

In vitro studies of the submerged angiosperm *Ruppia maritima*: auxin and cytokinin effects on plant growth and development

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Abstract. Axenic tissue cultures of *Ruppia maritima* L. were established and propagated clonally in vitro from terminal rhizome segments collected from Tampa Bay, Florida, USA. Cultures were maintained in a base medium consisting of synthetic seawater supplemented with half-strength Murashige and Skoog salts and 1% sucrose at pH 5.6. The effects of five cytokins [6-furfurylamino-purine (kinetin), 6-benzylaminopurine (BAP), 2-isopentyladenine (2iP), 6-(4-hydroxy-3-methyl-but-2-enyl-amino) purine (zeatin), and N-phenyl-N'-1,2,3-thiadiazol-5'yl urea (thidiazuron)] and one auxin [naphthalene acetic acid (NAA)] on explant growth and development were investigated. Cytokinin additions resulted in a 3- to 4-fold increase in nodal production, branching, and biomass of *R. maritima* after 12 wk in culture. Cultures responded in a dose-dependent manner to 2iP but exhibited broad dose-response curves to kinetin, BAP, zeatin, and thidiazuron. NAA addition resulted in increased leaf and internodal lengths, but reduced the number of leaves per node and the rhizome biomass. The addition of NAA almost completely suppressed root growth in media without cytokinins and had an antagonistic effect on nodal production and branching in cytokinin-supplemented media.

Introduction

Little information exists on the presence or effects of plant-growth regulators (PGRs) on seagrasses. Auxin- and cytokinin-like activity is present in compounds occurring in seawater, marine sediments, and algal extracts (Buggeln and Craigie 1971, Abe et al. 1972, Pedersen 1973, Tay et al. 1985, Mooney and van Staden 1986). Maruyama et al. (1986, 1989) reported that bacteria-derived auxin and cytokinin activities were higher in estu-

arine waters compared to coastal waters. Within estuaries, activities increased near the sediment/water interface, probably as the result of settlement and accumulation of organic debris in the relatively stagnant near-bottom water. They also found that marine bacteria with the ability to produce extracellular auxins and cytokinins are distributed widely and occur frequently, and they suggested that bacterially-derived PGRs may affect marine vegetation.

The significance of PGRs in affecting seagrass growth and development can be defined only through axenic in vitro culture techniques, which eliminate problems associated with microbial utilization and/or production of inorganic and organic compounds (Wetzel and McGregor 1968, Moffler and Durako 1984). Ailstock (1986) reported that axenic cultures of *Potamogeton pectinatus* L., a submerged aquatic species that occurs in salinities up to 12‰, exhibited dose-dependent shoot-formation responses to kinetin and increased root growth with increased auxin (indole-3-acetic acid, IAA) concentrations. Surprisingly, given the value of clonal axenic culture systems (Gerloff and Krombholz 1966, Ailstock 1986), the application of in vitro culture techniques to seagrasses has been restricted in focus (Durako 1988a). The majority of previous in vitro culture research on submerged vascular plants has involved nutritional studies (Pringsheim and Pringsheim 1962, Forsberg 1966, Wetzel and McGregor 1968, Moffler and Durako 1984, Seeliger et al. 1984, Thursby 1984, Durako and Moffler 1987).

The purpose of the present investigation was to develop a sterilization and culture protocol that produces axenic cultures with high rates of clonal propagation from a field-collected seagrass and then to examine the effects of exogenously-supplied PGRs on seagrass growth and development. In vitro clonal propagation is the most advanced application of plant tissue culture (Thorpe and Patel 1984), and it is a prerequisite for most applied uses of plant tissue culture, including somatic hybridization and genetic modification (Vasil and Vasil 1980). Although the principles of in vitro propagation are universal, each species has particular requirements that can

