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Nutritional requirements of the submerged angiosperm *Ruppia maritima* in algae-free culture*

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ABSTRACT: *Ruppia maritima* has the potential to become a model laboratory organism for studies with submerged aquatic vascular plants. The present study demonstrated that algae-free *R. maritima* grew well in a defined medium without sediment. Growth was a linear response to photon flux density over the range of light tested (up to 450 $\mu E\ m^{-2}\ s^{-1}$). Vitamins may be a necessary addition in artificial seawater. Trace metals caused little or no increase in growth during short-term (3 wk) growth studies, but appear to be required for long-term cultivation. Iron also caused no increase in growth, at the concentrations tested, but plants were greener in 1.46 µM iron. A nitrate concentration of 110 µM and a phosphate concentration of 2.3 µM were sufficient for maximum growth. However, 4.5 µM phosphate eliminated occasional $CaCO_3$ precipitation in stock cultures. Critical tissue nitrogen content was between 2.5 and 3.0 %, and critical phosphorus content between 0.25 and 0.35 %. A comparison with field data suggests that *R. maritima* was deficient in both nitrogen and phosphorus during much of the summer. Final dry weight and number of leafy shoots per plant correlated positively ($r^2 = .92$). A census of leafy shoots could therefore be a useful non-destructive technique for following the growth of individual plants. This study is the first record of algae-free cultures of *R. maritima*.

INTRODUCTION

Ruppia maritima L. (Potamogetonaceae), a submerged angiosperm, is most often found in shallow brackish water, ranging from coastal habitats to interior saline lakes (Setchell, 1946). To date, salinity and temperature have been the primary environmental parameters examined in the culture of *R. maritima*. This species grows best at salinities less than that of full seawater (Bourn, 1935; Mayer, 1967; Verhoeven, 1979), but tolerates salinities as high as 74 ppt (McMillan and Moseley, 1967). The temperature optimum for *R. maritima* lies between 20 and 25 °C (Setchell, 1924). An earlier attempt at examining the effect of light on the growth of *R. maritima* in the laboratory was unsuccessful because of algal contamination (Koch et al., 1974). No definitive work has been done on nutritional requirements for *R. maritima*.

This is the first published record of *Ruppia maritima*, or any other submerged marine angiosperm, in algae-free culture. *R. maritima* propagated easily and grew well in a defined medium. Algae-free material allowed an accurate assessment of its nutritional requirements in culture. Experiments were performed to determine the influence of light and different concentrations of vitamins, trace metals, iron, nitrate and phosphate on growth.

MATERIALS AND METHODS

All experiments were performed with algae-free material. Seeds of *Ruppia maritima* L. were collected in July 1980 from the north shore of the west basin of Ninigret Pond, a coastal lagoon in Rhode Island, USA (41°30'N × 71°30'W). Clonal cultures of *R. maritima* were started from seeds surface sterilized 15 to 20 min in 20 % Chlorox. Seeds were held in low light (ca. 50 $\mu E\ m^{-2}\ s^{-1}$) at 20 to 22 °C and 10 ppt salinity. The first seeds germinated after ca. 6 wk. Algae-free condition was verified by microscopic examination. All experimental plants were propagated from 1 clone.

Preliminary experiments verified that this isolate of

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Ruppia maritima grew in the laboratory over a broad range of temperatures and salinities, and preferred lower salinities and moderate temperatures. Maximum growth was at 20 °C and 10 ppt. Stock cultures were maintained at this temperature and salinity without sediment in natural seawater in aerated, 3.8 l glass jars with 3 l of medium and ca. 100 μE m^{-2} s^{-1} of cool white fluorescent light. The medium was one fourth strength medium 'f', minus silica (Guillard and Ryther, 1962). Experiments were performed in the same vessels and at the same temperature and salinity as the stock cultures.

Experiments lasted 3 wk, and media were changed once a week. Under the above conditions plants could be kept in logarithmic growth over the course of a 3 wk experiment (Fig. 1). Except for experiments with artificial seawater (Kester et al., 1967), all water was from lower Narragansett Bay, Rhode Island. Each jar was inoculated with 5 plants (consisting of segments with 1 leafy shoot). The initial dry weight was ca. 3.3 mg $plant^{-1}$. Starting plants for any given experiment came from the same stock culture. Growth was measured as the number of leaf clusters per plant, dry weight or both. For most experiments each plant was separated into leaves, roots, and rhizomes before drying and weighing. Variation among jars within the same treatment was minimal (Fig. 1) and only representative data are reported.

