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EFFECT OF SUCROSE CONCENTRATION ON PROLIFERATION OF *Bucephalandra* Sp. 'RED-MINI' CULTURES

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Abstract: *Bucephalandra* sp. 'Red Mini' is an aquatic flowering plant endemic to Borneo Island. The plant is popular among aquarists due to its magnificent appearance underwater submergence. Its vegetative propagation is relatively slow. Thus, applying the tissue culture technique could increase the production of diseased-free plantlets in large quantities within a short period. To date, limited reports are available on the effects of sucrose on the multiplication of *Bucephalandra* sp. 'Red Mini'. Therefore, this study investigated the effects of sucrose concentration on the number of shoot tips and reduced sugar content of *Bucephalandra* sp. 'Red Mini' cultures. The plantlets were cultured in MS medium containing various sucrose concentrations 1%, 3%, or 5% (w/v) added with 0 or 0.5 mg/L of Thidiazuron (TDZ), respectively. The newly formed shoot tips were recorded every two weeks intervals for 10 weeks and subsequently measured for the reducing sugar content. Results revealed that the new shoot tip number produced and the reduced sugar content were influenced by sucrose concentration and the presence of TDZ in the media. The highest shoot tip was recorded in the treatment medium containing 5.0% (w/v) sucrose added with 0.5 mg/L TDZ. In this medium, the shoot tip produced and reducing sugar content were 23.3 ± 4.0 shoot tips/culture and 0.35 ± 0.01 mM/culture, respectively. The finding suggested that sucrose concentration and TDZ synergistically regulate the formation of new shoot tips of *Bucephalandra* sp. 'Red Mini' and the reduced sugar content in the explants.

Keywords: Aquatic plant, reducing sugar, plantlets, shoot tips, culture medium.

Introduction

The *Bucephalandra* is an aquatic genus belonging to the Araceae family (Wong & Boyce, 2010). This herbaceous plant displays a remarkable morphological diversity and is a popular ornamental plant favoured by aquarists due to the leaf colours in an aquarium (Petruzzella *et al*., 2018). Notably, this plant occupies an epiphytic ecosystem with a shallow water body and grows along the water body edges (Madsen & Wersal, 2017; O'Hare *et al*., 2018). In addition, the plant has various life forms, leaf morphology from basic to complex, and particularly divided colours and inflorescence characteristics (Petruzzella *et al*., 2018).

Wong and Boyce (2016) described the *Bucephalandra* plants as obligate aquatic plants with small (2 cm) to medium-sized (6 cm) sizes. They also have dense roots, stabilising them in the shallow soil of river banks and enabling them to survive submerged. The plants also exhibit various leaf shapes, such as wavy edges, oval, flat edges, and straight and upon submergence in water, the leaves exhibit more intense colouration (Wong & Boyce, 2016).

In nature, *Bucephalandra* is known to have a low proliferation rate through vegetative, in which shoot tips are produced from the pseudo-rhizome. However, deforestation and uncontrolled collection activities from natural habitats to fulfil the market demand are the major traits of aquatic plants that might face extinction (Mashhor *et al*., 2012; Gaveau *et al*., 2014). Building on this, tissue culture

techniques provide the best alternative approach to producing many disease-free plantlets within a short period (Gaikwad *et al*., 2017). In plant tissue culture practices, sucrose is the culture medium's most commonly used organic carbon form. In plants, the primary photosynthetic products are carbohydrates, mainly monosaccharides (glucose and fructose), disaccharides (sucrose), and polysaccharides (starch) that provide essential energy sources and matter for survival and growth (Li *et al*., 2024).

Moreover, sucrose is the essential transported form of carbohydrate in plants from the leaves (source) to other plant parts for storage as well as cell development (Braun, 2022). Moreover, the sucrose concentration required varies among the plant species, and optimisation must be carried out (Sumaryono *et al*., 2012; Karatas & Aasim, 2014; Chandrasekara *et al*., 2015; Martins *et al*., 2015; Sotiropoulos *et al*., 2016).

N-phenyl-N'-l, 2, 3-thidiazol-5-urea, known as Thidiazuron (TDZ) is a phenyl-urea compound that is highly effective in inducing morphogenesis in plant tissue culture (Murthy *et al*., 1998). TDZ possesses both auxin- and cytokinin-like activities (Murthy *et al*., 1998) and has been used in the proliferation of a few aquatic plant species such as *Hemianthus callitrichoides* 'Cuba' (Barpete *et al*., 2015), *Bacopa monnieri* L. Pennel (Karatas & Aasim, 2014), *Xanthosoma sagittifolium* (Sama *et al*., 2012), *Cryptocoryne elliptica* (Norhanizan & Aziz, 2018) *Aglaonema* (Chen *et al*., 2018), and *Colocasia esculenta* (Du *et al*., 2006).

