is important to determine the endogenous ability of oil palm embryogenic calli in using mannose as an energy source, as well as to identify the maximum selection pressure of mannose in stopping the regeneration of oil palm embryogenic calli without or at a low concentration of sucrose. The effect of mannose on oil palm growth was examined by culturing oil palm embryogenic calli on media containing various combinations of mannose:sucrose concentrations, ranging from 0.5 to 30 g litre<sup>-1</sup>. Figures 1a and 1b show the effect of different combinations of mannose:sucrose on oil palm embryogenic callus regeneration recorded for the first three months of treatment. Significant effects could only be seen after four months of culturing on different combinations of mannose and sucrose

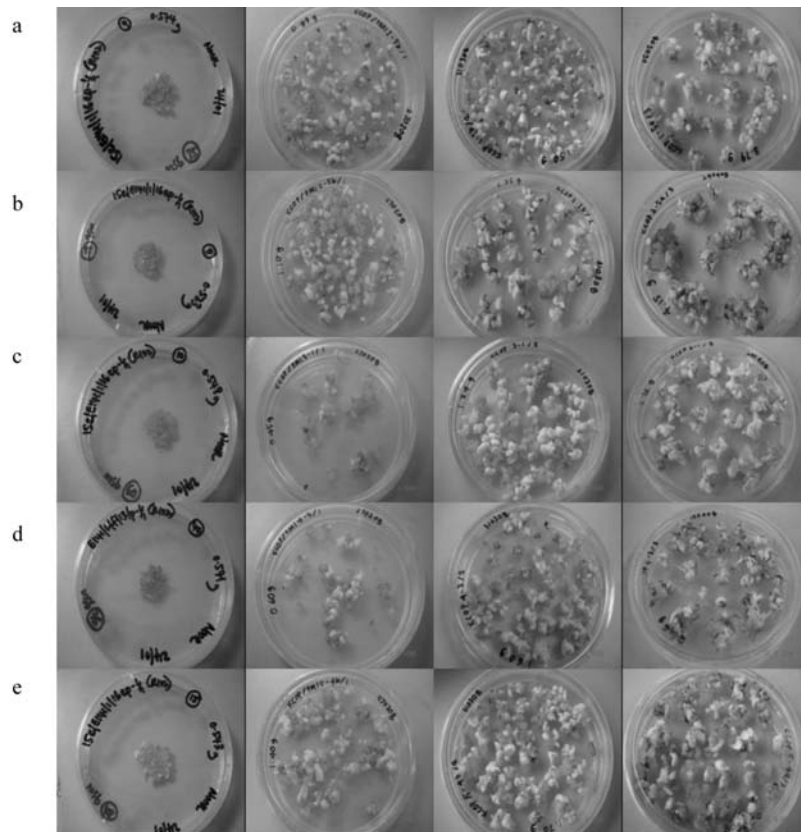


Figure 1a. Proliferation of non-bombarded oil palm embryogenic calli on media containing various concentrations of mannose:sucrose – a. 0:30 g litre<sup>-1</sup>, b. 5:25 g litre<sup>-1</sup>, c. 10:20 g litre<sup>-1</sup>, d. 15:15 g litre<sup>-1</sup> and e. 20:10 g litre<sup>-1</sup>. From left: before selection, one, two and three months after selection.