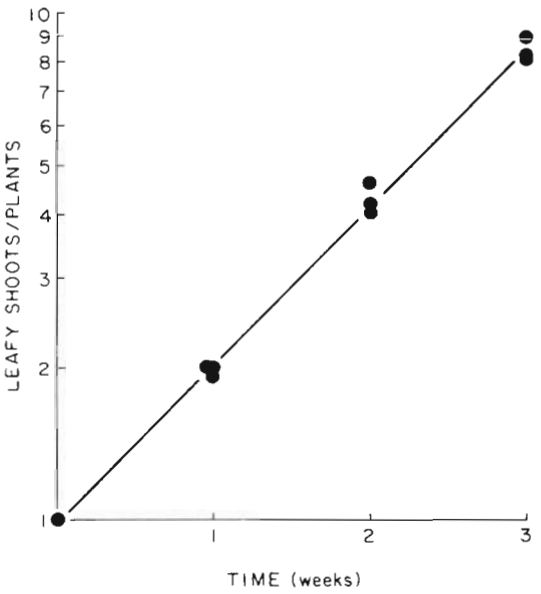

Fig. 1. *Ruppia maritima*. Growth as a function of time. Each point represents the mean of 5 plants. The medium was one fourth strength medium 'f', minus silica; 10 ppt salinity; 20 °C; 150 μE m^{-2} s^{-1}, cool white fluorescent light on a 16 : 8, light : dark cycle

The effect of photon flux density on growth was tested at 50 to 450 μE m^{-2} s^{-1} (at 50 μE m^{-2} s^{-1} intervals. Four 8 foot very-high-output, cool white fluorescent bulbs supplied light on a 16 h : 8 h, light : dark cycle. Cultures were placed at various distances from the light source to obtain the different light levels (measured with a LiCor quantum sensor, Model LI-185A*).

Various concentrations of vitamins, trace metals, iron, nitrate ($NaNO_3$) and phosphate ($NaH_2PO_4 \cdot H_2O$) were tested for optimal growth of *Ruppia maritima*. Concentrations tested were based on dilutions of the concentrations in medium 'f'. Vitamins (thiamine · HCl, biotin, cyanocobalamin), as well as trace metals (Co, Cu, Mo, Mn, Zn), were tested as a unit. Experiments with different concentrations of nitrate and phosphate were run in Kester's artificial seawater (KSW). The effect of vitamins was tested in both natural and artificial seawater. Unless otherwise noted, all nutrients not being tested were added to give one-fourth strength medium 'f'. Cool white fluorescent light was supplied at 350 μE m^{-2} s^{-1} on a 16 h : 8 h light : dark cycle.

To avoid precipitation, the original formula for KSW requires that it be autoclaved in 2 separate solutions that are combined after cooling and setting for 2 d. However, KSW was autoclaved as a single solution without precipitation by omitting NaH_2CO_3. The NaH_2CO_3 was autoclaved as a dry powder, and added after the KSW cooled.

Leaves from the nitrate and phosphate growth experiments were analysed for total tissue nitrogen (CHN analysis; Perkin Elmer elemental analyser, Model 240B) and phosphorus (Menzel and Corwin, 1965), respectively. In addition, leaf tissue nitrogen and phosphorus concentrations were determined for field samples of *Ruppia maritima* from the north shore of Ninigret Pond collected between June 1979 and July 1980.

RESULTS

Neither trace metals nor vitamins were essential additions for growth of *Ruppia maritima* in natural seawater, and results obtained with the Kester's artificial seawater, only suggested vitamins were necessary. Because there were not large differences in the growth response among the various vitamin additions, the lowest concentration tested (f/8) was chosen as the optimum.

There were no differences in growth among the different iron treatments (0.73, 1.46, 2.92, 5.84 μM), however, 1.46 μM (f/16) was chosen as the optimal iron concentration. Plants from the 0.73 μM treatment were

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noticeably paler than those from the other three treatments. Plants grown with 2.92 or 5.84 µM as the initial iron concentration had a flocculent material present in the culture and on the roots (presumably ferric hydroxide; Epstein, 1972).

