Agr. Nat. Resour. 53 (2019) 600–607

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Effects of plant growth regulators on *in vitro* seed germination and seedling development of *Enhalus acoroides*, a tropical seagrass

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Article Info

Article history:
Received 1 September 2017
Revised 3 August 2018
Accepted 7 October 2018
Available online 26 December 2019

Keywords:
Axenic culture,
Callus,
Development,
in vitro Germination,
Seagrass meadow

Abstract

The effects of plant growth regulators (PGRs) on seed germination and seedling development of *Enhalus acoroides* were investigated under axenic conditions. Seeds were cultured in sterile liquid medium supplemented with different concentrations of 6-benzyladenine (BA) × α-naphthylacetic acid (NAA) and N-phenyl-N'-(1,2,3-thidiazol-5-yl) urea (TDZ) × 2,4-dichlorophenoxyacetic acid (2,4-D). All seeds fully germinated (100% germination rates) within 1 d on liquid media without PGRs, while germination of seeds in liquid media supplemented with PGRs was delayed with 94–100% germination within 2–7 d. Embryogenic callus was obtained after 2 wk when cultured in liquid medium supplemented with 18 µM BA and 160 µM NAA. The addition of TDZ and 2,4-D inhibited root emergence. The results showed a significant effect of the culture medium on seedling development, but not on seed germination.

Introduction

Seagrass meadows support diverse faunal assemblages (Balestri et al., 1998). The distribution of seagrasses depends on several factors including temperature, precipitation and wind strength (Phillips and Meñez, 1988). The threats to seagrasses have been widely considered as due to humans and nature and seagrass losses have increased almost ten-fold over the last 40 yr in both tropical and temperate regions (Orth et al., 2006). Seagrass loss leads to a loss of the exuberance in the coastal zone (Duarte, 2009). Therefore, the conservation of seagrass meadow is extremely important to the coastal ecosystem.

Enhalus acoroides is the largest of the seagrasses and provides a variety of valuable ecological benefits to coastal areas where this species is widely distributed in the Indo-Pacific region (Green and Short, 2003). *E. acoroides* leaves occupy the water column from the seafloor to 1–2 m above and play a very important role in the marine ecosystem. Leaves and detritus of *E. acoroides* are food sources and shelter for sea turtles, fish, waterfowl and other marine animals especially dugong (Hines et al., 2005). Nine species of seagrass including *E. acoroides* have been found among the stomach contents of dugong (Adulyanukosol et al., 2001).

The previous studies on *E. acoroides* have mostly focused on ecology (Lewmanomont et al., 1996), species distribution, shoot density (Rattanachot, 2008; Rattanachot et al., 2015), and phenology (Walker et al., 2001). Successful propagation *in vitro* of *E. acoroides* has been difficult in the past. Little information exists on the effects of plant growth regulators (PGRs) on *E. acoroides*. The previous studies on the development of axenic seedlings of *E. acoroides* have focused attention on raising *E. acoroides* without adding PGRs (Thangaradjou

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https://doi.org/10.34044/j.anres.2019.53.6.06

and Kannan, 2008; Dagapioso and Uy, 2011). Thangaradjou and Kannan (2008) found that axenic culture of seedlings of *E. acoroides* was established without adding any growth regulators. Although the culture medium without PGRs could increase the growth of roots and leaves of *E.acoroides*, it could not induce callus and multiple shoots. The use of PGRs in axenic cultures of seagrass is unnecessary for the growth of roots and leaves of some seagrasses (Henry, 1998; Thangaradjou and Kannan, 2008), but it could raise callus and branch production and provide a rapid alternative to the more conventional means of propagation (Koch and Durako, 1991; Bird et al., 1994; Subhashini et al., 2014). The significance of PGRs in affecting seagrass growth and development can be defined only through axenic *in vitro* culture techniques (Koch and Durako, 1991). Consequently, a method of *in vitro* culturing and transplanting of *E. acoroides* has been developed (Phillips, 1980). Transplanting techniques, such as turf, plug and seed planting, have been used for conservation purposes (Thorhaug, 1986; Fonseca et al., 1988; Thom, 1990; Fonseca et al., 1998). Nevertheless, tissue culture has been shown to be successful for the rapid cloning of plants. In addition, PGRs are widely used to produce thousands of new plants and it is very important to germplasm conservation and establishing plantlets (Bird et al., 1994). To date, the only seagrass species that have been successfully cultured are: *Ruppia maritima* (NAA and TDZ supplement to the medium greatly increased rhizome and shoot growth rates of explants; Koch and Durako, 1991), *Halophila decipiens* (10 µM BA stimulated shoot and branch production of *H. decipiens*; Bird et al., 1998) and *Halodule pinifolia* (the formation of callus was reported when *H. pinifolia* was treated with 9.05 µM 2,4-D; Subhashini et al., 2014). Consequently, the current work was implemented to evaluate the seed development performance of *E. acoroides* as affected by various concentrations of PGRs. *in vitro* Culturing of *E. acoroides* is important for the long-term restoration and conservation of seagrass meadows. In addition, the current study attempted to develop axenic culture of the tropical seagrass, *E. acoroides*, which is very important in marine and coastal ecosystems of Thailand.