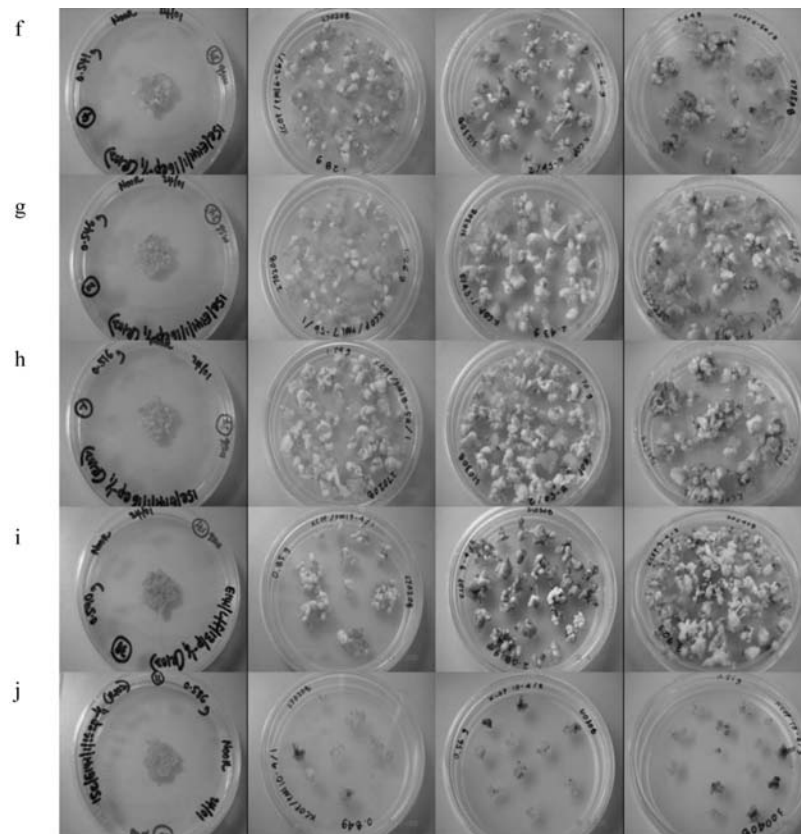


Figure 1b. Proliferation of non-bombarded oil palm embryogenic calli on media containing various concentrations of mannose:sucrose – f. 25:5 g litre<sup>-1</sup>, g. 30:0 g litre<sup>-1</sup>, h. 10:30 g litre<sup>-1</sup>, i. 20:30 g litre<sup>-1</sup> and j. without sucrose and mannose. From left: before selection, one, two and three months after selection.



(Figure 2). However, embryogenic calli cultured on a medium without sugar (treatment J) were found to die by the third month. The high tolerance of oil palm embryogenic calli to mannose may have been due to the endogenous ability of oil palm tissues to partially utilise mannose as a carbohydrate source (Herold and Lewis, 1977). Dekeyser *et al.* (1989) have suggested that other media components, mainly the amino acids glutamine, asparagine and arginine, may influence the selective effect of mannose which allows growth of untransformed cells in the presence of mannose.

**Effects of Various Mannose Concentrations on Embryogenic Callus Growth**

The optimal concentration of mannose required for efficient selection against control tissues was determined by weighing the tissues before subculture on fresh medium. The medium containing 0:30 g litre<sup>-1</sup> mannose:sucrose, the positive control, was used in the regeneration media of oil palm. The mean fresh weight of the treated

tissues was compared to that of the control (Figure 2). The fresh weight of treated tissues ranged from 130 g at 5:25 g litre<sup>-1</sup> mannose:sucrose to 98 g at 30:0 g litre<sup>-1</sup> mannose:sucrose. The embryogenic calli weight at the highest concentration of mannose (30 g litre<sup>-1</sup>) was about half that of the control (0 g litre<sup>-1</sup> mannose) at 154 g. For the 30:0 mannose:sucrose medium, the embryoids did not appear normal. They looked brownish and watery, and had less turgor than the embryoids on the control medium containing no mannose. However, the embryoids still survived and formed shoots. The shoots which formed in the medium with 0:30 g litre<sup>-1</sup> mannose:sucrose (control) were consistently larger than those formed in the medium with 30:0 g litre<sup>-1</sup> mannose:sucrose. Figure 3 shows the shoot formation capability of embryogenic calli in shoot selection medium. Adding mannose to the normal culture medium, even at concentrations of up to 30 g litre<sup>-1</sup>, did not efficiently arrest the growth of the embryogenic calli. Simultaneously, the number of regenerated shoots increased as well. However, the results indicate that the shoots exhibited extremely

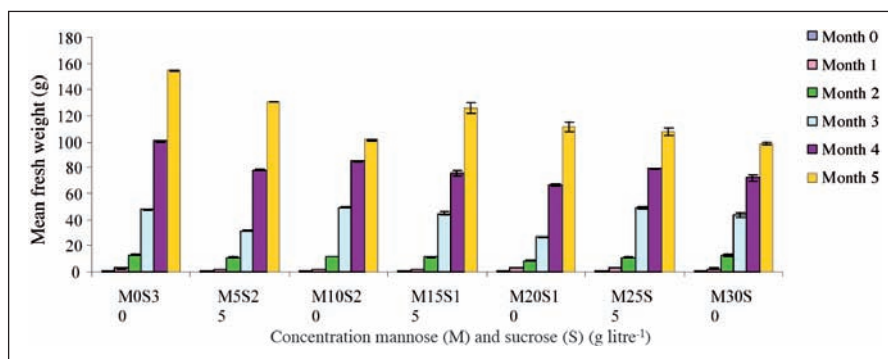


Figure 2. The mean fresh weight of non-transformed oil palm embryogenic calli in MS medium containing different combinations of mannose and sucrose concentrations over five months. The error bars denote the standard error of the mean.