To our knowledge, no study has been conducted regarding the effects of sucrose concentration and the application of TDZ on the genus *Bucephalandra.* Wang and Ruan (2013) stated that sugar and auxin regulate the division and expansion of plant cells. Therefore, it was hypothesised that sucrose concentration with the presence of TDZ could alter the shoot tip proliferation and reduce sugar content in *Bucephalandra* sp. 'Red Mini' cultures.

The current study objective was to determine the effects of sucrose concentration on shoot tip number produced by *Bucephalandra* sp. 'Red Mini' plantlets. In addition, the reduced sugar content in the treated explants was also monitored. The findings of this experiment can benefit the proliferation of aquatic plant species and ornamental aquatic plant providers in fulfilling industry demands.

Materials and Methods

Source of Explants and Cultural Conditions

In this study, an established in vitro plantlet of *Bucephalandra* sp. 'Red Mini' maintained in a phytohormone-free Murashige and Skoog basal medium (Murashige & Skoog, 1962) was used as an explant. Meanwhile, the plantlets were obtained from the Biotechnology Laboratory at the Universiti Malaysia Terengganu. A single plantlet, approximately 2.0 cm in height was excised from a one-month-old plantlet and aseptically transferred into the treatment medium. All cultures were placed in a plant culture room at a temperature of 28°C. The fluorescent lamp was used to provide a 12-hour photoperiod during the treatment.

Treatment Medium

The experimental design used was a Complete Randomised Design (CRD) with 10 replications of cultures for each treatment medium. The Murashige and Skoog basal salt (Murashige & Skoog, 1962) supplemented with B5 vitamin (Gamborg *et al*., 1968) was used as a treatment medium. Then, sucrose was added to the media at concentrations of 1.0%, 3.0%, and 5.0% (w/v), respectively.

All media were adjusted to pH 5.7 to 5.8 with 0.1 M NaOH or 0.1 N HCl. The media were solidified by adding 2.5 g/L Phytagel® before autoclaving at 121°C for 15 minutes. TDZ was filter sterilised using cellulose nitrate (0.2 µM) before being added into a sterilised media at concentrations of 0 and 0.5 mg/L.

Shoot Tips Counting and Total Reducing Sugar Analysis

The number of newly formed shoot tips and

reducing sugar contents were measured every two weeks internally for 10 weeks. At harvest, three cultures were randomly sampled and analysed for Total Reducing Sugar (TRS) content according to the Somogyi-Nelson method (Hatanaka & Kobara, 1980). The sample was homogenised in phosphate buffer (pH 7.0) and centrifuged at 2,600 x *g* for eight minutes. Accordingly, 0.2 ml of supernatant was mixed with 1.8 ml distilled water and 1.0 ml Nelson reagent (Hatanaka & Kobara, 1980) and boiled in a water bath for 10 minutes. After cooling to room temperature, 1.0 ml of Arsenomolybdic acid reagent and 6.0 ml distilled water were added. Consequently, absorbance was measured with a UV spectrophotometer at 510 nm, and the reduction of sugar was calculated based on a standard curved plotted solution using 0.5 mM glucose stock.

Statistical Analysis

Data on shoot tips produced and reducing sugar content were subjected to a one-way Analysis of Variance (ANOVA). Subsequently, the data on the effects of sucrose concentrations were analysed using a T-test ($p \leq 0.05$) for mean significant differences in the treatments.

Results

Effects of Sucrose Concentration on Shoot Tip Number

Results in Figure 1 displayed the effect of the treatment media, the TDZ-free [Figure 1 (A)] and TDZ-containing [Figure 1 (B)] media with different concentrations of sucrose on the number of shoot tips produced after 10 weeks of culture. Generally, the number of shoot tips increased with the culture period. They differed significantly ($p < 0.05$) among the media containing different sucrose concentrations and TDZ. In the TDZ-free medium [Figure 1 (A)], the shoot tip number sharply increased after six weeks of culture in the medium containing 1.0% and 3.0% (w/v) sucrose.

After 10 weeks of culture, it was noticed that there were no significant differences in the number of shoot tips produced by explant in TDZ-free media added with 3% and 5% sucrose [Figure 1 (A)]. Note that the number of shoot tips in these two sucrose concentrations was the highest (11.0 ± 2.0 shoot tips/explant). On the other hand, in 5% (w/v) sucrose medium, the shoot tip number produced remained unchanged. After 10 weeks of culture, the shoot tip number produced remained lower, approximately three per culture [Figure 1 (A)].