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evaluate effects of plant growth regulators on explant growth and development. NAA is a synthetic auxin with similar properties to the naturally-occurring auxin indole acetic acid (IAA). The latter was not used because it has relatively low activity and is readily inactivated in some tissue cultures (Murashige 1974). Concentrations of 0, 5, 10, and 20 mg/l each of 6-furfurylaminopurine (kinetin), 6-benzylaminopurine (BAP), 2-isopentyladenine (2iP), 6-[4-hydroxy-3-methyl-but-2-enylamino] purine (zeatin) were tested. Also tested were concentrations of 0, 10^{-9} , 10^{-7} , and 10^{-5} M of N-phenyl-N'-1,2,3-thiadiazol-5-yl urea (thidiazuron), a defoliant that induces a variety of cytokinin-like responses (Thomas and Katterman 1986). Six replicates of each treatment combination were initiated.

Eight weeks after establishment in axenic culture, plants were transferred to individual Magenta GA-7 flasks containing 200 ml of treatment medium. Cultures were terminated after 12 wk. At this time, culture axenicity was tested by standard inoculation and incubation techniques (Moffler and Durako 1984, Durako 1988a). Growth and development of the plant material were assessed using morphometric and biomass measurements. The number of nodes (short-shoots) and branch-points were counted; total rhizome length and lengths of leaves originating from five haphazardly selected nodes were measured. Mean internodal length was calculated by dividing the rhizome length by the number of nodes. Each plant was divided into rhizome, shoot, and (if present) root fractions, which were dried at 60°C for 48 h and weighed.

Results

The multi-step sterilization protocol outlined in Fig. 1, yielded 98 axenic *Ruppia maritima* cultures from the original 192 field-collected explants (51%). Contamination, when present, was mostly due to aerophilic fungi, which tended to grow on the surface of the medium. Slow-growing endophytic bacteria, presumably residing in the relatively large explants, may also have been present because, in several cultures, the medium did not become cloudy until after several weeks of culture.

In all media, initial bud expansion leading to leaf emergence was rapid, occurring within 1 wk of culture initiation (Fig. 2). Callus tissue was not observed on explants in any of the media tested. Explants formed roots only in media without cytokinins, and root growth was considerably suppressed by auxin addition (Table 2).

Cytokinin supplements to the base medium (i.e., control treatment) greatly increased rhizome and shoot growth rates of explants. In most cytokinin-supplemented media, this was reflected in rhizome and shoot biomass that was three to four times higher than biomass of control media after 12 wk (Table 2). Branching, node production, and rhizome lengths of explants were also generally increased 3- to 4-fold relative to the base medium (Figs. 3, 4). Auxin addition was usually antagonistic to cytokinin activity; it suppressed rhizome and shoot biomass, nodal production, and branching in cytokinin-supplemented media. Cytokinin addition reduced numbers of leaves per short-shoot (node) and shortened leaf and internode lengths, with few exceptions (Fig. 4). Auxin was again antagonistic to cytokinin, increasing leaf lengths and internode lengths.

All five of the cytokinins tested had similar effects on plant growth and development, although optimal concentrations for maximum propagation differed. Only 2iP stimulated growth in a dose-dependent manner; dose-de-

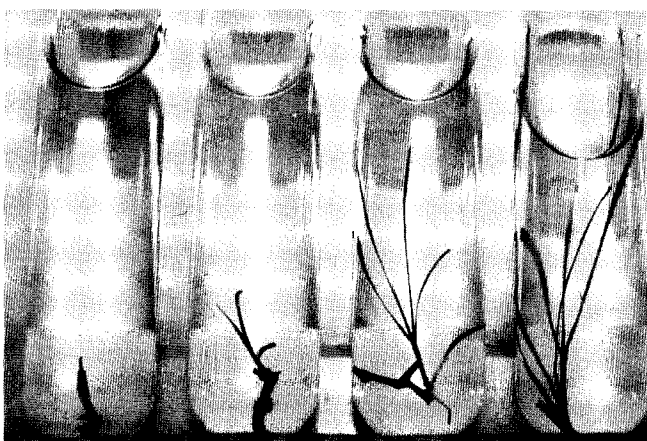


Fig. 2. *Ruppia maritima*. Terminal rhizome explants after (left to right) 1, 2, 3, and 4 wk in culture

