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MINIMAL INHIBITORY CONCENTRATION OF HYGROMYCIN FOR SELECTING TRANSFORMED OIL PALM EMBRYOGENIC CALLI

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ABSTRACT

Determination of optimum concentrations for a selection agent is important in obtaining true transformants during the selection stage. In this study, the minimal inhibitory concentrations of hygromycin for four different types of oil palm embryogenic calli (suspension, fine, yellowish and whitish) that were cultured on proliferation, maturation and regeneration media were assessed. The embryogenic calli were cultured on media supplemented with different concentrations of hygromycin. Data on embryogenic callus weight increment was recorded monthly for six months during subculturing. Our results demonstrated that oil palm embryogenic calli cultured on regeneration media (EC) were very sensitive to hygromycin. The growth of embryogenic calli was affected at a relatively low concentration of hygromycin as compared to suspension calli that were cultured on proliferation media (L-1). The regeneration of fine, yellowish and whitish embryogenic calli was completely inhibited at 5-6 mg litre^{-1} of hygromycin. In contrast, higher concentration of hygromycin at 10 mg litre^{-1} is needed to inhibit the proliferation of suspension calli cultured on proliferation media. The hygromycin concentrations determined for each of the embryogenic callus types can be used as a guideline to select true transformants in future oil palm transformation works.

Keywords: hygromycin selection, oil palm, embryogenic calli.

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INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.), a West African origin, has now become a major economic crop in Malaysia and the most efficient yielding oil bearing crop in the world (Kushairi *et al*., 2017). Being a major economic crop, it is important for it to remain competitive and sustainable. Biotechnology has been identified as a tool to improve the value of oil palm as it could produce high value fatty acids and metabolites which could not be achieved or required a very long process through conventional plant breeding. Furthermore with the long regeneration period (7-10 years) of oil palm breeding, biotechnology could effectively reduce the time to genetically improve oil palm (Sambanthamurthi *et al*., 2009). Biotechnology of oil palm aims at modifying the composition of the fatty acid, especially increasing the oleic acid, stearic acid, palmitoleic acid and ricinoleic acid as well as synthesising novel metabolites such as lycopene (carotenoid) and polyhydroxybutyrate, a biodegradable plastics (Masura *et al*., 2017; Parveez *et al*., 2015a). Biotechnological efforts require a genetic transformation system to transfer foreign genes into oil palm.

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Genetic transformation of monocot was started as early as 1980s by the production of transgenic *Asparagus officinalis* (Bytebier *et al*., 1987). Since then, tremendous efforts have been made to transform other monocots including oil palm with useful genes to enhance the plant quantitative traits. Those plants were usually transformed either using particle bombardment or *Agrobacterium*-mediated transformation. The method to transform oil palm via particle bombardment and *Agrobacterium*-mediate gene transfer was developed by Parveez and Christou (1998) and Masli *et al*. (2009), respectively. Both techniques are routinely used to incorporate different genes of interest into oil palm target tissue. Bombardment technique was successfully used to transform polyhydroxybutyrate (Parveez *et al*., 2008; 2015b; Yunus *et al*., 2008), herbicide Basta or glufosinate ammonium resistant (Parveez *et al*., 2000; Yunus and Kadir, 2008), phosphomannose isomerase (PMI) (Bahariah *et al*., 2013), and green fluorescent protein (Majid and Parveez, 2007; 2016; Parveez and Majid, 2008) into oil palm tissues. Meanwhile, 2-deoxyglucose-6-phosphate phosphatase (DOG^R1) (Izawati *et al*., 2015) and *bar* genes (Masli *et al*., 2009) were transformed via *Agrobacterium*-mediated gene transfer technique. Prior to the transformation of the desired genes, preliminary experiments are often conducted to determine minimal inhibitory concentration (MIC) of selection agent required for efficient selection under established culture condition. For oil palm, a number of MIC studies have been done using different types of selection agents such as kanamycin, geneticin G-418, neomycin, hygromycin, herbicide Basta, phosphomannose isomerase, DOG^R1, bialaphos and glufosinate ammonium (Parveez *et al*., 2007; Bahariah *et al*., 2012; Masli *et al*., 2012; Nurfahisza *et al*., 2016). Based on the MIC result, the putatively transformed tissues will be selected and regenerated on the specific concentration of the desired selection agent.

At present, Basta selection has been frequently used in oil palm transformation studies. As reported in previous oil palm transformation works using Basta as selection agent, about 1%-1.5% and 0.7% of transformation efficiency was obtained via particle bombardment (Parveez *et al*., 2000) and *Agrobacterium*-mediated transformation (Masli *et al*., 2009), respectively. Even though the transformation efficiencies obtained from both oil palm transformation techniques are acceptable for recalcitrant plant species, the oil palm transformation efficiency is still low compared to other monocots (Izawati *et al*., 2015; Parveez *et al*., 2015a). The low transformation efficiency is often associated with the presence of false positive or escapes plants. Generation of escapes or chimeras during selection and regeneration of transformed tissues cultured on culture media containing a selection agent is a major problem in most transformation systems (Miki and McHugh, 2004).

The type of selection agent, optimum concentration of selection agent and optimum period of selection phase are all the prerequisites that must be known for efficiently selecting true transformants. Usually, only minor parts of the target tissues are transformed, while the vast majority remains non-transformed. The chances to recover the transgenic lines without selection are usually low. Thus, it is necessary to determine the optimum parameters for selection as a less stringent selection could result in the regeneration of escapes (d'Erfurth *et al*., 2003; Gutierrez *et al*., 1997). The problem of escapes has been reported in early transformation works in rice (Christou and Ford, 1995), sorghum (Casas *et al*., 1997), tobacco (Park *et al*., 1998) and wheat (Altpeter *et al*., 1996). Similar problem has also been reported in oil palm transformation suggesting the escapes phenomenon in oil palm could be due to the use of low concentration of herbicide Basta during selection (Nurfahisza *et al*., 2014). Recently, Itaya *et al*. (2018) reported that regeneration of escapes transgenic soyabean plants could be eliminated through prolonged selection on hygromycin selection medium.

In this study, we evaluated hygromycin as an alternative to the Basta selection. Hygromycin is the second most widely used selection agent in plant transformation study (Miki and McHugh, 2004). It inhibits protein synthesis by causing mistranslation and interferes with protein translocation (Gonzalez *et al*., 1978). Hygromycin has been commonly used in monocot transformation such as rice (Htwe*et al*., 2014; Shimamoto *et al*., 1989) and wheat (Raja *et al*., 2010). In a previous study on oil palm, hygromycin at 20 mg $litre^{-1}$ was identified as minimal inhibitory concentration for completely prevented the growth of immature embryos (Abdullah *et al*., 2005; Parveez *et al*., 2007). Parveez *et al*. (2007) mentioned that, as early as five weeks of culture on 20 mg $litre^{-1}$ of Basta and hygromycin was effective to completely kill immature embryo and they are the most suitable selection agents to be applied when immature embryos is used as target tissue compared to kanamycin, paromomycin and geneticin G418. However, information on the optimal hygromycin concentration for selecting oil palm transformants derived from different tissues and developmental stages has not been reported.

