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Effects of organic additives and different carbohydrate sources on proliferation of protocorm-like bodies in *Dendrobium* Alya Pink

N. Nambiar[1], C.S. Tee[2] & M. Maziah[1*]

[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Biological Science, Faculty of Science, Universiti Tunku Abdul Rahman, 31900, Kampar, Perak, Malaysia

*** Corresponding author: maziahm@biotech.upm.edu.my**

Abstract

The purpose of this study was to investigate the effect of the addition of different organic additives and sugar types as carbon source on the growth and proliferation of protocorm-like bodies (PLBs) of a very new *Dendrobium* hybrid known as *D*. Alya Pink (DAP). PLBs were supplemented with homogenates of banana, tomato, and coconut water at various concentrations. Proliferation rate was recorded on a fresh weight basis. The effect of various types of local banana homogenate was also used to evaluate their efficiency in proliferation of PLBs. Six types of sugar were used to evaluate their effect on the growth of DAP PLBs. Results showed that banana and tomato homogenate was not effective for PLB proliferation DAP as control gave the highest fresh weight of 0.48 ± 0.02g. Coconut water was found to be the best organic additive for the proliferation of DAP PLBs which showed a four-fold increase of fresh weight (0.59 ± 0.03g) compared to the initial weight of 0.15g in just four weeks. Highest growth was recorded in PLBs supplemented with glucose, fructose and sucrose with 0.94 ± 0.55 g, 9.1 ± 0.82 g and 6.51 ± 0.52 g of PLBs respectively. Galactose, mannitol and sorbitol were not suitable to promote the growth of PLBs. In this study, coconut water was selected to be the best organic additive and glucose as the suitable carbohydrate source for the proliferation of *D*. Alya Pink PLBs when compared to the other homogenates and sugar types.

Keywords: *Dendrobium*, organic additive, PLBs, proliferation, sugar.
Abbreviations: DAP_*Dendrobium* Alya Pink; FW_fresh weight; MS_Murashige and Skoog; PLBs_protocorm-like bodies.

Introduction

Malaysia is a tropical country with high humidity. The climate during the day is moderately hot where the temperature can range from 20 to 30^{o}C. *Dendrobium* orchids can grow well at this type of weather making it one of the most popular types of orchids to be commercialized in tropical countries such as Malaysia and Thailand (Akter et al. 2007). *Dendrobium* hybrids occupy the foremost position in floriculture trade especially in ornamental flower industry because of its attractive colours, capability of flowering continuously and a prolonged post-harvest life when compared with other orchid species. They have high potential to be used as cut flowers, potted plants and also for landscaping purposes. The success of plant tissue culture is highly influenced by the growth regulators and nutrition supplied in the media. The media used for tissue culture of orchids is generally high in salt, mineral, vitamins, growth regulators and water (Murdad et al. 2010). Another important component in plant tissue culture media is the carbon source because they supply energy to the plants especially when they are not ready to photosynthesize their own food during the early stage of tissue culture (Al-Khateeb, 2008). The growth of the plants is also influenced by the presence of carbon source. Carbon source can be in the form of simple or complex sugars (Akter et al. 2007). Normally, sucrose is used as the carbon source in plant tissue culture. It is advisable to use organic additives in orchid culture medium as this has been reported to be an easy way to improvise the current plant tissue culture media towards commercial production (Ichihashi and Islam 1999). Various kinds of organic additives have been used in plant tissue culture to promote the growth of the plants including coconut water, banana pulp, potato homogenate and juice, honey, date palm syrup, corn extract, papaya extract and also beef extract (Islam et al. 2003; Murdad et al. 2010). Organic additives help in producing more PLBs, shoots and leaves (Akter et al. 2007), increases the size of somatic embryos (Al-Khateeb 2008), also promotes growth and development of asymbiotic seeds and regeneration of plantlets (Tawaro et al. 2008) . The reason organic additives are added into culture medium besides being a natural source of carbon is because they contain natural vitamins, phenols, fiber, hormones and also proteins (Gnasekaran et al. 2010). It was mentioned in a report by Al-Khateeb (2008) that organic additives contained not only sugar but also other nutrients such as proteins, lipids and minerals. Here in this study, a few types of organic additives have been used in order to investigate the effect of the additives being an alternative carbon source to replace sucrose on the behavior of *in vitro* cultures based on the PLB proliferation of *D*. Alya Pink (DAP). Six different sugar types were added into the culture media to observe the response of PLBs from DAP. DAP is a new *Dendrobium* hybrid formed as a result of cross between a tall and sturdy orchid type, *D*. Tengku Anis with a petite-dwarf hybrid known as *D*. bigibbum. DAP produces little light pink to purple coloured flowers suitable to be used as potted plant especially as decorative item and gift.

