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EFFECTS OF PLANT GROWTH REGULATORS ON SEED GERMINATION AND CALLUS INDUCTION OF *HYLOCEREUS COSTARICENSIS*

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Abstract

Dragon fruit (*Hylocereus costaricensis*) belongs to the family Cactaceae and are climbing vines which have received worldwide attention in recent years. However, there are still lack of information on the protocols for the establishment of *In vitro* culture system. In the present study, seed germination percentage were determined by culturing seeds on semi-solid Murashige and Skoog medium (MS) supplemented with 1 ppm 6-Benzylaminopurine (BAP) together with either 0, 0.5 or 0.8 ppm of Indole-3-butyric acid (IBA). Germination percentage was the highest by using plant growth regulators (PGRs) combination of 1 ppm BAP and 0 ppm IBA (93.33%). Subsequently, the cotyledons from seedlings of the germinated seeds were used for subsequent callus induction. Small pieces of cotyledons were excised and cultured on MS medium fortified with 0.45, 0.9, 1.8, 2.7, 3.6, and 4.5 ppm of 2,4-Dichlorophenoxyacetic acid (2,4-D) together with either 0, 0.9 or 1.8 ppm of BAP. Callus induction percentage was highest using the plant growth regulators (PGRs) combination of 3.6 ppm 2,4-D and 1.8 ppm BAP (75%). Hence, 3.6 ppm of 2,4-D and 1.8 ppm BAP was the best combination for callus induction of *Hylocereus costaricensis*.

Key words: Seed germination, Callus, *Hylocereus costaricensis*,

Introduction

Dragon fruit *H. costaricensis* also commonly known as 'pitahaya' which is popular for its distinct purple-red flesh. It is a native plant of South America and has been a well established crop in other countries such as China, Vietnam, Malaysia and Taiwan (Gunasena *et al*., 2006). The genus *Hylocereus* contains 16 species (Innes *et al.*, 1992) and becomes an important commodity among the local communities and even fetches higher price than 'The King of Fruits'-Durian (Gunasena *et al*., 2006).

Hylocereus costaricensis can be found in arid, semi-arid, subtropical as well as tropical climates. They are epiphytic or hemi-epiphytic in nature (Barbeau, 1990). They have triangular stems and requires a trellis for climbing. They are long day plants and flowers only bloom at night. The attractive, large white flowers open rapidly at 7 pm and completely opens by 10 pm. They gained recognition as an ornamental plant and is also well known for their edible fruits (Le Bellec *et al*., 2006).

Though the fruits are well known for its juiciness and sweetness, its composition has not been well characterized and studied. Despite that, the fruit has been used in pastries, juice, jam, sherbets, yoghurt and jelly. Many reports confirmed that red-fleshed varieties of dragon fruit have high levels of antioxidants and anti-proliferation properties (Wu *et al.*, 2006; Esquivel *et al.*, 2007; Nurliyana *et al.*, 2010; Kim *et al.*, 2011). In addition, *H. costaricensis* have higher nutritional levels which reduce cholesterol, diabetes, control blood pressure and promote dental health as compared to its counterparts. They have the ability to neutralize certain heavy metals too (Gunasena *et al*., 2006). The pulp of dragon fruit can be used as natural food colouring in food industries (Gao-Xi & Wan, 2004). *H. costaricensis* is rich in betalain pigments. Generally, it was well known that the main sources of betalain pigments were concentrates and powders obtained from beet root. Other sources of obtaining betalain concentrates were from the *Opuntia spp*., *Amaranthaceae* plants and the closely related species *H. polyrhizus*. The attempts of chemically synthesizing these compounds involve multiple steps resulting in low yields. Obtaining betalain pigments through plant cell and tissue cultures are an alternative

Dragon fruit plants can be propagated through seed propagation, vegetative propagation and grafting. Currently, the three conventional methods such as seed propagation and vegetative means through stem cuttings and grafting could not meet the current market demand (Gunasena *et al*., 2006). There is also the lack of a comprehensive *In vitro* propagation protocol of this particular species to obtain large amount of elite plants with desirable qualities. Vegetative propagation methods such as stem cutting and grafting can be used to overcome the shortcomings of seed propagation. However, callus induction is the first step of establishing *In vitro* cultures to obtain secondary metabolites in various plants.

