Callus Development of the Seagrass *Enhalus acoroides* (L.f.) Royle

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ABSTRACT

Enhalus acoroides (L.f.) Royle is one of the most important seagrass species found in the coastal areas of Thailand. This study aimed to obtain an efficient plant growth regulator (PGR) for *E. acoroides* propagation. The callus development was induced and described here for the first time. To produce explants, Murashige & Skoog (MS) basal medium was prepared by using 28 psu artificial seawater instead of freshwater. The axenic seeds were cultured in MS basal medium containing different concentrations of PGRs. The survival rate of seeds ranged from 87% to 100%. All explants produced leaves after seven days. The most effective medium for explant growth and development was MS basal medium supplemented with 30 µM α-naphthalene acetic acid (NAA) in combination with 10 µM Thidiazuron (TDZ). To produce calluses, the hypocotyls of *E. acoroides* were taken from 16-week-old seedlings and cultured in MS media supplemented with various concentrations of PGRs. The medium containing 4.0 µM Kinetin in combination with 10.0 µM 2,4-dichlorophenoxyacetic acid (2,4-D) under dark conditions (12 weeks) produced the highest percentage of callus formation. Callus developed when the hypocotyl was transferred to MS basal medium with 30 µM NAA in combination with 10 µM TDZ under light conditions. These findings can be used to guide in vitro propagation of *E. acoroides*.

Keywords: Callus, *Enhalus*, Plant growth regulator, Propagation, Seagrass

INTRODUCTION

Seagrass beds provide significant ecological functions in coastal ecosystems, such as maintaining coastal biodiversity and fisheries productivity. They also play a significant role in carbon sequestration (Fourqurean *et al*., 2012; Stankovic *et al*., 2012; Lyimo and Hamisi, 2023). *Enhalus acoroides*, one of the most important seagrass species found in coastal areas, is widely distributed in the Indo-Pacific region (Green and Short, 2003). Its leaves and detritus are food sources and provide shelter for living creatures in the sea, especially dugong (Hines *et al*., 2005). In Thailand, *E. acoroides* has been found among the stomach contents of dugong (Adulyanukosol *et al*., 2001).

Human activities pose a direct threat to seagrasses (Orth *et al*., 2006), such as increased turbidity caused by sediments from land erosion (Spalding *et al*., 2003; Orth *et al*., 2006) and plastic pollution in the sea (Balestri *et al*., 2017). Moreover, seagrasses are also threatened by shallow-net trawling, inappropriate fishing gear, boating, anchoring, and shipping activities (Spalding *et al*.,

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2003). These threats have major impacts on seagrass meadows, resulting in the loss of many seagrass areas (Spalding *et al*., 2003; Grech *et al*., 2012). Furthermore, the rate of sexual reproduction of the seagrass was less than 10% per year (Hemminga and Duarte, 2000), which has limited seagrass population growth. Consequently, methods to restore and rehabilitate seagrass populations have been developed (Short and Coles, 2001). Transplanting techniques, such as sod, turf, plug and seed planting, have been used for conservation purposes since 1980 (Phillips, 1980; 1982; Thorhaug, 1986; Lewis, 1987; Fonseca *et al*., 1988; 1998; Thom, 1990). Most seagrass restoration programs take clonal vegetative stock from a donor seagrass bed and transplant it to the restoration site (Biber *et al*., 2009). This method could disturb the donor bed, especially for large seagrasses such as *E. acoroides*, which recover slowly (Calumpong and Fonseca, 2001).

Previous studies on *E. acoroides* have mostly focused on ecology, species distribution, and phenology (Nakaoka and Supanwanid, 2000; Kanmarangkool *et al*., 2022). Tissue culture has been shown to be successful for the rapid cloning of plants. However, previous studies on tissue culture of *E. acoroides* has mainly focused on traditional culture methods, without adding plant growth regulators (PGRs) (Thangaradjou and Kannan, 2008; Dagapioso and Uy, 2011). There have been no studies examining the callus induction or *in vitro* propagation of *E. acoroides*. Accordingly, the purpose of this research was to identify an efficient PGR for callus induction of *E. acoroides*. *In vitro* propagation of *E. acoroides* is important for long-term restoration and conservation of seagrass meadows in coastal ecosystems.

