OPTIMISATION OF THE BOMBARDMENT PARAMETERS FOR TRANSFORMATION AND REGENERATION OF STABLE OIL PALM TRANSFORMANTS

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ABSTRACT

Genetic transformation approaches by using particle bombardment have been an integral part in efforts to improve palm oil quality. The bombardment parameters were previously evaluated to efficiently deliver deoxyribonucleic acid (DNA) during bombardment. However, the evaluation was carried out at transient stage and therefore the results may not be very precise. In order to improve the DNA delivery and regeneration of the oil palm carrying transgenes, a study at stable stage was performed and discussed in this article. Oil palm embryogenic calli were bombarded at several parameters using pAHC25 plasmid. Selection on media containing 1 mg litre^{-1} of bialaphos was initiated after one month of bombardment. Then, the concentration of bialaphos was increased to 2 mg litre^{-1} and subsequently to 3 mg litre^{-1} to effectively eliminate the regeneration of untransformed cells. Generated shoots were subjected to polymerase chain reaction (PCR) analysis. The best bombardment condition was determined based on the highest number of shoots carrying the transgene. The bombardment at 1100 psi helium pressure with 7.5 cm distance between stopping plate to tissue and one time bombardment using 2.0 µg DNA per bombardment was obtained as the best condition for DNA delivery and also minimised tissue damage. Hence, the results of this study will be useful as a guideline to generate transformed oil palm.

INTRODUCTION

Oil palm is the most important agricultural crop in Malaysia. Every part of oil palm such as fruits, leaves (Yusof *et al.*, 2016) and trunk (Sulaiman *et al.*, 2012) has their usefulness, which make this plant very valuable agricultural crop for the country. The main product is its oil which is not only useful for food stock but also for non-food applications such as industrial chemicals, pharmaceuticals, and nutraceuticals (Kushairi *et al.*, 2019). However, palm

oil needs to compete with other vegetable oils such as rapeseed, sunflower, olive, corn and others. In order to remain competitive, the palm oil industry has to increase its yield as well as to improve the palm oil quality and produce novel products (Kushairi *et al.*, 2018). The conventional method to improve palm oil quality is limited because of long regeneration time, narrow gene pool and open pollination behaviour of oil palm (Parveez *et al.*, 2015b). Thus, another approach, namely genetic engineering was embarked on to introduce specific genes into genomes and speeding up modification process of palm oil quality (Masani *et al.*, 2018).

Various ways to deliver foreign deoxyribonucleic acid (DNA) into the oil palm genome have been

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evaluated such as bombardment (Parveez *et al.*, 2000; 2015a), *Agrobacterium*-mediated transfer (Izawati *et al.*, 2015; 2012), polyethylene glycol (PEG)-mediated transfection and DNA microinjection (Masani *et al.*, 2014). DNA delivery by bombardment has been widely used and proven in many plants such as soyabean (Khalafalla *et al.*, 2005), rice (Tian *et al.*, 2015; Zhao *et al.*, 2011; Higuchi *et al.*, 2001; Datta *et al.*, 2000), wheat (Melchiorre *et al.*, 2002) and banana (Mahdavi *et al.*, 2014; Sreeraman *et al.*, 2005). However, the transformation efficiency of oil palm by bombardment was low at about 1%-1.5% (Parveez, 2000). Meanwhile, as reported by Nurfahisza *et al.* (2014), there were escapes and chimera detected in the regenerated transformed oil palm plantlets. In addition, bombardment method could also result in the integration of multiple copies of transgenes which could subsequently lead to co-suppression (Reddy *et al.*, 2003; El-Shemy *et al.*, 2004).

The physical (Parveez *et al.*, 1997) and biological (Parveez *et al.*, 1998) bombardment parameters affecting DNA delivery in oil palm based on transient expression of *gus* gene have been evaluated and successfully determined. However, these studies did not show the ability of transformed cells to regenerate which might not be directly translated into stable transformation. Thus, this article evaluates several bombardment parameters to determine the best condition for optimum DNA delivery and subsequently increase the ability to generate oil palm with integrated transgenes. This was basically determined by the presence of transgene into the regenerated shootlets.