Results

Effect of organic additives

In this study, the proliferation of DAP was carried out on half-strength MS medium (solid) supplemented with homogenates of various types of local bananas, tomato and young coconut water. The type and concentrations of organic additives influenced the response of PLBs in terms of proliferation. From the present study (Fig.1a), banana homogenate added to half-strength MS media at 5, 10, 20 and 30% was not effective in enhancing the growth of PLBs because by the end of week four, media without banana homogenate added showed higher fresh weight of PLBs (0.48 ± 0.02g) when compared to the initial fresh weight of 0.15g. Banana homogenate at 10% gave a final fresh weight of 0.37 ± 0.01g followed by 0.19 ± 0.01g (5%) banana homogenate and finally a decrease of 13.33% on the fresh weight of PLBs for the concentration of 30% banana homogenate. In a separate experiment, a comparison was done between the homogenates of a few selected local banana cultivars with a commercial banana powder to observe if there were any differences on PLB growth (shown in Fig. 1b). Observing the effects of various kinds of banana homogenate on PLB proliferation, Rastali (AAB) and Emas (AA) recorded the highest fresh weight increase compared to other types of local banana homogenate. Lowest proliferation of PLBs was recorded when a commercial banana powder was used. Generally, fresh weight of PLBs cultured in media containing fresh banana homogenates were higher when compared to the fresh weight of PLBs supplemented with the commercial banana powder. Control treatment produced an average of 0.48 ± 0.02g of PLBs when compared to the initial weight of 0.15g. Other local banana homogenates showed an average PLB fresh weight at the range of 0.4 to 0.48g except for Berangan homogenate which gave a lower fresh weight (0.37 ± 0.03g) after a month. High concentration of banana homogenate was not suitable to enhance the growth of DAP PLBs. PLBs were found to be not healthy when supplemented with banana homogenates especially at higher percentage. In this study, we observed the same type of problem when at the end of week eight, PLBs under the treatment of 20 and 30% failed to survive due to necrosis causing the fresh weight of PLBs to reduce in media containing 20 and 30% of banana powder (data not shown). PLBs in media containing 10-30% of banana homogenate began to form some discoloration on the surface that eventually caused the whole tissue to change into brown colour (Fig. 2). Although banana homogenate has been widely used in plant tissue culture, addition of this organic additive was not found to be a good option to improve PLB proliferation from DAP. Based on Fig.1c, tomato homogenate at all concentrations did not promote the proliferation DAP PLBs as they showed a decrease in the fresh weight at the end of week 4 except for the control. PLBs cultures in media containing tomato homogenate are shown in Fig. 3. It appears from the present study that among the different organic additives of various concentrations, coconut water at 10% was the most effective in promoting the growth of PLBs (Fig. 1d). Fresh weight of PLBs increased up to an average of four fold when 10% of coconut water was used (Table 1). Fresh weight of PLBs cultured in media containing 5% of coconut water were recorded to be 0.45 ± 0.02g,0.59 ± 0.01g (10%), 0.58 ± 0.03g (20%) and finally 0.56g (30%). PLBs cultured in media that had 10 and 20% of coconut water began to produce more shoots and roots and had begun to show signs of regeneration. Higher concentration of coconut water was found to be inhibitory towards early regeneration of DAP PLBs. PLBs grown in coconut water treatment is shown in Fig.4. PLBs in 30% of coconut water were observed to be greener and denser but did not produce shoots as in the previous two concentrations. Also in this study, average glucose content in the PLBs cultured on banana homogenate, tomato homogenate and coconut water was determined after a month of culture. Fig. 5a shows as the concentrations of banana homogenates in the media increased, the glucose content in the PLBs also increased ranging from 38.3 ± 1.3 mg/gFW to 74 ± 1.0 mg/g FW. Here, glucose content was found to be the lowest in control (30.8 ± 1.2 mg/gFW). A different trend was observed in the glucose content in PLBs cultured in media containing tomato homogenates (Fig. 5b). The glucose content in the PLBs in 10% of tomato homogenates was recorded to be the lowest with an average of (27.1 ± 1.0 mg/g FW). Glucose content in PLBs supplemented with 10-20% of tomato homogenate was not significantly different from control. Exceptionally, 30% of tomato homogenates gave the highest glucose content in the PLBs (59.4 ± 0.9 mg/g FW). Previously, we mentioned that based on this study, coconut water was the best organic additive in enhancing the growth of DAP PLBs. However, based on Fig. 5c, the glucose content in the PLBs was the lowest in PLBs supplemented with coconut water ranging from 15.0 ± 1.2 mg/g FW to 30.8 mg/g FW of glucose when compared with other organic additives used. Coconut water was more effective in maintaining the culture for a longer term when compared to banana homogenate. A very simple histological study was carried out to observe the difference in the PLBs cultured in media supplemented with 10% of banana homogenate, tomato homogenate and coconut water. The images are shown in Fig. 6.

Effect of carbohydrate sources

An experiment was carried out to compare the effect of six types of sugar on PLBs fresh weight and this result is shown in Fig.7(a). Among six types of sugar tested, glucose, fructose and sucrose produced higher fresh weight of PLBs with 9.94 ± 0.55 g, 9.1 ± 0.82 g and 6.51 ± 0.52 g respectively. Fresh weight of PLBs was the lowest in media containing galactose by producing only 0.22 ± 0.02 g after a month compared to the initial 0.15g of PLBs. Detrimental effects were found in PLBs in medium supplied with galactose as the main carbohydrate. When the sugar content in the PLBs was measured, we observed the highest sugar content in PLBs culture in media with galactose (76.57 ± 0.02 µg/ FW). Sugar content in PLBs supplied with other sugar types ranged from 6.52 ± 0.15µg/FW to 15.95 ± 1.19 µg/ FW. Chlorophyll content in the PLBs was also different when supplied with different sugar types as the carbon source. Total chlorophyll content was high in PLBs grown in media with sucrose (111.86 ± 3.25 µg/FW) followed by mannitol (109.04 ± 2.2 µg/FW). The total chlorophyll content in PLBs from media with fructose, glucose, galactose and sorbitol ranged from 22.45 ± 1.55 µg/FW to 57.79 ± 1.25 µg/FW.