There is currently no widely available information on a systematic set of protocol for the establishment of *In vitro* and callus induction of this particular species of dragon fruit (Dahanayake & Ranawake, 2012). Therefore, this study was aimed to establish callus culture of *Hylocereus costaricensis*. To achieve this objective, germinated seeds were grown on medium fortified with various concentrations of PGRs for callus induction.

Materials and Methods

The first part of this study involved attempts to improve seed germination using different combinations of PGRs. The effects of different combination and concentrations of PGRs on seed germination percentage were studied. Subsequently, different PGRs and their combinations were used to study the effects on the callus induction percentages of *H. costaricensis* by using cotyledon explants.

Seed source and culture establishment: Seeds of *H. costaricensis* were extracted from a fresh, ripe fruit purchased from a local market in Jelutong, Penang. The fruit was stored at room temperature few days prior to seed extraction since lower temperatures significantly decreases the viability of the seeds (Dahanayake & Ranawake, 2012). Freshly extracted seeds were subjected to the surface sterilization protocol by Mineo (1990) with slight modification by dipping in 70% ethanol for two minutes, immersed in a mixture of both 1% Clorox and a few drops of Tween 20 followed by three series of rinsing in distilled water. The last stage was blotting dry the sterile seeds on a filter paper. Explants were inoculated on MS medium. The medium consisted of MS basal salts, iron source, vitamins, sucrose (30g/L), and Gelrite (2.75g/L) supplemented with different concentrations and combinations of BAP and IBA according to the treatments summarized in Table 1. The pH was adjusted to 5.7-5.8 prior to autoclaving. All media were sterilized by autoclaving under 121ºC for 15 minutes. For every concentration, five seeds were cultured on the semi-solid Murashige & Skoog (1962) MS medium in a single culture jar. The experiment was repeated three times which made up a total of 15 replicates (5 explants × 3 jars). Cultures were maintained on a white wooden rack in a culture room with 16-hour photoperiod under fluorescent light at 25 ± 2ºC.

Table 1. Seed germination treatments with different plant growth regulator combinations

Seed germination treatment	Plant growth regulator combination
G_0	Control
G_1	1ppm BAP + 0ppm IBA
G_2	1ppm BAP + 0.5ppm IBA
G_3	1ppm BAP + 0.8ppm IBA

Sources of explants: Forty-day old cotyledons from seedlings of the previously germinated seeds were used as explants for subsequent callus induction experiment. The excised cotyledons (0.3 cm × 0.3 cm) were cultured on half-strength MS medium. Different concentrations of 2,4-D and BAP were used to study the callogenic responses of the cotyledons of previously grown *In vitro* seedlings according to the treatments summarized in Table 2. For each treatment, four explants were cultured on a semi-solid MS medium in a single jar. The experiment was then repeated four times to obtain a total of 16 replicates (4 explants × 4 jars) for each combination of PGR concentrations. Finally the cultures were kept in total darkness in a box at 25 ± 2ºC.

Table 2. Callus induction treatments with different plant growth regulator combinations

Callus induction treatment	Plant growth regulator combination
C_1	0.45ppm 2,4-D
C_2	0.9ppm 2,4-D
C_3	1.8ppm 2,4-D
C_4	2.7ppm 2,4-D
C_5	3.6ppm 2,4-D
C_6	4.5ppm 2,4-D
C_7	0.45ppm 2,4-D + 0.9ppm BAP
C_8	0.9ppm 2,4-D + 0.9ppm BAP
C_9	1.8ppm 2,4-D + 0.9ppm BAP
C_{10}	2.7ppm 2,4-D + 0.9ppm BAP
C_{11}	3.6ppm 2,4-D + 0.9ppm BAP
C_{12}	4.5ppm 2,4-D + 0.9ppm BAP
C_{13}	0.45ppm 2,4-D + 1.8ppm BAP
C_{14}	0.9ppm 2,4-D + 1.8ppm BAP
C_{15}	1.8ppm 2,4-D + 1.8ppm BAP
C_{16}	2.7ppm 2,4-D + 1.8ppm BAP
C_{17}	3.6ppm 2,4-D + 1.8ppm BAP
C_{18}	4.5ppm 2,4-D + 1.8ppm BAP

Data recording and statistical analysis: Seed germination rate and seed germination speed were calculated based on Aldhous (1972).