MATERIALS AND METHODS

The fruits of *Enhalus acoroides* were collected from the Andaman Sea Coast, Thailand. All fruits were cleaned and kept at 4 ºC during transport to laboratory. Three experiments were conducted in this study:

Experiment 1: Development of explant

Surface sterilization was conducted by shaking fruits in 70% (v/v) ethanol for 30 s, followed by adding 1.50% sodium hypochlorite (NaOCl) for 15 min, and the shaking in 0.60% NaOCl for 5 min. The sterilized fruits were rinsed three times with 28 psu sterilized artificial seawater under a laminar airflow cabinet (Tongkok *et al*., 2019).

The MS (Murashige and Skoog, 1962) basal medium was prepared by using 28 psu artificial seawater (AquaRaise® Reef Salt) instead of freshwater. Axenic seeds were cultured in MS basal medium containing different concentrations of α-naphthalene acetic acid (0, 30, 60, 90, 120, and 200 µM NAA) and thidiazuron (0, 1, 2, 5, and 10 µM TDZ) under a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 16 h light photoperiod provided by white fluorescent lamps for 16 weeks. Number and length of roots and leaves were measured and recorded.

Experiment 2: Induction and development of callus

Three hundred and fifty hypocotyls of *Enhalus acoroides* from Experiment 1 (16-week-old seedlings) were cultured in MS media for 12 weeks with 35 treatments, each with replication of 10 explants (n = 10). The explants were transplanted to MS basal media containing plant growth regulators. Thirty-six different culture media were tested in Experiment 2, consisting of Kinetin (0, 4, 8, 20, 40 or 60 µM KIN) in combination with 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 5, 10, 20, 40 or 60 µM 2,4-D). All media were adjusted to pH 5.8 prior to autoclaving at 103 kPa and 121 ºC for 15 min. All cultures were kept at 25±2 °C in a dark culture room for 12 weeks. Callus development in terms of its diameter was observed and recorded.

After 12 weeks, the callus was transferred onto MS medium with 30 µM NAA in combination with 10 µM TDZ in a clean culture room under light conditions (light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 16 h light photoperiod provided by white fluorescent lamps) for four weeks. Callus development was examined again after 16 weeks of culture. Formation of the shoot was observed after 21 weeks.

Experiment 3: Histological analysis

The selected callus specimens were fixed in Weak Chrome-acetic solution (Sass, 1958) for 24 h at room temperature. The specimens were washed three times in distilled water, then three times in 50% ethanol. The specimens were then dehydrated using a tert-butyl alcohol (TBA) series (50%, 70%, 85%, 95%, and 100% at 12 h each) and immersed three times in pure TBA (12 h each). The specimens were then transferred into a mixture of paraffin oil and pure TBA (1:1) for 12 h and immersed three times in 60 °C paraplast (12 h each). The specimens were embedded in paraffin blocks and sectioned to a thickness of 10–15 µm by a rotary microtome (Thermo, HM 325, UK). Staining of the specimens was done with safranin and fast green, after which they were mounted with permount. The permanent slides were observed under a compound light microscope (Zeiss, Axioskop 2, Germany). Starch grain was observed under a compound light microscope after staining with iodine-potassium iodide solution for 2 min.

Statistical analysis

Data were subjected to analysis of variance (ANOVA) and reported as means±SD. When significant differences in ANOVA were found, means were compared using the Tukey's post hoc test at the 5% probability level.

RESULTS AND DISCUSSION

The survival rate of seeds ranged from 87% to 100% across all concentrations of PGRs (Table 1). The highest ($p<0.05$) average root number was 2.40±0.62 in MS medium with 60 µM NAA without TDZ after 16 weeks of culture, whereby the other treatments produced only a single root (Table 2). Average root length was highest in MS medium supplemented with either 30 µM NAA plus 10 µM TDZ (root length = 4.60±0.34 cm) or 60 µM NAA alone (root length = 4.10±0.81 cm). Root development is important for both *in vitro* and *ex vitro* propagation, as the primary function of the root is absorption of water and dissolved nutrients necessary for plant development (Robert *et al*., 2015).

