Effects of N^6-(2-Isopentenyl) Adenine (2iP) on the Growth of Tropical Seagrass *Enhalus acoroides* after Germination

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ABSTRACT

The influence of a cytokinin, N^6-(2-Isopentenyl) adenine (2iP), on growth of *Enhalus acoroides* was examined in controlled laboratory experiments. Seeds of *E. acoroides* were collected from Haad Chao Mai National Park, Trang Province, Southern Thailand, on the Andaman Sea side. The seeds were cultured in sterile MS liquid medium containing 25, 50, 75, and 100 µM 2iP in glass culture bottles. The cultures were stored under 25±2˚C in axenic conditions for 64 days. The results showed that 2iP inhibits the initiation of *E. acoroides* root. The highest average root length was 19.10±1.13 mm in controlled MS medium. The treatment with increasing 2iP concentration showed positive effects on hypocotyl and leaf developments. The highest average hypocotyl length, 1.60±0.00 cm, was found in seedlings from 50 µM 2iP medium. The hypocotyl diameter recorded in controlled MS medium was not significantly different from those in 50 and 100 µM 2iP media. The highest average leaf length was 5.36±0.67 cm in 100 µM 2iP medium. This culture knowledge is a step forward to the *in vitro* seagrass propagation and restoration studies.

Keywords: : *Enhalus acoroides*, N^6-(2-Isopentenyl) Adenine (2iP), seedling development, seagrass tissue culture

INTRODUCTION

Enhalus acoroides (L.f.) Royle is the biggest tropical seagrass species with distribution from the shallow water above lowest low water (LLW) to a depth of 4 m (Nakaoka and Supanwanid, 2000). Because of the large size and very long leaves, *E. acoroides* occupies the water column from the seafloor to 1-2 m above the LLW and plays a very important role in the marine ecosystem. Its leaves and detritus are a source of food for sea turtles, fish, waterfowl and marine animals, especially dugong (Adulyanukosol and Poovachiranon, 2003).

Human activities have a direct threat to seagrasses (Orth *et al*., 2006), for example, increase in turbidity caused by sediment from land erosion or coastal development (Spalding *et al*., 2003; Orth *et al*., 2006), and plastic pollution in the sea (Balestri *et al*., 2017). Seagrasses are also threatened by shallow-net trawling, using inappropriate fishing gear, boating and shipping activities (Short and Waycott, 2010). These threats have major impacts on seagrass meadows as many seagrass areas have been lost (Spalding *et al*., 2003; Grech *et al*., 2012). Furthermore, the sexual reproduction of the seagrass is less than 10% per year of reproduction (Hemminga

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and Duarte, 2000), this might limit the population growth of seagrasses. Consequently, a method of restoration and rehabilitation of seagrass 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; Thom, 1990; Fonseca *et al.,* 1988, 1998). Thereafter, the seagrass *Ruppia maritima* had been cultured in cytokinin sucrose base medium under axenic conditions (Koch and Durako, 1991). Tissue culture has shown to be successful in rapid cloning of plants. In addition, tissue culture is widely used to produce thousands of new plants and it is very important to germplasm conservation and establishes plantlet (Bird *et al*., 1994). However, little information exists on the presence of plant growth regulators (PGRs) in seagrasses and their effects (Balestri *et al*., 1998). The only seagrass species that have been successfully cultured are *R. maritima* L. (Bird *et al*., 1996), *Halophila engelmannii* (Jewett-Smith and McMillan, 1990; Koch and Durako, 1991), and *Halodule pinifolia* (Subhashini *et al*., 2014).

Previously, *E. acoroides* studies have focused on ecology, distribution (Lewmanomont *et al*., 1996), effect of shoot density on reproduction (Rattanachot 2008; Rattanachot *et al*., 2015), and effect of desiccation periods on seed development (Dagapioso and Uy, 2011). Knowledge of *in vitro* cultures of *E. acoroides* is rare. Therefore, the purpose of this study was to investigate seed germination performance and seedling development of *E. acoroides* on various concentrations of plant growth regulator, N^6-(2-Isopentenyl) adenine (2iP), *in vitro* culture environment. This work provides a basis for *in vitro* propagation of *E. acoroides* which will be vital for seagrass restoration efforts.