Discussion

Effects of organic additives

Researchers have shown the usage of banana as organic additives in tissue culture media. It was proved to be the best for the organogenesis of *Dendrobium* orchid as it was able to

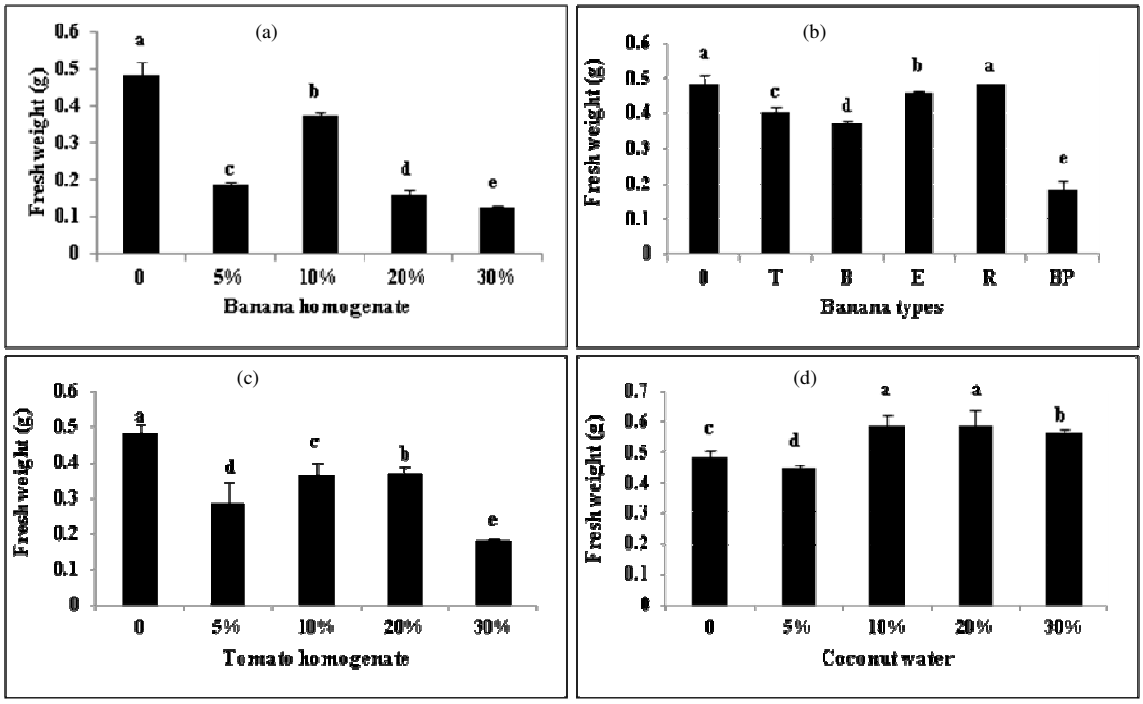

Fig 1. The effect of various concentrations of (a) banana homogenate (b) 10% of local banana homogenate and banana powder (c) tomato homogenate and (d) coconut water ; 0-control, T-Tanduk, B-Berangan, E-Emas, R-Rastali and BP-commercial banana powder) on the fresh weight of PLBs at the end of week four. Values shown are Mean ± SE.

increase the fresh weight of PLBs, effective in producing longer shoots and leaves and in promoting the growth of roots (Kong et al. 2007). Growth rate and plant vigor of *D. strongylanthum Rchb. f.* increased when mashed banana was added into the culture medium. However, banana homogenate as organic additive was not effective for the proliferation and maintenance of DAP PLBs. This was very obvious especially in the longer run where the PLBs at higher concentrations faced necrosis problem. It has been reported that addition of organic additives at higher concentrations can cause necrosis of the plant material (Ichihashi and Islam 1999). The same situation was observed in *Solanum laciniatum* where the plants supplemented with banana component in the media faced a significant decrease in its growth suspected to be due to toxic compound release into the media (Indrayanto et al. 1995).PLBs cultured in lower concentrations of banana homogenate did not proliferate more and failed to produce shoots even after eight weeks. This result was in contrast with the result obtained by Akter et al. (2007) which showed that *Dendrobium* PLBs obtained from media containing banana pulp and charcoal showed the highest growth when compared with other organic additives. This could be because of the synergistic effect of banana pulp and charcoal or merely because of the different variety of bananas used (Akter et al. 2007). In a study carried out by Tawaro et al. (2008) showed that activated charcoal was a better organic additive than banana homogenate, and coconut water to increase germination of *Cymbidium findlaysonianum* Lindl seedlings. From the present study, among the homogenates of different banana cultivars, 10% of Emas and Rastali homogenates were the best in replacing sucrose in the media. PLBs of *Phalaenopsis violaceae* also showed best response of proliferation rate under the supplementation of Emas type of banana extract to the half-strength MS basal media (Gnasekaran et al. 2010). Gnasekaran et al. (2010) had reported that banana homogenate is rich in minerals such as iron and potassium, vitamins and amino acids that play an important role in promoting the growth of PLBs. They also mentioned that the positive effect of banana homogenate is also due to the ability to act as a pH buffer of the medium. In this way the pH of the media is maintained to further stimulate PLB growth. As for tomato homogenate, the present study showed that the fresh weight of PLBs was the lowest in media with 5 and 30% of tomato homogenate. Similarly, Gnasekaran et al. (2010) also reported these two concentrations of tomato extracts that gave low growth rate for *Phalaenopsis violacea* PLBs. Contrastingly, Ichihashi and Islam (1999) showed in their report an improvement in the callus growth of orchids when tomato extract was used in the range of 5-20% but this study was carried out on *Phalaenopsis, Doritaenopsis and Neofineti*, not on *Dendrobium.* Our result was also not in agreement with the report of Gnasekaran et al. (2010) who stated that the interaction between half-strength MS and tomato extract enhanced the proliferation of *Phalaenopsis violaceae* PLBs. Coconut water at 10% could be used as a potential organic additive in the culture media for a normal growth of DAP PLBs. Coconut water at 10% was also found to be the most effective in proliferation of *Phalaenopsis violacea* PLBs (Gnasekaran et al. 2010). In some date palm varieties, coconut water at as low as 5% promoted the number of *in vitro* embryo formation and this increased as the coconut water concentration was increased (Al-Khayri, 2010). Therefore, we can also speculate that coconut water was effective in producing higher mass of PLBs as compared to