Results in [Figure 1 (B)] illustrated the effects of TDZ and sucrose concentrations on the number of shoot tips after 10 weeks of culture. The shoot tip number produced was significantly increased ($p < 0.05$) until the end of the experiment. Among the sucrose concentration treatments, the number of shoot tips produced by explants in 3.0% and 5.0% (w/v) sucrose was significantly higher ($p \leq 0.05$) compared to those in 1.0% (w/v) of sucrose after four weeks of culture [Figure 1 (B)].

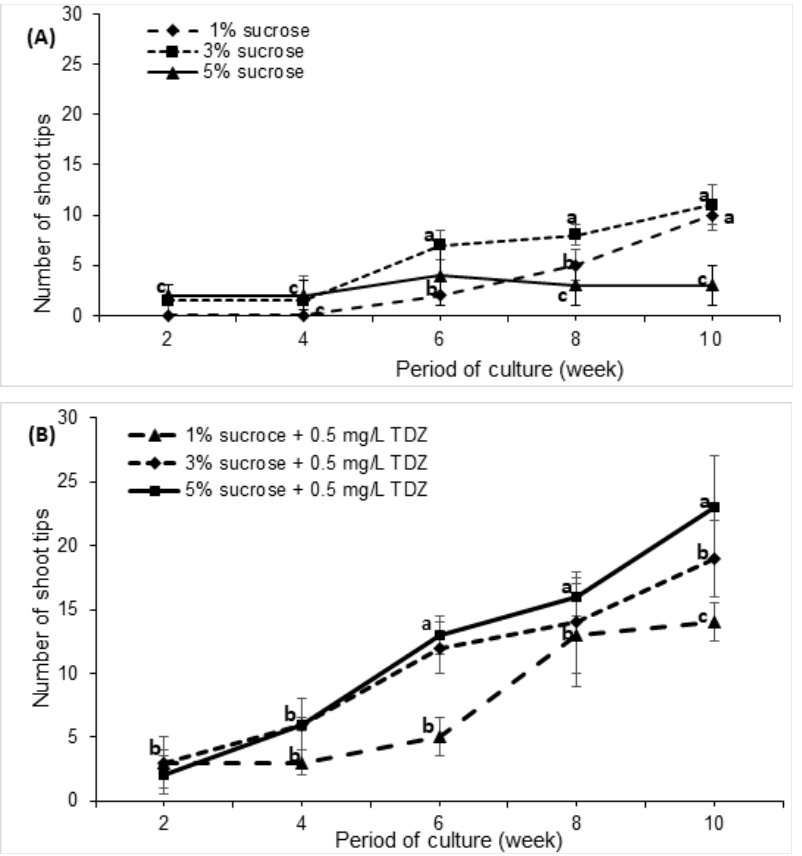

Figure 1: (A) Effects of sucrose concentration on the shoot tip number produced after 10 weeks of culture in TDZ-free medium and (B) TDZ-containing medium. The error bar indicates the range of value, $n = 3$. Value with different small letters indicates the differences between treatments ($p < 0.05$) using a T-test

After 10 weeks, it was observed that cultured in 5.0% (w/v) sucrose produced a significant number of shoot tips ($p < 0.05$), with the highest shoot tip number being 23 ± 4 shoot tips/explant. This was followed by explants cultured in 3.0% (w/v) sucrose (19 ± 3 shoot tips/explant). Explant culture in 1.0% (w/v) sucrose produced the lowest shoot tips among the treatments, which were approximately 14.0 ± 2.0 shoot tips/culture [Figure 1 (B)]. These findings indicated that sucrose and TDZ were synergistically involved in the shoot tip proliferation of *Bucephalendra* sp. var. 'Red Mini'.

The Appearance of Cultures

Figure 2 displays the appearance of the explant after it is cultured in the treatment media. It was noted that excessive shoot tips with a light-green colour were formed on the explants after six weeks transferred into the treatment media, particularly those cultures on medium containing a higher concentration of sucrose, which 3% and 5% (w/v) [Figure 2 (C) to (F)]. Clear and uniform shoot tips were observed on the explant cultured in a TDZ-containing medium as compared to the TDZ-free medium [Figure 2 (D) and (F)]. After 10 weeks of culture, the shoot tips produced in these media (TDZ + 3% sucrose and TDZ + 5% sucrose) were expanded and can be separated from the explants as a new single plantlet. On

the other hand, the newly emerging shoots from cultures in TDZ-free medium were ununiform and challenging to identify as completed single new plantlets [Figure 2 (C) and (E)].

