Journal of Oil Palm Research Vol. 24 April 2012 p. 1296-1302

OPTIMISATION OF 2-DEOXYGLUCOSE CONCENTRATION FOR IDENTIFYING THE SENSITIVITY LEVEL FOR OIL PALM EMBRYOGENIC CALLI

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ABSTRACT

In this study, the sensitivity of oil palm calli to 2-deoxyglucose (2-DOG) was evaluated to determine the optimal concentration to be used as a selection agent for oil palm embryogenic calli. It was reported earlier that 2-DOG has been used for selecting transformants using the DOG^{R}1 *gene which encodes 2-deoxyglucose-6-phosphate (2-DOG-6-P) phosphatase. In plants, 2-DOG is converted into 2-DOG-6-P by endogenous hexokinase which results in the inhibition of cell growth. Therefore, an experiment to determine the optimal concentration of 2-DOG required to fully inhibit the regeneration of untransformed oil palm embryogenic calli was carried out. The untransformed embryogenic calli were cultured on embryogenic callus (EC) medium supplemented with different concentrations of 2-DOG, ranging from 0 to 1000 mg litre^{-1}. Results show that 400 mg litre^{-1} of 2-DOG led to nearly complete inhibition (>80%) of oil palm embryogenic calli regeneration. This was demonstrated by a reduction in the calli weight and in the ability to form whitish embryoids. This optimal concentration of 2-DOG is recommended to be used for the selection and regeneration of transformed oil palm embryogenic calli after transformation using the* DOG^{R}1 *gene in future experiments.*

Keywords: 2-deoxyglucose, selection agent, untransformed calli, oil palm embryogenic calli.

Date received: 20 September 2010; **Sent for revision:** 20 October 2010; **Received in final form:** 24 January 2011; **Accepted:** 2 February 2012.

INTRODUCTION

The prerequisites for producing transgenic plants are the availability of a suitable gene transformation system, the existence of an efficient transformant selection system and a regeneration system to fully regenerate the transformed plant cells (Parveez, 1998). A good selection system enables transgenic plants or cells to be distinguished, individually isolated and selectively regenerated into whole plants after transformation. The expression of a selectable marker gene results in a product that allows the survival of the transformed cell in the presence of a selective agent that prevents the regeneration of the untransformed cell (Brasileiro and Dusi, 1999). The selection system can be divided into two, namely the negative and positive selection systems. The most common negative selection marker genes are those that confer resistance to herbicides or antibiotics which will kill non-transgenic tissues in the presence of the selection agent (Darbani *et al.*, 2007). Examples of negative selectable marker genes are the *bar* gene which mediates resistance to the herbicide phosphinothricine (Lohar *et al.*, 2001) and the *nptII* gene which confers resistance to the antibiotic kanamycin (Burris *et al.*, 2007). The selection agent used is usually toxic to the untransformed cells. Depending on the plant species, the selection method is not always effective and could frequently

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affect transgenic plant regeneration. Sometimes, the transformed cells may not generate into plants because the neighbouring dying untransformed cells could release growth inhibitors and toxic substances, and compromise the uptake of essential minerals and vitamins from the culture medium (Penna *et al.,* 2002).

Transfer of the selectable marker gene into the host genome confers advantage to the transformed cell or tissue enabling it to grow in the presence of the antibiotic or herbicide. The use of the negative selection system has resulted in public concern over the use of existing negative marker genes that encode for herbicide and antibiotic resistance (Putcha, 2000). One of the concerns is the possibility of escape of the foreign gene through pollen or seed dispersal from the transgenic plants to the wild type plants of the same species, or to other cultivars or weed relatives of the crop, which could result in genetic pollution (Jaiwal *et al.,* 2002).

The antibiotic resistance gene may also get passed into the genome of microorganisms in the gastrointestinal tract or in the soil, making them resistant to treatment with such antibiotics. Through the horizontal gene transfer (HRT) mechanism, the antibiotic resistance marker gene from genetically modified (GM) crops may be transferred to the gut and to soil bacteria, or to the cells of animals which eat these plants (FAO/WHO, 2000; Thompson, 2000). However, this has not been reported for any transgenic crops.