The aim of this study is to determine the MIC of hygromycin for selecting transformed oil palm embryogenic calli at different stages of culture such as suspension, fine, yellowish or whitish calli. Suspension and fine calli are normally used as target tissues for oil palm transformation (Parveez *et al*., 2008; 2015b; Masli *et al*., 2009; Bahariah *et al*., 2013; Izawati *et al*., 2015). Meanwhile, the yellowish and whitish calli represent slightly advance developing

stages of the embryogenic calli during tissue culture. The concentration of hygromycin depends largely on genotype, type of explants, explant sizes and even within different stages of development of the same species (Milojević *et al*., 2012). Therefore, it is important to determine the MIC for each tissue culture stage to tighten the selection process. This outcome is critical to eliminate the possibility of obtaining escape and chimeric plant. The information gained from this study will improve the selection process in oil palm transformation work to successfully select and regenerate true transformants.

MATERIALS AND METHODS

Preparation of Culture Media

In this study, callus proliferation (L-1), callus maturation (MSB) and callus regeneration (EC) media were used. The composition of these media is listed in *Table 1*. Hygromycin (Sigma) was supplied as sterile-filtered solution in 50 mg ml^{-1} stock and it was added directly to the media. The media were autoclaved at 121°C for 15 min and cooled down to 50°C before hygromycin was added to the needed concentrations.

Preparation of Plant Materials

Plant materials were obtained from Clonal Propagation Group, MPOB. Embryogenic calli established from *tenera* oil palm cabbage were used as the starting material. The embryogenic calli consist of suspension, fine, yellowish and whitish calli. The first part of the experiment was carried out to determine the minimal hygromycin concentration that inhibits proliferation of suspension (immature) calli cultured on proliferation media, L-1. The suspension calli aged 8-month old were obtained from liquid culture (*Figure 1A*). The suspension calli were filtered from L-1 liquid culture medium using 300 mm stainless steel mesh. The filtered calli (0.5 g) were cultured on L-1 solid media containing various concentrations of hygromycin (0, 2, 4, 6, 8 and 10 mg $litre^{-1}$) and incubated in the dark at 28°C.

The second part of experiments was conducted to determine the optimum hygromycin concentration that inhibits regeneration of fine, yellowish and whitish calli (collectively named as mature calli) on regeneration media, EC. Fine calli refers to the suspension calli that were previously cultured in maturation liquid media (MSB) for a month and then freshly transferred on EC solid media (*Figure 1B*). While, the yellowish and whitish calli were derived from fine calli that were cultured for a month on EC

TABLE 1. LIST OF CULTURE MEDIA USED IN THIS STUDY

Media	Composition
L-1	MS (Murashige and Skoog, 1962) + Y3 vitamins + 0.0375 g $litre^{-1}$ NaFeEDTA + 0.1 g $litre^{-1}$ myo-inositol + 3% sucrose, 0.93 mg $litre^{-1}$ α-Naphtaleneacetic acid (NAA) + 1 mg $litre^{-1}$ 2, 4-Dichlorophenoxyacetic acid (2,4-D) + 0.8 % agar, pH 5.7
EC	L-1 media without 2, 4-D
MSB	MS basal media

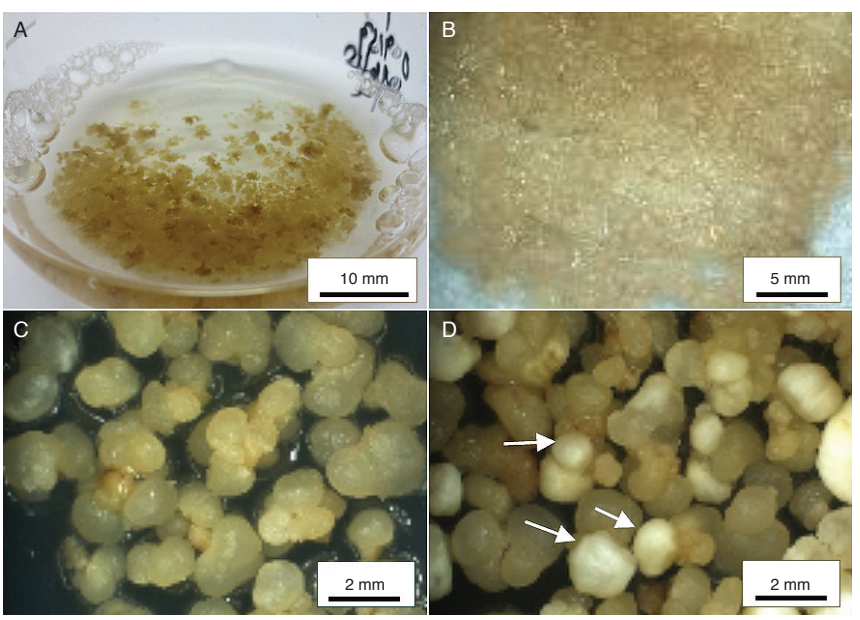

Figure 1. Types of oil palm calli used in this study. A: suspension calli; B: fine calli; C: yellowish calli; D: whitish calli (arrow).

media (*Figures 1C* to *1D*). Those embryogenic calli were cultured on EC solid media supplemented with different concentrations of hygromycin (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mg litre^{-1}). Approximately 0.5 g of embryogenic calli was cultured on each plate and the plates were incubated for 16 hr photoperiod at 28°C. Each treatment was conducted in five replicates. The embryogenic calli were subcultured every four-week interval onto fresh media containing the same concentration of hygromycin for six months.

Measurement of Proliferation Rate

Callus development on media added with different hygromycin concentrations was compared to the control (embryogenic calli on hygromycin-free media). Weight increment of embryogenic calli cultured on different concentrations of hygromycin was measured and recorded at a monthly interval. Callus proliferation rate was calculated as the percentage of weight increment using the following equation:

$$\text{Callus proliferation rate} = \left(\frac{\text{cwHx, m1} - \text{CwHx, m0}}{\text{cwH0, m1} - \text{CwH0, m0}} \right) \times 100\%$$

where:

CwHx - weight of embryogenic calli cultured on hygromycin-media.

CwH0 - weight of embryogenic calli cultured on hygromycin free-media.

m1 - in current month

m0 - in previous month

The control treatment was defined as 100% growth in a particular data set, assuming that there was no inhibitory element affecting the callus proliferation (Parveez *et al*., 1996). The optimal concentration of hygromycin was determined based on the concentration of hygromycin that completely inhibited the proliferation and regeneration of untransformed embryogenic calli.