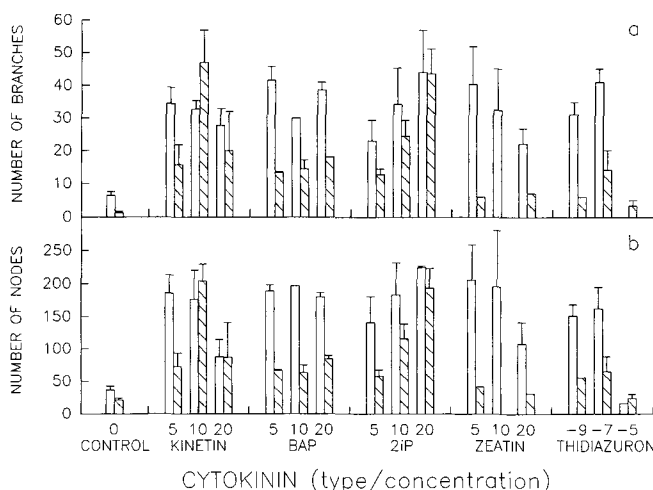


Fig. 3. *Ruppia maritima*. Effects of auxin and cytokinin treatments on (a) branching and (b) node production of axenic explants initiated from field-collected terminal rhizome explants cultured in liquid media for 12 wk (see "Materials and methods - Effects of auxin and cytokinins" for cytokinin concentrations). Open histograms = no auxin, hatched histograms = 1 mg/l naphthalene acetic acid. Means + standard errors

pendency was observed in branch and node production and in rhizome growth (Table 2; Figs. 3, 4). Kinetin, zeatin, and thidiazuron usually elicited greater growth responses at the lower treatment levels (i.e., 5 and 10 mg/l). Thidiazuron without NAA actually inhibited growth and development relative to the base medium at the highest concentration tested (10^{-5} M).

Discussion

Standard sterilization procedures commonly used for terrestrial plant material were insufficient for obtaining axenic *Ruppia maritima*. Surface and endophytic bacteria, as well as fungi, are prevalent on seagrasses (Sieburth et al. 1974, Newell and Fell 1980, Kuo et al. 1981, Smith and Hayasaka 1982, Schmidt and Hayasaka 1985). This

Table 2. *Ruppia maritima*. Biomass characteristics of axenic explants initiated from field-collected terminal rhizome explants after 12 wk in culture in liquid media supplemented with plant growth regulators. Data are means (SD); nd: no data due to contamination

Auxin (mg/l)	Cytokinin (mg/l)	n	Root dry wt (mg)	Rhizome dry wt (mg)	Shoot dry wt (mg)
Control					
0	0	6	2.2 (0.7)	21.0 (4.6)	58.5 (11.3)
1	0	6	0.3 (0.3)	16.7 (2.6)	35.9 (4.5)
Kinetin					
0	5	4	0	68.2 (7.7)	167.1 (24.8)
0	10	4	0	77.0 (26.2)	133.4 (40.6)
0	20	4	0	35.4 (8.2)	64.5 (17.8)
1	5	3	0	46.0 (14.6)	130.0 (43.2)
1	10	2	0	93.1 (13.6)	233.7 (48.1)
1	20	2	0	29.6 (12.0)	84.4 (41.2)
BAP					
0	5	3	0	106.8 (15.4)	200.0 (23.6)
0	10	1	0	96.6	247.6
0	20	3	0	81.1 (4.3)	172.5 (20.0)
1	5	3	0	55.8 (1.2)	122.2 (15.3)
1	10	5	0	49.9 (11.5)	109.3 (26.5)
1	20	2	0	39.4 (4.8)	100.2 (2.3)
2iP					
0	5	4	0	65.8 (7.1)	136.5 (27.6)
0	10	4	0	76.3 (12.3)	173.1 (36.7)
0	20	4	0	91.2 (12.2)	231.1 (9.6)
1	5	5	0	52.3 (8.3)	151.2 (26.5)
1	10	5	0	72.2 (11.2)	193.2 (27.8)
1	20	5	0	89.1 (8.8)	193.6 (24.3)
Zeatin					
0	5	3	0	51.6 (7.8)	214.0 (59.8)
0	10	2	0	51.6 (13.9)	149.2 (62.6)
0	20	4	0	36.1 (10.5)	94.4 (30.8)
1	5	1	0	33.3	122.1
1	10	0	nd	nd	nd
1	20	1	0	34.0	77.8
Thidiazuron^a					
0	-9	4	0	68.4 (13.3)	120.3 (21.3)
0	-7	2	0	59.4 (0.9)	138.5 (13.4)
0	-5	1	0	4.83	8.4
1	-9	1	0	31.7	58.8
1	-7	3	0	45.9 (16.3)	99.7 (38.8)
1	-5	3	0	16.5 (3.6)	23.5 (9.4)