The growth response of *Ruppia maritima* with respect to photon flux density is shown in Fig. 2. The

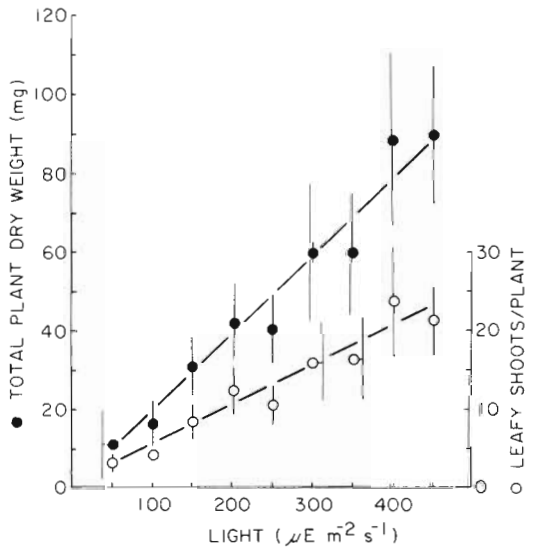

Fig. 2. *Ruppia maritima*. Growth (mean ± S.D., n = 5) after 3 wk at various levels of cool white light on a 16 : 8, light : dark cycle

response was linear over the range of light tested, and was the same whether measured as dry weight or number of leafy shoots per plant.

The final 2 experiments measured the effects of different concentrations of nitrate (Fig. 3A) and phosphate (Fig. 3B) on growth of *Ruppia maritima*. The lowest initial nitrate concentration that did not cause a decrease in growth was 110 µM (f/16). The phosphate growth curve showed that the minimal level of added phosphate needed to maintain optimal growth was 2.3 µM (f/32). Growth is plotted against total nitrogen and total phosphorus in the leaf tissues from the above 2 experiments in Fig. 4. Critical nitrogen content was between 2.5 and 3.0 %, critical phosphorus between 0.25 and 0.35 %. The total nitrogen and phosphorus contents of field collected material were below these critical nutrient values during the summer (Fig. 5).

Dry weight and number of leafy shoots were highly correlated (Fig. 6). Thus, the results within any experiment were the same whether growth was measured as dry weight or number of leafy shoots per plant. In addition, the individual growth responses of leaves, roots, and rhizomes followed the same pattern as that of the total plant. This was true because leaves, roots and rhizomes were a constant percentage of the total

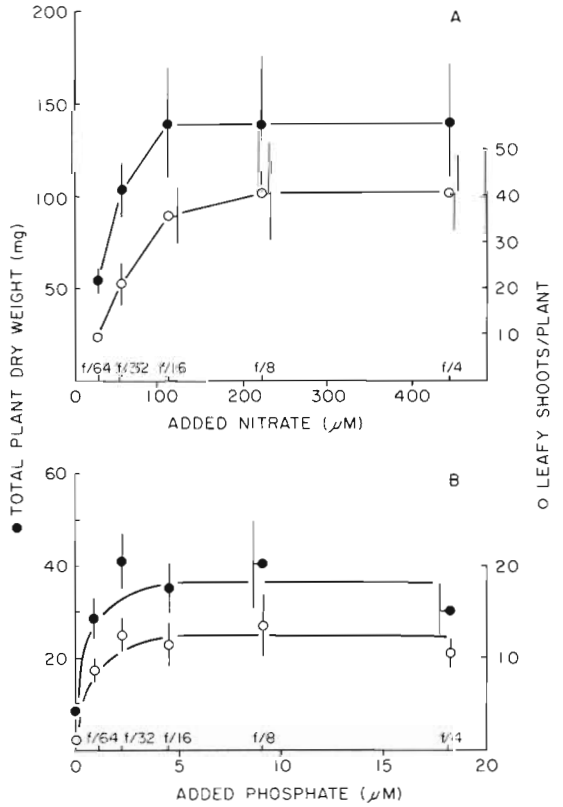

Fig. 3. *Ruppia maritima*. Growth (mean ± S.D., n = 5) after 3 wk at different concentrations of nitrate (A) and phosphate (B). For the nitrate experiment initial phosphorus concentration was 18.2 µM (f/4); both Fe and EDTA were supplied at 1.46 µM (f/16); vitamins were at f/8 strength; no trace metals were added. For the phosphate experiment the conditions were the same, with nitrate supplied at 110 µM (f/16)

