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Research Articles

Development of axenic seedlings of the seagrass, *Enhalus acoroides* (L.f.) Royle

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Abstract

Axenic culture of the seagrass, *Enhalus acoroides* (L.f.) Royle was successfully established using seeds which were aseptically dissected out from the fruits. The seedlings were raised in four culture media devoid of growth hormones. Axenicity of the seedlings was analysed by adopting standard marine bacterial / fungal isolation procedures and also by scanning electron microscopy. Of the four media used, medium 1 (10 % filter sterilized coconut water + 1 % charcoal + 15 g agar + filter sterilized UV treated seawater) promoted comparatively good growth of both leaf and root. About 90 % of the seedlings raised in the laboratory in media 1 and 3, (5 % bentonite clay + 15 g agar + filter sterilized UV treated water) grew upto the fourth leaf stage. Hence, medium 1 is recommended for raising axenic cultures of the seagrass, *E. acoroides*.

Keywords : aquatic plants, axenic culture, *Enhalus,* media, seagrasses

INTRODUCTION

In vitro culture and other micropropagation methods of higher plants are powerful tools in plant conservation. However, these techniques are being applied to submerged aquatic angiosperms only recently. In fact, only at the First International Seagrass Workshop conducted in Leiden, the Netherlands, in 1973, axenic culture of seagrasses was recommended as an area of new research (McRoy, 1973). Though it would be difficult to obtain axenic cultures of submerged macrophytes (Ailstock, 1986), it is highly essential to develop tissue cultures of seagrasses.

Muffler and Durako (1984) successfully developed axenic cultures of *Thalassia testudinum* by adopting a two step sterilization technique. Since then, many works were carried out on axenic seagrass cultures and only in a very few studies, successful results were obtained. For e.g. Jewett-Smith and McMillan (1990) developed *Halophila engelmannii,* Loques *et al.* (1990) raised *Posidonia oceanica,* Ellender (1991) raised callus from rhizome explants of *Halodule wrightii,* Koch and Durako (1991) developed axenic *Ruppia maritima,* Balestri *et al.* (1998) raised *Posidonia oceanica* and Bird *et al.* (1998) developed axenic *Halophila decipiens*. However, they were mostly on the temperate seagrass species.

We attempted to develop axenic culture of the tropical seagrass, *Enhalus acoroides* (L.f.) Royle, which is very important in terms of biomass production in the Gulf of Mannar Marine Biosphere Reserve, India. We have also focused attention on raising seagrass cultures without adding plant growth regulators in order to reduce the cost of culture.

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MATERIALS AND METHODS

Axenic culture of *E. acoroides* was made following the culture method given by Muffler and Durako (1984) using the media suggested by Jewett Smith and McMillan (1990) with some modifications. Four different culture media were tried for the present experiment, to develop the axenic culture of *E. acoroides*.

Media composition

Medium 1: 10 % filter sterilized coconut water + 1% charcoal + 15 g agar + filter sterilized UV treated seawater

Medium 2: 1 % charcoal + 15 g agar + filter sterilized UV treated seawater

Medium 3: 5 % bentonite clay + 15 g agar + filter sterilized UV treated seawater

Medium 4: 10 % filter sterilized coconut water + 15 g agar + filter sterilized UV treated seawater

In all, the media were prepared using the filtered natural seawater, having a salinity of 32 ‰ and a pH of 7.8. Then, the sterilized medium was poured into individual, sterile 75 ml culture tubes and 500 ml conical flasks.

Sterilization of seeds

Mature fruits of *E. acoroides* were collected from the Gulf of Mannar during low tide regimes and the fruits were disinfected with 0.1 % mercury chloride solution for 2-3 minutes and washed two to three times using filtered natural seawater. The disinfected fruits were then transported to the laboratory by keeping them in between ice bags.

In the laboratory, the fruits were dissected out by keeping them inside a laminar flow and the seeds were removed. These seeds were treated with 5 % sodium hypochlorite for 10 minutes and washed with filtered, sterilized seawater thrice. Again, these seeds were treated with 70 % ethanol for 10 seconds followed by washing them three times using filtered seawater. Then, all the seeds were kept in sterile seawater with 200 units / ml of penicillin plus streptomycin for 10 minutes, washed again and the antibiotics treated seeds were used for inoculation. Sterilized seeds were transferred to the test tubes and 500 ml conical flasks containing the medium, which were filled with filtered and UV treated seawater with a salinity of 32 ‰ and a pH of 7.8. Cultures were kept in front of fluorescent lamps (2000 lux), with a 10: 14 hour light: dark photoperiod at a temperature of 27 +1°C and maintained in a clean culture room.