MATERIALS AND METHODS

Specimen collection

Seeds of *Enhalus acoroides* (L.f.) Royle were collected from Haad Chao Mai National Park, Trang Province, southern Thailand, on the Andaman Sea side.

Specimen sterilization

The prepared mature fruits were washed with 70% ethanol (v/v) and followed by surface sterilization with 1.50% sodium hypochlorite (NaOCl) for 15 minutes. The sterilized fruits were rinsed 3 times in sterilized artificial seawater under laminar airflow cabinet.

Medium and culture preparation

The seeds of *E. acoroides* were cultured in the MS (Murashige and Skoog, 1962) liquid medium as a controlled medium and in MS liquid medium supplemented with 25, 50, 75 and 100 µM N^6-(2-isopentenyl) adenine (2iP) as treatments. Each seed was placed at the bottom of the glass culture bottles (6 cm in diameter and 11 cm height) containing 20 ml of media. The pH of medium was adjusted to 6.5. The cultures were incubated under a photoperiod of 16 hours and 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity at 25±2˚C (Bhojwani and Razdon, 1996; Kaveeta, 1997) for 64 days. Completely Randomized Design (CRD) was used for the experiment with 10 replicates. Root, leaf, hypocotyl, and seedling wet weight in all treatments were measured and morphological changes were recorded at 2, 4, 8, 16, 32 and 64 days of culture.

Statistical analysis

Data were statistically analyzed using SPSS. One-way analysis of variance (ANOVA) was used to test the effect of 2iP on seedling growth and development. When the results were significantly different between treatments, a Tukey's post-hoc test was used for multiple comparisons of treatments at $P<0.05$.

RESULTS

One hundred percent of seed cultured in controlled medium and 2iP media can germinate at day four. There was no effect of 2iP on seed germination but it affected seedling growth and morphological development.

Root development

The mature seed has a radicle that forms the primary root. The primary root emerges from the area of the base of embryo to the base of hypocotyl. The primary root was observed at day eight in all treatments (Figure 1). After 16 days, the adventitious roots occurred from the area between hypocotyl and cotyledon (Figure 2). Seedlings grown in controlled media could produce the adventitious roots before those in 2iP media. After 32 days, seedlings in all treatments could produce at least one adventitious root (Figure 1). At the end of experiment, root number was a marked different in controlled medium compared to 2iP media. The highest average number of root per plant is 3.20± 0.20 in controlled medium (Table 1). Root number tended to decrease in higher 2iP concentration treatments. The highest average root length of 19.10±1.13 mm was obtained in controlled medium. However, there was no significant difference with those in 75 and 100 µM 2iP media (Table 1).

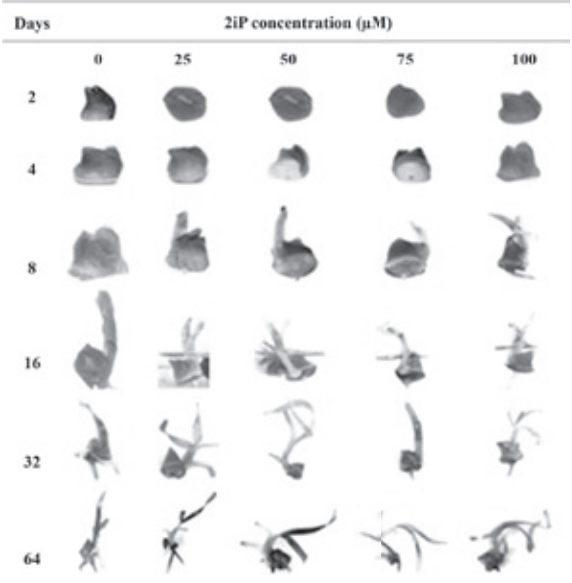

Figure 1. Seedlings of *E. acoroides* in controlled medium and 2iP media showing primary root emerging from the area of the base portion of hypocotyl at day eight, and adventitious root after 32 days. The leaves of seedling in all treatments were produced from plumule between cotyledon and hypocotyl. All seedlings showed ribbon like leaves with round apex when leaves elongated after 16 days.