The means were compared using Duncan's multiple range test using SPSS (version 20). The total amount of callus formed was recorded and the callus induction percentages (CIP) were calculated using the formula below:

$$\text{Callus induction percentage} = \frac{\text{Number of explants with callus}}{\text{Total number of explants used}} \text{ X } 100\ \%$$

Data were analyzed by Analysis of Variance (ANOVA) to detect significant differences between means of each groups. Means that significantly differed were compared using Multiple Comparisons Table containing Post Hoc Test at 5% level of significance with SPSS Version 20 to determine where the differences lie.

Results

Seed germination: Seeds cultured on seed germination treatment G_1 recorded the highest germination percentage at 93.33% followed by G_2 at 73.33%, and G_0 with 66.67% germination percentage. G_3 produced lowest germination percentage of 40%. Seeds from the G_0 group germinated at the fastest rate of 4 days. However, seeds from G_1, G_2 and G_3 generally required 5 days to germinate (data not shown). Analysis of variance between the groups indicated that there was statistical significance ($p<0.05$) in the germination percentage of the seeds between the four groups (Figs. 1 and 2a-e).

Callus induction: Results from Table 3 showed that callus induced from cotyledons of *H. costaricensis* was highest under C_{17} callus induction treatment (75%), closely followed by callus induced with C_{14} and C_{16} treatments, both recording the same callus induction percentages (68.75%). Results of CIP obtained from C_1 to C_6 callus induction treatments supplemented with only 2,4-D were generally lower than C_7 to C_{12} and C_{13} to C_{18} cotyledon explants treated with various combination and concentrations of 2,4-D and BAP. There were higher callogenic responses of explants in both the presence of 2,4-D and BAP. Callus formed were generally compact in morphology and yellow in colour (Fig. 3a-f).

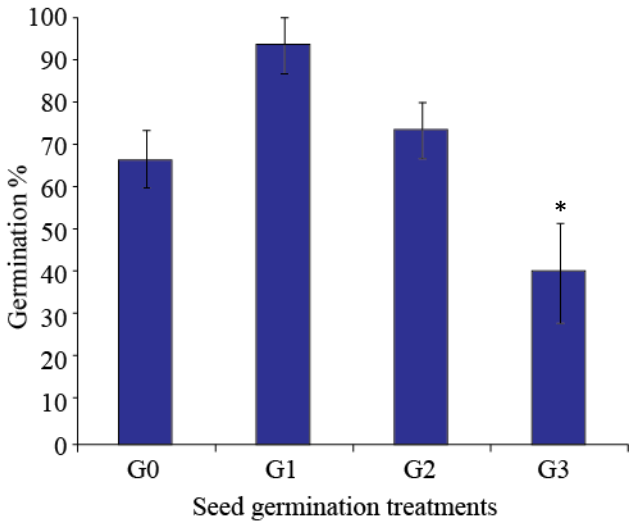

Fig. 1. Germination percentages of seeds from *Hylocereus costaricensis* fruit in response to different seed germination treatments of plant growth regulators.

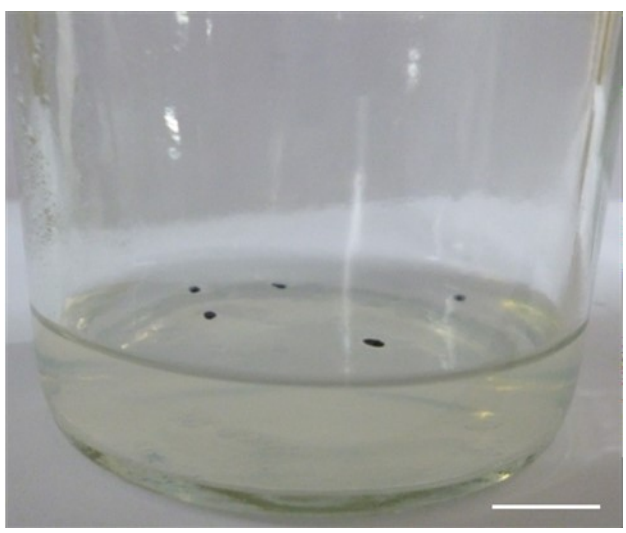

Fig. 2(a). Seeds inoculated on half-strength MS medium (1 day old). Bar = 1cm.