^a Concentrations of thidiazuron in 10^{-9} , 10^{-7} , and 10^{-5} M media

diversity of associated microorganisms, coupled with the characteristic lack of a well-developed cuticle in submerged macrophytes (Martin and Juniper 1970), makes effective, non-deleterious surface sterilization difficult (Durako 1988a). The major obstacle for developing tissue cultures of submerged macrophytes is the difficulty in obtaining axenic cultures (Ailstock 1986, Gusev et al. 1987). The multi-step sterilization protocol used here produced axenic plants in about half (51%) of the explants; however, even the contaminated explants were viable. Optimal explants of *R. maritima* consisted of an apical meristem (i.e., youngest node) and the next two to three nodes; single-node explants did not develop. Addi-

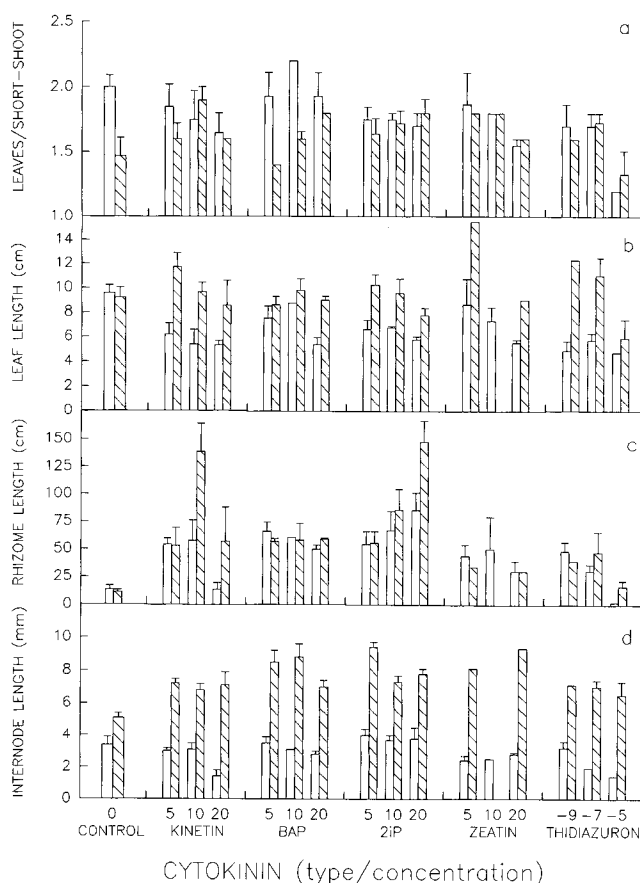


Fig. 4. *Ruppia maritima*. Effects of auxin and cytokinin treatments on (a) number of leaves per shoot, (b) leaf length, (c) rhizome length, and (d) internode length of axenic explants initiated from field-collected terminal rhizome explants cultured in liquid media for 12 wk. Further details as in legend to Fig. 3

tional nodes may act as a "lacunal buffer", protecting the apical tissue from damage resulting from direct contact with the various chemicals utilized in the sterilization protocol. This suggests a balance must be struck between the minimum size explant that will be viable and the maximum size that can be effectively sterilized.