MATERIALS AND METHODS

Plant Materials

Oil palm embryogenic calli received from the Breeding and Tissue Culture Unit of the Malaysian Palm Oil Board (MPOB) were derived from cabbages. The cabbages were cultured on callus induction media [MS salts (Murashige and Skoog, 1962) + Y_3 vitamins (1 g litre^{-1} thiamine HCl + 1 g litre^{-1} pyridoxine HCl + 1 g litre^{-1} nirotinic acid) (Eeuwans, 1976) + 0.1 g litre^{-1} myo-inositol and L-glutamine + 3% sucrose + 5 μM 2,4-dichlorophenoxyacetic acid (2, 4-D) + 0.25% activated charcoal + 0.7% agar] and incubated in the dark at 28°C. The calli were subcultured every four weeks (Parveez *et al.*, 2000) until embryogenic calli were formed.

Plasmid Construct and DNA-microcarrier Precipitation

The bombardment was performed using pAHC25 plasmid which contains *gus* and *bar* genes, both driven by maize ubiquitin promoter (*Figure 1*). The *bar* gene confers resistance to bialaphos. Precipitation of plasmid DNA onto microcarrier was performed according to the modified method developed by Parveez (1998). While vortexing, 10 μl of pAHC25 plasmid DNA (1 μg μl^{-1}), 50 μl of CaCl$_2$ (2.5 M) and 20 μl of spermidine (0.1 M) were sequentially added to the suspension of gold particles (50 μl at 60 μg ml^{-1}). The mixture was spun for 1 min at 8000 xg and the supernatant was
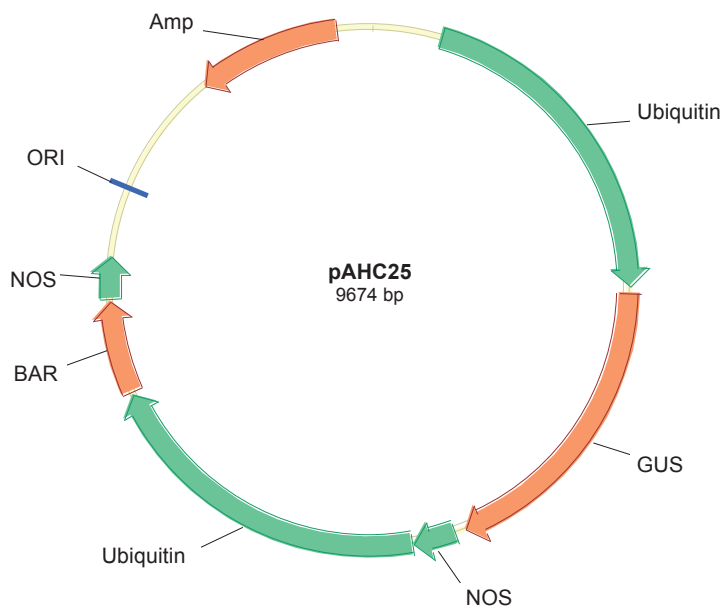
Source: Modified from Christensen and Quail (1996).

Figure 1. Map of pAHC25 plasmid. The plasmid (9674 bp) contains the bar and gus genes under the control of ubiquitin promoter and nos as terminator. The bar gene confers resistance to bialaphos.

discarded. Then, the mixture was washed using 250 μl of absolute ethanol and spun again at 8000 xg for 40 s. The supernatant was again discarded and the washing step was repeated three times. Finally 60 μl of absolute ethanol was added to resuspend the DNA-microcarrier mixture. For bombardment, 7 μl of DNA-microcarrier mixture was loaded onto the centre of macrocarrier and air-dried before bombardment was performed.

Particle Bombardment

The standard bombardment was performed according to Parveez *et al.* (1997; 1998). Four parameters (*Table 1*) were examined independently while the standard conditions (mark with*) were used during the bombardment. The experiment was carried out in five replicates for each treatment.