Materials and Methods

Collection of plant materials

Mature fruits of *Enhalus acoroides* (L.f.) Royle were collected from Haad Chao Mai National Park, Trang Province, southern Thailand. The mature fruits were 4−6 cm in diameter and had a ribbed, hairy skin. The collection site was at 7°23.4' N and 99°20.16' E. In total, 350 seeds were used in 35 treatments with 10 replicates of one seed each.

Specimen preparation

The mature fruits of *E. acoroides* were kept in an ice bucket and were then transported to the laboratory. In the laboratory, the mature fruits were washed and cleaned with seawater. Then, the cleaned fruits were rinsed once with sterilized artificial seawater (38% salinity), a mixture of dissolved mineral artificial salt and distilled water. The artificial seawater was sterilized by autoclaving at 103 kPa and 121°C for 15 min. The mature fruits were washed with 70% (volume per volume; v/v) ethanol, followed by surface sterilization.

Specimen sterilization

The first step in surface sterilizing the fruits consisted of shaking them for 30 s in 70% (v/v) ethanol, followed by surface sterilization using 1.50% sodium hypochlorite (NaOCl) for 15 min and then 0.60% NaOCl for 5 min. The sterilized fruits were rinsed three times with sterilized artificial seawater under a laminar airflow cabinet.

Medium preparation and seed culturing

The seeds of *E. acoroides* were removed from the sterilized fruits inside a laminar flow. These seeds were then transferred into 30 mL of liquid MS (Murashige and Skoog, 1962) medium containing plant growth regulators. Thirty-five different culture media were tested in the experiment, consisting of 6-benzyladenine (BA; 0, 2, 9 and 18 µM BA) in combination with α-naphthylacetic acid (NAA; 0, 20, 80 and 160 µM NAA), N-phenyl-N'-(1,2,3-thidiazol-5-yl) urea (TDZ; 0, 0.1, 0.2, 0.3 and 0.4 µM TDZ) in combination with 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 4.5, 9 and 18 µM 2,4-D). All media were adjusted to pH 5.8 prior to autoclaving (121°C for 15 min at 103 kPa). All cultures were kept at 25 ± 2°C under a light intensity of 40 µmol $m^{-2}s^{-1}$ and a 16 h of light photoperiod provided by white fluorescent lamps and maintained in a clean culture room. Seed germination was recorded every day. Percentages of germination were calculated at 7 d of culture. A seed was considered to have germinated when the first leaf and root had emerged and from then on it was considered a seedling (Fig. 1). Survival rates of seedlings were assessed at 60 d of culture. Development and phenological parameters of seedlings (the average number of roots and leaves, the maximum length of roots and leaves, and the fresh weight) were also recorded at 60 d of culture.

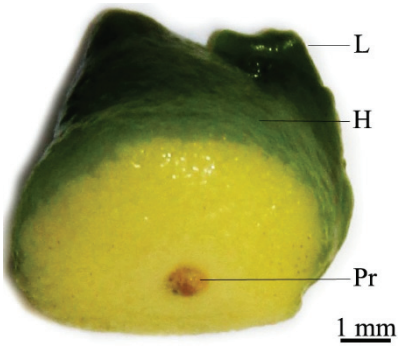

Fig. 1 Morphology of germinating seed on first day of culture showing leaf, primary root and large hypocotyl fused with the cotyledon (L = leaf, H = hypocotyl, Pr = primary root)

Statistical analysis

A completely randomized design was used with one factor (combination of PGRs added in the culture media) as the treatments which consisted of 35 combinations. Data were subjected to analysis of variance (ANOVA) and reported as the mean ± SD. When significant differences in ANOVA were found, means were compared using Tukey's post-hoc test at the 5% probability level.