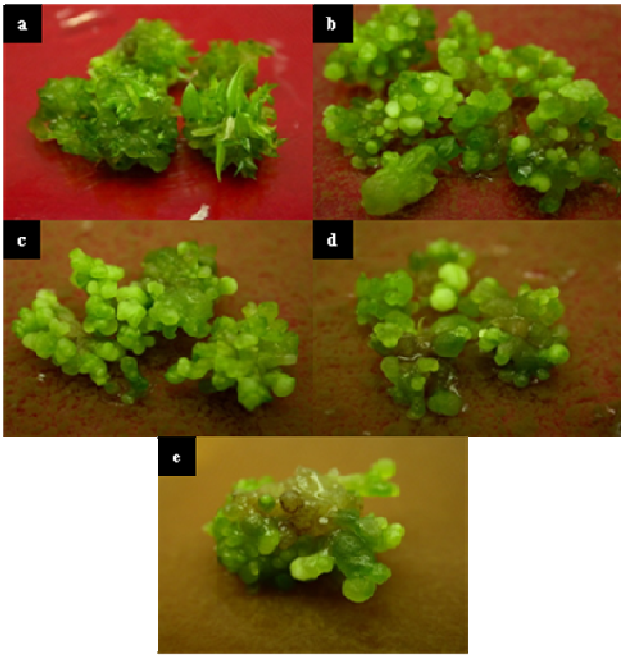

Fig 2. The effect of banana homogenate on the proliferation of PLBs. (a) control (b) 5% (w/v) (c) 10% (w/v) (d) 20% (w/v) and (e) 30% (w/v).

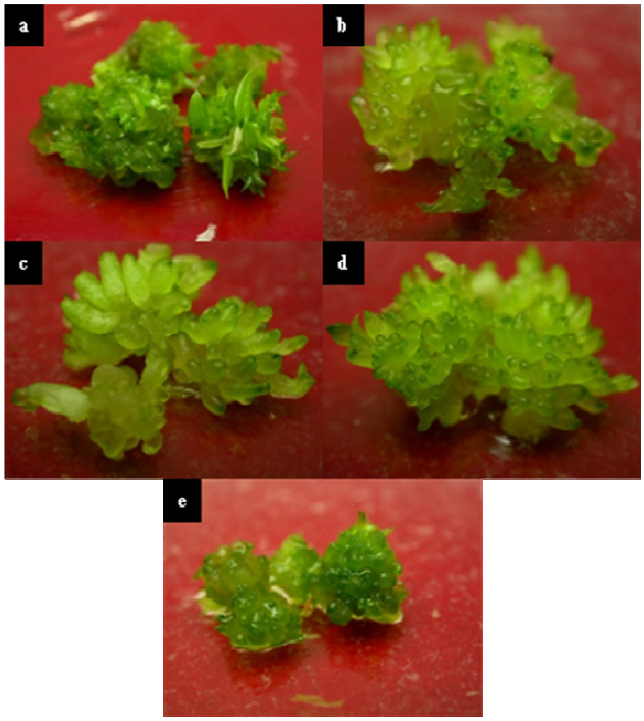

Fig 3. The effect of tomato homogenate on the proliferation of PLBs. (a)control (b) 5% (w/v) (c) 10% (w/v) (d) 20% (w/v) and (e) 30% (w/v)

other organic additives because basically coconut water are not only high in glucose and fructose but also contains various amino acids, fatty acids and minerals (Santoso et al. 1996). Coconut water also contains diphenyl urea which functions as cytokinin that can enhance growth of PLBs by inducing cell division (Gnasekaran et al. 2010; Texeira da Silva et al. 2006) and this could be the reason for early plant regeneration through PLBs supplemented with optimum concentration of coconut water.

In a study carried out by Nasib et al. (2008), coconut water was found to be able to increase the number of shoots and its length and the number of nodes. A study by Agampodi et al. 2009 also showed that shooting was prominent in canes treated with coconut water. Coconut water at 10% was also proved to be effective in increasing seed germination frequency and the leaf length of *Paphiopedillum villosum* showing the ability of this additive to stimulate shooting (Long et al. 2010). PLBs supplemented with lower concentrations of coconut water produced shoots and roots faster than PLBs cultured in media containing other organic additives. When coconut water was supplied together with the culture media, *Calanthe* plantlets showed an increase in the size of shoot, fresh weight and dry weight of shoots and roots and also in leaf area (Baque et al. 2011). In our study, coconut water at higher percentage inhibited early regeneration and this was in agreement with results obtained in a study by Gnasekaran et al. (2010), when coconut water at concentrations of 20 and 30% showed inhibitory effect on PLB growth. This was further supported by a research carried out on *Calanthe* hybrids by Baque et al. (2011) when coconut water at a high concentration produced abnormal plants and affected the growth and morphological characteristics. The regenerated plantlets from PLBs given the supply of coconut water also produced roots. Addition of coconut milk to culture medium produced plantlets with a vigorous growth and root production (Kong et al. 2007). The process of regeneration of plantlets was recorded to be faster in PLBs given the supply of coconut water because coconut water naturally contains cytokinins and auxins (Agampodi and Jayawardena 2009). In fact, IAA extracted from coconut water was used to stimulate the growth of adventitious root development in the propagation of *Dracaena purplecompacta* L. (Agampodi and Jayawardena 2009). Sucrose is the main component that plays essential role in producing a healthy and more number of embryos in plant tissue culture (Al-Khateeb 2008). Sometimes, we can observe an increase in the glucose content in the PLBs but with a decrease in the fresh weight of PLBs. This could be due to the accumulation of sugar in the PLBs taken up from the media. Al-Khateeb (2008) had mentioned in his report that these sugars kept accumulating in the tissue resulting in the increase in simple sugar content but somehow this sugar does not promote the growth in the tissue. This phenomenon could be seen in the effect of banana homogenate especially at higher concentrations. In the case of banana homogenate, the accumulation of sugar in the tissues caused toxicity and lead to death of tissues after some time. This result is in agreement with the results obtained by Gnasekaran et al. (2010). They observed a reduced PLB proliferation rate when 20 and 30% of banana homogenate was used. Reduction in fresh weight of PLBs sometimes can also be caused by the presence of complex sugars in the organic additives that could not be absorbed and transported in the system of PLB tissues. Addition of banana as additive into the media could also cause an increase in the sugar level of the media. Photosynthetic capability of plant tissue is affected due to accumulation of carbohydrate in the media due to high sugar