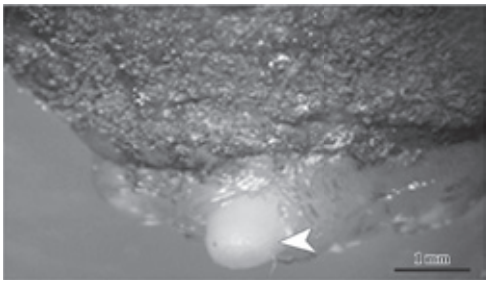

Figure 2. Surface view of hypocotyl and root tip of seedling in controlled medium at day 16th of culture, showing first adventitious root (arrow) that emerged between hypocotyl and cotyledon

Table 1. Number and length of *E. acoroides* root cultured in 2iP media.

2iP concentration	Root number			Root length (mm)		
	Min.	Max.	mean±S.E.	Min.	Max.	mean±S.E.
0	2.00	4.00	3.20 ± 0.20^{a}	15.00	27.00	19.10 ± 1.13^{a}
25	1.00	2.00	1.10 ± 0.10^{b}	0.10	13.00	4.52 ± 1.37^{b}
50	1.00	1.00	1.00 ± 0.00^{b}	6.00	21.00	7.85 ± 0.73^{b}
75	1.00	1.00	1.00 ± 0.00^{b}	2.00	21.00	14.60 ± 2.65^{a}
100	1.00	2.00	1.20 ± 0.16^{b}	9.00	21.00	18.10 ± 1.45^{a}

Remark: Similar superscripts within columns were not significantly different (P>0.05) from each other according to Tukey's post-hoc test at 5% probability level

Hypocotyl development

Hypocotyl of *Enhalus acoroides* can be produced before detaching from the fruit, which is called viviparous. The hypocotyl is dome shaped and fused with cotyledon and embryo. The hypocotyl has a provascular bundle, which extends from embryo to the base portion and its radicle (Figure 3). The seedling germinates and grows by using a nutrient source from the hypocotyl. The hypocotyl length and diameter at the initial day of experimentis 1.01±0.09 and 1.10±0.11 cm, respectively. At the end of experiment, there was a marked difference in the 50 µM 2iP medium compared to all other media. The highest average hypocotyl length, 1.60±0.00 cm, was found in seedling from MS medium supplemented with 50 µM 2iP. The hypocotyl diameter recorded in controlled medium (1.38±0.05 cm) was not significantly different from 50 and 100 µM 2iP media, as determined by one-way ANOVA (Table 2).

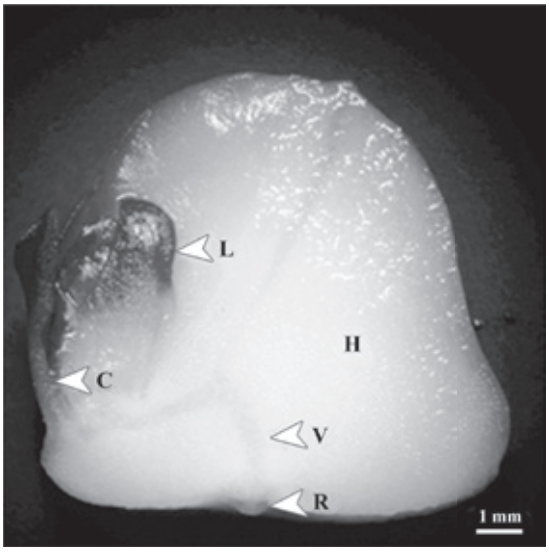

Figure 3. Transverse section through the seedling of *E. acoroides* showing a provascular bundle (V), hypocotyl (H), radicle (R), leaf (L), and cotyledon (C)