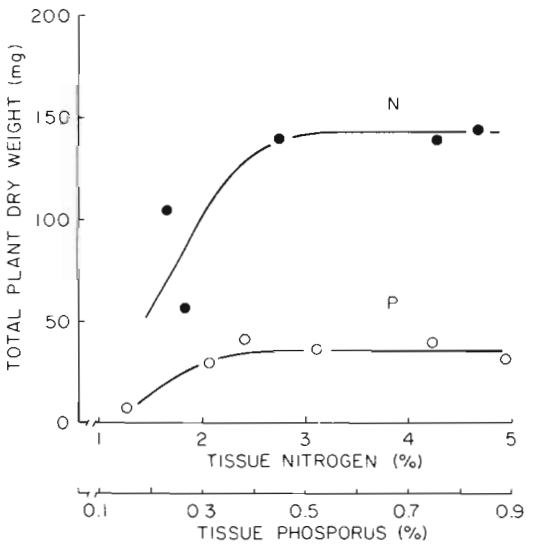

Fig. 4. *Ruppia maritima*. Growth plotted against total tissue nitrogen (N) and total tissue phosphorus (P). Each point represents the mean of 5 plants

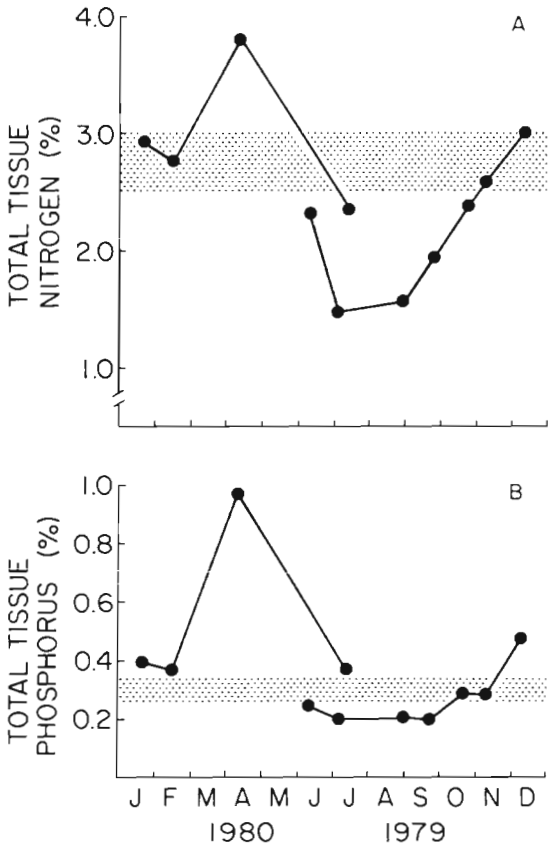

Fig. 5. *Ruppia maritima*. Seasonal variation in total tissue nitrogen (A) and phosphorus (B). Plants were collected from the north shore of the west basin of Ninigret Pond in Rhode Island. The stippled areas represent the range for the critical nutrient content

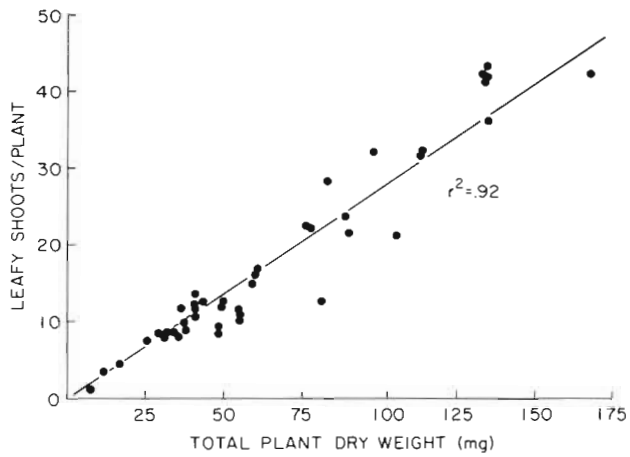

Fig. 6. *Ruppia maritima*. Correlation of number of leaf clusters per plant with final total dry weight. Graph includes all experiments for which total dry weights were determined

dry weight (38, 16 and 46 %, respectively) regardless of the experimental treatment (as long as the plants grew).

DISCUSSION

Results of this study show that *Ruppia maritima* can be cultured successfully without sediment and free from algal contaminants. Clonal material minimized influence of genetic differences among starting plants. Plants harvested from the same stock culture for each experiment minimized variation from differences in pre-conditioning. The availability of algae-free material allowed an accurate assessment of the influence of light and nutrients on growth.

The importance of algae-free material was emphasized with the light experiment. Koch et al. (1974) found that *Ruppia maritima* grows best with light between 200 and 450 foot-candles. They observed growth inhibition at levels of light > 450 foot-candles, and suggested that this was due to enhanced growth of epiphytes. By contrast, using algae-free material, the growth of *R. maritima* in this study was not saturated at the highest light tested (ca. 2000 foot-candles).