As compared to negative selection, a positive selection system allows for the identification and advantage selection of genetically transformed cells without damaging or killing the untransformed cells. In addition, this system promotes the growth of transformed cells (Miki and McHugh, 2004). Usually, the positive selection agent is not directly toxic to plant cells (Wenck and Hansen, 2005). Untransformed cells either grow slowly or not at all as compared to transformed cells. The transformed cells will have a metabolic advantage such as the capability of consuming a rare sugar, or other competitive advantages for stimulating cell growth over the untransformed cells, such as a response to a particular hormone and adaptation to extreme temperature. Examples of positive selection agents are 2-deoxyglucose (*DOG^{R}1* gene), betaine aldehyde (*badh* gene), D-xylose (*xylA* gene), D-mannose (*manA*/*pmi* gene), benzyladenine-N-3-glucoronide (*uidA*/*gusA* gene), L-cysteine (*ocs* gene), methotrexate (*dhfr* gene), 4-methyltryptophan (*tdc* gene) and s-aminoethyl (*dhps* gene) (Miki and McHugh, 2004).

The 2-deoxyglucose (2-DOG) is an example of a positive selectable marker system for plant transformation (Kunze *et al.*, 2001). The 2-DOG is a glucose analogue which has the 2-hydroxyl group replaced by hydrogen which prevents it from glycolysis. The 2-DOG is also known as 2-deoxy-D-glucose, 2-deoxy-D-mannose, 2-deoxy-D-arabino-hexose and 2-DG. The molecular formula, molar mass and melting point of 2-DOG are $C_6H_{12}O_5$, 164.16 g mol^{-1} and 142°C-144°C, respectively (Greenwood and Earnshaw, 1997).

This glucose analogue is similar to mannose and xylose in that it is non-toxic to plant cells. However, 2-DOG will prevent cell growth and development when it is converted by hexokinase. Therefore, the uptake of this glucose will give toxic effects to all living cells. The 2-DOG cannot be metabolised by untransformed cells and will be converted into 2-DOG-6-P through phosphorylation by endogenous hexokinase in the cytosol. When 2-DOG is added into the culture medium, plant growth is reduced due to 2-DOG-6-P accumulation, resulting in inhibition of glycolysis, protein synthesis, cell wall polysaccharide synthesis and also interference in protein glycosylation (Kunze *et al.*, 2001). The toxicity effects of 2-DOG-6-P may be similar to the toxicity of mannose-6-phosphate which leads to apoptosis or programmed cell death (Stein and Hansen, 1999). It also causes phosphate and ATP starvation, two substances which are involved in cell division and elongation, giving rise to growth inhibition (Aragão and Brasileiro, 2002).

The addition of 2-DOG inhibits root growth of many plants such as flax, vetch, clover, rye, barley, maize and oats (Farrar, 1995). The growth of *Nicotiana tabacum* cells and *Picea excelsa* (spruce) cells in tissue culture is strongly inhibited by 2-DOG (Zemek *et al.*, 1975; 1976). The reaction products of 2-DOG in these higher plants, *i.e.*, 2-DOG-1-P, 2-DOG-6-P, UDP-2-DOG and 2-DOG containing di- and oligosaccharides, were detected (Stanek *et al.*, 1963). The formation of these metabolites gives the inhibitory effect of 2-DOG. It had been reported that toxic compounds such as antibiotics which are used as selection markers in plant transformation also affect plant metabolism (Penna *et al.*, 2002). The addition of certain antibiotics frequently results in the regeneration of transgenic plants exhibiting changes in physiological and morphological aspects.