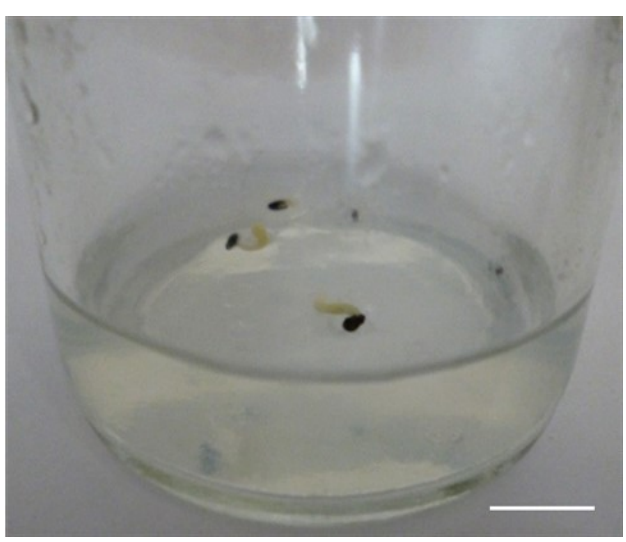

Fig. 2(b). Seedlings germinated on half-strength MS medium (7 days old). Bar = 1cm.

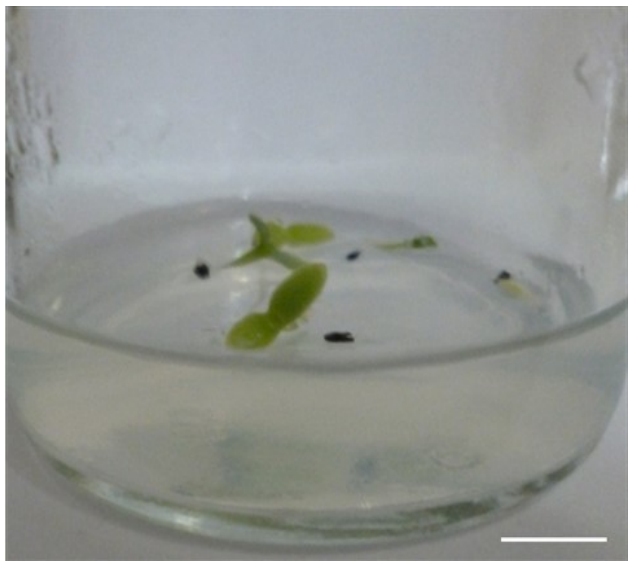

Fig. 2(c). Seedlings (14 days old). Bar = 1cm.

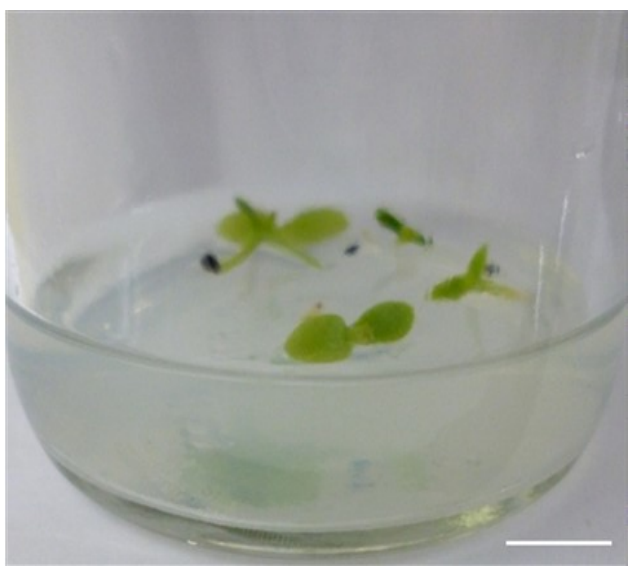

Fig. 2(d). Seedlings (21 days old). Bar = 1cm.