Changing the media as often as once a week may explain the lack of major differences among trace metal and vitamin treatments for cultures of *Ruppia maritima* in natural seawater. Background concentrations of vitamins and trace metals in natural seawater may have been sufficient to maintain good growth. However, approximately 10 mo after the trace metal experiment, during which time trace metals were not added to stock cultures (and the media were changed weekly), stock cultures experienced a period of reduced vigor. Adding trace metals (at f/8 concentrations) eliminated the problem. Plants presumably were able to store enough trace metals to grow for short time intervals without any additions to the medium. However, trace metals appeared to be an essential addition for long-term maintenance of cultures in the laboratory. The absences of a vitamin deficiency response may also be explained by the presence of bacteria since the cultures were not axenic.

The critical nitrogen and phosphorus content of *Ruppia maritima* leaves exceeds those reported for other aquatic vascular plants (Gerloff and Krombholz, 1966; Gerloff, 1975). The critical nutrient concentration of a plant is the internal concentration of that nutrient that is just limiting its growth. Internal concentrations lower than the critical concentration indicate nutrient deficiency (Gerloff, 1975). The low tissue concentrations of nitrogen and phosphorus for *R. maritima* in the field during summer suggests this species was deficient in both nutrients during this period. This is the time of year that *R. maritima* exhibits rapid growth in Ninigret Pond (Conover, 1966). Although sediments contain large reservoirs of nutrients (Iizumi et al., 1982; Kenworthy et al., 1982), diffusion to the roots (and subsequent uptake) may not equal demand. Seagras-

ses can be nutrient limited even when growing in nutrient-rich sediments (Orth, 1977; Harlin and Thorne-Miller, 1981). In addition, both ammonia and phosphate stimulate the growth of *Zostera marina* in the field (Harlin and Thorne-Miller, 1981), suggesting simultaneous deficiency in nitrogen and phosphorus. On the other hand, the low tissue content of nitrogen and phosphorus in *R. maritima* may not represent a period of nutrient deficiency. *R. maritima* is flowering during this period and it may be translocating large amounts of nitrogen and phosphorus from vegetative structures to the flowering shoots, a common phenomenon among flowering plants (Bieleski, 1973; Pate, 1980).

Table 1 summarizes the nutrient concentrations for the optimal growth of *Ruppia maritima* in culture. The

Table 1. Composition of enriched seawater for culturing *Ruppia maritima* (per liter of 10 ppt Kester's artificial seawater)

$NaNO_3$	9.35 mg	(110 μM)
$NaH_2PO_4 \cdot {}_2O$	0.62 mg	(4.5 μM)
Iron (as Cl^{-*})	81.5 μg	(1.46 μM)
EDTA (disodium salt)	543 μg	(1.46 μM)
Vitamins		
Thiamine · HCl	25 μg	
Biotin	0.12 μg	
Cyanocobalamin (B_{12})	0.12 μg	
Trace Metals		
$CoCl_2 \cdot 6H_2O$	2.5 μg	
$CuSO_4$ $5H_2O$	2.5 μg	
$MnCl_2 \cdot 4H_2O$	45 μg	
$NaMoO_2 \cdot 2H_2O$	15.8 μg	
$ZnSO_4 \cdot 7H_2O$	5.5 μg	

* A 1 mg ml^{-1} stock solution was prepared by dissolving 1 g iron powder in 10 ml concentrated HCl and diluting to 1 l with deionized water

EDTA concentration (1.46 μM = f/16) was selected to maintain the iron to EDTA ratio of medium 'f'. The optimal phosphate concentration for cultures was selected as 4.5 μM (f/16). At 2.3 μM phosphate (f/32), the optimum from the growth experiment, stock cultures occasionally exhibited a precipitate (presumably $CaCO_3$) on the roots. This may have occurred because there was more biomass per jar in the stock cultures than in the experiments. A 4.5 μM concentration for phosphate eliminated the precipitate and maintained the N to P ratio of medium 'f'.

Ruppia maritima has the potential to become a model laboratory organism for studies with submerged aquatic vascular plants. The present study demonstrated that *R. maritima* grows well in a defined medium. Plants are relatively small, therefore large culture volumes are not essential. Clonal material can be propagated rapidly because of its relatively rapid growth rate in culture. A census of the number of leafy shoots can be used as a non-destructive measure of growth to follow the growth of individual plants.

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