Figure 2: The appearance of the *Bucephalandra* sp. 'Red Mini' explant after 10 weeks cultured in the treatment media containing various sucrose concentrations; TDZ-free medium with 1% (w/v) sucrose (A), 3% (w/v) sucrose (C), and 5% (w/v) sucrose (E), TDZ-containing medium with 1% (w/v) sucrose (B), 3% (w/v) sucrose (D), and 5% (w/v) sucrose (F)

Effects of Sucrose on Total Reducing Sugar Content

Results in Figure 3 presented the effects of sucrose concentration applied into media on the TRS content in the explant after 10 weeks of culture. In the TDZ-free medium, the TRS content was increased accordingly with the period of culture times until eight weeks but drastically dropped after 10 weeks of culture [Figure 3 (A)]. The highest TRS content was in the cultures in treatment media containing 3.0% (w/v) sucrose after eight cultures, which was 8.6 ± 2.3 mg/g of the culture. This was followed by 5.0% (w/v) sucrose and 1.0% (w/v) sucrose, which was 5.6 ± 1.4 and 3.1 ± 0.5 mg/g culture, respectively [Figure 3 (A)].

On the other hand, in a TDZ-containing medium, the TRS content in the cultures was increased until 10 weeks of treatment [Figure 3 (B)]. Nevertheless, the TRS content in *Bucephalandra* sp. 'Red Mini' plantlets cultured in TDZ-containing medium exhibit a similar trend to the TDZ-free media, where the highest TRS content ($p < 0.05$) was also from cultures in 5.0% (w/v) sucrose (9.9 ± 2.5 mg/g of culture) followed by the cultures in 3.0% (w/v), which was 7.5 ± 2.3 mg/g of culture. A significantly lower ($p < 0.05$) TRS was noticed in cultures from 1.0% (w/v) sucrose, which was 2.8 ± 0.4 mg/culture [Figure 3 (B)]. These findings might

indicate that the stored TRS was not influenced by the presence of TDZ in the treatment medium; however, it depends on the amount of sucrose added into the medium and the age of the culture.

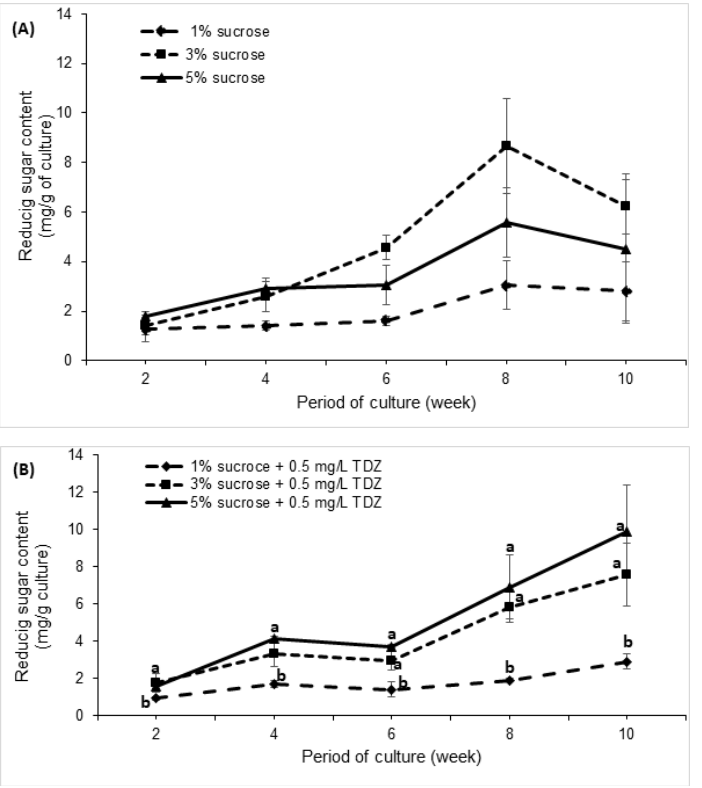

Figure 3: Effects of sucrose concentrations on the total reducing sugar content in Bucephalandra sp. 'Red Mini' after 10 weeks of cultures; cultures in TDZ-free MS media (A) and cultures in TDZ-containing medium (B). The bars represent the range of value, $n = 3$. Value with different small letters indicates the differences between treatments ($p < 0.05$) using a T-test

Discussion

Different from animals, plants generate organs throughout their life cycle. In plants, post-embryonic development relies on stem cell reservoirs localised in specialised tissues known as meristems (Cardenas-Aquino *et al*., 2023). The development of above-ground tissues and organogenesis is initiated and tightly coordinated by the Shoot Apical Meristem (SAM). SAM gives rise to leaves, axillary buds, flowers, and the stem. Notably, the establishment and maintenance of SAM allow the lifelong growth of plants are regulated by one key regulator known as SHOOT MERISTEMLESS (STM), a sugar-dependent from the TALE family (Long *et al*., 1996; Fujita & Kawaguchi, 2011; Lopes *et al*., 2023). Therefore, satisfactory sucrose concentration must be provided to the plant culture medium to enhance the cell proliferation and production of excessive new shoot tips.