Biomass allocation and growth of terminal rhizome segments of axenic *Ruppia maritima* in sucrose-enriched base medium without plant growth-regulators were similar to those reported for *R. occidentalis* S. Wats. in situ (Husband and Hickman 1985), although root growth in *R. maritima* was considerably less. *Thalassia testudinum* also exhibits reduced root growth in a sucrose-enriched medium (Durako 1988b). Initiation and rapid growth of roots of axenic *R. maritima* subcultured in base medium without sucrose (Koch and Durako unpublished data) suggest that reduced allocation to nutrient-absorptive root tissues may be indicative of nutrient sufficiency (Grime 1979).

As stated previously, there is almost nothing known regarding the effects of plant-growth regulators on seagrass growth and development. Auxins and cytokinins act as growth regulators and growth promoters, and both stimulate growth in several seaweeds in vitro (Pedersen 1968, 1973, Mishra and Kefford 1969, Dawes 1971, Fries

1977). Auxins typically promote root formation in terrestrial and aquatic plants (Thorpe and Patel 1984, Ailstock 1986). The suppression of *Ruppia maritima* root development by auxin was, therefore, unexpected. Kelly et al. (1971) observed increased root and shoot production in transplanted sprigs of *Thalassia testudinum* soaked for 1 h in 10% NAA. In contrast, Van Breedved (1975) found that a combination of auxin treatments on the same species had no effect. Auxin supplements reduced the rate of rhizome elongation and the number of rhizoids in the coenocytic green alga *Caulerpa sertularioides* (Gmelin) Howe (Mishra and Kefford 1969). However, Dawes (1971) observed that response in *C. prolifera* (Forsskal) Lamouroux was very specific to auxin concentration, with inhibition at levels greater than 10^{-5} M. A primary effect of added auxin is a lowering of the pH of the medium that permeates the plants' cell walls (Varner and Tuan-Hua Ho 1976). Therefore, the variability of marine plant responses to added auxin may reflect differences in the buffering capacity of various seawater media or it may be due to differences in species-specific sensitivities.

All five cytokinin additions altered the patterns of differentiation in *Ruppia maritima* through increased branching and decreased root formation and generally stimulated a 3- to 4-fold increase in rhizome and shoot growth. Relatively high cytokinin levels were employed in this study because studies by Ailstock (1986) and Kane et al. (1988) showed that responses of aquatic species consistently increased over the ranges tested (typically 0.1 to 10 mg/l), preventing determination of optimal concentrations. *R. maritima* lacked a typical dose-dependent response to kinetin, BAP, zeatin, and thidiazuron, suggesting a relatively broad response-curve for these compounds. In contrast, 2iP, a cytokinin isolated from coastal seawater and sediments (Pedersen 1973, Kentzer et al. 1980, Maruyama et al. 1986), stimulated growth and development in a dose-dependent manner. Responses to kinetin, zeatin, and thidiazuron indicated that growth stimulation becomes saturated at low levels, whereas with higher concentrations of thidiazuron, growth is actually inhibited. Similarly, Klaine (1986) observed growth stimulation in the freshwater aquatic plant *Hydrilla verticillata* Royle when low concentrations of thidiazuron were added, but he observed dramatic growth inhibition when concentrations were greater than 10^{-6} M.