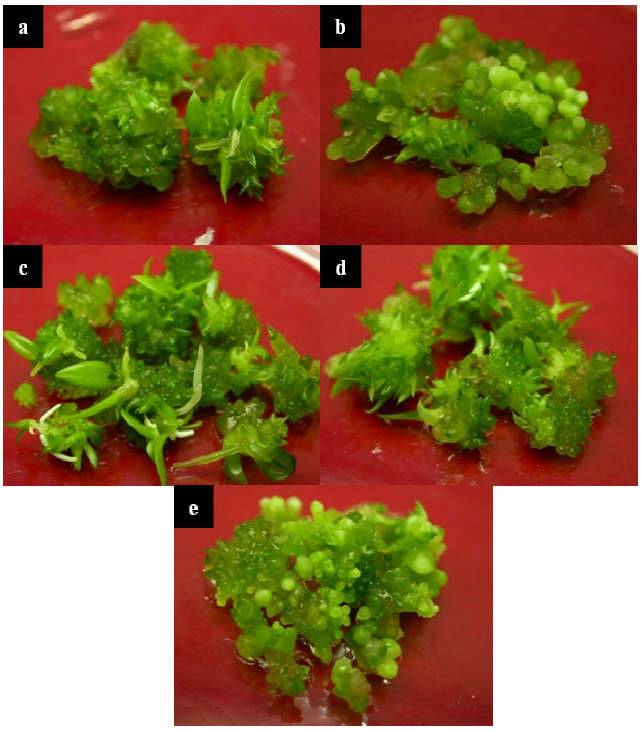

Fig 4. The effect of coconut water on the proliferation of PLBs. (a) control (b) 5% (v/v) (c) 10% (v/v) (d) 20% (v/v) and (e) 30% (v/v).

content (Faria et al. 2004). In another study, it was reported that addition of commercial banana powder into the culture medium increased alkaloid production and chlorophyll content in *Solanum laciniatum* but significantly reduced the growth index of the shoots believed to be due to toxic effects caused by certain components found in banana powder (Indrayanto et al. 1995). Based on the histological study carried out (as shown in Fig. 6), we observed PLBs supplemented with as low as 10% of banana homogenate in the culture media to show plasmolysis of the cells. This could be the reason why PLBs exhibited symptoms of dying especially at higher concentrations. The cytoplasm in the cells were not as dense as observed in PLBs from the control media The difference of the osmotic pressure in the plant tissue and the surrounding media could have caused the intercellular fluid to be drained out and therefore reducing the fresh weight of the tissue. From the results, we observed lower reducing sugar content in PLBs cultured in media with coconut water and this was in agreement with the fact that these PLBs could be maintained longer in media supplemented with coconut water compared to other organic additives. In a study by Nasib et al. (2008), kiwifruit cultures grown with the addition of coconut water in the media survived for two months without subculture but cultures without the addition of coconut water faced browning problem after just a month and had to be subcultured. We observed that coconut water at 10% enhanced shooting of PLBs in DAP but recorded the lowest reducing sugar content in the PLBs showing the possibilities of some mechanism involving the sugar metabolism and translocation with the shoot formation. Besides, the relatively lower amount of reducing sugar in PLBs with coconut water added media could be another reason these PLBs can last longer without being subcultured, suspected to be due to less stress caused by a higher sugar level in the plant tissues. In this study, the shooting effect was evident in PLBs supplemented with coconut water. The leaf primordial rich in stomata could be seen clearly under the microscope. Shooting was enhanced when DAP PLBs were cultured in media containing coconut water. The amount of reducing sugar in PLBs with tomato homogenate added into the media was highest at 30% of tomato homogenate and lowest in 10%. However, we could not relate this pattern with the growth rate of PLBs. Probably, an extensive study need to be conducted to understand in which mechanism does the tomato homogenate helps in PLBs proliferation. But, the shape of PLBs provided with tomato homogenate was different from PLBs in other organic additives. Long oblong shaped PLBs with meristematic area rich in chlorophyll especially at the shooting region were observed when DAP PLBs were cultured onto medium containing tomato homogenate. Although organic additives are proven to be able to stimulate the induction and growth of plants from various explants, they were also found to show inhibitory effects to the plants especially when supplied at certain levels. In this study, we found that banana homogenate at more than 10% caused the PLBs to experience necrosis on at least some parts of the cultured plant tissue. Islam et al. (2003) reported that source of plant, types of plants or cultivars and the choice of basal media plays an important role in determining the effect of organic extracts on PLBs proliferation. In addition, the optimum concentration of organic additives needed by plants vary among plant species.