The 2-DOG system works well in different varieties of potato and was effective under field conditions (Kunze *et al.*, 2001). No phenotype differences were detected between the transgenic plants and the control plants. The 2-DOG can be used as an effective selectable marker because it is: (a) soluble in the plant culture medium; (b) easily absorbed by plant cells; (c) easily available; and (d) safe (Todd and Tague, 2001). Therefore, it appears that the use of this marker for selecting transgenic plants is promising. At present, in negative selection, the herbicide Basta and the antibiotic hygromycin were shown to be effective selection agents for oil palm transformation;

however, at a low concentration of 40 mg litre^{-1}, the growth of oil palm embryogenic calli was inhibited (Parveez *et al.*, 1996). On the other hand, for positive selection, only the phosphomannose isomerase (PMI) system has been tested in the oil palm transformation system. It was reported that 30 mg litre^{-1} of mannose without sucrose was suitable to select and regenerate the untransformed oil palm embryogenic calli (Bahariah, 2010).

In this study, we evaluated the possibility of using 2-DOG as a selectable marker for oil palm by determining the optimal concentration that effectively suspended the growth of the untransformed cells.

MATERIALS AND METHODS

Regeneration System of Untransformed Oil Palm Explants into Full Plants

Oil palm young leaf cabbages were used as the source of explants to initiate calli. The calli were multiplied in embryogenic calli (EC) liquid medium [MS salts (Murashige and Skoog) + Y_3 vitamins + 0.0375 g litre^{-1} NaF eEDTA + 0.1 g litre^{-1} myo-Inositol + 0.1 g litre^{-1} L-glutamine + 0.1 g litre^{-1} L-asparagine + L-arginine + 3% sucrose + 5 µM α-naphtaleneacetic acid (NAA) + 0.8% agar, pH 5.7] using the suspension culture technique (Paranjothy *et al.*, 1989). The calli were transferred onto solidified EC medium, were continuously sub-cultured onto fresh EC medium every month, and incubated at 28°C in the dark until shoots appeared. The explants were then transferred onto rooting medium (RM) [MS salts + Y_3 vitamins + 0.0375 g litre^{-1} NaFeEDTA + 0.1 g litre^{-1} myo-Inositol + 0.3 g litre^{-1} L-glutamine + 6% sucrose + 9 µM α-naphtaleneacetic acid (NAA) + 0.15% activated charcoal, pH 5.7] to initiate the growth of roots (Paranjothy *et al.*, 1989). Explants with strong shoots and roots were grown and maintained in soil.

Determination of 2-DOG Concentration for Untransformed Oil Palm Embryogenic Calli

Five replicates of 0.5 g oil palm embryogenic calli were cultured onto EC medium supplemented with increasing concentrations of 2-DOG (0, 50, 100, 150, 200, 300, 400, 500, 600 and 1000 mg litre^{-1}). The calli were transferred onto fresh EC medium every four weeks. The weight of the embryogenic calli was recorded every month for a total of five months. After five months, the percentage of calli proliferation (in terms of weight and size) was measured using the formula modified from Dennehey *et al.* (1994). In this study, the growth rate of the untreated control was used as the standard and was considered to have 100% proliferation assuming that there was no inhibition or stress which would reduce the proliferation rate. Thus, the increased weight of the treated tissues would give the percentage of proliferation for each treatment relative to the control.

$$\text{\% of calli proliferation} = \frac{\text{Final weight - initial weight of selected tissues (g)}}{\text{Final weight - initial weight of control tissues (g)}} \times 100$$

RESULTS AND DISCUSSION

Regeneration of Untransformed Oil Palm Explants

Before determining the effects of 2-DOG on oil palm calli, the regeneration of untransformed oil palm calli was observed as demonstrated in *Figure 1*. This was used as the benchmark for comparing the effects of 2-DOG on oil palm development. The oil palm young leaf cabbage (*Figure 1a*) started to produce calli after two to three months of culture. In order to obtain an adequate amount of calli in a short time, the calli were multiplied in liquid routine medium using the suspension culture technique (*Figure 1b*). The calli were transferred onto solidified EC medium for a period of one month (*Figure 1c*). This step allowed the calli to grow into whitish embryoids after one to two months of culture on EC medium (*Figure 1d*). The whitish embryoids that turned into greenish polyembryoids were separated to allow further production of primary shoots (*Figures 1e* to *1g*). After about one to two months, the primary shoots started to elongate (*Figures 1h* and *1i*), followed by the initiation of roots (*Figures 1j* and *1k*). Finally, the regenerated explants were transferred and maintained in soil in polybags (*Figure 1l*). The regeneration process, starting from the embryogenic calli to full explants took between 12 and 18 months.