Literature cited

- Abe, H., Uchiyama, M., Sato, R. (1972). Isolation and identification of native auxins in marine algae. *Agric. biol. Chem.* 36: 2259–2260
- Ailstock, M. S. (1986). Clonal propagation of *Potamogeton pectinatus* in axenic culture. In: Webb, F. J. (ed.) *Proceedings of the 13th Annual Conference on Wetlands Restoration and Creation*. Hillsborough Community College, Tampa, Florida, p. 11–17
- Buggeln, R. G., Craigie, J. S. (1971). Evaluation of evidence for the presence of indole-3-acetic acid in marine algae. *Planta* 97: 173–178
- Dawes, C. J. (1971). Indole-3-acetic acid in the green algal coenocyte *Caulerpa prolifera* (Chlorophyceae, Siphonales). *Phycologia* 10: 375–379
- Durako, M. J. (1988a). Turtle grass (*Thalassia testudinum* Banks ex König) – a seagrass. In: Bajaj, Y. P. S. (ed.) *Biotechnology in agriculture and forestry*. Vol. 6. Crops II. Springer-Verlag, Berlin, Heidelberg, New York, p. 504–520
- Durako, M. J. (1988b). Growth responses of light- and dark-cultivated axenic *Thalassia testudinum* seedlings to organic carbon amendment and pH. *EOS Trans., Am. geophys. Un.* 69: p. 1115
- Durako, M. J., Moffler, M. D. (1987). Nutritional studies of the submerged marine angiosperm *Thalassia testudinum*. I. Growth responses of axenic seedlings to nitrogen enrichment. *Am. J. Bot.* 74: 234–240
- Forsberg, D. (1966). Sterile germination requirement of seed of some water plants. *Physiologia Pl.* 8: 128–137
- Fries, L. (1977). Growth regulating effects of phenylacetic acid and p-hydroxyphenylacetic acid in *Fucus spiralis* L. (Phaeophyceae, Fucales) in axenic culture. *Phycologia* 16: 451–456
- Gerloff, G. C., Krombholz, P. H. (1966). Tissue analysis as a measure of nutrient availability for the growth of angiosperm aquatic plants. *Limnol. Oceanogr.* 11: 529–537
- Grime, J. P. (1979). *Plant strategies and vegetation processes*. Wiley & Sons, New York
- Gusev, M. V., Tambiev, A. H., Kirikova, N. N., Shelyastina, N. N., Aslanyan, R. R. (1987). Callus formation in seven species of agarophyte marine algae. *Mar. Biol.* 95: 593–597
- Husband, B. C., Hickman, M. (1985). Growth and biomass allocation of *Ruppia occidentalis* in three lakes differing in salinity. *Can. J. Bot.* 63: 2004–2014
- Kane, M. E., Sheehan, T. J., Ferwerda, F. (1988). In vitro growth of American lotus embryos. *HortSci.* 23: 611–613
- Kelly, J. A., Fuss, C. M., Hall, J. R. (1971). The transplanting and survival of turtle grass *Thalassia testudinum*, in Boca Ciega Bay, Florida. *Fish. Bull. U.S.* 69: 273–280
- Kentzer, T., Synak, R., Burkiewicz, K., Barnas, A. (1980). Cytokinin-like activity in sea water and *Fucus vesiculosus* L. *Biologia Pl.* 22: 218–225
- Klaine, S. J. (1986). Influence of thidiazuron on propagule formation in *Hydrilla verticillata*. *J. aquat. Pl. Mgmt* 24: 80–82
- Kuo, J., McComb, A. J., Cambridge, M. L. (1981). Ultra-structure of the seagrass rhizosphere. *New Phytol.* 89: 139–143
- Martin, J. T., Juniper, B. E. (1970). *The cuticles of plants*. Edward Arnold Press, London
- Maruyama, A., Maeda, M., Simidu, U. (1986). Occurrence of plant hormone (cytokinin)-producing bacteria in the sea. *J. appl. Bact.* 61: 569–574
- Maruyama, A., Maeda, M., Simidu, U. (1989). Microbial production of auxin indole-3-acetic acid in marine sediments. *Mar. Ecol. Prog. Ser.* 58: 69–75
- Mishra, A. K., Kefford, N. P. (1969). Developmental studies on the coenocytic alga, *Caulerpa sertularioides*. *J. Phycol.* 5: 103–109
- Moffler, M. D., Durako, M. J. (1984). Axenic culture of *Thalassia testudinum* Banks ex König (Hydrocharitaceae). *Am. J. Bot.* 71: 1455–1460
- Mooney, P. A., van Staden, J. (1968). Algae and cytokinins. *J. Pl. Physiol.* 123: 1–21
- Murashige, T. (1974). Plant propagation through tissue cultures. *A. Rev. Pl. Physiol.* 25: 135–166
- Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Pl.* 15: 473–497
- Newell, S. Y., Fell, J. W. (1980). Mycoflora of turtle grass (*Thalassia testudinum* König) as recorded after seawater incubation. *Botanica mar.* 23: 265–275
- Pedersen, M. (1968). *Ectocarpus fasciculatus*: marine brown alga requiring kinetin. *Nature, Lond.* 218: p. 776
- Pedersen, M. (1973). Identification of a cytokinin, 6-(3-methyl-2-butenylamino) purine, in seawater and the effects of cytokinins on brown algae. *Physiologia Pl.* 28: 101–105
- Pringsheim, E. G., Pringsheim, O. (1962). Axenic cultures of *Utricularia*. *Am. J. Bot.* 49: 898–901
- Schmidt, M. A., Hayasaka, S. S. (1985). Localization of a dinitrogen-fixing *Klebsiella* sp. isolated from root-rhizomes of the seagrass *Halodule wrightii* Aschers. *Botanica mar.* 27: 437–442