Results

Seed germination

Under axenic conditions, seeds were able to germinate and grow on liquid medium without PGRs and with PGRs. Seeds fully germinated on the first day when cultured in the liquid medium without PGRs, while seed germination in liquid medium supplemented with PGRs started on days 2–7. At 7 d of culture, the average germination was in the range 94.5–100% but there were no significant differences between treatments (Table 1). At the end of the experiment (after 60 d of culture), the survival rates were 94–100%; there were no significant differences in seedling survival between treatments (Table 1).

Table 1 Seed germination and seedling growth at 60 d of culture of *E. acoroides* on Murashige and Skoog media containing various concentrations of plant growth regulators

Treatment		Germination		Number of roots per explant	Root length (mm)	Number of leaves per explant	Leaf length (cm)	Survival rate (%)
Cytokinin (µM)	Auxin (µM)	Time	Rate (%)					
Control		1st day	100	3.20±0.20^{a*}	19.1±1.13^{a}	5.20±0.13^{a}	4.31±0.29^{d}	100
BA	NAA							
0	20	7th day	100	1.00±0.00^{c}	1.20±0.13^{c}	3.00±0.00^{b}	4.35±0.27^{d}	100
0	80	7th day	100	1.00±0.00^{c}	2.20±0.13^{c}	2.90±0.10^{bc}	4.62±0.31^{cd}	100
0	160	7th day	98.2	1.00±0.00^{c}	2.00±0.26^{c}	2.40±0.16^{b}	4.66±0.37^{cd}	98
2	0	4th day	100	1.00±0.00^{c}	1.20±0.13^{c}	3.00±0.00^{b}	4.35±0.27^{d}	100
2	20	6th day	100	1.00±0.00^{c}	1.20±0.13^{c}	3.00±0.00^{b}	4.35±0.27^{d}	100
2	80	6th day	100	1.00±0.00^{c}	2.20±0.13^{c}	2.90±0.10^{bc}	4.62±0.31^{cd}	100
2	160	6th day	96.5	1.00±0.00^{c}	2.00±0.26^{c}	2.40±0.16^{c}	4.66±0.37^{cd}	96
9	0	4th day	100	1.00±0.00^{c}	1.00±0.00^{c}	2.80±0.20^{b}	3.49±0.18^{d}	100
9	20	6th day	100	1.00±0.00^{c}	1.00±0.00^{c}	2.80±0.20^{b}	3.49±0.18^{d}	100
9	80	6th day	100	1.00±0.00^{c}	2.30±0.33^{c}	2.70±0.15^{bc}	4.38±0.55^{d}	100
9	160	6th day	95.3	1.00±0.00^{c}	1.70±0.15^{c}	2.50±0.17^{c}	4.94±0.38^{cd}	95
18	0	4th day	100	2.00±0.00^{ab}	6.20±0.81^{b}	3.00±0.15^{b}	4.43±0.25^{d}	100
18	20	6th day	100	2.00±0.00^{ab}	6.20±0.81^{b}	3.00±0.15^{b}	4.43±0.25^{d}	100
18	80	6th day	100	1.00±0.00^{c}	2.80±0.33^{c}	2.90±0.10^{b}	3.84±0.16^{d}	100
18	160	6th day	94.5	1.00±0.00^{c}	1.40±0.22^{c}	2.50±0.17^{c}	4.17±0.32^{cd}	94
TDZ	2,4-D							
0	4.5	2nd day	100	0.00±0.00^{d}	0.00±0.00^{d}	4.60±0.16^{ab}	9.05±0.84^{a}	100
0	9	2nd day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.50±0.22^{b}	7.73±0.57^{ab}	100
0	18	2nd day	95.8	0.00±0.00^{d}	0.00±0.00^{d}	3.50±0.17^{b}	9.87±0.97^{a}	95
0.1	0	2nd day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.50±0.17^{b}	7.48±0.44^{ab}	100
0.1	4.5	4th day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.60±0.22^{b}	7.49±0.45^{ab}	100
0.1	9	4th day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.80±0.13^{b}	5.58±0.67^{bcd}	100
0.1	18	4th day	96.6	0.00±0.00^{d}	0.00±0.00^{d}	4.20±0.13^{ab}	5.19±0.64^{bcd}	96
0.2	0	2nd day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.80±0.13^{b}	4.29±0.42^{d}	100
0.2	4.5	4th day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.90±0.10^{b}	4.30±0.42^{d}	100
0.2	9	4th day	100	0.00±0.00^{d}	0.00±0.00^{d}	4.00±0.21^{ab}	5.09±0.57^{bcd}	100
0.2	18	4th day	96.5	0.00±0.00^{d}	0.00±0.00^{d}	3.80±0.13^{b}	4.83±0.38^{cd}	96
0.3	0	2nd day	100	0.00±0.00^{d}	0.00±0.00^{d}	4.40±0.22^{ab}	5.19±0.44^{bcd}	100
0.3	4.5	7th day	100	0.00±0.00^{d}	0.00±0.00^{d}	4.50±0.22^{ab}	5.24±0.46^{bcd}	100
0.3	9	7th day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.80±0.13^{b}	3.34±0.34^{d}	100
0.3	18	7th day	94.7	0.00±0.00^{d}	0.00±0.00^{d}	3.80±0.13^{b}	4.30±0.62^{d}	94
0.4	0	2nd day	100	0.00±0.00^{d}	0.00±0.00^{d}	3.90±0.23^{b}	4.44±0.49^{d}	100
0.4	4.5	7th day	100	0.00±0.00^{d}	0.00±0.00^{d}	4.00±0.21^{ab}	4.46±0.49^{d}	100
0.4	9	7th day	100	0.00±0.00^{d}	0.00±0.00^{d}	4.50±0.17^{ab}	5.92±0.77^{bcd}	100
0.4	18	7th day	95.5	0.00±0.00^{d}	0.00±0.00^{d}	3.80±0.13^{b}	5.50±0.39^{bcd}	95