Table 2. Size of *E. acoroides* hypocotyl cultured in 2iP media

2iP concentration	Hypocotyl length (cm)			Hypocotyls diameter (cm)		
	Min.	Max.	mean±S.E.	Min.	Max.	mean±S.E.
0	0.90	1.20	1.07±0.04^{d}	1.20	1.70	1.38±0.05^{a}
25	1.10	1.40	1.31±0.04^{c}	0.90	1.20	1.11±0.00^{b}
50	1.60	1.60	1.60±0.00^{a}	1.30	1.30	1.30±0.00^{a}
75	1.20	1.60	1.45±0.06^{b}	1.10	1.20	1.17±0.00^{b}
100	1.20	1.50	1.47±0.03^{b}	1.10	1.30	1.25±0.00^{a}

Remark: Similar superscripts within columns were not significantly different (P>0.05) from each other according to Tukey's post-hoc test at 5% probability level

Leaf development

Leaf of *E. acoroides* is simple leaf without ligule. Leaf is ribbon like with round apex and entire margin. Leaf arrangement is alternate. Venation is palmately parallel. The leaf is produced from plumule between cotyledon and hypocotyl (Figure 3).

The result showed that leaves of seedlings were developed within day eight in all treatments (Figure 1). The highest average leaf number was 5.20±0.13, found in controlled medium. However, the leaf numbers of seedling at the end of experiment in controlled medium and all 2iP media were not significantly different. Leaf length and width increased when the concentration of 2iP increased. The highest average leaf length was 5.36±0.67 cm in 100 µM 2iP medium. There was a marked difference (P<0.05) in 100 µM 2iP medium compared to control medium (Table 3). The highest average leaf width was 0.64±0.13 cm in 75 µM 2iP medium. There was no statistical differences among 75, 100 µM 2iP media and control (Table 3). The result showed that cytokinin 2iP could promote leaf development especially leaf elongation of seedling.

Seedling weight

The seedlings have a distinct leaf primordium which is protected by cotyledon and enlarged hypocotyl. At day eight, all seedlings had leaves which were longer than their cotyledon. The leaf tended to continuously increase from day eight to the end of experiment. At the end of experiment, the weight of above ground part, shoot and leaf, was highest in 100 µM 2iP medium (0.29±0.02 g). The above ground weight of seedling

Table 3. Number and size of *E. acoroides* leaf cultured in 2iP media.

2iP concentration	Leaf number			Leaf length (cm)			Leaf width (cm)		
	Min.	Max.	mean±S.E.	Min.	Max.	mean±S.E.	Min.	Max.	mean±S.E.
0	5.00	6.00	5.20±0.13^{a}	3.10	6.30	4.31±0.29^{b}	0.40	0.60	0.49±0.02^{ab}
25	2.00	6.00	4.40±0.47^{a}	0.70	9.50	4.29±1.16^{b}	0.30	0.50	0.37±0.02^{b}
50	3.00	6.00	4.80±0.38^{a}	3.10	6.10	4.59±0.35^{b}	0.40	0.50	0.42±0.01^{b}
75	2.00	6.00	4.90±0.48^{a}	1.60	6.00	5.15±0.45^{a}	0.30	1.40	0.64±0.13^{a}
100	2.00	6.00	4.80±0.53^{a}	1.10	7.60	5.36±0.67^{a}	0.30	0.50	0.52±0.02^{a}

Remark: Similar superscripts within columns were not significantly different (P>0.05) from each other according to Tukey's post-hoc test at 5% probability level

cultured in 100 µM 2iP medium was significantly different ($P<0.05$) from all other media, as determined by one-way ANOVA (Table 4). The above ground weight tended to increase when the concentration of 2iP increased. The underground average weight including root and hypocotyls was highest at 1.01±0.04 g in controlled medium. However, the underground weight of seedlings grew in controlled medium and all 2iP treatments were not significantly different.