All seeds produced leaves after seven days. One seed developed to a shoot with 2 to 7 leaves. The response of leaf growth to different PGRs was quite different from that of root growth, whereby the highest number of leaves (7.00±0.40 leaves), with moderate leaf length was obtained from MS medium supplemented with 5.0 µM TDZ alone. The maximum leaf length was 10.60±0.55 cm in MS medium supplemented with 30 µM NAA and 10.0 µM TDZ (Table 1 and Figure 1). The number of explant leaves is very important for explant survival under *in vitro* culture that has limited nutrient sources and gases. Therefore, increasing leaf length can increase photosynthesis and be the mainspring for their development. The number and length of roots and leaves under these combinations were significantly different from PGR-free medium. Although TDZ at both 1.0 and 10 µM promoted seedling growth, the length of roots and leaves was highest in the medium with the combination of 10 µM TDZ and 30 µM NAA. The response of seedlings to different concentrations of NAA and TDZ was not the same. The growth was stimulated under high concentrations of TDZ, but not at lower concentrations (Figure 2). In contrast, Koch and Durako (1991) found that growth was inhibited in the seagrass *Ruppia maritima* when high concentrations of TDZ were added. The variability of marine plant responses to added PGRs may be due to species-specific sensitivity.

Although the seeds of *Enhalus acoroides* are currently suitable for culture, collection is limited by the timing and success of sexual reproduction. However, propagation using seed is not the only process to achieve rapid cloning of plants in a tissue culture laboratory.

This is the first report of callus development of *E. acoroides*. To induce callus formation, the basal MS medium was supplemented with combinations of Kinetin and 2,4 D. Although callus can be induced from any part of the plant, in this experiment, it was apparent that the hypocotyl produced callus. The calluses gradually developed and, at week 12, they were grown suitably for cell differentiation in the next step. Eleven PGR combinations (T8, T9, T10, T14, T15, T16, T20, T21, T22, T26, and T27) were found to induce the

Table 1. Growth and development of seeds of *Enhalus acoroides* at 16 weeks of culture on Murashige and Skoog basal medium containing various concentrations of NAA and TDZ.

	Treatment		No. of roots per explant	Root length (cm)	No. of leaves per explant	Leaf length (cm)	Survival rate (%)
	NAA (µM)	TDZ (µM)					
T0	0	0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	2.00±0.00 ^{f}	0.50±0.00 ^{g}	100 ^{a}