Determination of 2-DOG Concentration for Untransformed Oil Palm Embryogenic Calli

Based on percentage of proliferation. In order to determine the lowest concentration of 2-DOG which effectively inhibited the growth of untransformed oil palm calli and at the same time only allowed the transformed calli to grow, the calli were cultured onto EC medium supplemented with various concentrations of 2-DOG. The embryogenic calli which were obtained from the suspension culture were cultured onto embryogenic calli medium for a period of one month, followed by sub-culturing them onto EC media containing 0 to 1000 mg litre^{-1} of 2-DOG. *Figure 2* shows the percentages of oil palm embryogenic callus

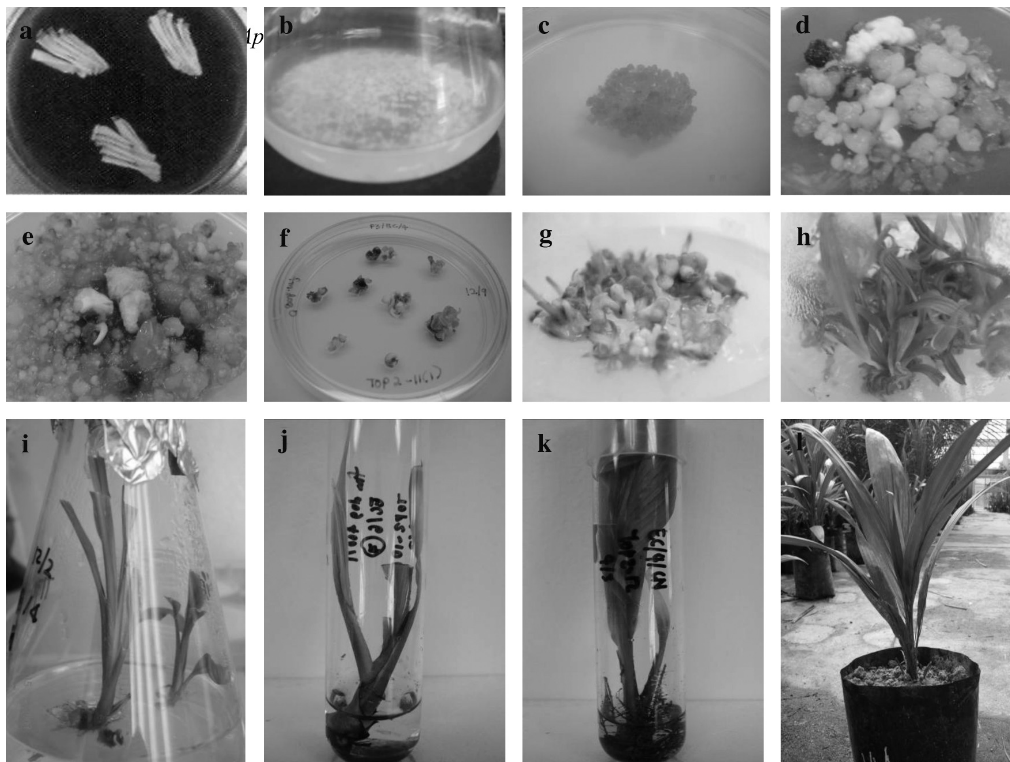

Figure 1. Regeneration of untransformed oil palm embryogenic calli. a: oil palm young leaf cabbage; b: oil palm calli in suspension culture; c: calli on solidified embryogenic calli (EC) medium; d: whitish embryoids appeared after one month of culture; e: whitish embryoids turned into greenish embryoids; f: greenish embryoids were separated and cultured on EC medium; g: polyembryoids with primary shoots; h: shoot development; i: shoot elongation; j and k: root development; l: oil palm plant in polybag.