Table 4. Seedling weight of *E. acoroides* in 2iP media

	Above ground weight (g) (shoot and leaf)			Below ground weight (g) (root and hypocotyl)		
2iP concentration	**Min.**	**Max.**	**mean±S.E.**	**Min.**	**Max.**	**mean±S.E.**
0	0.12	0.31	0.19±0.02^{b}	0.90	1.24	1.01±0.04^{a}
25	0.00	0.38	0.13±0.04^{b}	0.59	1.09	0.79±0.05^{a}
50	0.11	0.27	0.19±0.02^{b}	0.63	0.86	0.79±0.03^{a}
75	0.03	0.23	0.17±0.02^{b}	0.72	1.04	0.86±0.04^{a}
100	0.02	0.35	0.29±0.02^{a}	0.78	1.00	0.85±0.03^{a}

Remark: Similar superscripts within columns were not significantly different ($P>0.05$) from each other according to Tukey's post-hoc test at 5% probability level

DISCUSSION

The study of growth of *Enhalus acoroides* seeds showed that plant growth regulator 2iP had no effect on seed germination. This indicates that seed of *E. acoroides* could germinate under different medium conditions. Moreover, *E. acoroides* produces viviparous seeds that store nutrients in the seeds and they could immediately germinate just after detachment from the parental plant (Balestri *et al*., 1998). This adaptation could promote seed dispersal and distribution within the seagrass meadow environment. However, the ability of seed development depends on various environmental conditions.

N^6-(2-isopentenyl) adenine (2iP) affects root, hypocotyl and leaf developments of *E. acoroides*. The plant growth regulator 2iP is a naturally occurring cytokinin that is a precursor of the cytokinin Zeatin. In natural plants, the cytokinin is mainly synthesized in roots and translocated to shoots and leaves via the xylem flow. 2iP has been used in tissue cultures to support *in vitro* propagation such as stimulating callus differentiation, promoting axillary bud and stimulating cell division (Einset, 1986; Zhang *et al*., 2014). It can inhibit root formation and root number but promote root length. Root length tended to increase when the concentration of 2iP increased (Table 1). In addition, the plant growth regulator, 2iP, also stimulate hypocotyl and leaf length. Similar results were also reported on *Ruppia maritima* (Kock and Durako, 1991), *R. megacarpa*, *Halophila ovalis* (Henry, 1998), *Sophora tonkinensis* (Jana *et al*., 2013), and bilberry (Jaakola *et al*., 2001). The previous studies showed that increasing 2iP concentration promoted nodal production, growth, and axillary shoots. In addition, 2iP could promote cell division in plants (Skoog and Miller, 1957) and induce elongation of shoots in *Cymbopogon nardus* (Chan *et al*., 2005). However, Kock and Durako (1991) suggested that a positive effect of 2iP depends on its concentration. Suitable 2iP concentration also varies depending on plant species. From this study, the results showed that 75 to 100 µM 2iP were the optimal concentrations for growth and elongation of *E. acoroides* seedlings.

Root length increasing is important for both *in vitro* and *ex vitro* propagation. The primary functions of root are absorption of water and dissolved nutrients necessary for seed development

(Robert *et al*., 2015). In addition, leaf of explant is also important for surviving *in vitro* culture environment that has limited nutrient sources and gas. Therefore, increasing leaf length can increase photosynthesis process. From this study, there was a correlation between hypocotyl length and leaf length ($P<0.05$, $R^2=0.5299$, n=240) (Figure 4). This analysis may indicate that increasing hypocotyl size is related to nutrient storage in seedlings which could cause leaf to lengthen. Kuo and den Hartog (2006) reported that the hypocotyl of *E. acoroides* acted as nutrient storage source for seed development. An abundance of starch grains was observed in the hypocotyl of some seagrasses species including *E. acoroides*. This is an important adaptation of *E. acoroides* seedlings, which is crucial for seedling growth in the field or seagrass meadows.