T1	0	1.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	6.00±0.00 ^{b}	0.50±0.27 ^{g}	100 ^{a}
T2	0	2.0	1.00±0.00 ^{b}	2.20±0.21 ^{b}	6.00±0.00 ^{b}	8.30±0.31 ^{b}	100 ^{a}
T3	0	5.0	1.00±0.00 ^{b}	2.00±0.30 ^{b}	7.00±0.40 ^{a}	6.00±0.37 ^{d}	100 ^{a}
T4	0	10.0	1.00±0.00 ^{b}	2.10±0.01 ^{b}	5.00±0.00 ^{c}	7.10±0.27 ^{c}	100 ^{a}
T5	30	0	1.00±0.00 ^{b}	2.40±0.31 ^{b}	4.00±0.10 ^{d}	4.50±0.31 ^{e}	93 ^{a}
T6	30	1.0	1.00±0.00 ^{b}	2.40±0.37 ^{b}	5.00±0.16 ^{c}	5.10±0.37 ^{de}	100 ^{a}
T7	30	2.0	1.00±0.00 ^{b}	2.20±0.28 ^{b}	5.00±0.20 ^{c}	7.60±0.18 ^{bc}	100 ^{a}
T8	30	5.0	1.00±0.00 ^{b}	2.40±0.28 ^{b}	5.00±0.20 ^{c}	5.40±0.18 ^{de}	100 ^{a}
T9	30	10.0	1.00±0.00 ^{b}	4.60±0.34 ^{a}	6.00±0.15 ^{b}	10.60±0.55 ^{a}	100 ^{a}
T10	60	0	2.40±0.62 ^{a}	4.10±0.81 ^{a}	6.00±0.15 ^{b}	5.60±0.25 ^{d}	100 ^{a}
T11	60	1.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	6.00±0.15 ^{b}	8.50±0.25 ^{b}	100 ^{a}
T12	60	2.0	1.00±0.00 ^{b}	2.20±0.21 ^{b}	4.00±0.10 ^{d}	4.10±0.16 ^{e}	93 ^{a}
T13	60	5.0	1.00±0.00 ^{b}	2.05±0.30 ^{b}	4.00±0.17 ^{d}	5.20±0.32 ^{de}	93 ^{a}
T14	60	10.0	1.00±0.00 ^{b}	2.10±0.00 ^{b}	5.00±0.20 ^{c}	5.60±0.32 ^{d}	100 ^{a}
T15	90	0	1.00±0.00 ^{b}	2.44±0.62 ^{b}	5.00±0.22 ^{c}	4.10±0.57 ^{e}	100 ^{a}
T16	90	1.0	1.00±0.00 ^{b}	2.40±0.32 ^{b}	5.00±0.17 ^{c}	4.90±0.97 ^{e}	100 ^{a}
T17	90	2.0	1.00±0.00 ^{b}	2.20±0.57 ^{b}	5.00±0.17 ^{c}	4.50±0.44 ^{e}	100 ^{a}
T18	90	5.0	1.00±0.00 ^{b}	2.42±0.20 ^{b}	5.00±0.22 ^{c}	7.50±0.45 ^{bc}	100 ^{a}
T19	90	10.0	1.00±0.00 ^{b}	2.25±0.34 ^{b}	4.00±0.13 ^{d}	8.80±0.67 ^{b}	93 ^{a}
T20	120	0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	4.00±0.13 ^{d}	6.10±0.42 ^{d}	93 ^{a}
T21	120	1.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	4.00±0.10 ^{d}	5.20±0.42 ^{de}	93 ^{a}
T22	120	2.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	4.00±0.0010 ^{d}	4.50±0.57 ^{e}	87 ^{a}
T23	120	5.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	3.00±0.13 ^{e}	2.10±0.38 ^{f}	87 ^{a}
T24	120	10.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	3.00±0.12 ^{e}	7.10±0.44 ^{c}	87 ^{a}
T25	200	0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	4.00±0.13 ^{de}	9.00±0.34 ^{b}	93 ^{a}
T26	200	1.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	4.00±0.13 ^{de}	9.00±0.62 ^{b}	93 ^{a}
T27	200	2.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	4.00±0.23 ^{de}	4.50±0.49 ^{e}	93 ^{a}
T28	200	5.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	3.00±0.11 ^{e}	6.20±0.49 ^{d}	87 ^{a}
T29	200	10.0	1.00±0.00 ^{b}	0.10±0.00 ^{c}	3.00±0.17 ^{e}	5.70±0.77 ^{d}	87 ^{a}