Cultures were tested for axenicity after 40th and 60th days of culture. The possible marine bacterial contaminants were checked by standard inoculation and incubation in peptone yeast extract marine medium and glycerine marine medium. Fungal contaminants were tested by using the Potato Dextrose Agar medium. In addition, Scanning Electron Microscopic (SEM) investigations were also carried out to detect surface microbial colonies.

Measurement of growth

Growth of the axenically raised *E. acoroides* seedlings was recorded by measuring total leaf area, green leaf area and root length. Growth parameters were recorded at an interval of 15 days and monitoring was done up to 90th day of the culture. The total leaf area which is related to productivity (Hent, 1978) was measured using a mm scale. Measurement was taken behind or alongside the culture tubes / flasks. Since the *E. acoroides* leaves are linear and simple, leaf length and breadth measurements could be obtained easily to calculate the total area of the leaf. After the 30th day of culture, senescence of some leaves was noticed in all the tubes. Hence, after the 30th day, both mean total leaf area (MTLA-includes both senescent and green leaves) and mean green leaf area (MGLA-includes only green leaves) were measured. Root length was measured after taking out the seedlings from the culture tubes / flasks. Photographs were also taken to ascertain the growth of leaves and roots.

In order to determine the differences in growth rate (leaf area) in different media, the growth was statistically analyzed. Differences in media was determined by Duncan's multiple range test ($P<0.05$) using SPSS / MS Windows package.

RESULTS

Growth of *E. acoroides* (leaf area) in different media are given in table 1 and variations in mean total leaf area and mean green leaf area and root length recorded in *E. acoroides* seedlings grown in different media (1, 2, 3 & 4) are shown in Figures 1 to 5 and Plate 1. Seedlings raised in medium 1 showed more mean total leaf area (970 mm^2) than the seedlings grown in other media. As stated earlier, from the 30th day onwards in all the test tubes, yellowing of leaves was noted followed by leaf falling. Hence, from the 45th day, the mean total leaf area and green leaf area were measured. Again, only in medium 1, maximum green leaf area was noticed. Medium 1 also promoted root growth to the extent of 360 mm but the root growth was greater in culture medium 2 (385 mm). Medium 2 showed good root growth (385 mm) as compared to all the other three media. However, the mean total leaf area of the seedlings grown in medium 2 (620 mm^2) was the lowest of the 4 media types. In medium 3, seedlings registered good leaf growth (710 mm^2) but the root promotion in this medium was very poor (40 mm). Mean total leaf area noted was next only to that of culture medium 1. Culture medium 4 was equally good in promoting leaf growth as that of medium 3. But the root growth was not well pronounced in this medium and in some seedlings, there was no root initiation. The maximum mean total leaf area recorded was 690 mm^2 on the 90th day of culture.

Aseptically inoculated seeds showed 90-95 % germination in all the media tested. About 90 % of the seedlings raised in the laboratory in two media *viz.*, culture media 1 and 3, grew up to the sixth (including senescent leaf) leaf stage. When the seedlings raised in all the four culture media were tested for microbial contamination on the 40th and 60th days of development, nearly 30% of the seedlings showed visible contaminants. Tubes, which did not show any visible microbial colonies, were tested for axenicity.

Inoculation and incubation of the leaf lamina of seedlings (randomly selected) of all 4 culture media were done in Peptone Yeast Extract Marine Medium and Glycerine Marine Medium for bacteria. At the same time, leaf laminas were also inoculated in Potato Dextrose Agar Medium for testing the presence of fungal contaminants. However, no bacterial and fungal contaminants were noticed even after 72 hours of incubation.

Further, SEM photographs were taken to check the presence of surface bacterial colonies on the leaf lamina taken from 60-day-old cultures. There were no microbial colonies on the leaf lamina as evidenced by the SEM pictures (Plate 2). All these tests proved that the seagrass cultures established during the present study were axenic in nature.