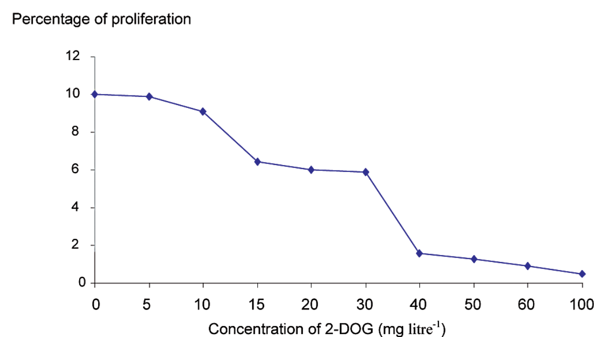

Figure 2. Proliferation percentages of oil palm embryogenic calli after five months at different concentrations of 2-deoxyglucose.

TABLE 1. MEAN WEIGHTS OF OIL PALM EMBRYOGENIC CALLI AFTER FIVE MONTHS AT DIFFERENT CONCENTRATIONS OF 2-DEOXYGLUCOSE

2-deoxyglucose (g litre^{-1})	Mean
0	28.41±0.49^{g}
50	28.15±0.21^{g}
100	25.92±0.24^{f}
150	18.41±0.09^{e}
200	17.32±0.03^{d}
300	17.04±0.02^{d}
400	5.03±0.06^{c}
500	4.14±0.03^{c}
600	3.05±0.01^{b}
1 000	1.86±0.00^{a}

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 means ±S.E of five replications after five months.

proliferation after five months in different concentrations of 2-DOG. At 50, 100, 150, 200 and 300 mg litre^{-1} of 2-DOG, the proliferation rates were 99%, 91%, 64%, 60% and 59%, respectively. Generally, from 0 to 300 mg litre^{-1}, the weights increased within five months, but started to decrease slowly at 400 to 1000 mg litre^{-1}. The weight increment may have been due to the availability of a sugar supply from the medium, or that low concentrations of 2-DOG did not affect the growth ability of the calli. At 400, 500 and 600 mg litre^{-1}, the proliferation percentages decreased to 16%, 13% and 9%, respectively. At these concentrations (400-600 mg litre^{-1}), the growth of the calli was affected, causing the weight to start to decrease. At the highest concentration of 2-DOG (1000 mg litre^{-1}), callus growth was inhibited by almost 95%. The growth inhibition was presumably due to absence of the enzyme 2-DOG-6-P phosphatase which was needed to negate the toxic effect of 2-DOG-6-P. The accumulation of this toxic compound affected the proliferation process of the calli, causing the reduction in weight. The significant difference in the average weight of oil palm embryogenic calli in the media containing 10

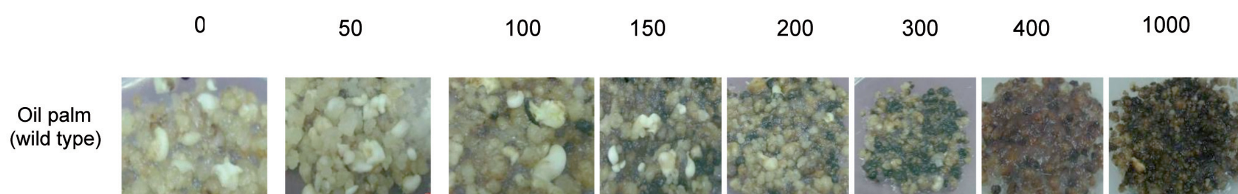

Figure 3. Effect of 2-deoxyglucose on formation of whitish embryoids at different concentrations. Whitish embryoids were produced and remained healthy at concentrations of 0-150 mg litre^{-1}. At concentrations of 200 and 300 mg litre^{-1}, embryoids started to turn dark and die. As the concentrations increased from 400-1000 mg litre^{-1}, all the embryoids were killed and turned dark.

different concentrations of 2-DOG was confirmed using the Duncan Multiple Range Test. The results are summarised in *Table 1*. From *Table 1*, the test concentrations of 2-DOG produced significant differences in calli weight within five months. As explained earlier, the proliferation percentage was reduced as the concentration of 2-DOG increased.