Statistical Analysis

All data were statistically tested by analysis of variance (ANOVA) using SPSS software. The least significant differences at $p \leq 0.05$ indicated treatment effect was significant.

Histological Analysis

Histological analysis was carried out for each developmental stage of embryogenic calli according to Fisher (1968) and Mari *et al*. (1995) with minor modifications. The embryogenic calli were fixed in glutaraldehyde-paraformaldehyde-caffeine (GPC) fixative buffer [50 ml 0.2 M phosphate buffer, pH 7.2; 20 ml 10% paraformaldehyde; 4 ml 25% glutaraldehyde (Sigma G-6257); 1 g caffeine; 26 ml distilled water] for 24 to 48 hr at room temperature. The samples were then dehydrated in ascending ethanol percentage: 30% (30 min); 50% (45 min); 70% (45 min); 80% (60 min); 90% (60 min); 95% (60 min) and twice in absolute ethanol for 60 min each. The samples were further bathed in butanol three times for a minimum of 24 hr per bath, and then in impregnation-butanol solution for a minimum of 48 hr. The tissues were then prepared for filtration with basic resin (Leica Historesin Embedding Kit) for 24 hr at 4°C under slight vacuum. The specimens were embedded in impregnation-hardener II solution, where the samples were left to dry in the mold overnight. The resin was allowed to be fully polymerised after which holders were attached and 5 μm sections were sliced using a Leica microtome (Leica, Germany). Slides were stained in Periodic acid-Schiff reagent for 20 min and Naphthol blue black at 60°C for 5 min. Distilled water, pH 4.5 was applied in between staining steps to wash off excess dye. The Periodic acid-Schiff (red stain) specifically stains polysaccharide (starch reserves and walls) while Naphthol blue-black specifically stains soluble or reserve proteins in blue-black (Fisher, 1968). Images of the slides were viewed and photographed with a camera attached to AZ100 microscope (NIKON).

RESULTS AND DISCUSSION

Preliminary Study

An initial experiment was carried out to test hygromycin concentrations ranging from 10-100 mg litre^{-1} using untransformed fine, yellowish and whitish calli. The experiment was conducted up to six months on the same selection agent containing medium. It was discovered that all the callus growth was fully inhibited even at the lowest concentration of hygromycin tested, 10 mg litre^{-1} (*Figure* 2). Hygromycin at 10 mg litre^{-1} could be too high as it could negatively affect the embryogenic callus development. The use of a very high concentration of selection agent would be too stringent which may result in the failure of moderately resistant calli to survive and regenerate (Ijaz *et al*., 2012). Therefore, a further study was conducted to determine the optimum concentration of hygromycin on oil palm callus types by focusing on concentration between 1-10 mg litre^{-1}.

Determination of Hygromycin Concentration using Suspension Calli

The L-1 callus culture is the first stage of oil palm regeneration via embryogenic calli. It is crucial to determine the MIC at this stage, when

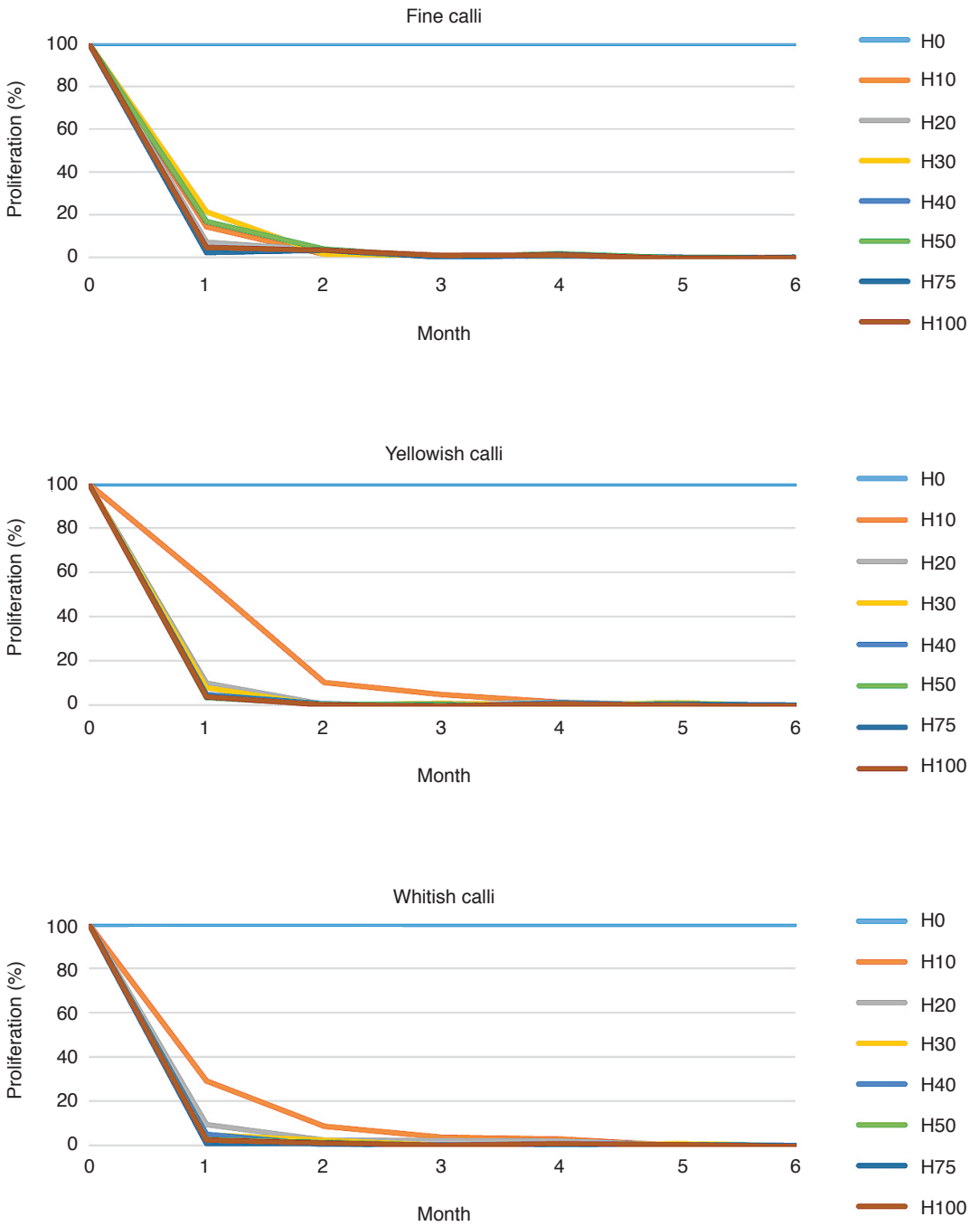

Figure 2. Preliminary minimal inhibitory concentration (MIC) experiments were performed using untransformed fine, yellowish and whitish calli cultured on 10-100 mg litre^{-1} of hygromycin up to six months.