DISCUSSION

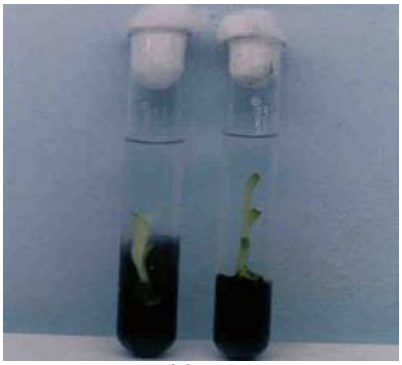
Kuo *et al.* (1981), Smith and Hayasaka (1982) and Schmidt and Hayasaka (1985) are of the opinion that the standard sterilization procedures commonly used for terrestrial plant materials are insufficient for obtaining the

Plate 1. Variations in the growth of *E. acoroides* seedlings grown in different media (see "Materials and Methods" section for description of media)

(a)

(b)

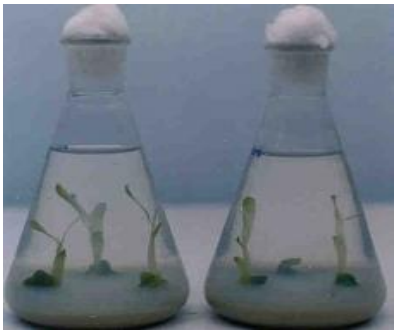
Medium 1



(a)

(b)

Medium 2



(a)

(b)

Medium 3

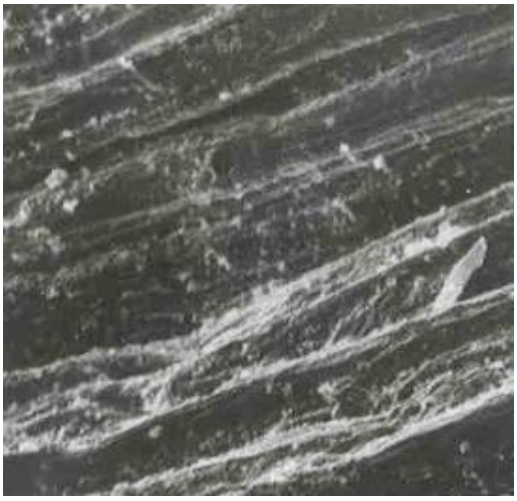
(a)

(b)

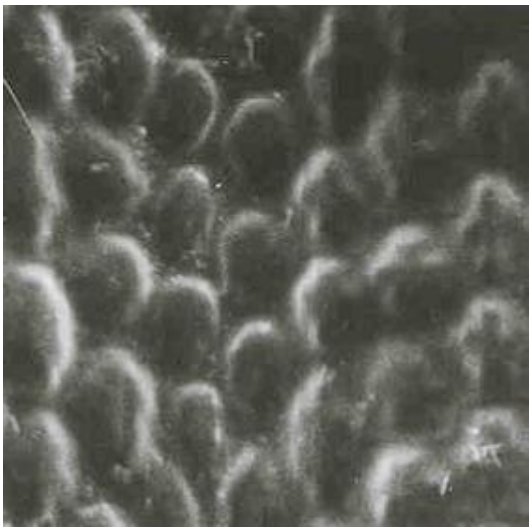
Medium 4

Plate 2. SEM photograph of axenically cultured *E. acoroides* seedlings of a. seed surface (300 X), b. root surface (500 X) and c. leaf surface (1000 X)

(a)



(b)



(c)

Table 1. Growth of *E. acoroides* (leaf area) in different media (values expressed in mm^2 are mean ± *SD*)

Medium	Days of culture (growth in mm^2)						
	0	15	30	45	60	75	90
1	45[a] +2.44	270[a] +11.22	580[a] +8.64	660[a] +1.63	720[a] +4.96	860[a] +8.48	970[a] +11.52
2	40[b] +2.16	220[c] +7.07	480[c] +8.52	500[c] +15.57	540[c] +3.55	590[c] +3.26	620[c] +12.08
3	45[a] +0	250[a] +1.22	520[b] +4.96	540[b] +5.09	580[b] +5.71	660[b] +5.88	710[b] +4.96
4	40[b] +2.16	260[a] +7.25	530[b] +4.89	550[b] +0.81	575[b] +9.27	670[b] +11.51	690[b] +4.08

Means with different superscripts are statistically different, $P < 0.05$; Duncan's multiple range test