Selection and Regeneration

Bombarded calli were cultured on fresh embrogenic calli (EC) agar medium [MS salts (Murashige and Skoog, 1962); Y3 vitamins; 0.0375 g litre⁻¹ NaFeEDTA; 0.1 g litre⁻¹ myo-Inositol; 0.1 g litre⁻¹ L-glutamine; 0.1 g litre⁻¹ L-asparagine; 0.1 g litre⁻¹ L-arginine; 3% sucrose; 5 μM α-Naphtaleneacetic acid (NAA); 0.8% plant agar; pH 5.7] for about four weeks. The bombarded calli were then transferred onto EC agar media containing 1 mg litre⁻¹ bialaphos followed by 2 mg litre⁻¹ and 3 mg litre⁻¹ for the third month and onward.

Molecular Analysis of Putative Transgenic Plantlets

Leaves from putative transgenic plantlets were cut and subjected to genomic DNA isolation by using the method of Doyle and Doyle (1990) with a modification using 16 000 xg of centrifugation speed. The genomic DNA obtained was used as template for PCR analysis by using primers as listed in *Table 2*. An internal control gene was amplified by using a programme described by Nurfahisza *et al.* (2014) (data not shown). This is to ensure that the DNA samples were suitable for PCR amplification (Parveez and Majid, 2018). Later, the amplifications of transgenes were carried out by using primers UbibarF1 and BarR2 which would amplify a part of ubiquitin promoter and *bar* gene. The expected size of the amplified band was 1.2 kb.

The number of shoots so called transformant carrying the *bar* gene was identified and counted. After that, the number of transformant was divided with five (the number of replicates per treatment) to calculate the average transformant for each treatment. Finally, the value of average transformant obtained for each treatment was plotted into graph.

RESULTS AND DISCUSSION

Optimising the transformation parameters is one of the crucial studies that needs to be carried out. Parveez *et al.* (1997; 1998) have successfully determined at transient level the optimised parameters for oil palm transformation via particle bombardment by using *gus* gene. The putative transgenic oil palm plants have been generated by using these optimised parameters. However, analysis on putative transgenic obtained showed that most of the plants were not true transformants (Nurfahisza *et al.*, 2014). This might be due to the expression of transgenes at transient level which

TABLE 1. LIST OF PARTICLE BOMBARDMENT PARAMETERS EVALUATED IN THIS WORK

Parameters	Treatments
Helium pressure (psi)	650, 900, 1 100*, 1 350, 1 550
Distance of stopping plate to tissue (cm)	6.0, 7.5*, 9.0, 10.5, 12.0
Number of bombardment	1*, 2*, 3
DNA quantity (μg)	0.50, 1.00, 1.25, 1.50*, 1.75, 2.00

Note: *Standard condition according to Parveez *et al.* (1997; 1998).

TABLE 2. PRIMERS AND THEIR SEQUENCES USED FOR POLYMERASE CHAIN REACTION (PCR) ANALYSIS

Primers	Gene	Sequences	Expected size (kb)
Por-12	Internal	5'-CCCTCATCCATAGCACA-3'	1.1
Por-38	control	5'-CAGGGAGCAAAGAAGCA-3'	
UbiF1	Bar	5'-CCGCAGACGGGATCGATTTC-3'	1.2
BarR2		5'-GTCATGCCAGTTCCCGTGCT-3'	

Note: Internal control gene was amplified using Por-12 and Por-38 primers. Transgene was amplified using UbiF1 and BarR2 primers.

does not reflect the ability of the transformed cells to regenerate. Transient expression can occur whether or not the transgenes integrated into the genome of target tissue. Thus, a few bombardment parameters were evaluated at stable transformation level and discussed in this article. The determination of the most optimum parameters at stable transformation level based on PCR analysis on generated shootlets was much more convincing as it represents the integration of transgene into the plant genome.