BA = 6-benzyladenine; NAA = α-naphthylacetic acid.

*Mean values (±SD) with different lowercase superscripts letters within each column denote significant ($p < 0.05$) differences between groups.

Seedling development

Development of the root and leaf of seedlings was assessed at 60 d of culture. Root numbers of seedling in the MS medium supplemented with different combinations of BA and NAA inhibited the formation and elongation of roots (Fig. 2) but stimulated callus formation. The parenchymal cells in the base portion of the hypocotyl developed into embryogenic callus after 2 wk in the MS media containing different concentrations of BA and NAA. Callus was induced at all concentrations of NAA (20, 80 and 160 µM NAA) with 18 µM BA (except at 0, 2, and 9 µM BA) as shown in Fig. 3. Roots of seedlings were not produced in the MS media supplemented with TDZ and 2,4-D (Fig. 4). The highest root number was 3.20 ± 0.2 roots in the MS medium without PGRs after 60 d of culture. The root numbers in the different media are shown in Table 1; there were significant differences (Table 2). The highest root length was 19.10 ± 1.13 mm in the MS medium without PGRs after 60 d of culture (Table 1); there were significant differences (Table 3). Seedlings had shoots with the average number of leaves varying from 2.4 to 5.2 leaves. The leaves of seedlings in all treatments were produced from a plumule between the cotyledon and hypocotyl (Fig. 5). All seedlings produced ribbon-like leaves with a round apex. The highest leaf number was 5.20 ± 0.13 leaves in the MS medium without PGRs. The maximum average leaf length was 9.87 ± 0.97 cm in the MS medium supplemented with 18 µM 2,4-D. The number of leaves per explant and the length of the leaves in the MS media with different combinations of PGRs were both significantly different (Tables 4 and 5, respectively).

Seedling fresh weight

The MS media with different combinations of PGRs increased the seedling fresh weight at 60 d of culture. The seedling fresh weight in the MS media supplemented with different combinations of BA and NAA were significantly different. The highest seedling weight was 2.13 ± 0.11 g in 18 µM BA and 160 µM NAA. The highest above ground weight (leaf and shoot) of seedlings was 0.36 ± 0.03 in 18 µM BA and 20 µM NAA. The highest below ground weight (root and hypocotyl) was 1.86 ± 0.12 g in 18 µM BA and 160 µM NAA (Fig. 6A). The seedling weight in the MS media with different combinations of TDZ and 2,4-D were significantly different. The highest seedling weight was 2.23 ± 0.17 g in 0.2 µM TDZ with 18 µM 2,4-D. The highest above ground weight of seedlings was 1.33 ± 0.14 g in 0.1 µM TDZ with 4.5 µM 2,4-D. The highest below ground weight of seedlings was 1.15 ± 0.13 g in 0.2 µM TDZ with 18 µM 2,4-D (Fig. 6B).