Fig. 2(e). Seedlings (30 days old). Bar = 1cm.

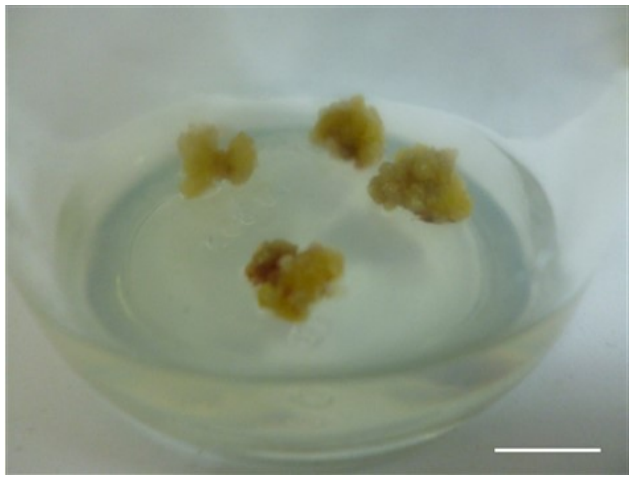

Fig. 3(a). C_{17}, callus induction in seedlings of dragon fruit (*Hylocereus costaricensis*) on half-strength MS medium after culture period of 6weeks. Cultures were kept in total darkness in a box with temperature regulated at 25 ± 2ºC.

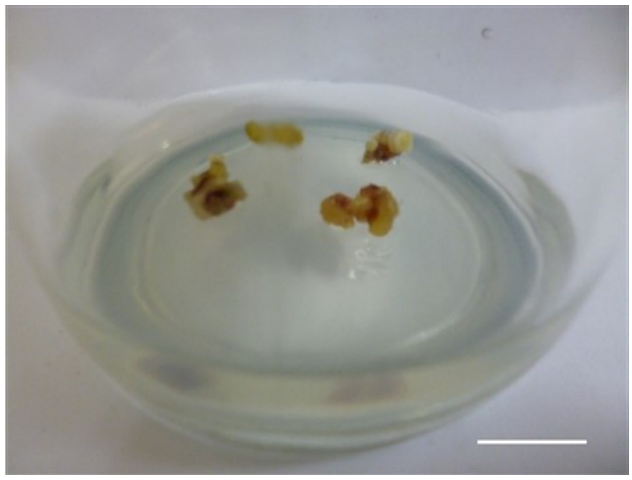

Fig. 3(d). C_{11}, callus induction in seedlings of dragon fruit (*Hylocereus costaricensis*) on half-strength MS medium after culture period of 6weeks. Cultures were kept in total darkness in a box with temperature regulated at 25 ± 2ºC. Bar = 1cm.

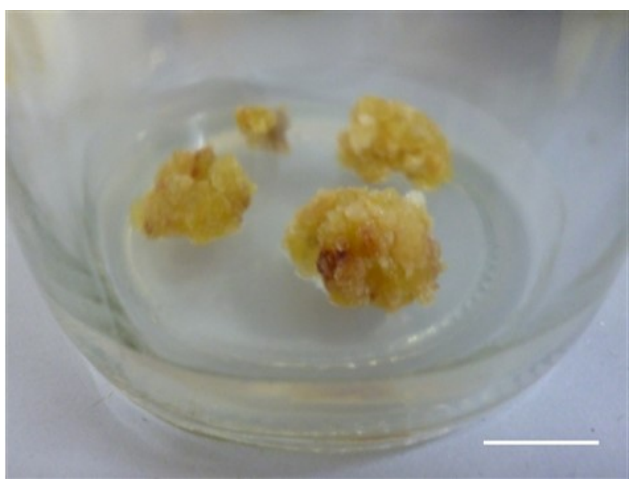

Fig. 3(b). C_{16}, callus induction in seedlings of dragon fruit (*Hylocereus costaricensis*) on half-strength MS medium after culture period of 6weeks. Cultures were kept in total darkness in a box with temperature regulated at 25 ± 2ºC. Bar = 1cm.