Vichkovitten *et al*. (2016) reported that *E. acoroides* transplantation success in Thai coastal waters depends on many physical and biological factors including low temperature, and low sulfide in sediments. In addition, the critical period of transplants was within the first three months when plants needed to acclimatize to their new environments. Therefore, soundness size of seedling has a great influence on seagrass growth, where larger seedlings could produce larger plants than smaller ones.

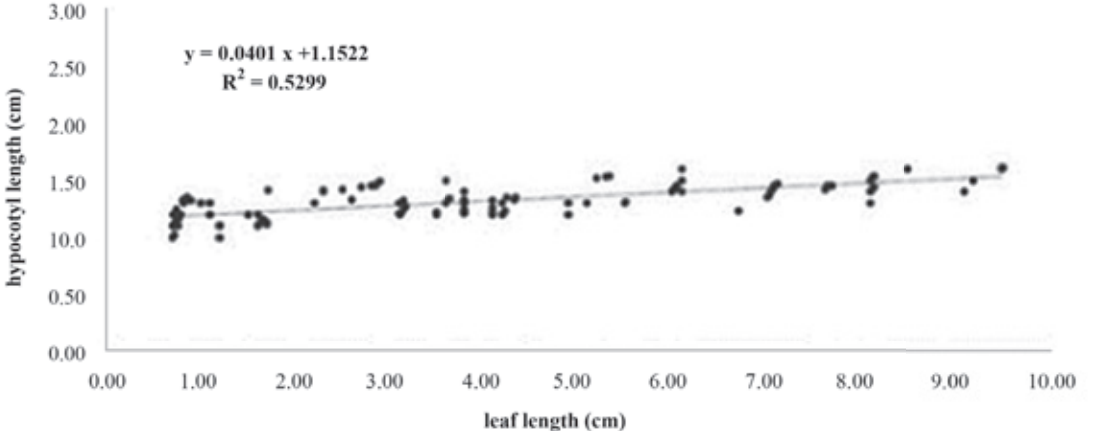

Figure 4. A correlation between hypocotyl length and leaf length of *E. acoroides* seedlings in all treatments

CONCLUSION

N^6-(2-isopentenyl) adenine (2iP) showed a negative effect on *E. acoroides* root initiation. However, the positive effects showed in *E. acoroides* hypocotyl and leaf development. The optimal concentrations of 2iP in cultured media range from 75 to 100 µM. These concentrations could stimulate the growth of hypocotyl that is important nutrient storage for seedling. The optimal 2iP concentrations can promote leaf elongation and is suitable for *E. acoroides* seedling culture during preparation period prior transplanting.

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LITERATURE CITED

Adulyanukosol, K. and S. Poovachiranon. 2003. **A pictorial key to general species for identification of seagrass cells in stomach contents of dugong from the Andaman Sea, Thailand.** Phuket Marine Biological Center, Thailand. 16 p.

Balestri, E., L. Piazzi and F. Cinelli. 1998. *In vitro* germination and seedling development of *Posidonia oceanica*. **Aquatic Botany** 60: 83-93.

Balestri, E., V. Menicagli, F. Vallerini and C. Lardicci. 2017. Biodegradable plastic bags on the seafloor: A future threat for seagrass meadows. **Science of the Total Environment** 606: 755-763.

Bhojwani, S.S. and M.K. Razdan. 1996. **Plant Tissue Culture: Theory and Practice, a revised edition.** Elsevier, Amsterdam, the Natherlands. 767 p.

Bird, K.T., J. Jewett-Smith and M.S. Fonseca. 1994. Use of *in vitro* propagated *Ruppia maritima* for seagrass meadow restoration. **The Journal of Coastal Research** 10: 732-737.

Bird, K.T., M.S. Brown, T.T. Henderson, C.E. O´Hara and J.M. Robbie. 1996. Culture studies of *Ruppia maritima* L. in bicarbonate and sucrose-based media. **Journal of Experimental Marine Biology and Ecology** 199: 153-164.

