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**RESTORING SEAGRASS BEDS:
SOME NEW APPROACHES WITH RUPPIA MARITIMA L.
(WIDGEON-GRASS)**

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ABSTRACT

Experimental seagrass plantings were conducted in three artificially created ponds in central Sarasota Bay. Planting units were produced from locally collected Ruppia maritima (Widgeon-grass) that had been clonally micropropagated in vitro. Explants used to produce the planting units were rhizome segments containing five short-shoot nodes. Explants were propagated axenically in sucrose-enriched media supplemented with the cytokinin 2-isopentyl adenine (2iP). Culture conditions resulted in high rates of node production and branching: nodes and branches exhibited doubling times of less than two weeks. We used a new planting technique in which the micropropagated plants were placed in biodegradable cotton-mesh bags that contained river-rock ballast in three configurations. This technique protected and anchored the fragile planting units prior to rooting. Leaves and roots rapidly grew through openings in the cotton-mesh bags, and plants became rooted before the bags decomposed (about two months). Survival of the plants in Pond #1 ranged from 60 to 97% after three to five months and was greatest in planting units with rock ballast secured to the bag corners. Survival ranged from 15 to 63% in Pond #2, which had less tidal flushing, rockier sediments, and a persistent accumulation of macroalgae. No planting units survived in Pond #3, seemingly because of unsuitable sediments and disturbance by humans. Plants expanded rapidly, and areal coverage doubled approximately every month. These preliminary results indicate that Widgeon-grass produced through micropropagation may be suitable for planting in situ. In addition, the use of the cotton-bag technique enhances establishment and survival of this structurally fragile species.

INTRODUCTION

Seagrass communities provide critical habitat and trophic structure for ecologically and economically important fishery species, and they are a major force in stabilizing coastal ecosystems (Thayer et al., 1984; Zieman and Zieman, 1989). Unfortunately, seagrasses have significantly declined in areal coverage and quality in many coastal areas. Losses of 80 to 90% of historical seagrass coverages have occurred in areas such as Tampa and Chesapeake bays (Lewis et al., 1985; Dennison et al., 1993). Much of this loss has been attributed to excessive nutrient loading and the resulting poor water quality. Reductions in nutrient loading in many coastal systems over the past ten years have contributed to improved water quality, and seagrasses have recolonized some shallow areas that had been barren for several decades (Johansson and Lewis, 1992). However, lack of recruitment stock may limit the extent to which natural recolonization can occur in suitable sites.

Because resource managers are recognizing the importance of seagrasses in the production and maintenance of living marine resources, much emphasis has been placed on developing techniques for restoring seagrass beds. Most previous seagrass-restoration projects have depended on the destructive and labor-intensive harvest of planting units from natural donor beds. The only nondestructive approaches have been based on the collection of Thalassia testudinum Banks ex König seedlings and sprigs from beach wrack (Thorhaug, 1974; Durako and Moffler, 1981, 1984; Derrenbecker and Lewis, 1983; Phillips and Lewis, 1983). In vitro micropropagation offers a potentially low-cost, highly efficient, and nondestructive technique for propagating seagrasses at rates that are much higher than those obtained with other methods of propagation. This biotechnological approach can also provide genetically uniform material for bioassaying various types of environmental stresses and allows for the identification, selection, and clonal propagation of stress-tolerant genotypes. In addition, the production of vigorous planting stock can be adjusted to meet the the sporadic demands of planting projects (Ailstock, 1986).

Ruppia maritima L. (Widgeon-grass) is a cosmopolitan seagrass that occurs throughout the world in a variety of ecological niches. In addition, Ruppia is characteristically an early and frequent recolonizer of sites previously occupied by climax seagrass species. Although Ruppia has not been widely used in previous seagrass planting studies, this species may be better suited for initial testing of restoration-site suitability than other species are because it has the broadest physiological tolerances of any seagrass. In this report, we describe the results of several trial plantings. We used a new planting technique in which micropropagated Ruppia was placed in ballasted, biodegradable cotton-mesh bags.

MATERIALS AND METHODS

Micropropagation

Ruppia maritima was micropropagated using the protocol described by Koch and Durako (1991), with some modifications. We modified the sterilization procedure to decrease contamination rates by including a vacuum treatment that infiltrates the lacunar spaces of the explants (i.e., field-collected rhizome sections) with sterilizing and antimicrobial agents.