Discussion

The results of the analysis of variance revealed that the different concentrations of PGRs had no effect on seed germination. However, the findings revealed that the seeds in the liquid medium without PGRs germinated on the first day while germination of seeds in liquid medium supplemented with PGRs was delayed. Nevertheless, all seeds had fully germinated within 1 wk. These results showed that the ability of *E. acoroides* seeds to germinate and develop on different MS media may represent an adaptation to the seed dispersion mechanism in this species. The seed of *E. acoroides* could germinate under various conditions, but the period of the seed germination, survival rates and development were affected by PGRs. The hypocotyl of *E. acoroides* is a nutrition source for the seedling. Nutrient storage in the form of starch grains was reported for another seagrass species (*Thalassia*; Kuo et al*.,* 1991). Seeds of seagrass also contain other nutrients such as vitamins, protein, carbohydrates, polyunsaturated fatty acids and antioxidants (Montaño et al*.,* 1999). Therefore, the seed germinates and grows under its own energy while still cultured until there is a lack of nutrient source in the seed. Balestri et al. (1998) suggested that seedling growth may depend on the nutrient stored in the seagrass seed and differences in the culture conditions. When seed is in an unsuitable environment, it adapts by reducing cell division and cell expansion.

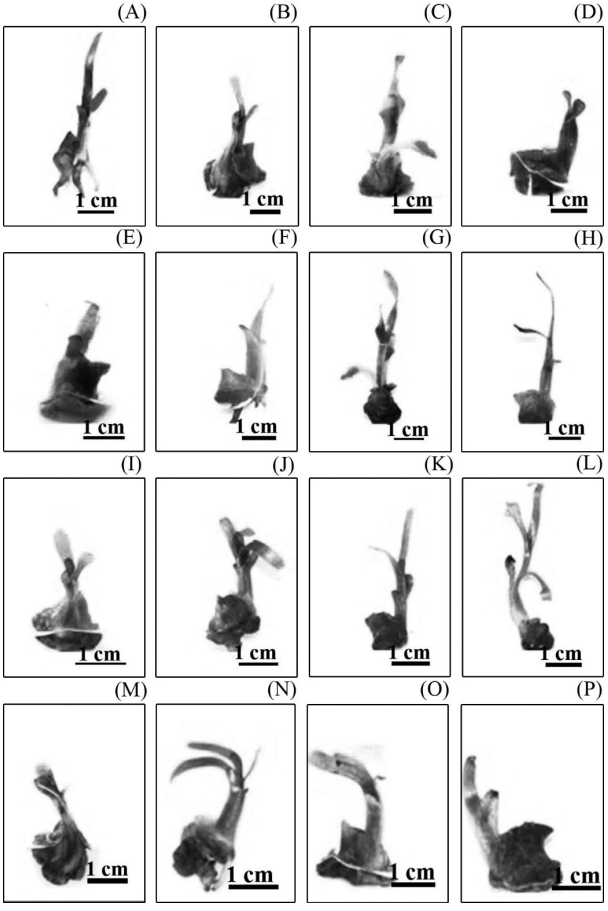

Fig. 2 Effects of 6-benzyladenine (BA) and α-naphthylacetic acid (NAA) on seedling growth at 60 d of culture of *E. acoroides*: (A) seedling in control cultures with no plant growth regulators; (B) seedling in 20 µM NAA; (C) seedling in 80 µM NAA; (D) seedling in 160 µM NAA; (E) seedling in 2 µM BA; (F) seedling in 2 µM BA and 20 µM NAA; (G) seedling in 2 µM BA and 80 µM NAA; (H) seedling in 2 µM BA and 160 µM NAA; (I) seedling in 9 µM BA; (J) seedling in 9 µM BA and 20 µM NAA; (K) seedling in 9 µM BA and 80 µM NAA; (L) seedling in 9 µM BA and 160 µM NAA; (M) seedling in 18 µM BA; (N) seedling in 18 µM BA and 20 µM NAA; (O) seedling in 18 µM BA and 80 µM NAA; (P) seedling in 18 µM BA and 160 µM NAA

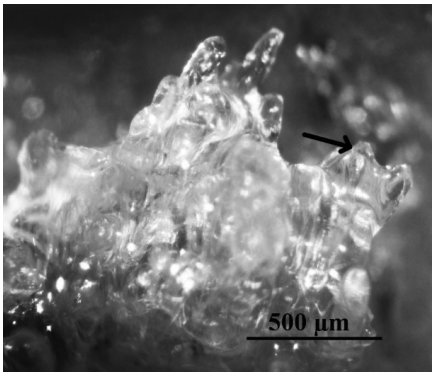

Fig. 3 Surface view of *E. acoroides* embryogenic callus showing globular somatic embryos and a heart-shaped somatic embryo (arrow) on Murashige and Skoog (1962) medium supplemented with 18 µM 6-benzyladenine and 160 µM α-naphthylacetic acid