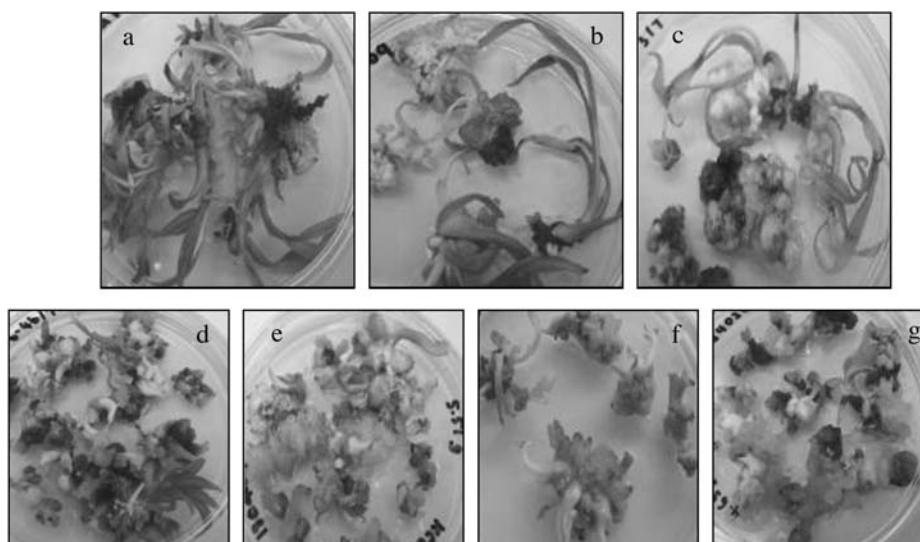


Figure 3. Comparison of shoot proliferation from non-transformed oil palm embryogenic calli on media containing various concentration of mannose:sucrose, a. 0:30 g litre<sup>-1</sup>, b. 5:25 g litre<sup>-1</sup>, c. 10:20 g litre<sup>-1</sup>, d. 15:15 g litre<sup>-1</sup>, e. 20:10 g litre<sup>-1</sup>, f. 25:5 g litre<sup>-1</sup> and g. 30:0 g litre<sup>-1</sup>.

slow growth on mannose when used as the sole carbohydrate source compared to growth on sucrose. This finding suggests the possibility of little or no PMI-like activity in the shoots, and thus their inability to use mannose as a carbon source.

When plant cells lacking the PMI enzyme are cultured on medium containing mannose, the cells convert mannose to mannose-6-phosphate, but are unable to isomerise mannose-6-phosphate to fructose-6-phosphate. The accumulation of mannose-6-phosphate inhibits phosphoglucose isomerase, causing a block in glycolysis and results in cell death (Goldsworthy and Street, 1965). It has been demonstrated that sugar-cane calli were unable to grow on mannose-containing medium (Gill *et al.*, 2004). This was probably due to a deficiency in endogenous PMI enzyme activity resulting in a decreasing energy supply through phosphorylation of mannose by hexokinase (Pego *et al.*, 1999). Alternatively, it could also be due to the presence of a mannose-induced endonuclease causing DNA laddering and eventual death of the plant cells (Stein and Hansen, 1999). In tomato, it was reported that the presence of mannose reduced the percentage of explants producing shoots (Bříza *et al.*, 2008). Complete inhibition of organogenesis was observed on a medium containing 10 g litre<sup>-1</sup> mannose, regardless of the sucrose content. In lettuce, it was observed that no explants produced shoots on media without or with a low concentration of sucrose (10 g litre<sup>-1</sup>), demonstrating their protective effect against mannose which caused inhibition of the organogenesis of cotyledonous leaves (Bříza *et al.*, 2010). Higher protection was obtained at an intermediate concentration of sucrose (20 g litre<sup>-1</sup>). Similar results have also been reported for tomato. The addition of sucrose to the selection medium reduced the inhibitory effect of mannose on shoot formation (Sigareva *et al.*, 2004).