Figure 1. Variations in mean total leaf area (MTLA) and mean green leaf area (MGLA) in *E. acoroides* seedlings grown in medium 1 (See "Materials and Methods" section for description of media)

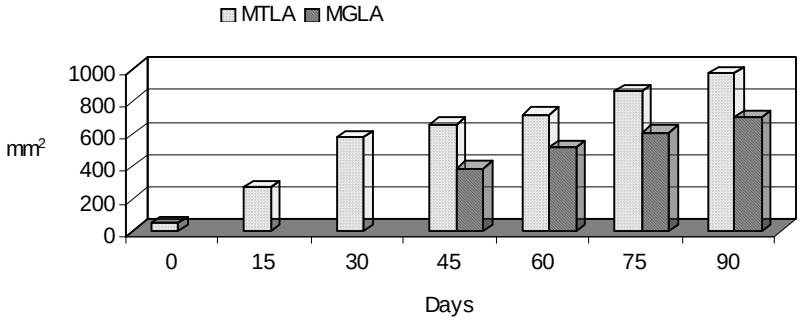


Figure 2. Variations in mean total leaf area (MTLA) and mean green leaf area (MGLA) in *E. acoroides* seedlings grown in medium 2 (See "Materials and Methods" section for description of media)

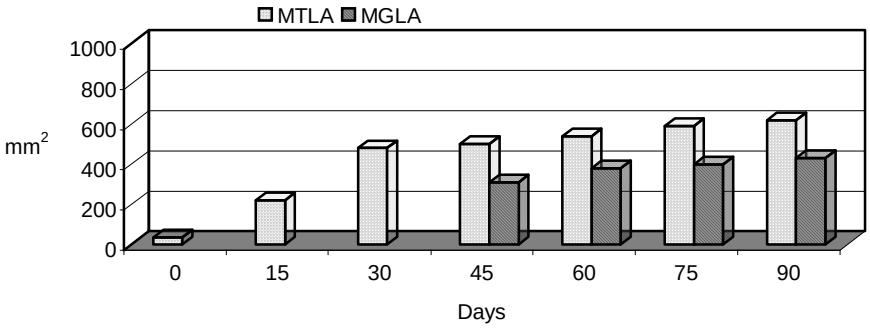


Figure 3. Variations in mean total leaf area (MTLA) and mean green leaf area (MGLA) in *E. acoroides* seedlings grown in medium 3 (See "Materials and Methods" section for description of media)

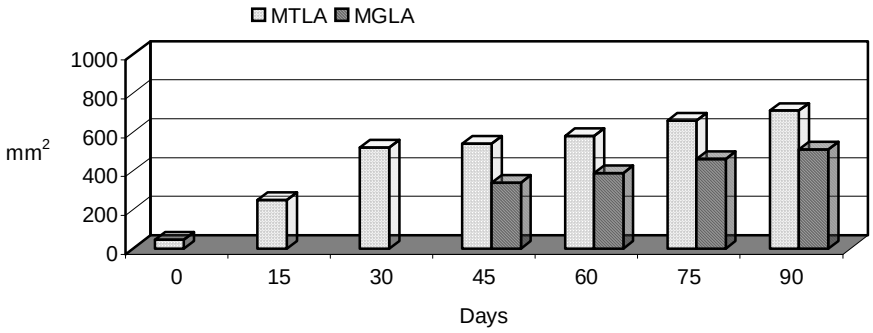


Figure 4. Variations in mean total leaf area (MTLA) and mean green leaf area (MGLA) in *E. acoroides* seedlings grown in medium 4 (See "Materials and Methods" section for description of media)