Plant material, consisting of rhizome sections with attached short-shoots, was collected from Sarasota Bay in February 1992. The sections were cut into single-branch segments having at least five shoots and were soaked in a fungicide solution (Captan, 1.5 g/L of 20 ppt Sterile Artificial Seawater [SASW]) overnight on an orbital shaker. Segments were then trimmed to three- to five-node explants using aseptic techniques in a laminar-flow hood. Batches of 10-20 explants were surface-sterilized by vacuum infiltration (40 cm Hg vacuum for 10 minutes) of a Clorox/SASW solution (1:5 vol/vol) with 0.1% Tween as a surface wetting agent. Following infiltration, explants were rinsed three times in sterile seawater.

Single, surface-sterilized explants were aseptically transferred to 24-well plates containing 2 ml of a sterile-filtered antibiotic solution (0.20 g/L each of rifampin and erythromycin in 20 ppt SASW). Explants were infiltrated (40 cm Hg vacuum for 30 minutes) with antibiotics on the orbital shaker and then were equilibrated to ambient pressure for 24 hours. Individual explants were then directly transferred to 24-well plates containing 2 ml of culture medium. The medium consisted of Koch and Durako basal salts (Sigma #K-1254), 1% (wt:vol) sucrose, and 10 mg/L 2iP (N^6 -[2-Isopentenyl]adenine) in 20 ppt SASW at pH 5.6. Cultures were illuminated (12 h light:12 h dark) by cool-white fluorescent lamps with a PAR (photosynthetically active radiation photon-flux density) of approximately $100 \mu E m^{-2} s^{-1}$ and were maintained at 26°C in a temperature-controlled culture room. After 10 days incubation, uncontaminated plants were aseptically transferred to sterile 25- x 150-mm culture tubes containing 40 ml of fresh media. The rapidly growing plants were micropropagated by subdividing into 5- to 10-node segments at monthly intervals.

One month prior to being planted into the field, plants were transferred to culture tubes containing media lacking sucrose to "harden" the plants and promote root production. Cultures were also placed in growth chambers with high-intensity lighting (500 - $700 \mu E m^{-2} s^{-1}$) to acclimatize them to sunlight.

Planting Site

Clonally micropropagated Ruppia maritima was planted in three ponds (designated Pond #1, #2, and #3) at the Sarasota BayWalk site on City Island in central Sarasota Bay, Florida. Sarasota BayWalk is a 4.5-acre habitat-restoration and enhancement site created as an Early Action Demonstration Project through the Sarasota Bay National Estuary Program (SBNEP). The objective of the BayWalk project was to create highly productive, diverse, and integrated habitats by removing nonnative plant species, lowering land elevations, and planting appropriate native coastal and wetlands plants (Roat et al., 1992). Pond #1 is adjacent to the SBNEP office and is the only natural pond on the site. This pond is bordered on the south side by a natural mangrove forest and on the north side by a planted Spartina alterniflora Loisel. fringe marsh. Pond #1 has a direct connection to Sarasota Bay via a small tidal creek; shallow channels connect Pond #1 to Pond #2 and Pond #2 to Pond #3. Ponds #2 and #3 were created to provide suitable habitat for fish. These ponds have rockier sediments, experience less tidal flushing, and have more variable salinities than Pond #1 does. The shoreline of Pond #2 consists of planted Spartina alterniflora. Pond #3 is the largest, deepest, and least flushed of the three ponds. The sediments in this pond consist of a thin veneer of sand over a compacted, rocky base. The shoreline of this pond has sparse plantings of Rhizophora mangle L. and some Spartina. Ponds #2 and #3 are not within sight of the SBNEP office and may have been subject to disturbance by humans.

Planting Units

Previous test plantings of Ruppia (Durako, unpublished data) showed that the staple technique (Fonseca et al., 1982), currently the most widely used method for planting seagrass, was inappropriate for Widgeon-grass because of its fragile rhizome. Thus, a new planting technique was developed in which biodegradable cotton-mesh (cheesecloth) bags that contained river-rock ballast were used. This technique anchored and protected the fragile planting units (PUs). Several bag configurations were tested:

Treatment I (T-I): bags contained loose stones and were tied with a knot or jute twine,

Treatment II (T-II): bags were constructed as in T-1 and were tied with jute twine to the inside corners of 25 cm x 25 cm PVC quadrats (four bags quad⁻¹) to protect the PUs from bioturbation, and

Treatment III (T-III): bags contained stones that were sewn into the corners.

All bags were approximately 8 cm x 10 cm.