Effect of carbohydrate sources

Sucrose has been widely used as the major carbohydrate source to supply energy to cells in plant tissue culture because of its efficiency in being transported across the plasma membrane (Kumaraswamy et al. 2010). *In vivo* sugar application also had shown to increase the nitrogen fixation ability performed by diazotrophs which is found in the root environment of rice (*Oryza sativa*) (Naher et al. 2011). However, pure research grade sucrose is expensive and therefore, to cut down the production cost, researchers nowadays replace sucrose with conventional table sugar and natural carbon sources (Kumaraswamy et al. 2010). In the present study, we obtained the highest average fresh weight of PLBs from media added with fructose followed by glucose and sucrose. Although the fresh weight of PLBs given the supplementation of glucose and sucrose was not significantly different, this value is important to show us that for DAP hybrid, these two carbon sources were more suitable in enhancing the growth. Besides, sucrose hydrolysis occurs at a faster rate when compared to the uptake rate of sugars into the plant tissue (Yu et al. 2000) and this could contribute to the higher fresh weight of PLBs in media with glucose and fructose. In potato plantlets, media with sucrose produced lesser nodes than plantlets in media with glucose (Rahman et al. 2010). According to Kumaraswamy et al. (2010) again, response in shoot multiplication of Patchoul (*Pogostemon cablin* Benth*)* was poor when the culture medium was added with glucose and fructose compared to other carbon sources.Based on the present study, the size of PLBs with the addition of glucose, sucrose and fructose were comparatively larger than the PLBs of other sugar treatments. This is because sugars especially glucose and sucrose encourages the growth of cells. Both glucose and sucrose play an essential role in accelerating cell division process by specifically enhancing cell expansion and encouraging the accumulation

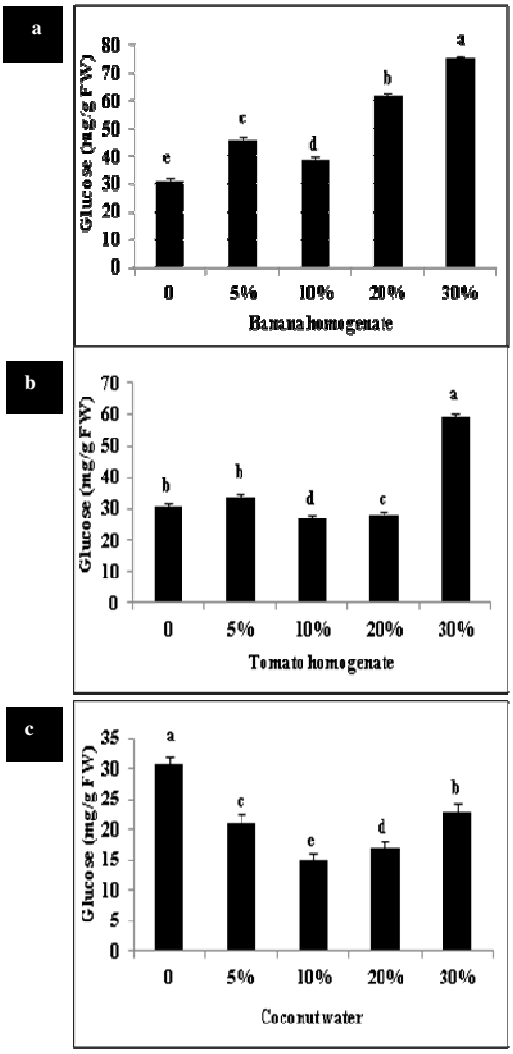

Fig 5. The effect of various concentrations of (a) banana homogenate (b) tomato homogenate and (c) coconut water on the glucose content (mg/g FW) in PLBs at the end of week four. Values shown are Mean ± SE.