In a previous study, Jo *et al.* (2009) reported that higher shoot tip proliferation of plants from the Araceae family can be achieved in a medium containing sucrose concentrations ranging from 5% to 8%. Hence, the current study examined the effect of sucrose concentrations from 1% to 5% on the number of shoot tips produced

by *Bucephalendara* sp. 'Red Mini' cultures. However, the results of the current study revealed that the effects of sucrose concentration on the number of shoot tips produced varied among the TDZ-free and TDZ-containing media (Figure 1).

The number of shoot tips produced depends on sucrose concentration and the TDZ presence in the media. As illustrated in Figure 1 (B), a higher number of shoot tips was obtained in 3% to 5% sucrose concentration. This was contradicted in the TDZ-free media, where higher shoot tips of *Bucephalendar*a sp. 'Red Mini' cultures were recorded in 1% sucrose [Figure 1 (A)]. The effect of sucrose concentration differs among plant species. For instance, 4% to 6% sucrose was reported to increase root formation in *Billbergia zebrina* cultures (Martins *et al*., 2015).

Despite the sucrose concentrations, the presence of TDZ in the culture medium also contributes to the appearance of the newly produced shoot tips by *Bucephalendara* sp. 'Red Mini' cultures (Figure 2). In a TDZ-free medium, a higher sucrose concentration (5% w/v) managed to induce excessive cell proliferation. However, it failed to form a complete plantlet [Figure 2 (E)]. In the TDZ-containing medium, the proliferated cells were managed to form a complete plantlet. These findings indicate that TDZ possesses a unique property to stimulate both the auxin- and cytokinin-like activities (Murthy *et al*., 1998) that can induce in vitro morphogenesis on *Bucephalendara* sp. 'Red Mini' cultures. Furthermore, a similar finding was reported on other aroid species such as *C. elliptica* (Norhanizan & Aziz, 2018), taro (Du *et al*., 2006; Fitriani *et al*., 2016), and *Alocasia amazonica* (Jo *et al*., 2009). In addition, TDZ was also reported to induce axillary bud and adventitious bud formation in sago palm (Sumaryono *et al*., 2012).

Under in vitro conditions, plants have low photosynthetic capability due to limited carbon dioxide existence in the culture vessel (Gamborg *et al*., 1968). The plant cells such as *Bucephalendara* sp. 'Red Mini' utilised the sucrose provided and catalysed glucose and fructose as carbon sources for organelles biosynthesis and development of new organs. According to Granot *et al*. (2013), hexose kinase enzymes play a role in sugar-sensing and plant development. This was clarified by a vigorous shoot tip formation in the medium that contained higher sucrose concentrations, particularly by the cultures that were supplied with TDZ [Figure 2 (D) to (F)]. Nevertheless, the TRS content in *Bucephalendara* sp. 'Red Mini' cultures varied among those TDZ-free and TDZ-containing cultures (Figure 3).

In the case of TDZ-free media, the highest TRS content was after eight weeks of culture and sharply declined after 10 weeks of culture [Figure 3 (A)]. Meanwhile, in TDZ-containing media, the TRS content steadily increased after 10 weeks of culture, with 5% (w/v) sucrose being the highest [Figure 3 (B)]. These results suggested that *Bucephalendar*a sp. 'Red Mini' bud outgrowth is regulated by plant growth regulators and sugars. Building on this, sugars behave as signalling entities that promote bud outgrowth through several sugar signalling pathways corresponding to the trehalose 6-phosphate, hexokinase-, glycolysis/ tricarboxylic acid, and oxidative pentose phosphate pathway-dependent signalling pathways (Jiang *et al*., 2022).

Conclusions

The current finding suggested that sucrose concentration and TDZ synergistically regulated cell proliferation and bud formation. In particular, the MS culture medium added with a high sucrose concentration (3% to 5% w/v) combined with a low concentration of TDZ (0.5 mg/l) is useful to enhance a higher number of *Bucepharandra* sp. 'Red mini' shoot tip production, which has more than 20 tips/ cultures. Accordingly, the production of massive and quality plantlets within a short culture period can be attained to fulfil the demand of the ornamental aquatic plant industry and protect the species from extinction.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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