The difference in mean fresh weights of oil palm embryogenic calli on media with seven different combinations of mannose and sucrose was confirmed statistically using DMRT. The results are summarised in the form of an ANOVA table (Table 1). The weight of embryogenic calli grown on 30 g litre<sup>-1</sup> mannose was significantly different from that of embryogenic calli grown on 30 g litre<sup>-1</sup> sucrose ( $P \leq 0.05$ ) as determined by ANOVA and DMRT.

In the present study, apart from determining the effect of mannose on the weight of the embryogenic calli, mannose concentration also had an effect on the proliferation rate of the calli. The percentage of calli proliferation was only 64% on the medium containing the highest concentration of mannose (30 g litre<sup>-1</sup>). This represents a reduction of about 36% compared to control. The percentage of proliferation was shown to increase steadily with the decrease in mannose level in the medium. The percentage slowly reduced when no sucrose was

TABLE 1. ANOVA FROM DIFFERENT COMBINATIONS OF MANNOSE AND SUCROSE IN THE MEDIA

	Media [Mannose: sucrose (g litre <sup>-1</sup> )]	Mean weight
1	0:30	154.45±0.29 <sup>e</sup>
2	5:25	130.51±0.26 <sup>d</sup>
3	10:20	101.13±0.32 <sup>ab</sup>
4	15:15	126.03±3.70 <sup>d</sup>
5	20:10	111.48±4.00 <sup>c</sup>
6	25:5	107.12±2.90 <sup>bc</sup>
7	30:0	98.46±0.97 <sup>a</sup>

Note: \* treatment means with the same letter are not significantly different at  $p=0.05$  according to Duncan's Multiple Range Test. Values represent the mean ±S. E. of five replicates over five months.

added to the culture medium. There was a linear decrease in the percentage of plant regeneration from the non-transformed embryogenic calli when they were grown for five months on various concentrations of mannose (Figure 4). Overall, the regeneration of whole plantlets from embryogenic calli was reduced with an increase of mannose level in the medium.

#### Effects of Various Mannose Concentrations on Shoot Formation

The combinations of mannose and sucrose produced mostly green polyembryogenic calli followed by regeneration of plantlets. Occasionally, the calli did not produce any plantlets. An increase in mannose concentration resulted in a higher number of calli producing shoots. Calli cultured on media containing only mannose as the source of carbohydrate grew normally and produced voluminous green callus, with a recovery rate of 61%. The medium containing sucrose as the sole source of carbohydrate produced relatively less calli (32%) (Table 2). However, in the medium containing only mannose, the shoots grew very slowly, were much smaller in size, and eventually turned watery and developed light brown colouration. The shoots also lost their vigour during the prolonged culture period. Even though the plants still survived in the presence of mannose, the development could potentially be arrested by carbohydrate starvation due to the absence of sucrose (Haldrup *et al.*, 1998a; Wang *et al.*, 2000). It was also reported that mannose selection did not result in a direct negative toxic effect as the control callus and shoots continued to grow, albeit very slowly and acquiring a brown colour (Lindsey and Gallois, 1990). Table 2 also shows the effect of various mannose concentrations on shoot length. Shoot length decreased progressively with the increase in mannose content. The shoots exhibited an extremely slow growth rate on the medium with mannose as the sole carbohydrate

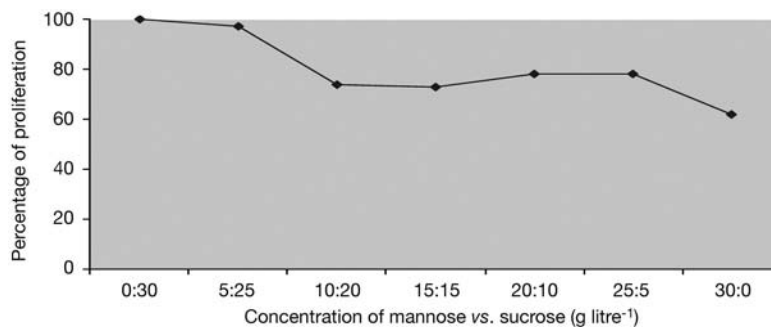


Figure 4. Proliferation percentage of oil palm embryogenic calli after five months at different concentrations of mannose vs. sucrose (g litre<sup>-1</sup>).

source; the growth of 2.5 cm shoot length was 3.5 times shorter than that of the shoots grown on the medium supplemented with only 30 g litre<sup>-1</sup> sucrose, *i.e.* the control (shoot length of 8.5 cm). This observation is in contrast to selection based on antibiotics or herbicides, which normally kills non-transformed cells immediately. The mannose-based selection strategy allowed transformed cells to survive while starving the non-transformed cells.