Note: NAA = α-naphthylacetic acid; TDZ = N-phenyl-N'-(1,2,3-thidiazol-5-yl) urea; *Mean values (±SD; n = 10) with different superscripts within each column denote significant ($p<0.05$) differences between groups.

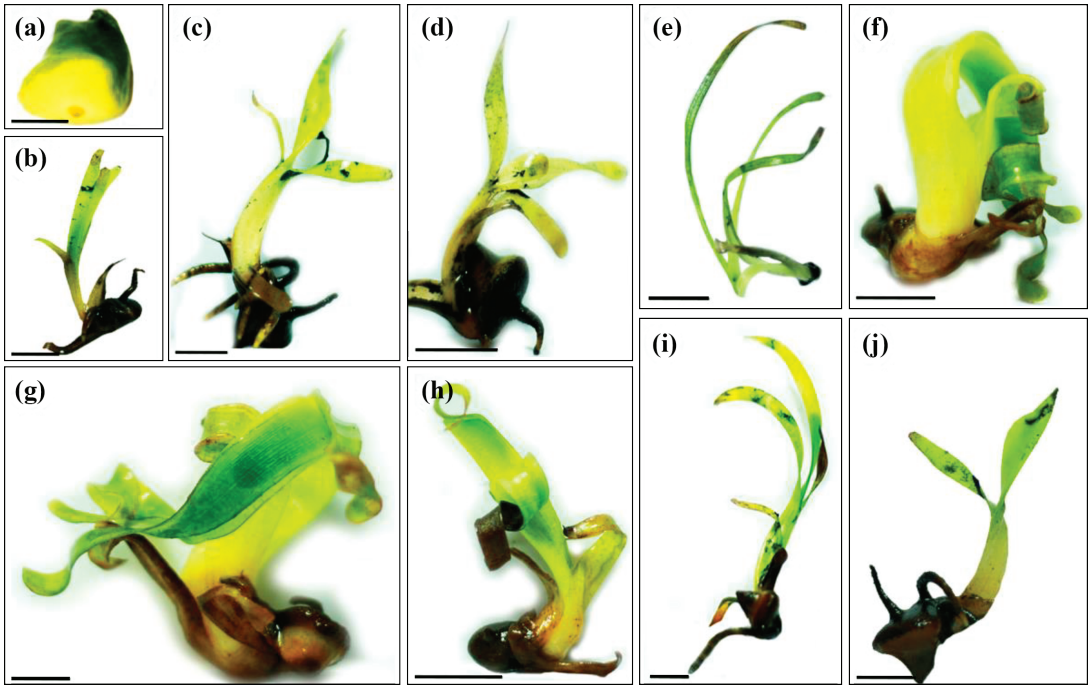

Figure 1. Seedlings of *Enhalus acoroides* cultured in MS media supplemented with various concentrations of NAA and TDZ: (a) control treatment with no plant growth regulators; (b) 30 µM NAA; (c) 60 µM NAA; (d) 90 µM NAA; (e) 200 µM NAA and 10 µM TDZ; (f) 10 µM TDZ; (g) 30 µM NAA and 10 µM TDZ; (h) 60 µM NAA and 10 µM TDZ; (i) 90 µM NAA and 10 µM TDZ; (j) 200 µM NAA and 10 µM TDZ. Scale bar: 1 cm

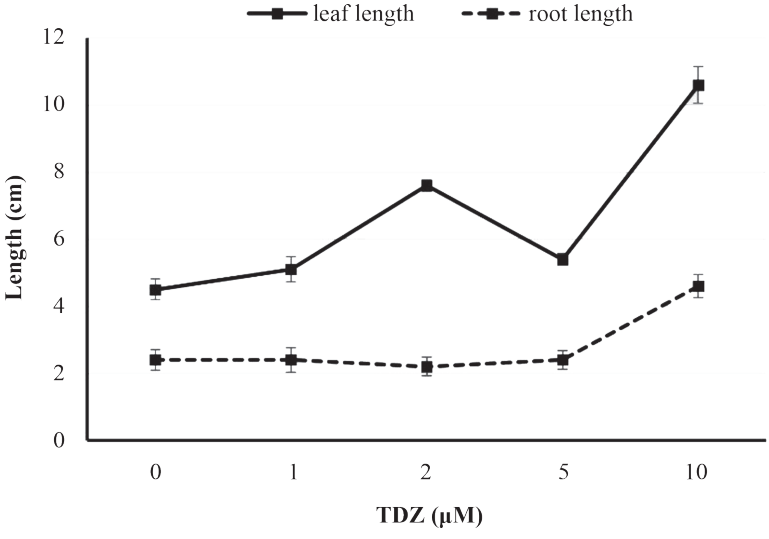

Figure 2. Mean leaf and root length of *Enhalus acoroides* at 16 weeks of culture supplemented with 30 µM NAA and different TDZ concentrations.

formation of callus (Table 2). Callus initiation occurred from the cut ends of the hypocotyl. Size of calluses was different among the eight culture media treatments. The induction percentage ranged from 10% to 100% (Table 2). Notably, all explants formed callus in the T8 medium (medium supplemented with 4 µM kinetin and 10 µM 2,4-D); this treatment also produced the highest callus diameter (17.23±3.28 mm) and friable callus texture.

Friable structure of callus covered most of the explant surface. The callus color varied slightly among treatments with different proportions of white, light yellow and brown (Figure 3), and the callus color was related to the color of explant and hypocotyl used in the experiment. It should be noted that the color of callus may differ among species; for example, callus of *Halodule pinifolia* is friable yellow (Danaraj *et al*., 2017). In rice, a terrestrial monocot, the optimal callus from mature seed has a creamy or yellow appearance which signifies capability for differentiation (Shim *et al*., 2020; Liang *et al*., 2021).