the suspension calli are used as a target tissue. An accurate determination of MIC at the early stage would also prevent unnecessary tissue culture procedures during regeneration of transformants by eliminating untransformed tissues at the proliferation stage. Determination of MIC for suspension calli was entirely based on the weight increment, since the embryogenic calli were continuously cultured on callus proliferation media. Hygromycin at the concentrations of 2-8 mg litre^{-1} was insufficient to fully inhibit the proliferation of suspension calli. The suspension calli were shown to continue to proliferate at 90.3%, 68.6%, 50.8% and 24.2%, at the concentrations of 2, 4, 6 and 8 mg litre^{-1}, respectively (*Figure 3*). The embryogenic calli were shown to continue to increase in weight, even at the sixth month of culture. However, the proliferation of suspension calli was completely stunted when cultured on the media containing 10 mg litre^{-1} of hygromycin, at the sixth month of culture. On 10 mg litre^{-1} of hygromycin, the tissue turned brownish (*Figure 4F*) and exhibited decreased cell growth

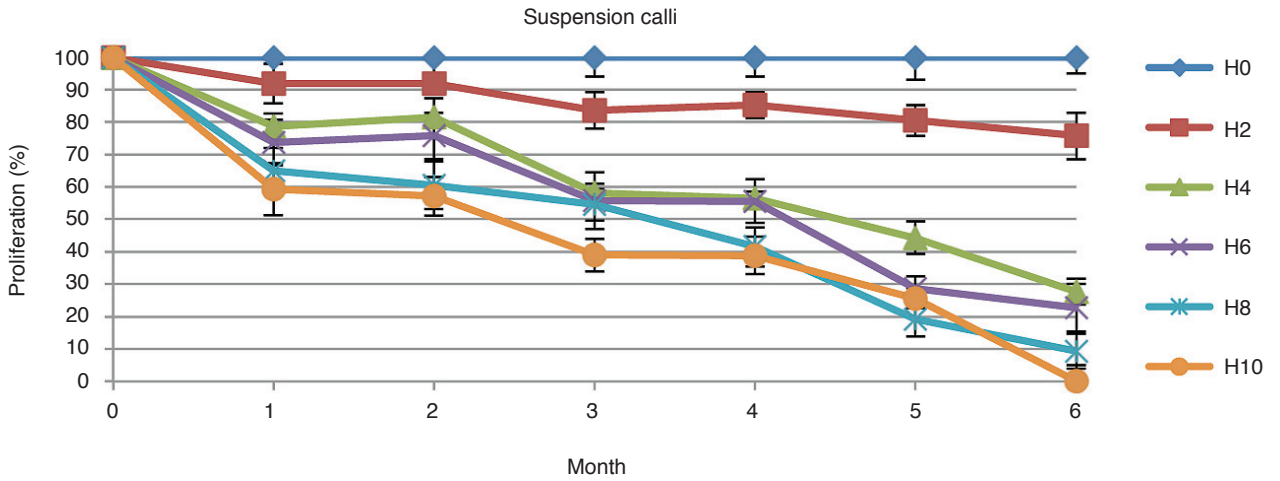

Figure 3. Proliferation percentage of suspension calli being cultured onto L-1 media containing different concentrations of hygromycin up to six months. H0: control without hygromycin; H2: 2; H4: 4; H6: 6; H8: 8 and H10: 10 mg litre^{-1} hygromycin. The bars indicate ± standard errors.

indicated by the smallest cell aggregates compared to cultures on lower concentrations of hygromycin (*Figures 4B* to *4E*). In contrast, the suspension calli cultured on media without hygromycin were yellowish and developed into bigger embryogenic masses (*Figure 4A*).

Determination of Hygromycin Concentration using Mature Calli

The suspension calli proliferated on maturation media (MSB) which later developed into yellowish and whitish calli when cultured on the EC media. Therefore, the MIC for each type of calli should be determined in order to efficiently eliminate untransformed tissues at every stage of callus development. Based on the plotted curves in *Figure 5*, it was clearly seen that 1 and 2 mg litre^{-1} of hygromycin were not effective in preventing callus proliferation in all types of embryogenic calli. For instance, the fine calli could still proliferate up to 96.85% and 91.19% on 1 and 2 mg litre^{-1} of hygromycin, respectively (*Figure 5A*). The proliferation of all mature embryogenic calli started to decline on media containing 3 mg litre^{-1} and onwards. As the concentration increased, the proliferation rate was decreased and finally inhibited. Hygromycin at 6 mg litre^{-1} was detrimental for fine calli (*Figure 5A*).

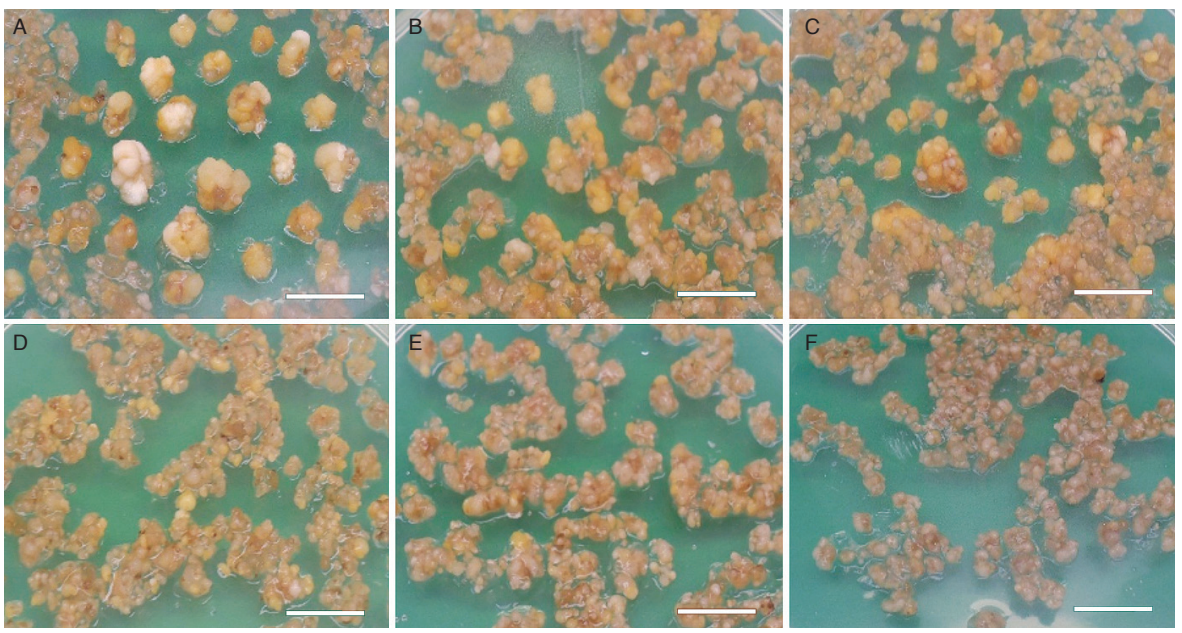

Figure 4. Proliferation of suspension calli after being cultured for six months on L-1 media containing 0-10 mg litre^{-1} hygromycin. A: Control without hygromycin; B: 2; C: 4; D: 6; E: 8 and F: 10 mg litre^{-1} hygromycin. At no and low concentration of hygromycin, the calli developed faster compared to the calli cultured on higher concentration of hygromycin as indicated by their bigger size (scale bar indicates 10 mm).