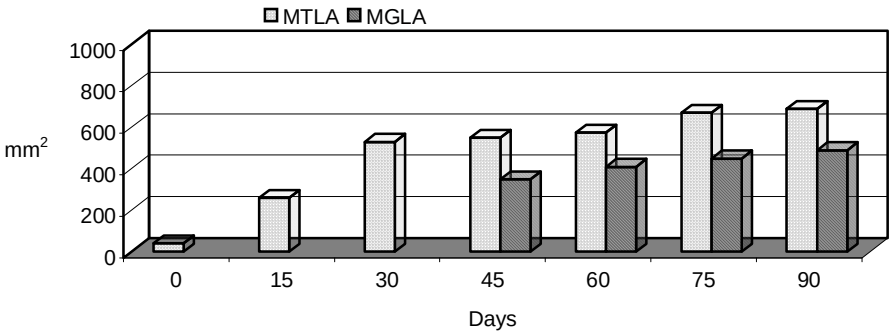
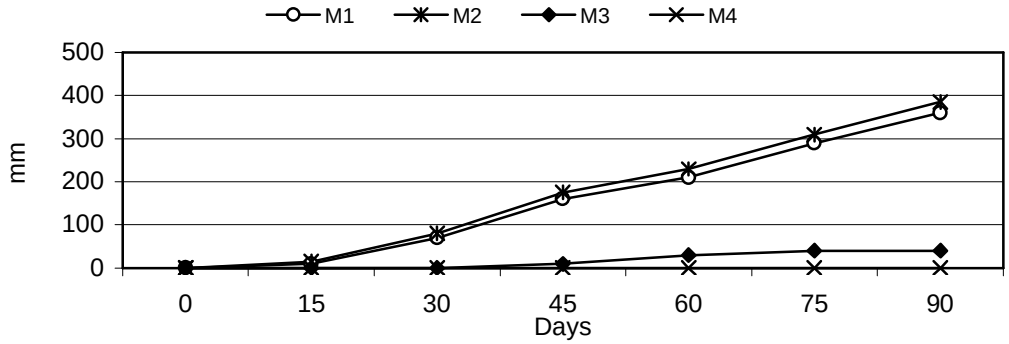


Figure 5. Variations in root length of *E. acoroides* seedlings grown in different media (m1 to m4). See "Materials and Methods" section for description of media.



seagrass, *Ruppia maritima* cultures and this might hold good for other seagrasses as well. In the present investigation, even after several sterilization steps, nearly 30 % of the culture tubes or conical flasks showed visible contaminations. However, this is a better result when compared to that of Koch and Durako (1991) who noticed about 49 % of contamination and Bird *et al.* (1996) who observed more than 50 % contamination. Earlier, Newell and Fell (1982) reported that the aerophilic fungi and slow growing endophytic bacteria are the causative organisms for such contaminations.

Results of the present study, clearly indicated a significant difference in the growth of *E. acoroides* (leaf area) (P<0.05; Duncans multiple range tests) between different media tested (Table 1). Especially medium 1 promoted good leaf growth but the root promotion in this medium was not up to that of the medium 2. However, the overall growth rate was comparably good in medium 1.

Leaf growth was more in media where filter sterilized coconut water was used *i.e.,* medium 1 falls in one group along with the media 4 and 3 in promoting leaf growth and the medium 2 falls in another group in promoting root growth. The good root promotion noticed in medium 2 was probably due to the presence of 1% charcoal in that. Bird and Jewett-Smith (1994) also opined that the promotion of root growth was more in *H. engelmanii* when the charcoal composition was increased from 0.5 to 1% in the medium. Durako (1988) explained that the better root growth seen in these kinds of rooting substrates could be due to their better ion-exchange capacity with the particulate matrix thus allowing increased nutrient uptake.

In media 3 and 4, though leaf growth was much better than that of the medium 2, root growth was poor. Increased amounts of carbon sources might reduce root growth in the case of seagrasses as opined by Durako (1988) in *T. testudinum* and by Koch and Durako (1991) in *Ruppia* along with increased nodal growth. Bird *et al.* (1996) also reported that the growth rates of leafy portions of seagrasses are higher in sucrose based media as compared to that of bicarbonate based media. From these reports, it could be concluded that carbon source is one of the important factors in raising *in vitro* seagrass cultures. From the present investigation, it is understood that the sterilization and medium composition play major roles in obtaining axenic *in vitro* cultures of seagrasses.

The present study has also indicated that the use of plant growth regulators in developing axenic cultures of seagrasses is unnecessary. Bird *et al.* (1998) reported that the auxins tested in raising *Halophila decipiens* cultures did not cause any significant increase in growth. However, cytokinins stimulated the shoot production in *H. decipiens*. Moreover, the studies of Koch and Durako (1991) revealed that cytokinin addition altered the patterns of differentiation in the plant by increasing the branching and decreasing the root formation. Koch and Durako (1989) reported morphological differentiation of growing segments of *R. maritima* in the higher concentrations of plant growth regulators. However, they concluded that a correct combination of 2ip and thidiazuron could produce plants that could be readily propagated *in vitro*. Loques *et al.* (1990) also noted that higher levels of kinetin are harmful for *in vitro* cultures. But the studies of Koch and Durako (1991) with *R. maritima* and Bird and Jewett-Smith (1994) with *H. engelmannii* showed an increase in growth with the increase in the concentrations of the auxins like IAA and NAA. So, development of axenic cultures using seeds as explants without adding any growth regulators would be an ideal and cost-effective method for seagrasses.