Helium Pressure

A few rupture discs corresponding to different helium pressure were evaluated. After the bombarded calli have been cultured for about 17 months on selection media, a few shoots were obtained (*Figure 2*). The regenerated shootlets showed normal growth and morphology compared to the wild type. All shoots were subjected to PCR analysis for the identification of the shoots that carry the transgene (data not shown). The highest number of transformants was obtained from calli that were bombarded at 1100 psi helium pressure, followed by 1350 psi, 650 psi and 1550 psi (*Figure 3*). However, no transformant was obtained from calli that were bombarded at 900 psi. The most optimum helium pressure obtained in this study (1100 psi) is similar to the results obtained from the transient study reported by Parveez *et al.* (1997). The pressure at 1100 psi was not too harsh and could not cause cell injury (Tadesse *et al.*, 2003; Parveez *et al.*, 1997) but strong enough for the microcarrier to penetrate into the targeted tissue (Parveez *et al.*, 1997; Mousavi *et al.*, 2009). Studies on single bud banana (Sreeramanan *et al.*, 2005), male flower of banana (Mahdavi *et al.*, 2014), dates (Mousavi *et al.*, 2009) and sorghum (Tadesse *et al.*, 2003) also found that pressure at 1100 psi was an optimum pressure for bombardment. However, there were also a few bombardments that have been carried out using other pressures such as 650 psi to bombard maize (Frame *et al.*, 2000), 1350 psi for papaya (Che Radziah *et al.*, 2011) and 1800 psi for peanut plant (Schnall and Weissinger, 1995). Nevertheless, too high helium pressure and other harsh bombardment conditions could cause injury to target tissues and subsequently to regeneration of transgenic plants.

Distance from Stopping Plate to Tissue

In addition to helium pressure, the distance from stopping plate to tissue was also evaluated in this work. The distance was set at five different values, namely 6, 7.5, 9, 10.5 and 12 cm. The regenerated shoots was subjected to PCR analysis to identify the transformants (*Figure 4*). The highest number of transformants obtained from calli that were bombarded using 7.5 cm distance (*Figure 5*). As for the helium pressure, the result was also similar to the findings reported by Parveez *et al.* (1997). Study by Ruma *et al.* (2009) on tomato also showed that 7.5 cm was the optimum distance for bombardment on shoot tips, hypocotyls and cotyledons of tomato. However, certain studies showed that different distances were more suitable for target tissues of other plants. For example, it was shown that 6 cm and 9 cm were the optimum distance for soyabean (Khalafalla *et al.*, 2005) and male flowers of banana (Mahdavi *et al.*, 2014), respectively. The distances from stopping plate to tissue would certainly affect the DNA delivery into the target tissue. The acceleration may be reduced with the distance (Parveez *et al.*, 1997) and this would also reduce the ability of DNA to reach and penetrate the target tissue. Meanwhile, if the distance was too close, the target tissue may be damaged due to the high acceleration of the microcarrier. Thus, distance at 7.5 cm was the suitable distance at which the DNA could reach the target tissue without causing harmful effects to the tissues.

Number of Bombardment

The number of bombardment is another parameter that has been shown to affect the transformation efficiency. In this work, the number of bombardment was varied at one, two and three times. Results indicated that only one time bombardment could generate transformed shoots (*Figure 6*). Although shoots were also generated for two times bombardment but they were not transformants. Meanwhile, no shoot was generated when bombardment was carried out three times. Based on transient study, Parveez *et al.* (1997) found that one and two times bombardment had no significant effect on the amount of signals obtained. However, there were a number of studies reported two times bombardment gave a better transformation efficiency such as in male flower of banana (Mahdavi *et al.*, 2014), soyabean (Khalafalla *et al.*, 2005), cassava (Zhang *et al.*, 2000) and maize (Petrillo *et al.*, 2008). Two times bombardment may increase the coverage area of target tissues. However, bombardment more than two times might cause mechanical injuries to the tissues (Sreeramanan *et al.*, 2005). The tissue injury could inevitably lead to a low transformation efficiency. For oil palm, one time bombardment was sufficient to deliver DNA into the target tissue and minimise the tissue injury. Meanwhile, multiple bombardment could cause difficulty for tissues to recover and generate into shoots.