Chan, L.K., P.R. Dewi and P.L. Boey. 2005. Effect of plant growth regulators on regeneration of plantlets from bud cultures of *Cymbopogon nardus* L. and the detection of essential oils from the *in vitro* plantlets. **Journal of Plant Biology** 48(1): 142-146.

Dagapioso, D.M. and W.H. Uy. 2011. Seed germination and seedling development of the seagrass *Enhalus acoroides* (L.f.) Royle *in vitro*: effect of burial depth and desiccation period. **Journal of Environmental Aquatic Resource** 2: 34-46.

Einset, J.W. 1986. Zeatin biosynthesis from N^6-(Δ^2-isopentenyl) adenine in Actinidia and other woody plants. **Proceedings of the National Academy of Sciences of the United States of America** 83: 972-975

Fonseca, MS., WJ. Kenworthy, GW. Thayer. 1988. Restoration and Management of Seagrass System: A Review. **The Ecology and Management of Wetlands** 2: 353-368.

Fonseca, MS., WJ. Kenworthy, GW. Thayer. 1998. **Guidelines for the Conservation and Restoration of Seagrasses in the United States and Adjacent Waters.** National Oceanic and Atmospheric Administration, Maryland. 222 p.

Grech, A., K. Chartrand-Miller, P. Erftemeijer, M. Fonseca, L. McKenzie, M. Rasheed, H. Taylor and R. Coles. 2012. A comparison of threats, vulnerabilities and management approaches in global seagrass bioregions. **Environmental Research Letters** 7(2012), DOI:10.1088/1748-9326/7/2/024006.

Hemminga, M.A. and C.M. Duarte. 2000. **Seagrass Ecology: An Introduction.** Cambridge University Press, Cambridge, 298 p.

Henry, M.G. 1998. **The *in vitro* propagation of seagrasses: *Halophila ovalis, Ruppia magacarpa* and *Posidonea coriacea*.** Edith Cowan University, Australia. 87 p.

Jaakola, L., A. Tolvanen, K. Laine and A. Hohtola. 2001. Effect of N^6–isopentenyl adenine concentration on growth initiation *in vitro* and rooting of bilberry and lingonberry microshoots. **Plant cell, Tissue and Organ Culture** 66: 73-77.

Jana, S., I. Sivanesan and B.R. Jeong. 2013. Effect of cytokinins on *in vitro* multiplication of *Sophora tonkinensis*. **Asian Pacific Journal of Tropical Biomedicine** 3: 549-553.

Jewett-Smith, J. and C. McMillan. 1990. Germination and seedling development of *Halophila engelmannii* Aschers (Hydrocharitaceae) under axenic conditions. **Aquatic Botany** 36: 167-177.

Kaveeta, R. 1997. **Plant Tissue Culture: Theory and Practice.** Kasetsart University. Bangkok. 219 p.

Koch, E.W. and M.J. Durako. 1991. *In vitro* studies of the submerged angiosperm *Ruppia maritima*: auxin and cytokinin effects on plant growth and development. **Marine Biology** 110: 1-6.

Kuo, J. and C. den Hartog. 2006. Seagrass Morphology, Anatomy, and Ultrastructure. *In*: **Seagrasses: Biology, Ecology and Conservation.** (ed. A.W.D. Larkum, R.J. Orth and C.M. Duarte), pp. 51-87. University of Sydney, Australia.

Lewis, RR. 1987. The restoration and creation of seagrass meadows in the southern United States. *In*: **Subtropical Searasses of the Southern United States** (ed. M.J. Durako), pp. 153-173. Department of Natural Resources Publication, Florida.

Lewmanomont K., S. Deetae, and V. Srimanobhas. 1996. Seagrass of Thailand. **Proceedings international workshop, Rottnest Island, Western Australia:** 21-26.

Murashige T. and F. Skoog. 1962. A revised medium for rapid growth bioassays with tobacco tissue cultures. **Physiologia Plantarum** 15: 473-497.