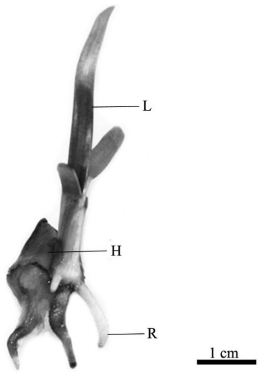

Fig. 5 Surface view of morphology of *E. acoroides* seedling at 60 d of culture showing shoot with leaf and large hypocotyl fused with cotyledon (L = leaf, H = hypocotyl, R = root)

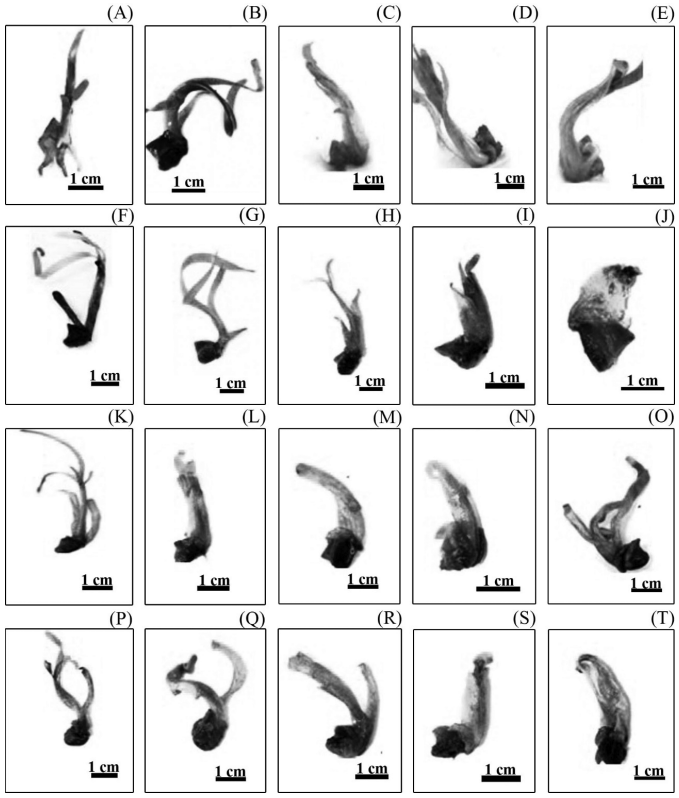

Fig. 4 Effects of N-phenyl-N'-(1,2,3-thidiazol-5-yl) urea (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D) on the seedling growth at 60 d of culture of *E. acoroides*: (A) seedling in control cultures with no plant growth regulators; (B) seedling in 0.1 µM TDZ; (C) seedling in 0.2 µM TDZ; (D) seedling in 0.3 µM TDZ; (E) seedling in 0.4 µM TDZ; (F) seedling in 4.5 µM 2,4-D; (G) seedling in 0.1 µM TDZ and 4.5 µM 2,4-D; (H) seedling in 0.2 µM TDZ and 4.5 µM 2,4-D; (I) seedling in 0.3 µM TDZ and 4.5 µM 2,4-D; (J) seedling in 0.4 µM TDZ and 4.5 µM 2,4-D; (K) seedling in 9 µM 2,4-D; (L) seedling in 0.1 µM TDZ and 9 µM 2,4-D; (M) seedling in 0.2 µM TDZ and 9 µM 2,4-D; (N) seedling in 0.3 µM TDZ and 9 µM 2,4-D; (O) seedling in 0.4 µM TDZ and 9 µM 2,4-D; (P) seedling in 18 µM 2,4-D; (Q) seedling in 0.1 µM TDZ and 18 µM 2,4-D; (R) seedling in 0.2 µM TDZ and 18 µM 2,4-D; (S) seedling in 0.3 µM TDZ and 18 µM 2,4-D; (T) seedling in 0.4 µM TDZ and 18 µM 2,4-D

Table 2 Effect of different concentrations of plant growth regulators on number of roots of *E. acoroides* after 60 d of culture. Probability values of one-way analysis of variance indicate that treatment means are significantly different ($p < 0.05$)

	Sum of Squares	Degrees of freedom	Mean Square	F-value	Significance
Between groups	195.817	34	5.759	269.968	0.000
Within groups	6.720	315	0.021		
Total	202.537	349			