In the laboratory, micropropagated plants were transferred from culture tubes and inserted into the bags, the open end of each bag was folded over and stapled, and the bags were placed in a cooler chest in layers separated by paper towels moistened with media. The cooler chest containing PUs was then transported to the field site. Planting units were installed in the ponds within three hours of initial preparation.

Field Planting

Test plantings were conducted on three dates. All PUs were installed on approximately 25-cm centers. Within a pond, PUs were installed in an area that was apparently uniform. On October 13, 1992 we installed a total of 177 PUs (A symbols in Fig. 1). In Pond #1, three rows of ten T-I bags were alternated with two rows of five T-II quadrats each (70 PUs). In Pond #2, a row of ten T-I bags was placed on either side of one row of five T-II quadrats (40 PUs). Pond #3 was planted in the same configuration as Pond #1, except that a single T-I bag replaced a T-II quadrat in one of the two T-II quadrat lines (67 PUs).

A second group of 50 PUs, consisting entirely of T-III bags, was installed on November 25, 1992 (B symbols in Fig. 1). A row of ten PUs was placed parallel to one of the outside T-I rows in Pond #1 (10 PUs). Two rows of ten PUs each were placed outside and parallel to the two outside T-I rows in both Pond #2 (20 PUs) and Pond #3 (20 PUs).

The final group of 138 PUs, again consisting entirely of T-III bags, was installed on December 16, 1992 (C symbols in Fig. 1). In this planting, a plot with three rows of eight PUs and two rows of seven PUs each was planted in Pond #1 (38 PUs), a 5- x 6-unit plot was planted in Pond #2 (30 PUs), and a 7- x 10-unit plot was planted in Pond #3 (70 PUs).

Survival of the PUs was monitored regularly until March 28, 1993. Areal coverage rates of T-I and T-III bags in Pond #1 were estimated by averaging the width (d, in cm) of the PUs on two perpendicular axes and by computing areal spread as

$$[\pi(d/2)^2]/\Delta\text{days} = \text{cm}^2/\text{day}$$

Because of the close proximity and rapid coalescence of the PUs within the quadrats, areal coverage rate could not be measured for Treatment II. Doubling time (DT) in days was calculated as

$$\text{DT} = .301/[(\log x_1 - \log x_0)/\Delta t]$$

where x_1 and x_0 are the number of nodes and branches for cultured plants or the area of a PU at t_1 and t_0 days; $\Delta t = t_1 - t_0$.

RESULTS AND DISCUSSION

More than 350 robust plants of Ruppia maritima were produced from fewer than 20 five-node rhizome segments using the in vitro micropropagation techniques described by Koch and Durako (1991). The axenic Ruppia cultures had high rates of growth and branching in vitro. Figure 2 illustrates characteristic rates of node production and branching during a month-long propagation cycle that was initiated with single-branch, five-node explants. The growth rates shown in this figure yielded doubling times of 13.5 days for nodes and 11.0 days for branches. These results are comparable to the two-week doubling times reported for axenic sago pondweed, Potamogeton pectinatus L., cultured in vitro in nutrient-enriched media (Ailstock et al., 1991).

Leaves rapidly grew through the ballasted cheesecloth bags used for planting the Ruppia, and the plants became rooted and started to spread before the bags deteriorated. Doubling times for the areal coverage of T-I and T-III plants in Pond #1 averaged from 25 to 37 days, and areal coverage rates averaged from 5 to 19 $\text{cm}^2 \text{ day}^{-1}$ (Fig. 3). The areal coverage rates for Ruppia fell within the range of values reported by Fonseca et al. (1987) for transplants of field-harvested PUs of shoal grass, Halodule wrightii Aschers. (11.4 $\text{cm}^2 \text{ day}^{-1}$ for the northern Gulf of Mexico to 74 $\text{cm}^2 \text{ day}^{-1}$ for the Florida Keys), and manatee grass, Syringodium filiforme Kützinger (4.7 $\text{cm}^2 \text{ day}^{-1}$ for the northern Gulf of Mexico to 40 $\text{cm}^2 \text{ day}^{-1}$ for the Florida Keys). Because these ranges are similar and our Ruppia data represent only fall and winter growth, the micropropagated material seems to be robust and has a high growth potential. The relatively long DT and low coverage rate of the December 16th plantings probably resulted from the low water temperatures (water temperatures declined from 27.2°C on 12/16/92 to about 14.5°C on 2/21/93) and short daylengths for this planting group. Even so, the 38-unit plot installed on December 16th completely coalesced within three months.