DNA Quantity per Bombardment

The final parameter evaluated in this work was the DNA quantity used in each bombardment.
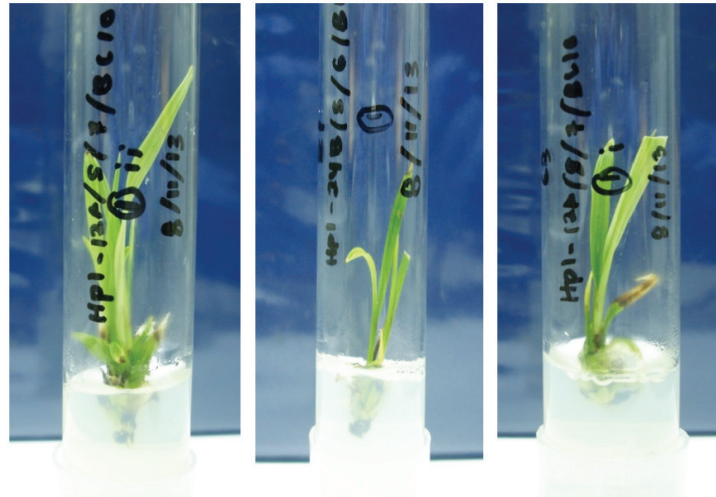
Figure 2. Shootlets generated from calli bombarded with pAHC25 plasmid after 17 months of selection. Bombarded calli were cultured on embryogenic calli (EC) media containing 1 mg litre^{-1} of bialaphos for a month, followed by culturing at increase bialaphos concentration (2 mg litre^{-1}) for a month. The calli were then cultured onwards on media with 3 mg litre^{-1} of bialaphos until shootlets generated.
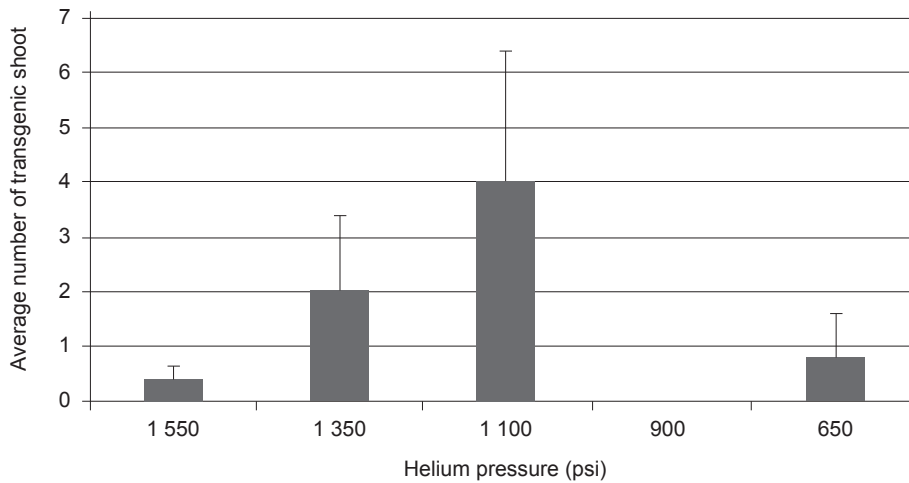