Table 3 Effect of different concentrations of plant growth regulators on root length of *E. acoroides* after 60 d of culture. Probability values of one-way analysis of variance indicate that treatment means are significantly different ($p < 0.05$)

	Sum of Squares	Degrees of freedom	Mean Square	F-value	Significance
Between groups	4012.789	34	118.023	69.613	0.000
Within groups	534.060	315	1.695		
Total	4546.849	349			

Table 4 Effect of different concentrations of plant growth regulators on number of leaves of *E. acoroides* after 60 d of culture. Probability values of one-way analysis of variance indicate that treatment means are significantly different ($p < 0.05$)

	Sum of Squares	Degrees of freedom	Mean Square	F-value	Significance
Between groups	171.297	34	5.038	11.367	0.000
Within groups	139.620	315	0.443		
Total	310.917	349			

Table 5 Effect of different concentrations of plant growth regulators on leaf length of *E. acoroides* after 60 d of culture. Probability values of one-way analysis of variance indicate that treatment means are significantly different ($p < 0.05$)

	Sum of Squares	Degrees of freedom	Mean Square	F-value	Significance
Between groups	759.370	34	22.334	6.025	0.000
Within groups	1167.625	315	3.707		
Total	1926.995	349			

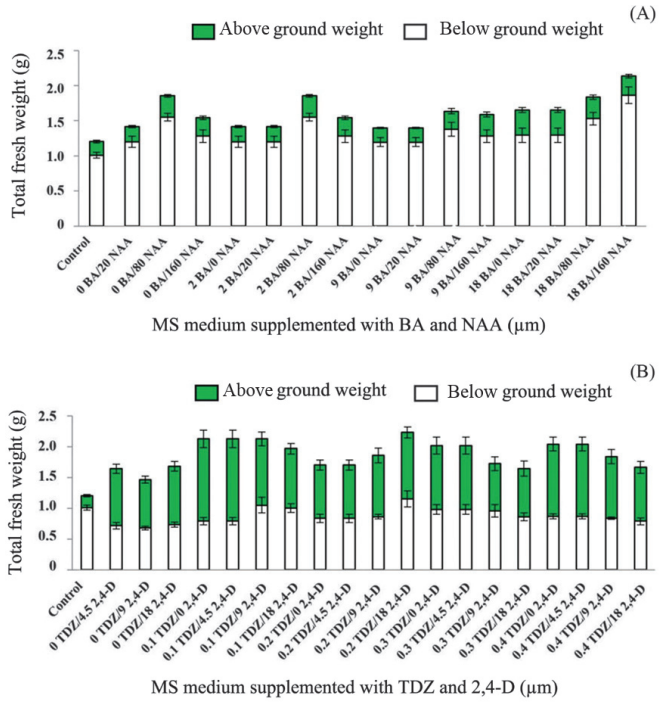

Fig. 6 Mean total fresh weight (± SD) of *E. acoroides* seedling at 60 d of culture: (A) seedlings in Murashige and Skoog (MS) media containing various concentrations of 6-benzyladenine (BA)and α-naphthylacetic acid (NAA) showing above ground weight (leaf and shoot) and below ground weight (root and hypocotyl); (B) seedling in Murashige and Skoog media containing various concentrations of N-phenyl-N'-(1,2,3-thidiazol-5-yl) urea (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D)

A significant difference was observed among the different concentrations of PGRs in terms of root and leaf developments after 60 d of culture. The different concentrations of TDZ in combination with 2,4-D and of BA in combination with NAA had negative effects on the formation and development of roots. The average numbers of roots under these combinations were significantly different from the average number of roots on the PGR-free medium. In addition, the average root lengths in the PGR media were lower than for the control medium. The response of seed to different hormone types and concentrations was not the same. The negative interaction of TDZ in combination with 2,4-D resulted in relatively fewer roots than for BA in combination with NAA, even though the MS media without PGRs had better root development than for the media with PGRs. These findings revealed that the PGR media promoted leaf development in *E. acoroides*. Responses to TDZ indicated that growth stimulation became saturated at low levels, whereas with higher concentrations of TDZ, growth was inhibited. Similarly, Koch and Durako (1991) observed growth inhibition in the seagrass *Ruppia maritima* when high concentrations of thidiazuron (TDZ) were added. BA with NAA actually inhibited the growth and development of leaves Therefore, the weight of below ground biomass was greater than the weight above ground. The addition of BA and NAA in the culture medium promoted hypocotyl elongation and the size of the hypocotyl. Callus formed from the hypocotyl when cultured in the MS media supplemented with BA and NAA. The medium supplemented with BA and NAA not only stimulated hypocotyl development, but also promoted root growth. In contrast, Bird et al. (1998) found that 10 µM BA stimulated shoot and branch production of *H. decipiens*. Therefore, the variability of marine plant responses to added PGRs may be due to differences in species-specific sensitivities.