The present study revealed that *E. acoroides* showed great capacity to produce callus, even though seagrasses are known to be difficult to regenerate *in vitro* because of fungi and bacteria living in the epidermal cells of their vegetative organ (Kuo *et al*., 1981). The present study has demonstrated capacity for callus formation from the hypocotyl. In fact, our group has attempted to

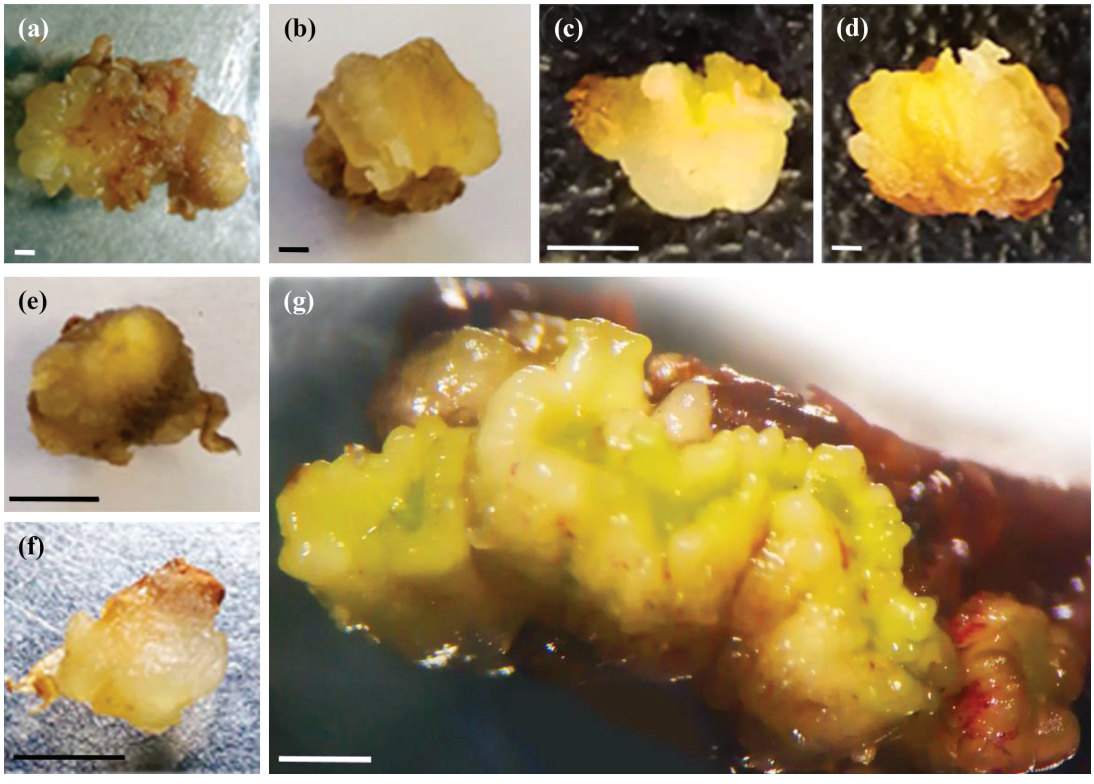

Figure 3. Callus of *Enhalus acoroides*: (a) callus formation from the cut ends of the hypocotyl at 12 weeks of culture under dark condition in 4 µM Kinetin and 10 µM 2,4-D (T13); (b) callus in 4 µM Kinetin and 20 µM 2,4-D (T14); (c) callus in 4 µM Kinetin and 40 µM 2,4-D (T15); (d) callus in 8 µM Kinetin and 10 µM 2,4-D (T19); (e) callus in 8 µM Kinetin and 20 µM 2,4-D (T20); (f) callus in 20 µM Kinetin and 10 µM 2,4-D (T25); (g) an outgrowth on callus under light condition after 16 weeks cultured in 30 µM NAA and10 µM TDZ. Scale bar: 1 mm

Table 2. Induction and development of callus in *Enhalus acoroides* at 12 weeks of culture on Murashige and Skoog basal media containing various concentrations of plant growth regulators.