Based on formation of whitish embryoids. Apart from determining the effects of 2-DOG concentration quantitatively, these effects were also evaluated visually. Based on *Figure 3*, the untransformed oil palm embryogenic calli were observed for their ability to form embryoids at increasing concentrations of 2-DOG, ranging from 0 to 1000 mg litre^{-1}. Proliferation of oil palm calli into whitish embryoids decreased with increasing concentrations of 2-DOG. Formation of whitish embryoids could be observed from 0 up to 200 mg litre^{-1}, which might have been due to the endogenous resistance of the embryogenic calli towards low concentrations of 2-DOG. At 300-1000 mg litre^{-1}, almost none of the embryogenic calli proliferated into whitish embryoids even though the amount of calli increased. We postulated that at 300 mg litre^{-1} of 2-DOG, accumulation of 2-DOG-6-P started to affect the growth ability of the embryogenic calli, causing them to fail in proliferation into embryoids. Further, as the concentrations increased up to 1000 mg litre^{-1}, this extensive exposure to 2-DOG caused the embryogenic calli to turn black and die.

In addition, it was observed that the embryogenic calli were still multiplying at 0-300 mg litre^{-1} 2-DOG. However, at 400-1000 mg litre^{-1}, cell multiplication slowly decreased with the increasing concentrations of 2-DOG. At 400 mg litre^{-1}, no whitish embryoid was obtained, therefore this concentration may be suitable for use in the selection of transformed embryogenic calli. As 2-DOG cannot be metabolised, it is thought that the calli gained the carbon source or food supply from the sucrose present in the culture medium. This promoted calli to multiply and increase in size. When all the carbon source had been used up, the calli were not able to multiply further and, therefore, the amount of calli decreased. The ability to produce embryoids was also affected. The accumulation of 2-DOG-6-P, a product resulting from phosphorylation of 2-DOG, led to toxicity to the embryogenic calli. As the amount of 2-DOG-6-P that accumulated increased, the production of whitish embryoids decreased. In addition, the colour of the calli also changed from yellowish to brownish, and then later into black as the 2-DOG concentration increased. Therefore, based on these observations, we concluded that the regeneration capacity of the calli decreased with increasing concentrations of 2-DOG.

In a study by Kunze *et al.* (2001), positive selection using 2-DOG was applied to tobacco and potato. The chosen concentrations of 2-DOG ranging from 0-700 mg litre^{-1} were tested on explants of both species. Formation of calli and shoot buds of untransformed tobacco and potato explants was strongly reduced at 500 mg litre^{-1} of 2-DOG. At 500 mg litre^{-1}, only 20% and 2% of the untransformed tobacco and potato explants were able to form calli and shoot buds, respectively. At higher concentrations, the explants turned yellowish and died. It was suggested that tobacco was more sensitive to 2-DOG as compared to potato tissues. This means that a higher concentration of 2-DOG is needed to inhibit the growth of potato explants.

On the other hand, oil palm appears to be more sensitive to 2-DOG as compared to tobacco and potato. This is because the growth of oil palm calli into embryoids were fully inhibited at 400 mg litre^{-1} of 2-DOG, whereas in tobacco and potato, concentrations of more than 500 mg litre^{-1} of 2-DOG were effective in inhibiting the formation of calli and shoot buds. By comparing these three different tissues, it seems that 2-DOG is tissue- or species-dependent (possibly, monocots *vs* dicots), whereby different tissues show different sensitivity towards different concentrations of 2-DOG.

CONCLUSION

In this study, we have successfully determined the optimal concentration of 2-DOG to be used as a selection agent for the transformation of oil palm. 2-DOG at 400 mg litre^{-1} was found to be optimum in killing untransformed oil palm embryogenic calli, and will be used for the selection of transgenic oil palm embryogenic calli in future experiments.

ACKNOWLEDGEMENT

We wish to thank the Director-General of MPOB for permission to publish this article. The authors would also like to thank Dr Ahmad Tarmizi Hashim and Ms Esther Leela from the Tissue Culture Group of MPOB for the supply of oil palm calli. Finally, we would like to acknowledge all staff of the Genetic Transformation Laboratory for their help.

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