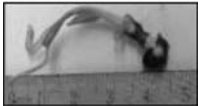




#### Optimal Mannose Concentration for Oil Palm Transformation

The results of this study show that the toxic effect of mannose on oil palm increased with decreasing sucrose concentration. The results suggest that the oil palm embryogenic callus cultures are able to partially utilise mannose as a carbon source as indicated by their ability to form shoots. Compared to other plant species, oil palm can be considered to be not very sensitive to mannose. The concentration of mannose and its combination with sucrose used for selection vary for each crop. For example, the selection and regeneration of maize and wheat calli have been shown to be effective on media containing 10 g litre<sup>-1</sup> mannose and 7 g litre<sup>-1</sup> mannose combined with 3 g litre<sup>-1</sup> sucrose, respectively (Wright *et al.*, 2001). On the other hand, Negrotto *et al.* (2000) set a combination of 5 g litre<sup>-1</sup> sucrose and 10 g litre<sup>-1</sup> mannose as the selection index for maize. However, in cassava, a mannose concentration of 20 g litre<sup>-1</sup> only allowed for a low frequency of organogenesis (Zhang and Puonti-Kaerlas, 2000) while in sugar beet, full inhibition of regeneration was observed on a medium containing 3 g litre<sup>-1</sup> mannose (Joersbo *et al.*, 1998). When the phytotoxic effect of mannose, in the presence of four different non-toxic saccharides, on the dry mass of sugar beet cotyledonary explants after three weeks of growth was tested, results show that the higher the sucrose concentration the lower was the mannose toxicity (Joersbo *et al.*, 1999). Inhibitory effects of mannose on the organogenesis of cotyledonous leaves were also reported for lettuce (Bríza *et al.*, 2010). It was

shown that no explants produced shoots on media containing mannose.

The current findings suggest that embryogenic callus medium containing 30 g litre<sup>-1</sup> mannose is suitable for use in oil palm transformation experiments. The concentration was demonstrated to allow only minimal plant growth. This observation is in contrast to other reports which indicated a combination of mannose and sucrose had to be used. Depending on the relative concentrations of sucrose used, the frequency of escapes seemed to increase (He *et al.*, 2006; Jain *et al.*, 2007). In this study, the optimal selection level of mannose for oil palm was higher than for other crops. A similar result has also been reported by Kim *et al.* (2002) on pepper. Shoot formation from the cotyledons of pepper was not completely inhibited until mannose concentration reached 50 g litre<sup>-1</sup> in the presence of sucrose. To date, this is the highest mannose concentration used for selection. Higher concentrations of sucrose had an additive effect resulting in reduced shoot formation rate in pepper. In this case, mannose itself did not seem to be the sole inhibitor of shoot development. It was shown that sucrose should also be present in the medium in order to generate selection pressure (Kim *et al.*, 2002). The very high level of mannose (50 g litre<sup>-1</sup>) in addition to sucrose also affected shoot formation, which may be due to an osmotic pressure effect. In potato, it was reported that the initial concentration of mannose used was very low, at 1 g litre<sup>-1</sup>. The mannose concentration was gradually increased to 2.5, 5 and 10 g litre<sup>-1</sup> (Bríza *et al.*, 2008). Furthermore, prolonged culture on 5 g litre<sup>-1</sup> mannose also resulted in higher transformation efficiency. A similar selection strategy using mannose has also been adopted for rice (Hoa and Bong, 2002). The concentration of mannose during selection was increased stepwise, starting from 25 g litre<sup>-1</sup> and progressing to 35 g litre<sup>-1</sup> for the last round of selection. Stepwise increase of the selection agent helped to protect the transformants from selection shock as well as to produce higher transformation efficiency.