Pond #1 seemed to provide the best conditions for survival and growth of the Ruppia (compare Figs. 4 and 5), perhaps because it is a natural, relatively well-flushed pond. The first group of bags (T-I) had an initial 37% loss of units that seemed to be due to a combination of factors related to the T-I bag design. The knots and jute twine used as bag closures attracted the attention of curious herons and egrets who picked up the bags by the knots and flung them out of the planting grid (personal observation). We later observed some of these "displaced" units growing outside the grid, so the true survival rate is higher than shown in Figure 4. Because the knots and twine tended to float up in the water column, they became fouled with

algae, and this may have shaded out the underlying Ruppia. This material was also relatively slow to break down; the twine persisted long after the cheesecloth had disappeared (after about two months).

Because biogenic effects have been reported to be one of the most frequent problems associated with the failure of seagrass restoration projects (Fonseca, 1990), PVC quadrats (the T-II treatment) were used to protect PUs from decapod excavations and from sediment blow-outs caused by rays. No loss of T-II PUs occurred in Pond #1 (Fig. 4) or Pond #2 (Fig. 5) for the first one and one-half months (perhaps the size of the quads protected the bags from "acute heron/egret displacement"). However, the twine that secured the PUs to the PVC quadrats became increasingly fouled, and we observed 35 to 40% mortality during the second month.

We found no evidence of bioturbation by benthic organisms or by rays, but we observed problems with the T-I and T-II PUs associated with the jute twine and knotted bag closures. Therefore, we redesigned the cheesecloth bags so they would lie flat on the bottom. This was accomplished by securing the rock ballast into the corners of the bags and stapling the bag closed. Figure 4 illustrates the increased survival rate of plants in bags of the T-III design over those in bags of the T-I and T-II designs for two Pond #1 plantings on November 25th (80% survival after 123 days) and December 16 (97% survival after 102 days). Because they lay flat on the bottom, the T-III bags had increased surface contact with the sediment, and the top side had better exposure to light. The T-III bags also deteriorated in a more uniform manner.

Compared to plant survival in Pond #1, survival in Ponds #2 (Fig. 5) and #3 (100% mortality or loss) was much lower for all PU treatments and for all three planting dates (Fig. 4). The last group of PUs in Pond #2, which were installed in an area with the least rocky sediments of the pond, had the best survival (63% after 102 days). Pond #2 experienced a sustained, pond-wide bloom of macroalgae that prevented us from measuring areal spread and eventually covered most of the PUs. No PUs survived more than about one month in Pond #3; at least some of the loss was associated with disturbance by humans. We found four of our PVC quadrats neatly stacked on the shoreline during one of our first monitoring visits, and we also observed evidence of foot traffic in the planting grids. The lower survival rates in Ponds #2 and #3 may also have been partially a result of sediment-related factors. Both ponds were artificially created and had coarser and rockier sediments than Pond #1 did. In Pond #3, the underlying sediments had been mechanically compacted, which may have prevented the PUs from rooting.

SUMMARY

Restoring and creating seagrass beds is of paramount importance in compensating for previous and current seagrass losses. Much information is still needed before we can effectively manage and restore seagrass systems (Fonseca, 1990). However, the newly developed micropropagation procedures and planting techniques for species such as Ruppia maritima (Koch and Durako, 1991) provide both ecologically and economically sound alternatives to the current seagrass restoration practices, which can be labor-intensive and destructive. Tissue culture and micropropagation have become well-established approaches for producing and rapidly multiplying many agriculturally and horticulturally important crops, but these approaches have only recently been applied to submersed aquatic angiosperms (Ailstock, 1986; Durako, 1988; Ailstock et al., 1991; Koch and Durako, 1991; Bird et al., 1993). One of the greatest benefits of these biotechnological approaches is that they may allow us to select and produce high numbers of vigorous planting units without damaging natural donor beds. The results of this study indicate that plant material produced by in vitro micropropagation is capable of vigorous growth in situ. The ballasted cheesecloth-bag technique introduced here provides a biodegradable alternative to the use of persistent metal staples for anchoring planting units. The bag technique may also reduce installation time because divers will not need to "plant" the units, and the bags may enhance survival of fragile transplants by reducing plant breakage.

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FIGURE DESCRIPTIONS

Figure 1. Diagram of the relative placement of micropropagated Ruppia maritima plants within planting areas in three ponds at Sarasota BayWalk. Letters refer to planting date: A = 10/13/92, B = 11/25/92, C = 12/16/92. The As signify T-I planting units (PUs), the As in boxes signify T-II quadrat PUs, Bs and Cs signify T-III PUs (see Materials and Methods).