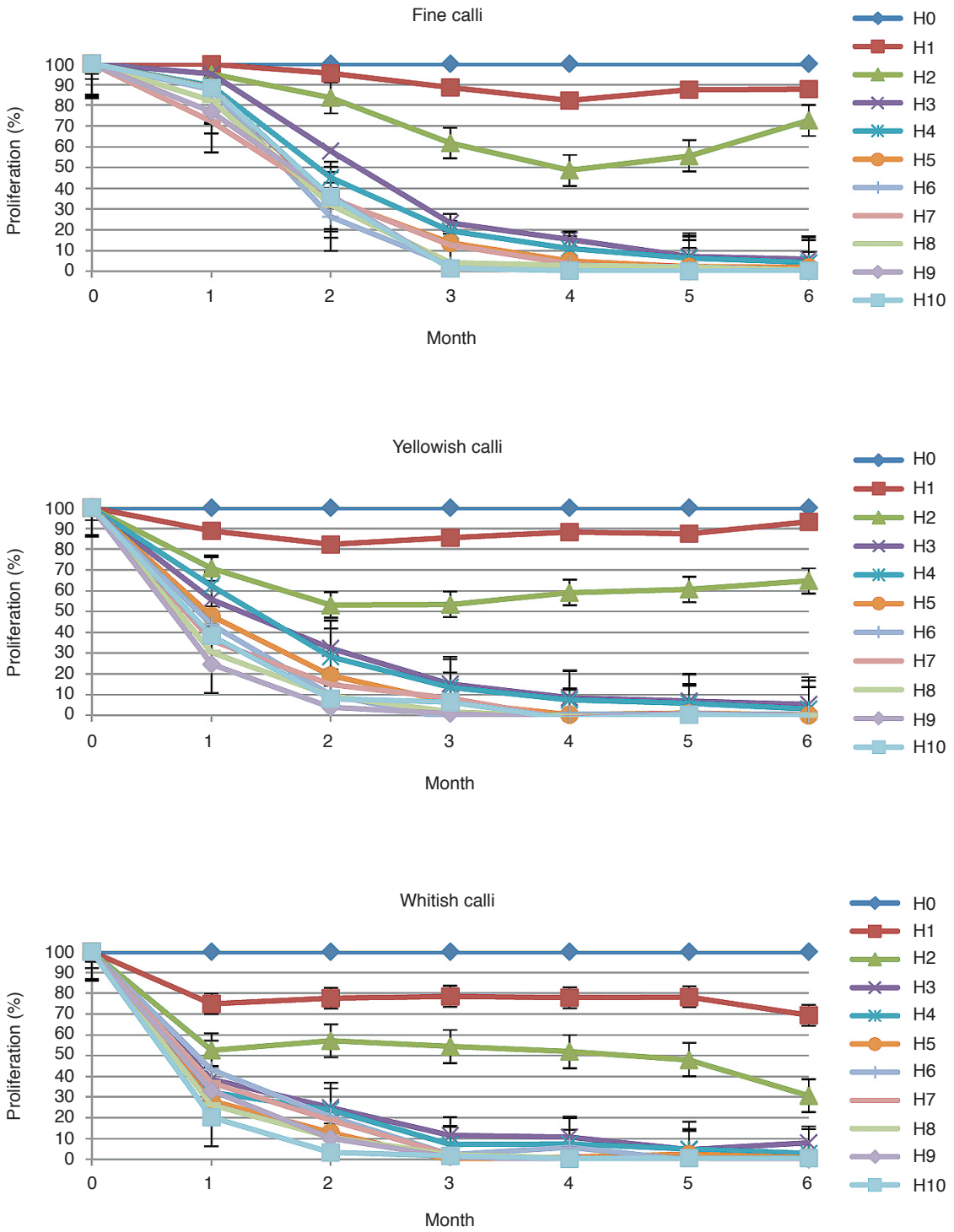

Figure 5. Proliferation percentage of embryogenic calli after being cultured for six months on callus regeneration (EC) media containing different concentrations of hygromycin. A: Fine calli; B: yellowish calli; C: whitish calli. H0: control without hygromycin; H1: 1; H2: 2; H3: 3; H4: 4; H5: 5; H6: 6; H7: 7; H8: 8; H9: 9 & H10: 10 mg litre^{-1} hygromycin. The proliferation percentage for the three types of calli cultured on EC media was significantly affected by the different concentrations of hygromycin tested ($p<0.05$).

Whilst, yellowish (*Figure 5B*) and whitish calli (*Figure 5C*) were fully inhibited on 5 mg litre^{-1} of hygromycin. Effects of hygromycin on the development of the three types of embryogenic calli for six months on EC media supplemented with different concentrations of hygromycin embryogenic calli are as shown in *Figures 6* to *8*.

Comparison of MIC between Immature and Mature Calli

Higher hygromycin concentration (10 mg litre^{-1}) was needed to inhibit the proliferation of suspension calli compared to fine calli (6 mg litre^{-1}), as well as yellowish and whitish calli (5 mg litre^{-1}).