TABLE 2. EFFECT OF VARYING MANNOSE CONCENTRATION ON SHOOT FORMATION

Expt. No. (Man:Suc) (g litre <sup>-1</sup> )	No. of explants	Shoot differentiation	% of greenish embryoids	No. of regenerated shoots min. – max.	Length of shoot (cm)
A (0:30)	38		32	10 – 18	8.5
B (5:25)	42		26	13 – 15	7.0
C (10:20)	54		28	5 – 9	6.0
D (15:15)	62		45	5 – 9	5.5
E (20:0)	72		53	8 – 10	5.0
F (25:5)	53		47	3 – 5	4.0
G (30:0)	59		61	4 – 7	2.5

Note: shoot height decreased with increasing mannose content in the medium. Embryogenic calli were placed on regeneration media supplemented with increasing concentrations of mannose and decreasing concentrations of sucrose: a. 0:30 g litre<sup>-1</sup>, b. 5:25 g litre<sup>-1</sup>, c. 10:20 g litre<sup>-1</sup>, d. 15:15 g litre<sup>-1</sup>, e. 20:10 g litre<sup>-1</sup>, f. 25:5 g litre<sup>-1</sup> and g. 30:0 g litre<sup>-1</sup>. The mean from three shoots/treatment was computed.

## CONCLUSION

Determination of the optimal concentration of mannose as a transformation selection agent for oil palm embryogenic calli has been successfully carried out. The results indicate that the embryogenic calli were able to survive selection on mannose-supplemented media. The optimal level of mannose to be used for selection was found to be 30 g litre<sup>-1</sup>, and was determined over five months of testing using multiple treatments. At this concentration, the explants were only able to form shorter shoots. However, the shoots failed to regenerate into healthy plantlets. For future experiments in regenerating transgenic oil palm, embryogenic calli will be transformed with the phosphomannose isomerase (*pmi*) gene, and subsequently selected on a medium

supplemented with 30 g litre<sup>-1</sup> mannose as the sole carbon source.

## ACKNOWLEDGEMENT

The authors would like to thank the Director-General of MPOB for permission to publish this article. We also thank Dr Ahmad Tarmizi Hashim from the Clonal Propagation Group, MPOB, for providing the oil palm embryogenic callus cultures. We thank Dr Omar Rasid and Dr Maizura Ithnin of MPOB for critically reviewing the manuscript. In addition, we thank Dr Omar Rasid, Ms Haiza Hazrina and all members of the Genetic Transformation Laboratory for their assistance.