Figure 2. Production (mean $\pm$ s.e.; n = 10) of nodes and branches for axenic micropropagated Ruppia maritima during a one-month propagation cycle.

Figure 3. Areal-coverage doubling time and rate of spread for T-I (10/13/92) and T-III (11/25/92 and 12/16/92) plants in Pond #1 as of 3/16/93.

Figure 4. Survival of micropropagated Ruppia maritima planted on three dates in Pond #1.

Figure 5. Survival of micropropagated Ruppia maritima planted on three dates in Pond #2.

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C C C C C
C C C C C
C C C C C
C C C C C
C C C C C
C C C

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A A	A A	A A	A A	A A
A A	A A	A A	A A	A A

A A A A A A A A A A

A A	A A	A A	A A	A A
A A	A A	A A	A A	A A

A A A A A A A A A A
B B B B B B B B B B

B B B B B B B B B B
 A A A A A A A A A A

A	A	A	A	A	A
A	A	A	A	A	A

 A A A A A A A A A A
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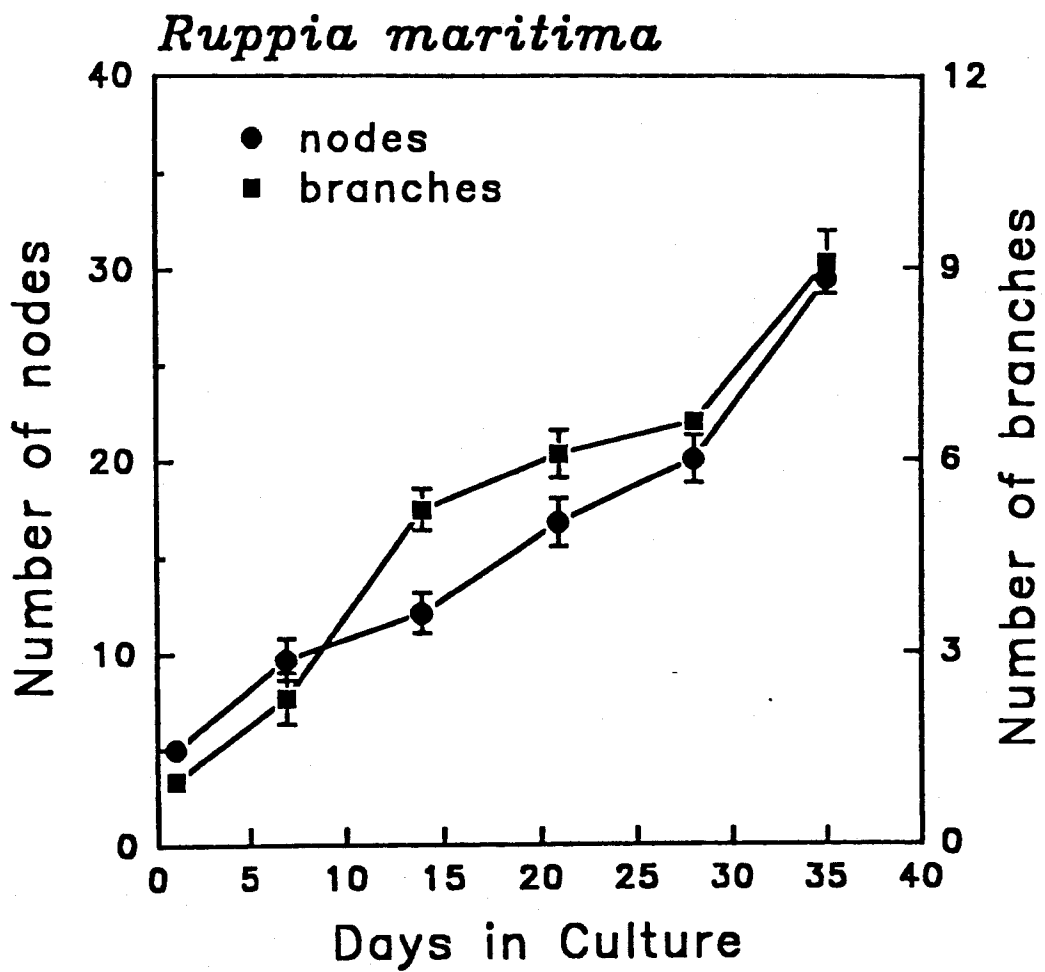
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A A	A A	A A	A A	A A
A A	A A	A A	A A	A A

A A A A A A A A
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A A	A A	A A	A A
A A	A A	A A	A A

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Pond #1 3/16/93