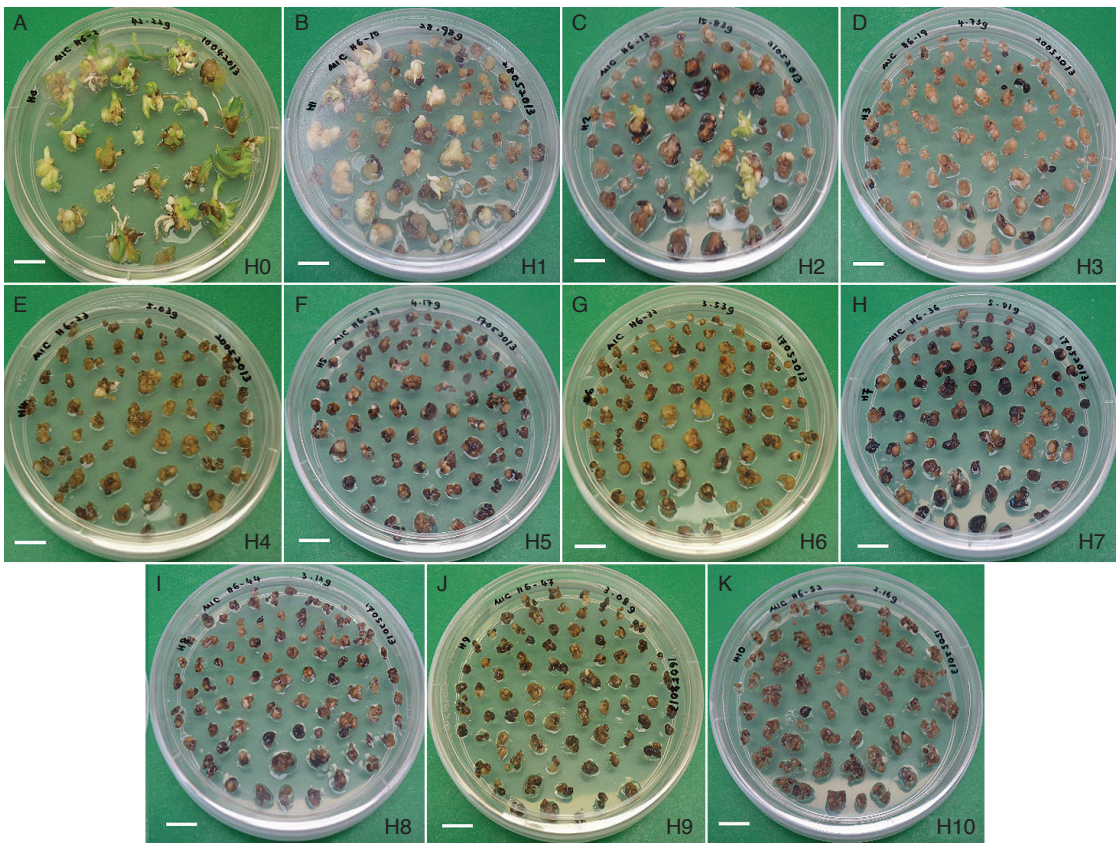

Figure 6. Development of fine calli after being cultured for six months on callus regeneration (EC) media supplemented with different concentrations of hygromycin. A: Control without hygromycin ; B: 1; C: 2; D: 3; E: 4; F: 5; G: 6; H: 7; I: 8; J: 9 and K: 10 mg litre^{-1} hygromycin (scale bar indicates 10 mm).

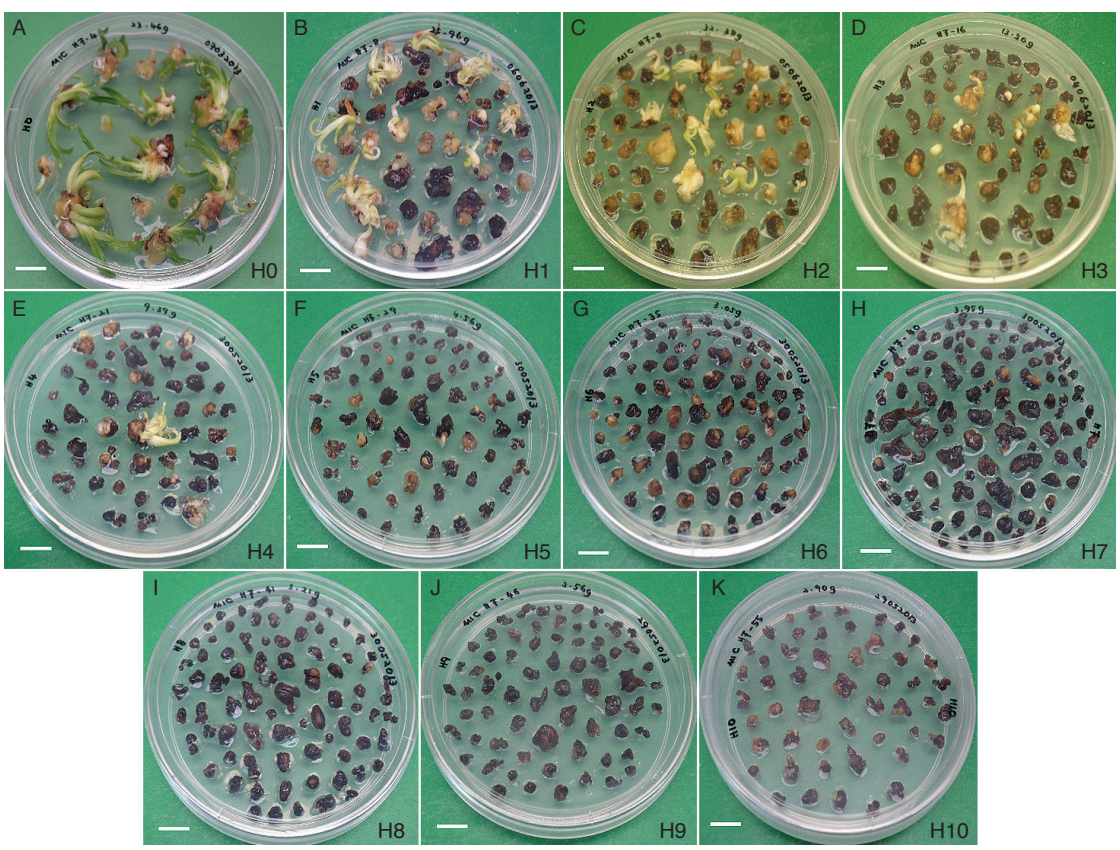

Figure 7. Development of yellowish calli after being cultured for six months on callus regeneration (EC) media supplemented with different concentrations of hygromycin. A: Control without hygromycin; B: 1; C: 2; D: 3; E: 4; F: 5; G: 6; H: 7; I: 8; J: 9 and K: 10 mg litre^{-1} hygromycin (scale bar indicates 10 mm).