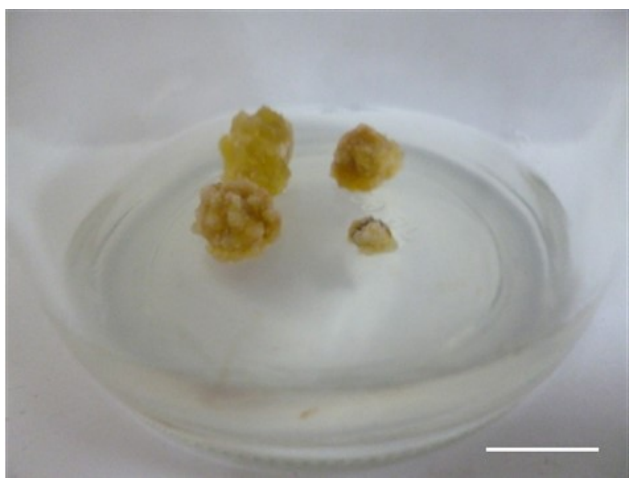

Fig. 3(e). C_{15}, callus induction in seedlings of dragon fruit (*Hylocereus costaricensis*) on half-strength MS medium after culture period of 6weeks. Cultures were kept in total darkness in a box with temperature regulated at 25 ± 2ºC. Bar = 1cm.

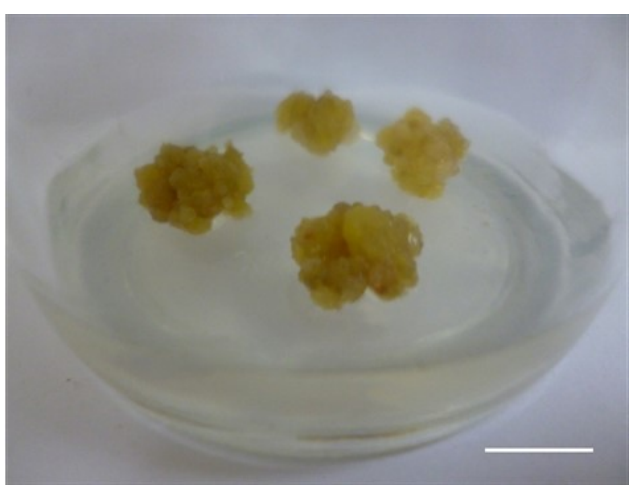

Fig. 3(c). C_{14}, callus induction in seedlings of dragon fruit (*Hylocereus costaricensis*) on half-strength MS medium after culture period of 6weeks. Cultures were kept in total darkness in a box with temperature regulated at 25 ± 2ºC. Bar = 1cm.

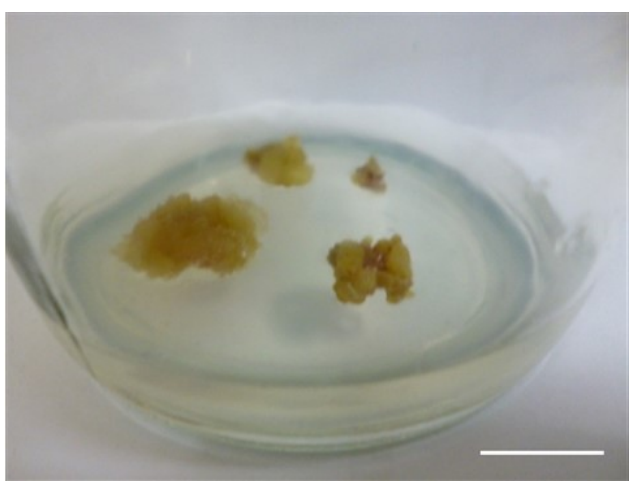

Fig. 3(f). C_{18}, callus induction in seedlings of dragon fruit (*Hylocereus costaricensis*) on half-strength MS medium after culture period of 6weeks. Cultures were kept in total darkness in a box with temperature regulated at 25 ± 2ºC. Bar = 1cm.