Present study revealed that the culture medium 1 (10 % filter sterilized coconut water + 1% charcoal + 15 g agar + filter sterilized UV treated seawater) is ideal for raising axenic cultures of the tropical seagrass, *E. acoroides* without adding any growth hormone. From the present investigation, it is also felt that in future, axenic seagrass seedlings could be raised through tissue culture of different plant parts such as shoot

apices, endosperms, epicotyl and hypocotyl.

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REFERENCES

Ailstock, M.S. 1986. Clonal propagation of *Potamogeton pectinatus* in axenic culture. *In:* Webb, F.J (Ed.), *Proceeding of the 13th Annual conference on wetland Restoration and Creation*. Hillsborough Community College, Tampa, Florida. P. 11 - 17.

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

Bird, K.T., Brown, M.S., Henderson, T.T., O'Hara. C.E. and Robbie, J.M. 1996. Culture studies of *Ruppia maritima* L. in bicarbonate and sucrose based media. *J. Exp. Mar. Biol. Ecol*., 199: 153 - 164.

Bird, K.T. and Jewett Smith, J. 1994. Development of a medium and culture system for *in vitro* propagation of the seagrass *Halophila engelmannii*. *Can. J. Bot*., 72: 1503 - 1510.

Bird, K.T., Johnson, J.R. and Jewett Smith, J. 1998. *In vitro* culture of the seagrass *Halophila decipiens*. *Aquat. Bot.,* 60: 377 - 387.

Durako, M.J. 1988. Turtle grass (*Thalassia testudinum* Banks ex Konig) - A seagrass. *In*: Bajaj, Y.P.S (Ed.) *Biotechnology in Agriculture and Forestry Vol. 6*, Crops II, Springer - Verlag, Berlin, P. 504 - 520.

Ellender, R.D. 1991. Initiation of callus cultures and plantlets regeneration from seagrasses and marine coastal plants, Final Report, Mississippi – Alabama Sea Grant Consortium. P.108.

Hent, R. 1978. *Plant Growth Analysis Studies in Biology No. 96*. Edward Arnold, London.

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

Koch, E.W. and Durako, M.J. 1991. *In vitro* studies of the submerged angiosperm *Ruppia maritima*: Auxin and cytokinin effects on plant's growth and development. *Mar. Biol*., 110: 1- 6.

Koch, E. W. and Durako, M.J. 1989. Seagrass micro-propagation: A step closer to restoration with *In vitro* plants (Florida). *Restor. Manage*., 7: 99 - 100.

Kuo, J., McComb, A.J. and Cambridge, M.L. 1981. Ultrastructure of the seagrass rhizosphere. *New Phytol*., 89: 139 - 143.

Loques, F., Caye, G. and Meinesz, A. 1990. Axenic culture of selected tissue of *Posidonia oceanica*. *Aquat. Bot*. 37: 171 - 188.

McRoy, C.P. 1973. Seagrass ecosystems: Research recommendations of the international seagrass workshop. *In*: *Proc. International Seagrass Workshop*; Leiden, Netherlands. P. 62.

Moffler, M.D. and Durako, M.J. 1984. Axenic culture of *Thalassia testudinum* Banks ex Konig (Hydrocharitaceae). *Amer. J. Bot.* 71: 1455 - 1460.

Newell, S.Y. and Fell, J.W. 1982. Surface sterilization and the active mycoflora of leaves of a seagrass. *Bot. Mar.,* 25: 339 - 346.

Schmidt, M.A. and Hayasaka, S.S. 1985. Localization of a dinitrogen - fixing *Klebsiella* sp. isolated from root - rhizomes of the seagrass *Halodule wirghtii* Aschers. *Bot. Mar.,* 27: 437 - 442.

Smith, G.W. and Hayasaka, S.S. 1982. Nitrogenase activity associated with *Halodule wrightii* root. *Appl. Environ. Microbiol*., 43: 1244 - 1248.