- Seeliger, U., Cordazzo, C., Koch, E. W. (1984). Germination and algae-free laboratory culture of widgeon grass, *Ruppia maritima*. *Estuaries* 7: 176-178
- Sieburth, J. M., Brooks, R. D., Gessner, R. V., Thomas, C. D., Tottle, J. L. (1974). Microbial colonization of marine plant surfaces as observed by scanning electron microscopy. In: Colwell, R. R., Morita, R. Y. (eds.) *Effects of the ocean environment on microbial activities*. University Park Press, Baltimore, p. 418-432
- Smith, G. W., Hayasaka, S. S. (1982). Nitrogenase activity associated with *Halodule wrightii* roots. *Appl. envirl Microbiol.* 43: 1244-1248
- Tay, S. A., MacLeod, J. K., Palni, L. M., Letham, D. S. (1985). Detection of cytokinins in a seaweed extract. *Phytochem.* 24: 2611-2614
- Thomas, J. C., Katterman, F. R. (1986). Cytokinin activity induced by thidiazuron. *Pl. Physiol.* 81: 681-683
- Thorpe, T. A., Patel, K. R. (1984). Clonal propagation: adventitious buds. In: Vasil, I. K. (ed.) *Cell culture and somatic cell genetics of plants*. Vol. 1. Academic Press, New York, p. 49-60
- E. W. Koch and M. J. Durako: In vitro studies of *Ruppia maritima*
- Thursby, G. W. (1984). Nutritional requirements of the submerged marine angiosperm, *Ruppia maritima* in algae-free culture. *Mar. Ecol. Prog. Ser.* 16: 45-50
- Van Breedveld, J. (1975). Transplanting seagrasses with emphasis on the importance of substrate. *Fla mar. Res. Publs* 17: 1-26
- Varner, J. E., Tuan-Hua Ho, D. (1976). Hormones. In: Bonner, J., Varner, J. E. (eds.) *Plant biochemistry*. Academic Press, New York, p. 713-770
- Vasil, I. K., Vasil, V. (1980). Clonal propagation. In: Vasil, I. K. (ed.) *Perspectives in plant cell and tissue culture*. Academic Press, New York, p. 145-165
- Verhoeven, J. T. A. (1979). The ecology of *Ruppia*-dominated communities in Western Europe. I. Distribution of *Ruppia* representatives in relation to their autecology. *Aquat. Bot.* 6: 197-268
- Wetzel, R. G., McGregor, D. L. (1968). Axenic culture and nutritional studies of aquatic macrophytes. *Am. Midl. Nat.* 80: 52-64