Table 3. Callus induction percentages from different callus induction treatments.

Callus induction treatment	Callus induction percentages
C_1	12.5%
C_2	31.25%
C_3	12.5%
C_4	18.75%
C_5	25%
C_6	31.25%
C_7	25%
C_8	12.5%
C_9	50%
C_{10}	N/A
C_{11}	62.5%
C_{12}	18.75%
C_{13}	37.5%
C_{14}	68.75%
C_{15}	62.5%
C_{16}	68.75%
C_{17}	75%
C_{18}	62.5%

Discussion

Dormant seeds are seeds that are not capable of germinating in a specific period of time under any combinations of physical environmental factors such as light intensity and temperature. The delay in seed germination may be due to the period of requirement for radicle emergence and embryo growth after the mature seeds have been dispersed. Freshly matured seed which develops during seed germination on the mother plant is termed as primary dormancy (Hilhorst, 1995 and 1998; Bewley, 1997a). On the other hand, a non-dormant seed is able to germinate over a wider range of environmental factors such as light and temperature. Scarification using sand is the mechanical process of cutting the seed coat using abrasion to break the seeds external dormancy and encourage germination. Seeds stand a better chance of germinating when the testa is scarified. The testa of seed is physically opened to allow air and moisture to penetrate and permeate the seed. Once the seeds are scarified, they do not store well and have to be cultured as soon as possible (Jerry & Carol, 2004). The germination of seeds starts in day four or day five and can be transferred to a pot after a month. Even though seed germination is simple, the quality of the new plant may be compromised due to the effects of cross pollination.

Previous studies on *In vitro* seed germination of *H. polyrhizus* on MS medium supplemented with 1 ppm BAP, 0 ppm IBA recorded 88% germination percentage (Kari *et al.*, 2010). In this study, only the group 1 ppm BAP, 0 ppm IBA was higher (93%) than the previous studies on *H. polyrhizus*.

Studies have shown that there was a significant difference in responsiveness between different source of explants. The proximal part of cotyledons of *Selenicereus megalanthus* performed better in callus induction when compared to epicotyl and hypocotyl regions on medium supplemented with thidiazuron (Pelah *et al.*, 2002).

Plant growth and developmental processes require the action of phytohormones including auxins and cytokinins (Bajguz and Piotrowska, 2009). Higher auxin to cytokinin ratio is important in promoting callus induction. By adjusting concentration of PGRs in the medium, callus formed from explants can be observed (Pierik, 1987; Ekiz & Konzak, 1997). Cytokinin played a vital part in inducing callus induction when used in combination with auxin. It was evident when callus induction treatments composed of both 2,4-D and BAP yielded higher CIP than auxin treated explants. The results were supported by Nordstrom *et al.* (2004) who suggested that auxins may regulate the level of cytokinin and metabolism and vice versa. It was reported that callus induction was associated with the application of high exogenous auxin to cytokinin ratio in certain plant species (Smulders *et al.*, 1988; Branca *et al.*, 1991). In this study, callus induced from auxin 2,4-D alone yielded lower CIP, however, a combination of both auxin and cytokinin resulted in higher CIP. Thus, this study was in agreement with Aitchison *et al.* (1977) suggesting that the presence of cytokinin is also generally required for callus induction.

H. costaricensis has a great potential of becoming a source of purple pigments known as betalain compounds as a potential secondary metabolite which may prove useful in many industries (Viñas *et al.*, 2012). Callus cultures may play an important role in the production and enhancement of betalains from this particular species of dragon fruit *H. costaricensis* as it may confer a stable betalain production after few subculture cycles. Furthermore, calluses showing steady production of betalain compounds may be used to establish cell suspension cultures allowing the recovery and easy isolation of large quantities of betalain compounds in future studies.

Conclusion

Both auxins and cytokinins play a role in promoting callus induction. Best callus induction was obtained by using cotyledons with C_{17} callus induction treatment for *H. costaricensis*. Callus obtained from cotyledon explants may have the potential in future attempts to obtain secondary metabolites from the *H. costaricensis* via cell suspension culture system.

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