	Treatment		Callus development		
	Kinetin (µM)	**2,4-D (µM)**	**Callus induction (%)**	**Callus diameter (mm)**	**Callus texture**
T0	0	0	0	-	-
T1	0	5	0	-	-
T2	0	10	0	-	-
T3	0	20	0	-	-
T4	0	40	0	-	-
T5	0	60	0	-	-
T6	4	0	0	-	-
T7	4	5	0	-	-
T8	4	10	100	17.23±3.28 [a]	Friable
T9	4	20	60	6.37±2.19 [b]	Friable
T10	4	40	20	1.66±0.48 c	Friable
T11	4	60	0	-	-
T12	8	0	0	-	-
T13	8	5	0	-	-
T14	8	10	60	5.67±2.01 [b]	Friable
T15	8	20	20	1.81±0.41 [c]	Friable
T16	8	40	10	1.76±0.43 [c]	Friable
T17	8	60	0	-	-
T18	20	0	0	-	-
T19	20	5	0	-	-
T20	20	10	40	2.03±0.49 [c]	Friable
T21	20	20	20	1.68±0.35 [c]	Friable
T22	20	40	10	1.63±0.38 [c]	Friable
T23	20	60	0	-	-
T24	40	0	0	-	-
T25	40	5	0	-	-
T26	40	10	10	1.66±0.35 [c]	Friable
T27	40	20	10	1.62±0.18 [c]	Friable
T28	40	40	0	-	-
T29	40	60	0	-	-
T30	60	0	0	-	-
T31	60	5	0	-	-
T32	60	10	0	-	-
T33	60	20	0	-	-
T34	60	40	0	-	-
T35	60	60	0	-	-

Note: 2,4-D = 2,4-dichlorophenoxyacetic acid; *Mean values (±SD) in the same column superscripted with different lowercase letters are significantly ($p<0.05$) different.

use other parts of *E. acoroides* for callus induction, however, without success. This may be explained by the fact that other parts of *E. acoroides* are composed of tannin cells which produce phenolic compounds for protection against competitors, predators and pathogens (Qi *et al*., 2008; Santoso *et al*., 2012). As such, these phenolic compounds might be produced when using those parts for callus induction, and thus resulted in failure to produce callus. However, the hypocotyl does not have tannin cells (Tongkok *et al*., 2019), and their absence may allow callus formation in this part of the plant.

In addition, during hypocotyl culture, callus was observed at the pores in the epidermis layer. The breaking up of epidermal layers is due to cell division of parenchyma cells in the inner tissues under the influence of PGRs. Callus from the hypocotyl was formed when the epidermis of the explant was opened. Callus was never formed on the surface, probably because this ability is confined to the vascular cells of the plant (López-Puc *et al*., 2006). The opened epidermis allows element exchange between the environment and the living tissues. The pores observed in the epidermis layer closely resemble lenticels, which are the openings formed during secondary growth of stems and roots. They are found on the epidermis of different plant organs, made up of parenchymatous cells and permit water vapor and gas exchange between environment and the internal tissue spaces of the organs (Carrillo-López and Yahia, 2019).

Calluses from T8 medium transferred to MS medium containing 30 µM NAA and 10 µM TDZ, one of the best combinations for growth and development of seeds in experiment 1, showed rapid development. The callus color varied from yellow to light green. A green spot and green nodule were produced on the surface of the callus, as can be seen in Figure 3g. TDZ promotes cell division and differentiation, while NAA seems to be crucial for the induction of organogenesis (Flaishman *et al*., 2005; Khierallah *et al*., 2017). This study demonstrated that the callus of *E. acoroides* could undergo organogenesis when cultured in a suitable medium, and that callus induction required a higher supply of TDZ than in other seagrasses such as *Cymodocea nodosa*, whose calluses were induced using only 1 µM TDZ in MS medium (García-Jiménez *et al*., 2006).

An outgrowth on callus from 30 µM NAA and 10 µM TDZ after 21 weeks was selected for histological analysis (Figures 4 and 5). The adventitious shoots were observed on the outgrowth of the callus. However, the shoot from these calluses died after subculture. Histological analysis confirmed the formation of shoots on the callus (Figure 4a). Formation of the shoot was observed from the meristematic center, growing leaf and arch of the vascular tissue (Figure 4b). Cells in the meristematic centers were characterized by clearly visible nuclei and thin cell walls. Histological analysis of the leaf revealed large intercellular