The most interesting finding of this study was the callus formation of the *E. acoroides* hypocotyl. Callus formation was observed from the hypocotyl when cultured in the MS media supplemented with BA and NAA after 2 wk. This was the first report of callus formation of *E. acoroides* and demonstrated that the hypocotyl is capable of being the initial explant for callus induction. Sarmast et al. (2009) reported that the potential of different parts (apical, middle and bottom parts of the leaf) of snake plant leaf affect the production of meristemoids. Using the bottom parts of the leaf for callus formation was better than the apical parts. This may have been due to the different amounts of plant hormones in different parts of plant. This result demonstrated that callus formation is only obtainable at the base portion of the hypocotyl that contained numerous starch grains (Fig. 7), while the apical portion of the hypocotyl consisted of parenchyma cells and air lacunae (lack of starch grain) when the anatomy of hypocotyl was examined before and after callus induction. However, the current anatomical studies clearly indicated that callus formation may be caused by not only the hormones but also by the carbohydrates. Thorpe and Murashige (1970) suggested that although sucrose in the medium is

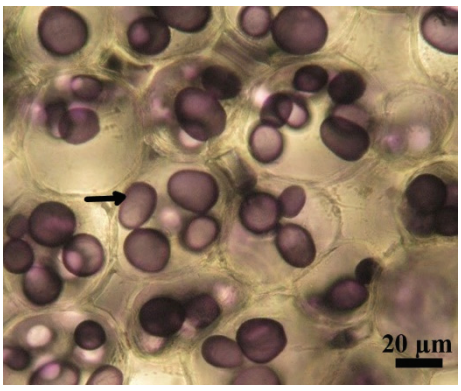

Fig. 7 Transverse section of parenchyma in base portion of *E. acoroides* hypocotyl showing starch grains (arrow)

the carbon source most frequently used for callus induction and plant regeneration, carbohydrates not only act as a source of carbon and energy, but also as an osmotic agent during organogenesis. The results obtained in the current study showed that the carbohydrates in plant tissue may play an important role in callus formation. In addition, auxin and cytokinin have been widely used to generate callus, but different species may require different media for callus induction (Smith, 2006). The current results showed that BA and NAA promoted callus induction, while TDZ and 2,4-D inhibited root formation. Suitable auxin and cytokinin concentrations vary depending upon plant organs and plant species. Accordingly, exogenous hormone is an important factor for regeneration in *E. acoroides*. Although the seeds of *E. acoroides* were currently the best explant for tissue culture establishment, the collection of seed was limited by the timing of sexual reproduction success. The successful surface sterilization of *E. acoroides* fruit was reported in the methods of the current study, which also suggested that seeds are the best explant for tissue culture establishment. The seed of *E. acoroides* in unopened fruit must be processed soon after collection to prevent fruit dehiscence. The establishment of axenic cultures of seagrass has proved difficult in previous study. All seagrass including *E. acoroides* submerge in water, all parts of the plant are naturally contaminated by microorganisms. As a result, explant sterilization is very difficult because there are fungi and bacteria living in the epidermal cells of vegetative organs (Kuo et al., 1981), especially in the rhizome and root. Successful axenic cultures of *E. acoroides* from vegetative organs have not been reported. Future research will be focused on the development of methods for the proliferation of seagrasses from all parts in both vegetative and reproductive organs. Therefore, development of a method for *in vitro* propagation is required for long-term restoration and conservation of seagrass meadows.

The current study demonstrated the successful induction of callus from the hypocotyl. The MS medium supplemented with 18 µM BA and 160 µM NAA was the best treatment for callus induction from the hypocotyl of *E. acoroides*.

Conflict of Interest

The authors declare that there are no conflicts of interest

Acknowledgements

This work was partially supported by the Center for Advanced Studies for Agriculture and Food, Institute for Advanced Studies, Kasetsart University, Bangkok, Thailand under the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Ministry of Education, Thailand (Center for Advanced Studies for Agriculture and Food, KU Institute for Advanced Studies (CASAF, NRU-KU, Thailand).

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