Figure 3. Effects of helium pressure on transformation and regeneration of oil palm transformant. Data presented is the mean of five replicates.
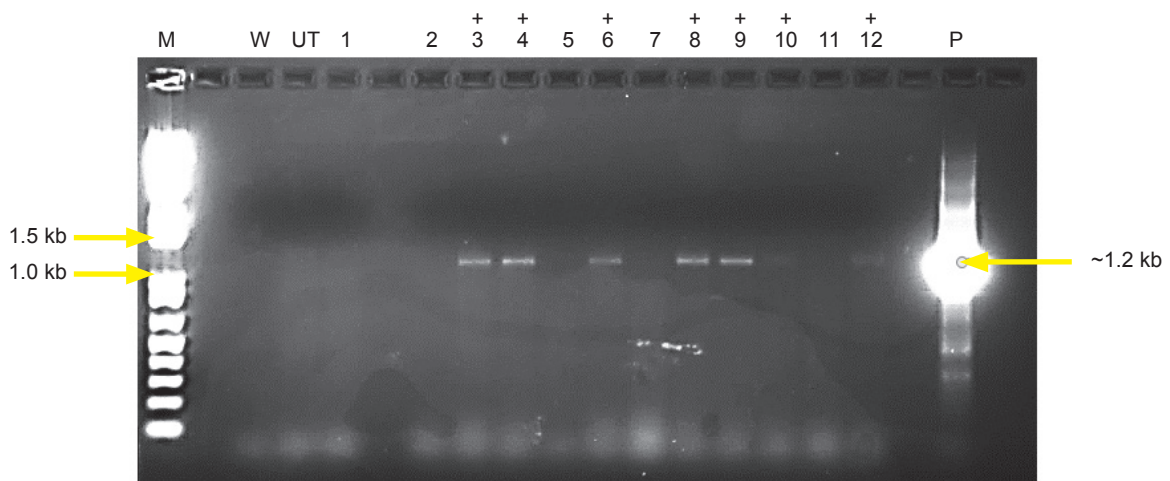

Figure 4. Polymerase chain reaction (PCR) analysis on regenerated shoots to detect the presence of bar *gene. Only seven out of 12 regenerated shoots showed the presence of* bar *gene (amplicon at ~1.2 kb). Lane M - 1 kb plus DNA ladder (Invitrogen); W - non-template control (water); UT - untransformed control; 1-12 - regenerated shoots and P - pAHC25 plasmid.*
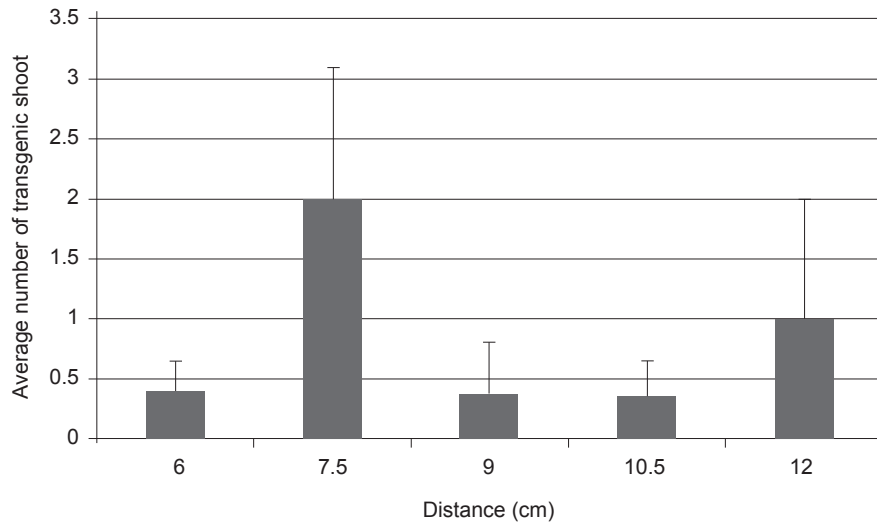
Figure 5. Effects of distance from stopping plate to tissue on transformation and regeneration of oil palm transformant. Data presented is the mean of five replicates.
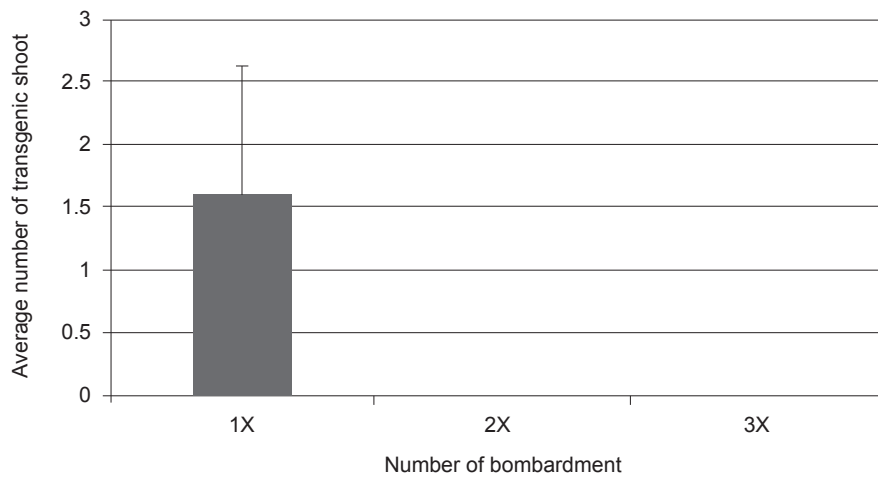