of reserves in plant embryos (Borisjuk et al., 2003). According to Borisjuk et al. (1998), glucose was found to be high in undifferentiated tissues undergoing mitosis but low in abundance in highly differentiated tissues. Sucrose on the other hand was found to be higher in cells undergoing active expansion and is correlated to the accumulation of starch. Embryogenesis in carnation was enhanced when sucrose was used because of its ability to supply nutrients to the plant cells and the osmotic potential possessed by this carbon source (Karami et al., 2008 ; Biahoua and Bonneau, 1999). High sucrose concentration in the medium increases osmotic stress to the plants but according to Karami et al. (2006), this is normal and helps in enhancing the somatic embryogenesis in carnation. Same type of observation was reported by Sié et al. (2010) stress caused by high osmotic pressure in media containing 3% sucrose successfully initiated callus from roselle. In carnation, mannitol was also able to enhance the embryogenesis process but only when combined with the addition of sucrose (Karami et al., 2008). In our study, mannitol was not a suitable carbon source for the proliferation of the PLBs but we would not deny the possibility of mannitol to be able to proliferate the PLBs when combined with other carbon sources. Various concentrations of sorbitol were used to increase regeneration efficiency of rice plants from its embryogenic calli (Geng et al., 2008). In wheat, sorbitol supplementation did not only reduce the number of days needed for plant regeneration from callus but at the same time produced more robust plants as the concentrations of sorbitol increased. Number and quality of wheat calli was proved to be better in sorbitol enriched media compared to media without sorbitol (Hassan et al., 2009). However, sorbitol did not contribute towards the increase of DAP PLBs fresh weight although it performed better than galactose and mannitol. Generally, different carbon sources perform differently depending on the types of plants involved in the study. For example, in *Panicum* spp, a type of turf grass, the *in vitro* shoot regeneration through somatic embryogenesis was the highest when maltose was supplied into the media but an inhibition in regeneration was observed when galactose, lactose, mannose and sorbitol were supplied (Seo et al., 2010). In an orchid known as *Gigantum speciasum*, growth rate of plants in media with maltose was double the growth rate obtained from media supplied with sucrose (Sopalun et al., 2010). Media containing galactose and mannitol showed the lowest growth rate in DAP PLBs. In our study, the fresh weight of PLBs in media added with galactose was the lowest following media which contained mannitol. Similarly, galactose showed a low callus induction rate in cotton plant and the reason was because galactose could not be absorbed by the plant (Michel et al., 2008). In our study also, we observed that the shapes of the PLBs varied when different types of carbon source were used. PLBs supplied with sucrose and fructose produced more globular shaped PLBs but when sorbitol was supplied, PLBs produced were more elongated in shape. Biahoua and Bonneau (1999) had mentioned in their work that sucrose at various concentrations induced globular-shaped embryo formation in plants. PLBs with mannitol supplementation were oval in shape. The size of PLBs produced with fructose addition was larger than PLBs with the addition of sucrose and mannitol. Number of shootlets regenerated from the PLBs were highest in media with sorbitol (87±16) followed by sucrose (49±12), glucose (47±17) and finally fructose (28±17). PLBs in control media and media added with galactose and mannitol did not generate any shoots after a month. Similar situations were observed in *in vitro* date palm cultures when shoot regeneration was influenced by different sources of carbon. No vegetative growth was observed when carbon sources were not supplied in the media further showing that shoot regeneration requires energy which is normally obtained from the carbon source present in the culture media (Al- Khateeb, 2008). When the total chlorophyll content in PLBs was determined, the results showed a lower chlorophyll content in PLBs from media containing glucose and fructose although these two treatments produced PLBs with higher fresh weight. Kumaraswamy et al. (2010) also showed in their report that chlorophyll content in Patchouli plants given the treatment of glucose and fructose was low because of hyperhydricity caused by these treatments. There is a possibility for the high fresh weight of PLBs being caused by excessive absorption of water from the sugar enriched media causing hyperhydricity of the cells. Different carbon sources affect the photosynthetic activity of a plant at a varied level

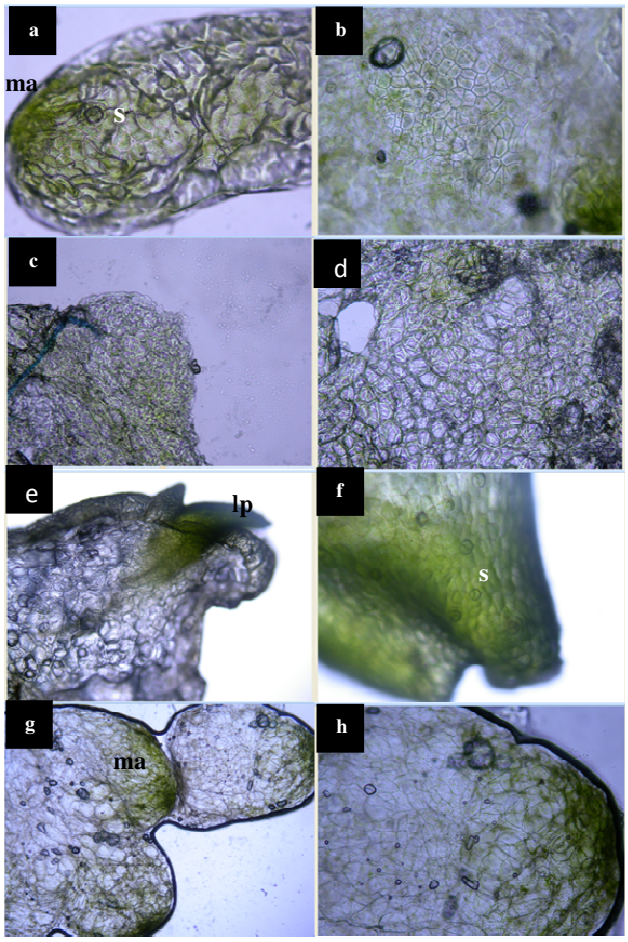

Fig 6. Histology of the selected PLB tissues after two months of culture in half strength MS media supplemented with 10% of various organic additives. (a) Control (40x); shoot apex with meristematic area (ma) (b) Control (100 x); (c) Banana homogenate (40x). (d) Banana homogenate (100x); arrow showing plasmolysis of the cell. (e) Coconut Water (40x); shoot apex with differentiation of peripheral meristematic area forming leaf primordial (lp). (f) Coconut Water (100 x); shoot apex magnified to show abundancy of stoma (s). (g) Tomato homogenate (40x) ; organization of meristematic area (ma) in the oblong shaped PLBs and emergence of growing points with a denser chlorophyll pigmentation and (h) Tomato homogenate (100x); pictures taken under normal light microscope.

(Kumaraswamy et al., 2010) even when they are found to be promotive towards proliferation resulting in a higher fresh weight of PLBs. This phenomenon can explain the varied pattern of chlorophyll content in the PLBs provided the supplementation of different source of carbon. A study showed that development of chloroplasts in *Arabidopsis thaliana* can be inhibited by glucose at a high concentration that affects a chloroplast –specific fatty acid formation which is the major constituent in the membranes of chloroplasts (To et al., 2003). However, this does not mean that glucose affects the chloroplast development directly but might include some other related mechanisms which we are not aware of. Expression of some genes related to chloroplast