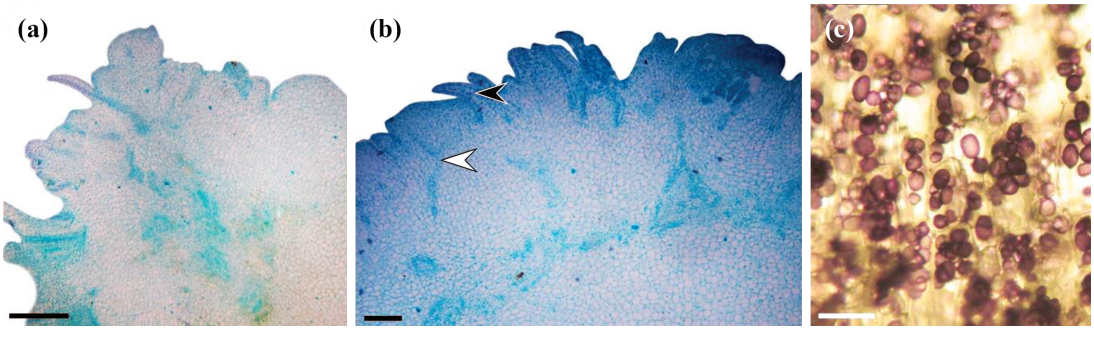

Figure 4. Histomicrographs of *Enhalus acoroides* callus from MS basal medium supplemented with 30 µM NAA and10 µM TDZ after 21 weeks; (a) presence of newly formed shoots on the callus; (b) formation of shoot, parenchyma cell in mesophyll layer of new leaf (black arrow) and vascular bundle (white arrow); (c) Starch grains around the vascular tissue. Scale bar: 200 µm (a), 100 µm (b), 50 µm (c).

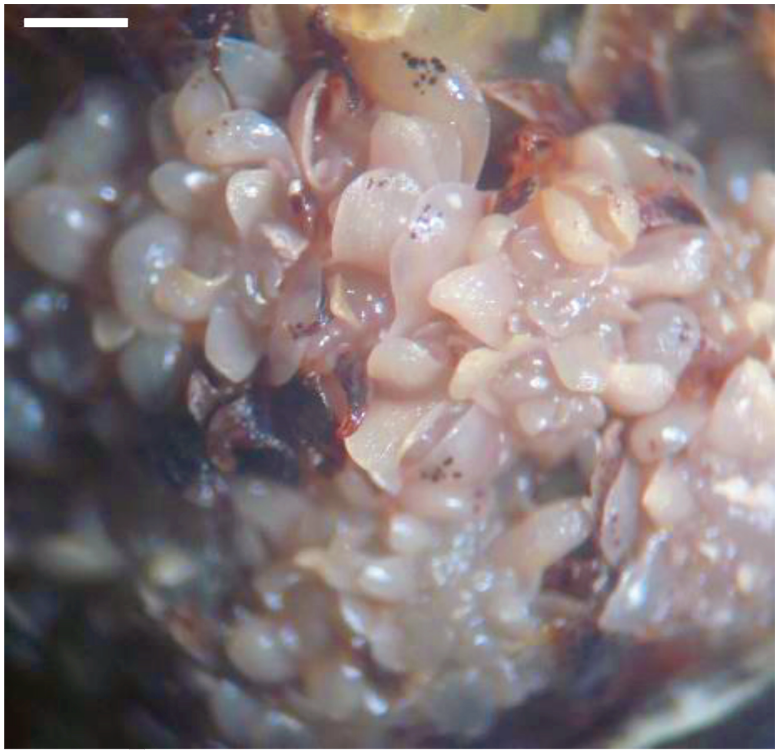

Figure 5. *Enhalus acoroides* shoot organogenesis from callus after 21 weeks of culture. Scale bar: 200 µm.

spaces and parenchyma cells in the mesophyll layer. Furthermore, the leaf was connected to the explant's vascular bundle via the vascular bundle of the new leaf. Starch grains were observed around the vascular tissue (Figure 4c). Interestingly, starch grain around the vascular tissue were also found on the outgrowth of the callus. Anatomical studies clearly indicated that induction and development of calluses may be caused not only by hormones but also by the carbohydrates from starch grains. Although the sucrose in the medium is the main carbon source most frequently used for callus induction and plant regeneration, carbohydrates also act as a source of carbon and energy, as well as an osmotic agent during organogenesis (Thorpe and Murashige, 1970).

In this research, we were able to induce callus formation in *E. acoroides* through tissue culture techniques. However, there are many more steps towards production of a viable plantlet from the callus. The embryogenic calluses and the improvement of *in vitro* propagation methods should be the focus of continued study to produce plantlets from tissue culture for seagrass transplantation and restoration in the future.

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