Nakaoka M. and C. Supanwanid. 2000. Quantitative estimation of the distribution and biomass of seagrasses at Haad Chao Mai National Park, Trang Province, Thailand. **Fisheries Research Bulletin** 22: 10-22.

Orth, R.J., T.J.B. Carruthers, W.C. Dennison, C.M. Duarte, J.W. Fourqurean, K.L. Heck JR., A.R. Hughes, G.A. Kendrick, W.J. Kenworthy, S. Olyarnik, F.T. Short, M. Waycott, and S.L. Williams. 2006. A global crisis for seagrass ecosystems. **Journal of Biosciences.** 56: 987-996.

Phillips, RC. 1980. **Plant and Propagation Guidelines and Techniques for Seagrasses of the United States and its Territories.** Seattle, WA. CERC Contract No. DAWC 72-79C-0030.

Phillips, RC. 1982. Seagrass Meadows. *In*: **Creation and restoration of Coastal Plant Families** (ed. RR. Lewis), pp. 173-202. CRC Press, Boca Raton, Florida.

Rattanachot, E. 2008. **Effect of Shoot Density on Growth, Recruitment and Reproduction of *Enhalus acoroides* (L.f.) Royle at Haad Chao Mai National Park, Trang Province, Thailand.** Prince of Songkla University, Songkla. 85 p.

Rattanachot, E., F.T. Short and A. Prathep. 2015. *Enhalus acoroides* responses to experimental shoot density reductions in Haad Chao Mai National Park, Trang Province, Thailand. **Marine ecology** 37: 411-418.

Robert, E.M.R., J. Oste, T.V. der Stocken, D.J.R. De Ryck, K. Quisthoudt, J.G. Kairo, F. Dahdouh-Guebas, N. Koedam and N. Schmitz. 2015. Viviparous mangrove propagules of *Ceriops tagal* and *Rhizophora mucronata*, where both Rhizophoraceae show different dispersal and establishment strategies. **Journal of Experimental Marine Biology and Ecology** 468: 45-54.

Short, F.T. and M. Waycott. 2010. ***Enhalus acoroides*. The IUCN Red List of Threatened Species 2010: e.T173331A 6992567.** http://dx.doi.org/10.2305/IUCN.UK.2010-3.RLTS.T173331A6992567.en.pdf (accessed on 27 April 2017).

Short, F.T. and R.G. Coles. 2001. **Global Seagrass Research Methods.** Elsevier Science B.V., London, UK. 473 p.

Skoog, F., and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. **Symposia of the Society for Experimental Biology** 11: 118–131.

Spalding, M., M. Taylor, C. Ravilious, F. Short and E. Green. 2003. Global Overview: The Distribution and Status of Seagrasses. *In*: **World Atlas of Seagrasses** (ed. E.P. Green and F. T. Short), pp. 5-26. University of California Press, Berkeley.

Subhashini, P., S. Raja and T. Thangaradjou. 2014. Establishment of cell suspension culture protocol for a seagrass (*Halodule pinifolia*): Growth kinetics and histomorphological characterization. **Aquatic Botany** 117: 33-40.

Thom, RM. 1990. A review of eelgrass (*Zostera marina* L.) transplanting projects in the Pacific Northwest. **Northwest Environment Journal** 6: 121-137.

Thorhaug, A. 1986. Review of seagrass restoration efforts. **Ambio** 15(2): 110-117.

Vichkovitten, T., A. Intarachart, K. Khaodon, and S. Rermdumri. 2016. Transplantation of Tropical Seagrass *Enhalus acoroides* (L.f.) in Thai Coastal Water: Implication for Habitat Restoration. **Greater Mekong Subregion Academic and Research Network International Journal** 10: 113-120.

Zhang, K., W.O. Novak, Z. Wei, M. Gou, X. Zhang, Y. Yu, H. Yang, Y. Cai, M. Strnad and C. Jun Liu. 2014. Arabidopsis ABCG14 protein controls the acropetal translocation of root-synthesized cytokinins. **Nature communications** 10: 1-12.