## REFERENCES

- ABDULLAH, R; ZAINAL, A; HENG, W Y; LI, L C; BENG, Y C; PHING, L M; SIRAJUDDIN, S A; PING, W Y S and JOSEPH, J L (2005). Immature embryo: a useful tool for oil palm (*Elaeis guineensis* Jacq.) genetic transformation studies. *Electronic. J. Biotechnol.*, 18: 24-34.
- ARAGÃO, F J L and BRASILEIRO, A C M (2002). Positive, negative and marker-free strategies for transgenic plant selection. *Braz. J. Plant Physiol.*, 14: 1-10.
- ASWATH, C R; MO, S Y; KIM, D H and PARK, S W (2006). *Agrobacterium* and biolistic transformation of onion using non-antibiotic selection marker phosphomannose isomerase. *Plant Cell Rep.*, 25: 92-99.
- BŘÍZA, J; PAVINGEROVÁ, D; PŘIKRYLOVÁ, P; GAZDOVÁ, J; VLASÁK, J and NIEDERMEIEROVÁ, H (2008). Use of phosphomannose isomerase-based selection system for *Agrobacterium*-mediated transformation of tomato and potato. *Biol. Plant.*, 52: 453-461.
- BŘÍZA, J; RŮŽIČKOVÁ, N; NIEDERMEIEROVÁ, H; DUSBABKOVÁ, J and VLASÁK, J (2010). Phosphomannose isomerase gene for selection in lettuce (*Lactuca sativa* L.) transformation. *ACTA Biochimica Polonica.*, 57: 63-68.
- DEKEYSER, R B; CLAES, M; MARICHAL, M; VAN MONTAGU, M and CAPLAN, A (1989). Evaluation of selectable markers for rice transformation. *Plant Physiol.*, 90: 217-223.
- DENNEHEY, B K; PETERSEN, W L; FORD-DANTINO, C; PAJEAU, M and ARMSTRONG, C L (1994). Comparison of selection agents for use with the selectable marker gene *bar* in maize transformation. *Plant Cell Tiss. Org. Cult.*, 36: 1-7.
- EEUWANS, C J (1976). Mineral requirement for growth and callus initiation of tissue explants from mature coconut palms (*Cocos nucifera*) cultured *in vitro*. *Physiol. Plant.*, 36: 23-28.
- FERBER, D (1999). GM crops in the cross hairs. *Science*, 286: 1662-1666.
- FU, D L; XIAO, Y M; MUTHUKRISHNAN, S and LIANG, G H (2005). *In vivo* performance of a dual genetic marker, *ManA-Gfp* in transgenic bentgrass. *Genome*, 48: 722-730.
- GADALETA, A; GIANCASPRO, A and BLANCO, A (2004). Phosphomannose isomerase, PMI, as selectable marker gene in durum wheat transformation. *Proc. of the XLVIII Italian Society of Agricultural Genetics*.
- GILL, A T; SNYMAN, S J; POTIER, B A M and HUCKETT, B I (2004). Towards antibiotic resistance-free transgenic sugarcane. *Proc. S. Afr. Sug. Technol. Ass.*, 78: 163-166.
- GOLDSWORTHY, A and STREET, H E (1965). The carbohydrate nutrition of tomato roots VIII. The mechanism of the inhibition by D-mannose of the respiration of excised roots. *Ann Bot.*, 29: 45-58.
- HALDRUP, A; PETERSEN, S G and OKKELS, F T (1998a). Positive selection: a plant selection principle based on xylose isomerase, an enzyme used in the food industry. *Plant Cell Rep.*, 18: 76-81.
- HALDRUP, A; PETERSEN, S G and OKKELS, F T (1998b). The xylose isomerase gene from *Thermoanaerobacterium thermosulfurogenes* allows effective selection of transgenic plant cells using D-xylose as the selection agent. *Plant Mol. Biol.*, 37: 287-296.
- HE, Z Q; DUAN, Z Z; LIANG, W; CHEN, F J; YAO, W; LIANG, H W; YUE, C Y; SUN, Z X; CHEN, F and DAI, J W (2006). Mannose selection system used for cucumber transformation. *Plant Cell Rep.*, 25: 953-958.
- HEROLD, A and LEWIS, D H (1977). Mannose and green plants: occurrence, physiology and metabolism, and use as a tool to study the role of orthophosphate. *New Phytol.*, 79: 1-40.
- HOA, T T C and BONG, B B (2002). *Agrobacterium*-mediated transformation of rice embryogenic suspension cells using phosphomannose isomerase gene, *pmi*, as a selectable marker. *Omonrice.*, 10: 1-5.
- JAIN, M; CHENGALRAYAN, K; ABOUZID, A and GALLO, M (2007). Prospecting the utility of a PMI/mannose selection system for the recovery of transgenic sugar cane (*Saccharum* spp. hybrid) plants. *Plant Cell Rep.*, 26: 581-590.
- JOERSBO, M; DONALDSON, I; KREIBERG, J; PETERSEN, S G; BRUNSTEDT, J and OKKELS, F T (1998). Analysis of mannose selection used for transformation of sugar beet. *Mol. Breeding*, 4: 111-117.
- JOERSBO, M; PEDERSON, S G and OKKELS, F T (1999). Parameters interacting with mannose selection employed for the production of transgenic sugar beet. *Physiol. Plant.*, 105: 109-115.
- KIM, J Y; JUNG, M; KIM, H S; LEE, Y H; CHOI, S H; LIM, Y P; MIN, B W; YANG, S G and HARN, C H (2002). A new selection system for pepper regeneration by mannose. *J. Plant Biotechnol.*, 4: 129-134.