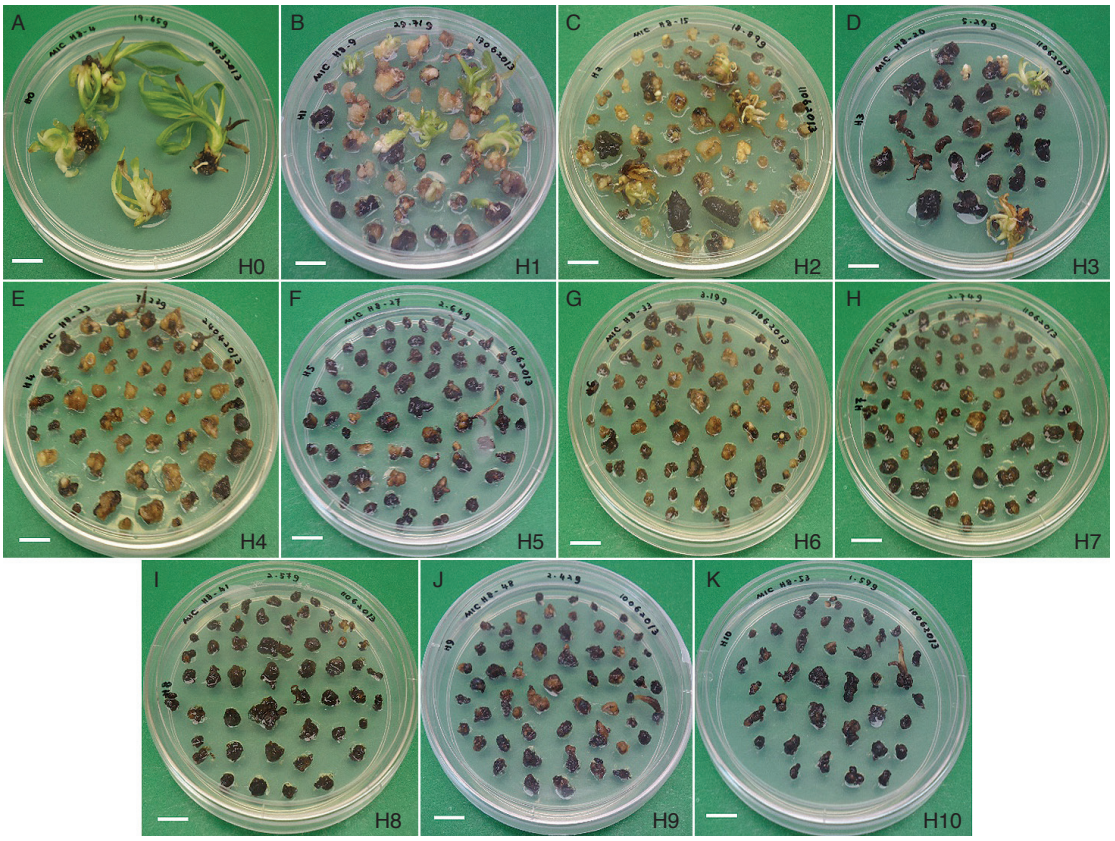

Figure 8. Development of whitish calli after being cultured for six months on callus regeneration (EC) media supplemented with different concentrations of hygromycin. A: Control without hygromycin; B: 1; C: 2; D: 3; E: 4; F: 5; G: 6; H: 7; I: 8; J: 9 and K: 10 mg litre^{-1} hygromycin (scale bar indicates 10 mm).

One possible reason for the different sensitivity between immature and mature embryogenic calli towards hygromycin was due to the different types of media used, which can lead to the differences in callus anatomy. The suspension and mature embryogenic calli were cultured on callus proliferation (L-1) and EC, respectively. The suspension calli are actively dividing cell cultures on the L-1 media which contain plant growth regulator 2, 4-Dichlorophenoxyacetic acid (2, 4-D). At this stage, oil palm suspension callus aggregates consist of round and actively dividing cells based on the histology analysis done by Teixeira *et al*. (1995). Previous studies have shown that 2, 4-D plays an important role in the dedifferentiation and cell division in somatic embryogenesis (Constantin *et al*., 2015; Meneses *et al*., 2005; Sané *et al*., 2006).

According to the analysis of variance, there were significant differences in the callus weight increment data obtained from the different hygromycin concentrations and types of embryogenic calli used (*Table 2*). Fluctuation of hygromycin even at 1 mg litre^{-1} has brought a lot of differences to the proliferation rates. Significant reduction on the growth rate was seen on 3 mg litre^{-1} (H3) and above. Approximately 50% reduction in the proliferation rate were recorded for H3 compared to the H2 treatment. Oil palm embryogenic calli can be considered as highly sensitive to the hygromycin since the regeneration of embryogenic calli was

TABLE 2. ANOVA OF HYGROMYCIN CONCENTRATION AND TYPE OF EMBRYOGENIC CALLI ON THE PROLIFERATION OF EMBRYOGENIC CALLI

Treatment	Variance of proliferation rate (%)		
	Mean square	F value	Sig.
Hygromycin concentration	23 018.953	72.947	0.000*
Type of embryogenic calli	6 922.631	21.938	0.000*

Note: *The proliferation rate was significantly affected by the different concentrations of hygromycin and types of embryogenic calli tested ($p<0.05$).
ANOVA – analysis of variance.

inhibited at as low as 5 mg litre^{-1} of hygromycin. The use of low hygromycin concentration (5 mg litre^{-1}) was reported in other plants such as *Oncidium* orchid (Ong *et al*., 2000; Liau *et al*., 2003) and *Jatropha curcas* (Li *et al*., 2008). Differential sensitivity towards hygromycin could be due to the variation of plant species and explants used such as single buds of banana at 20 mg litre^{-1} (Sreeramanan *et al*., 2006), immature embryo of barley at 50 mg litre^{-1} (Bartlett *et al*., 2008), immature embryos and embryogenic calli of rice at 30-50 mg litre^{-1} of hygromycin (Li *et al*., 1993).

Amongst the three types of mature embryogenic calli, fine calli were the least sensitive to hygromycin as compared to yellowish and whitish calli. The difference in response towards hygromycin is not known. Therefore, histology analysis of the all the four types of embryogenic calli was carried out to compare the nature of the embryogenic callus types and hopes to find the answer for the different level of sensitivity to hygromycin. Histological observation clearly indicated that there were differences in the anatomy between mature and immature embryogenic calli and also between mature embryogenic calli itself, as shown in *Figure 9*.

Suspension calli consist of globular and typically small cells with a dense cytoplasm, a nucleus with an evident nucleolus and a high nucleus/cytoplasm ratio (*Figures 8A* to *8B*). A high ratio of nucleus/cytoplasm indicated the presence of meristematic zones that could lead to the development of somatic embryos and the formation of shoots. The result obtained agrees with the morphological characteristics of somatic embryos from sugar cane (Alcantara *et al*., 2014), Pelargonium (Haensch, 2007) and date palm (Sané *et al*., 2006). When the suspension calli were transferred to the media devoid of 2, 4-D, they were differentiated and channelled to embryogenesis development. The embryogenic calli started to divide and develop into pro-embryos and globular embryos as seen in the fine callus stage (*Figures 9C* to *9D*). Later, more advanced stage of somatic embryo development was observed. At yellowish callus stage, the formation of heart shape was observed (*Figures 9E* to *9F*). Whilst, formation of vascular tissues, vascular bundle and apical meristem was clearly identified at the whitish calli stage (*Figures 9G* to *9J*). Indeed, the histological analysis showed that more advanced stage of development was observed in the yellowish and whitish calli compared to the fine calli. The presence of vascular system might assist the transportation and uptake of hygromycin more efficiently to other parts of the tissue, thereby making the whitish calli more sensitive to the added hygromycin compared to the fine calli. This could also answer the reason for obvious toxicity effect observed in whitish calli (*Figure 8*) compared to immature calli (*Figure 4*). The findings of histology along with the generated MIC data showed that the concentration of hygromycin needs to be gradually decreased throughout callus maturation process, to ensure that the transformed cells retain the ability to regenerate to normal plants. This could answer the lower concentration needed to completely inhibit the regeneration of yellowish and whitish calli compared to the fine calli.