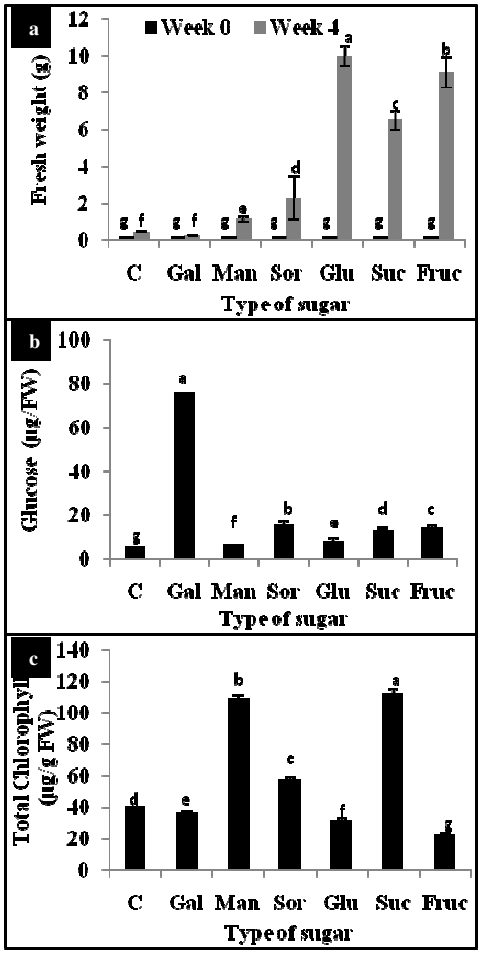

Fig 7. Effect of various sugar types on (a) fresh weight (g) (b) glucose content (µg/g FW) and (c) total chlorophyll content (µg/g FW) in PLBs at week four. Values shown are Mean ± SE

formation was also reported to be reduced when plants were exposed to environment with high sugar content (Bauer et al., 2001)

Materials and methods

PLBs originally initiated from the shoot tip of the mother plant were maintained and subcultured every month in hormone free half-strength MS media until further use. Half-strength MS media with no addition of organic additives was used as the control media. Half-strength MS media without sucrose added was used as the basal media for organic additive supplemented media. pH of the media was adjusted to 5.75 prior to autoclaving with 1N NaOH or HCl. Banana, and tomato homogenate was added on a weight per volume (w/v) basis whereas coconut water was added on a volume per volume basis (v/v). Five different concentrations of organic additives used in this experiment were 0 (control), 5, 10, 20 and 30 %. In the process of preparing banana and tomato homogenate, the appropriate amount is weighed, diced and blended with distilled water at a ratio of 1g:100 ml before adding them to the media. Coconut water was measured at the appropriate volume and poured into the

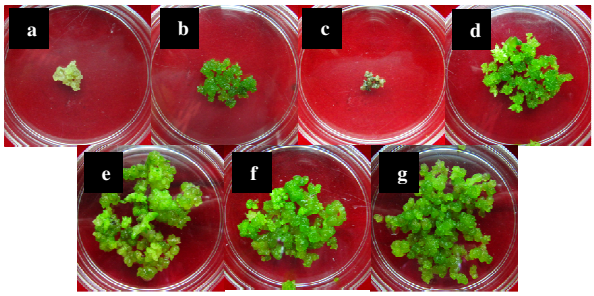

Fig 8. DAP PLBs after four weeks in (a) control (no sugar added) (b) mannitol (c) galactose (d) sorbitol (e) glucose (f) sucrose and (g) fructose.

media. All organic additives were prepared fresh and added immediately into the basal media. In another experiment, a comparison was done on the effect of a commercial banana powder (supplied by Sigma- Aldrich) and banana homogenate (variety Berangan) on the proliferation of PLBs. Here, the banana powder was weighed accordingly and added into the media. A comparison on the effect of some local banana varieties on the PLB proliferation was alsocarried out. Berangan (AAA), Emas (AA), Rastali (AAB) and Tanduk were the selected local banana cultivars used for this study. Media with zero percent organic additives were used as the control. To investigate the effect of different carbohydrate sources on the PLBs proliferation, basal media was supplemented with 2% (w/v) of sugar consisting of galactose, mannitol, sorbitol, glucose, sucrose and fructose. No sugar was added into the control media for this experiment.After preparing the media, 0.3% Gelrite was added for solidification. The media was autoclaved with a pressure of 1.16 kg/cm^2 at 121^{o}C for 15 minutes. Autoclaved media was poured into sterile petri dish in the laminar air flow.A total of 0.15g of two weeks old PLBs were weighed and cultured onto the media. All cultures were maintained at 25 ± 2^{o}C in the culture room condition under a 16-h photoperiod of 40 µmol m^{-2} s^{-1} light provided by cool white florescent tubes. The fresh weight of the PLBs was weighed after four weeks. The reducing sugar content was measured based on the Somogyi-Nelson method , 1944. Sample is homogenized with distilled water, incubated at 90^{o}C before being filtered to produce the crude fraction. 0.1 ml of the crude fraction was then added with 1ml of Somogyi-Nelson reagent, and then left in boiling water for 10 minutes. The sample was allowed to cool down before adding 1ml of arsenomolybdate reagent and 6ml of distilled water. The absorbance of this extract was then recorded at 510nm by using a spectrophotometer. Glucose was used as a standard reference to prepare the standard curve. The mean values were calculated from measurements of ten replicates and the SE of the means were determined. One way ANOVA and Duncan's multiple range test ($p<0.05$) was applied to determine the significance of the result between different treatments. All statistical analysis were done using SPSS version 11.0 for Windows.

Conclusion

From all the results obtained on the effect of the various organic additives on the fresh weight of DAP PLBs, the best organic additive for the proliferation of these particular PLBs is 10% coconut water. The suitable sugar type for DAP PLBs proliferation is fructose and glucose but considering the cost of fructose and glucose which are much higher than sucrose, the usage of sucrose as the carbohydrate source is acceptable.

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