Figure 6. Effects of bombardment number on transformation and regeneration of oil palm transformant. Each value is the means of five replicates.
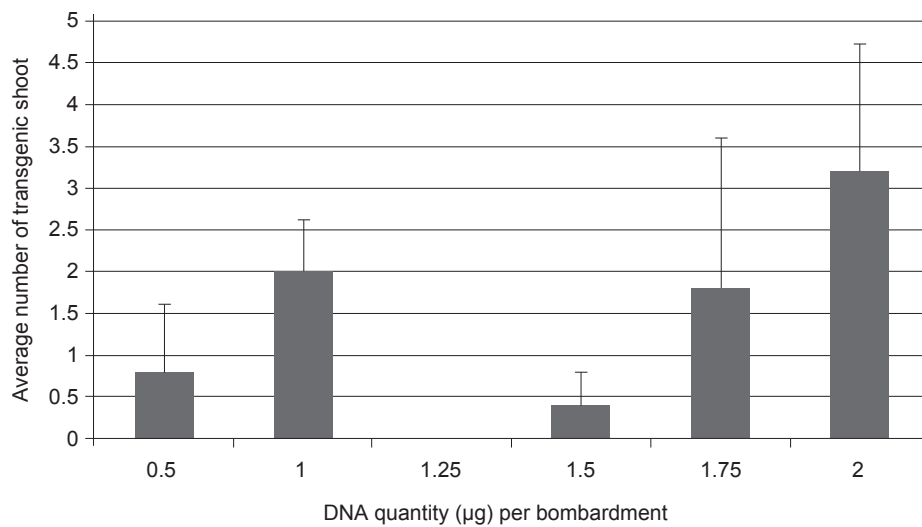

Figure 7. Effects of deoxyribonucleic acid (DNA) quantity per bombardment on transformation and regeneration of oil palm transformant. Each value is the means of five replicates.

Among five different treatments of DNA quantity per bombardment examined, 2.0 µg of DNA per bombardment was shown to produce the highest number of transformants (*Figure 7*). This result is in contrast to the findings of transient study by Parveez *et al.* (1998) which indicated that the optimum amount of DNA was at 1.5 µg. Study on rice (Carsono and Yoshida, 2008) and banana (Sreeramanan *et al.*, 2005; Mahdavi *et al.*, 2014) also used 1.5 µg of DNA for optimum bombardment. However, Mousavi *et al.* (2009) used a much higher DNA quantity per bombardment which was at 2.5 µg DNA. A sufficient amount of DNA used in bombardment could increase the transformation efficiency while a low DNA quantity would result in the insufficient ratio of DNA to microcarrier (Matroodi *et al.*, 2013).

CONCLUSION

The optimisation of biolistic parameters was crucial to increase the transformation efficiency. Numerous optimisation studies on different plants have been carried out as different tissues and plants need different conditions for bombardment. Previous study on oil palm which was based on transient expression showed a slight variation in results as compared to this study which was based on stably transformed shoot generated. Results obtained from this study showed that transformed plantlets were successfully regenerated through one time bombardment with 2.0 µg of DNA at pressure of 1100 psi and 7.5 cm distance between stopping plate to target tissue. The results could be used as a guideline in the effort to generate the stably transformed oil palm.

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