- KUNZE, I; EBNETH, M; HEIM, U; GEIGER, M; SONNEWALD, U and HERBERS, K (2001). 2-Deoxyglucose resistance: a novel selection marker for plant transformation. *Mol. Breed.*, 7: 221-227.
- LINDSEY, K and GALLOIS, P (1990). Transformation of sugar beet (*Beta vulgaris*) by *Agrobacterium tumefaciens*. *J. Exp. Bot.*, 41: 529-536.
- MALCA, I; ENDO, R M and LONG, M R (1967). Mechanism of glucose counteraction of inhibition of root elongation by galactose, mannose, and glucosamine. *Phytopathology*, 57: 272-278.
- MOHDBASRI, W (2009). Overview of the Malaysian oil palm industry 2008. [http://econ.mpob.gov.my/economy/EID\\_web.htm](http://econ.mpob.gov.my/economy/EID_web.htm).
- MURASHIGE, T and SKOOG, F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-497.
- NEGROTTO, D; JOLLEY, M; BEER, S; WENCK, A R and HANSEN, G (2000). The use of phosphomannose isomerase as a selectable marker to recover transgenic maize plants (*Zea mays* L.) via transformation. *Plant Cell Rep.*, 19: 798-803.
- O'KENNEDY, M M; BURGER, J T and BOTHA, F C (2004). Pearl millet transformation system using the positive selectable marker gene phosphomannose isomerase. *Plant Cell Rep.*, 22(9): 684-690.
- PARVEEZ, G K A (1998). *Optimization of Parameters Involved in the Transformation of Oil Palm Using the Biolistic Method*. Ph.D thesis. Universiti Putra Malaysia.
- PARVEEZ, G K A (2000). Production of transgenic oil palm (*Elaeis guineensis* Jacq.) using biolistic techniques. *Molecular Biology of Woody Plants* (Jain, S M and Minocha, S C eds.). Kluwer Academic Publishers. p. 327-350.
- PARVEEZ, G K A; NA'IMATUAPIDAH, A M; ALIZAH, Z and RASID, A O (2007). Determination of minimal inhibitory concentration of selection agents for selecting transformed immature embryos of oil palm. *As. Pac. J. Mol. Biol. Biotechnol.*, 15(3): 133-146.
- PEGO, J V; WEISBEEK, P J and SMEEKENS, S C M (1999). Mannose inhibits *Arabidopsis* germination via a hexokinase-mediated step. *Plant Physiol.*, 119: 1017-1023.
- RAJANAIDU, N and JALANI, B S (1995). World-wide performance of DXP planting materials and future prospects. *Proc. of the 1995 PORIM National Oil Palm Conference - Technologies in Plantation, the Way Forward*. PORIM, Bangi. p. 1-29.
- REED, J; PRIVALLE, L; POWELL, M L; MEGHJI, M; DAWSON, J; DUNDER, E; SUTTIE, J; WENCK, A; LAUNIS, K; KRAMER, C; CHANG, Y F; HANSEN, G and WRIGHT, M (2001). Phosphomannose isomerase: an efficient selectable marker for plant transformation. *In Vitro Cell. Dev. Biol. Plant.*, 37: 127-132.
- SHEU-HWA, C S; LEWIS, D H and WALKER, D A (1975). Stimulation of photosynthetic starch formation by sequestration of cytoplasmic orthophosphate. *New Phytol.*, 74: 383-392.
- SIGAREVA, M; SPIVEY, R; WILLITS, M; KRAMER, C and CHANG, Y F (2004). An efficient mannose selection protocol for tomato that has no adverse effect on the ploidy level of transgenic plants. *Plant Cell Rep.*, 23: 236-245.
- SONGUL, G; EKREM, G; RAJVINDER, K; JOSHUA, W; LING, M; HAN-QI, T and PEGGY, G L (2009). Efficient, reproducible *Agrobacterium*-mediated transformation of sorghum using heat treatment of immature embryos. *Plant Cell Rep.*, 28: 429-444.
- STEIN, J C and HANSEN, G (1999). Mannose induces an endonuclease responsible for DNA laddering in plant cells. *Plant Physiol.*, 121: 71-79.
- WANG, A S; EVANS, R A; ALTENDORF, P R; HANTEN, J A; DOYLE, M C and ROSICHAN, J L (2000). A mannose selection system for production of fertile transgenic maize plants from protoplasts. *Plant Cell Rep.*, 19: 654-660.
- WRIGHT, M; DAWSON, J; DUNDER, E; SUTTIE, J; REED, J; KRAMER, C; CHANG, Y; NOVITZKY, R; WANG, H and ARTIM-MOORE, L (2001). Efficient biolistic transformation of maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using the phosphomannose isomerase gene, PMI, as the selectable marker. *Plant Cell Rep.*, 20: 429-436.
- ZAI-SONG, D; MING, Z; YU-XIANG, J; LIANG-BI, L and TING-YUN, K (2006). Efficient *Agrobacterium*-mediated transformation of rice by phosphomannose isomerase/mannose selection. *Plant Mol. Biol. Reporter*, 24: 295-303.
- ZHANG, P and PUONTI-KAERLAS, J (2000). PIG-mediated cassava transformation using positive and negative selection. *Plant Cell Rep.*, 19: 1041-1048.