We noticed that oil palm needs different concentrations of hygromycin when the selection was applied throughout the period of cultivation. A higher concentration of hygromycin, 10 mg litre^{-1} was needed at the callusing stage and once the calli have entered maturation stage, the concentration needs to be reduced to 5-6 mg litre^{-1}. Similar observation was reported for *Setaria viridis*, where 40 mg litre^{-1} of hygromycin was needed in the callusing media and then reduced to 15 mg litre^{-1} in plant regeneration media (Van Eck *et al*., 2017). In wheat transformation, hygromycin at 150 mg litre^{-1} was applied during callus selection and then reduced to 50 mg litre^{-1} at later selection stage (Gils, 2017). This type of selection is also known as stepwise selection.

The application of stepwise selection, either as gradually increasing or gradually decreasing of hygromycin concentration during the selection period has been reported in some other plant species such as wheat (Mitić *et al*., 2014) and *Aloe vera* (Velcheva *et al*., 2010) to avoid the negative effect of high antibiotic concentration on shoot regeneration and to increase the selection efficiency. Combination of delayed and stepwise increasing of hygromycin selection significantly enhanced the transformation efficiency in wheat by 7% compared to previously achieved 0.41% (Mitić *et al*., 2004). In contrast, Li *et al*. (2013) demonstrated that for *Crambe abyssinica*, using a constant low concentration of selection agent or gradually decreasing the selection agent concentration does not influence the transformation efficiency obtained. The results from this study suggest that gradually decreasing the hygromycin selection should be applied in future oil palm transformation study when immature calli are used as the starting material.

Another parameter that should be considered in developing an efficient selection strategy is the optimum duration of selection phase. In this study, it shows that around six months are required to completely inhibit the growth of all the four types of embryogenic calli. In earlier oil palm transformation works, resistant bombarded callus appeared after around five to six months on hygromycin-containing media (Parveez and Christou, 1998). A similar response was observed in rice transformation (Li *et al*., 1997). They noticed that the fast-growing callus line could be distinguished among the bombarded embryogenic calli after three to four subcultures on selection media containing 30-50 mg litre^{-1} of hygromycin. Whilst, six to eight months of selection on hygromycin were sufficient to allow the regeneration of transformed *Alstroemeria*

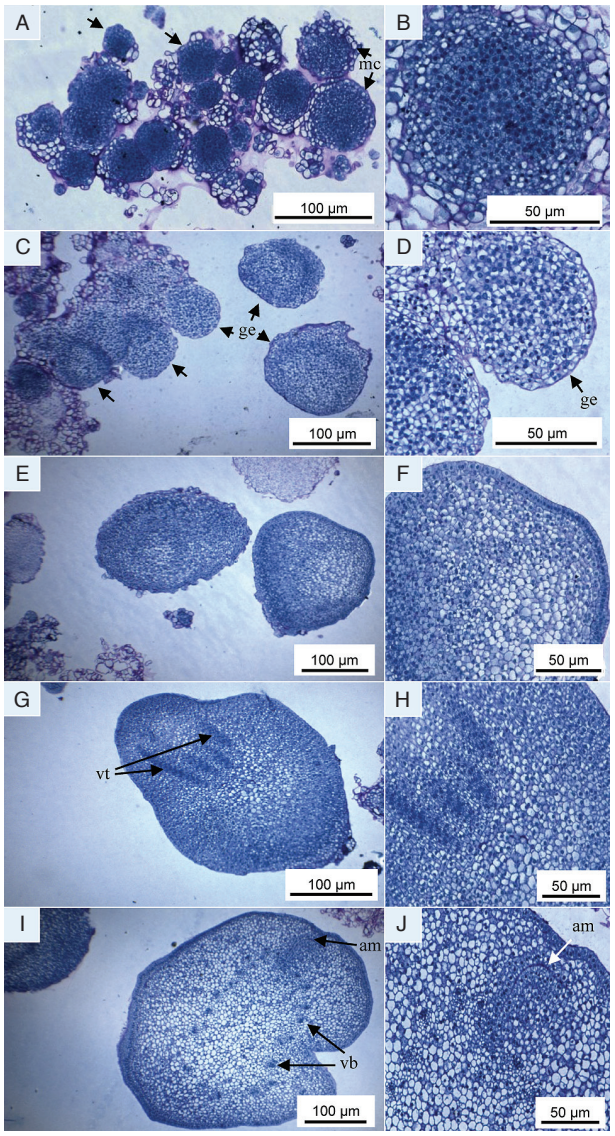

Figure 9. Histological observation of suspension, fine, yellowish and whitish calli. A-B: Suspension calli were made up of meristematic cells (mc); C-D: formation of pro-embryos and globular embryos (ge) were observed in fine calli; E-F: formation of heart shape in yellowish calli; G-J: formation of well-differentiated vascular tissues (vt), vascular bundle (vb) and apical meristem (am) observed in whitish calli.

embryogenic calli (Kim *et al*., 2007). Whereas, shorter culture period, about five weeks on a super-lethal dose of hygromycin, 200 mg litre^{-1} was sufficient to clearly identify hygromycin resistant of Russian wild rye (Wang *et al*., 2004).

CONCLUSION

Our result demonstrated that a higher concentration of hygromycin was needed to inhibit the proliferation of suspension calli compared to mature embryogenic calli. The mature embryogenic calli are very sensitive to hygromycin, since their proliferation and regeneration were inhibited at a relatively low concentration of hygromycin. Thus, to effectively select and regenerate the transformed oil palm embryogenic calli, the concentration of selection agent used during the selection should depend on the types of embryogenic calli used. Therefore, for oil palm transformation using suspension calli as target tissue, early stages of selection need to be carried out at a higher hygromycin concentration, 10 mg litre^{-1} and once the embryogenic calli mature to fine and whitish or yellowish, lower concentrations of hygromycin, 5-6 mg litre^{-1} will need to be applied for an efficient selection of transformants. This is the first report on the evaluation of the effects of hygromycin on different types of oil palm embryogenic calli and could be used as a guideline for the selection of transformants.

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