Journal of Coastal Research	10	3	732–737	Fort Lauderdale, Florida	Summer 1994

Use of *in vitro* Propagated *Ruppia maritima* for Seagrass Meadow Restoration

Kimon T. Bird†, Jerilyn Jewett-Smith‡ and Mark S. Fonseca§

†Center for Marine Science Research
University of North Carolina at Wilmington
Wilmington, NC 28403, U.S.A.

‡Department of Biology
University of Tampa
401 W. Kennedy Ave.
Tampa, FL 33606, U.S.A.

§National Marine Fisheries Service
National Oceanographic and Atmospheric Administration
Beaufort, NC 28516-9722, U.S.A.

ABSTRACT

BIRD, K.T.; JEWETT-SMITH, J., and FONSECA, M.S., 1994. Use of *in vitro* propagated *Ruppia maritima* for seagrass meadow restoration. *Journal of Coastal Research*, 10(3), 732–737. Fort Lauderdale (Florida), ISSN 0749-0208.

The use of *in vitro* propagated *Ruppia maritima* for seagrass meadow restoration was evaluated in two experiments. Experiment 1 compared two different planting methods for *in vitro* propagated plants. In one method, cultured plants were attached to metal staples which were then inserted into the sediment. Almost all of these transplants disappeared within one month at the four different planting sites. For the other method, *in vitro* propagated plants were first transferred to peat pots and grown in a flowing seawater system for six weeks. These transplants showed 20 to 80% survival. *Ruppia maritima* was still growing in experimental plots after 11 months at three of the four sites. There was an increase in the number of short shoots m and the percent cover. After 23 months, there was decreased cover of *R. maritima* and an increase in *Zostera marina* In Experiment 2, *R maritima* was propagated *in vitro* using a modified culture medium. Plants from these cultures were directly rooted *ex vitro* in peat pots during six weeks growth in a flowing seawater system. These planting units were transplanted to three sites. After 12 months, the experimental plots showed significant coverage of *R. maritima* at two sites. The other site was a more exposed location and had no *R. maritima* in the experimental plots from either Experiment 1 or 2, probably due to the severe winter storm of 1993. The increase in shoot numbers and areal coverage in the experimental plots suggests that *R maritima* can be propagated *in vitro* and used successfully for habitat restoration.

ADDITIONAL INDEX WORDS: *Micropropagation, restoration,* Zostera marina, in vitro *culture, seagrasses, biotechnology.*

INTRODUCTION

Coastal revegetation is important for stabilization of spoil banks, mitigation and ecosystem restoration. Revegetation projects include coastal dunes, mangrove areas, salt marshes and seagrass meadows (LEWIS, 1982). Seagrass meadows occur in areas ranging from estuaries and lagoons to tidal flats. Because seagrasses provide important habitat and trophic structure for marine fisheries, they have been the focus of considerable restoration efforts. Current restoration technology generally uses seagrass plugs or rhizome segments collected from a donor seagrass bed. There is some concern that large removals of seagrasses could adversely affect donor beds, especially of the slower growing species.

One alternative to the use of plants from donor seagrass meadows is propagated plants. The technologies for propagating seagrasses are still immature. There is some success with cultivation in tanks (KIRKMAN, 1978; SHORT, 1985). Another alternative is to use *in vitro* propagated plants. *In vitro* propagation, a type of plant tissue culture, is now widely used in the commercial horticulture industry (PIERIK, 1987). With the recent development of *in vitro* propagation techniques for the seagrass *Ruppia maritima* L. (KOCH and DURAKO, 1991; BIRD *et al.*, 1993), we decided to examine whether plants propagated using such techniques could be transferred back to the field. We considered this to be a critical step before continuing research on other seagrass species or implementation of large scale restoration projects.

In our first experiment, we propagated and rooted *R. maritima in vitro* at a salinity of 17 ppt. In this experiment, we compared the effectiveness of plants grown in peat pots before field transplanting to that of plants attached to staples.

In our second experiment, we propagated *R. maritima in vitro* using a salinity of 5 ppt which

93101 received 22 September 1993; accepted in revision 17 December 1993

led to faster growth in culture (BIRD *et al.*, 1993). Also, instead of using *in vitro* rooting techniques, plants were rooted *ex vitro* directly in peat pots before planting in the field. In this second experiment, there were no trials using cultured plants attached to staples.

MATERIALS AND METHODS

In Vitro Propagation

Ruppia maritima was propagated in Wilmington using existing cultures that were started from plants collected at Beaufort, North Carolina in 1990. Cultures used for Experiment 1 were grown using methods similar to KOCH and DURAKO (1991). The culture medium consisted of half strength Murashige and Skoog medium, artificial seawater (Instant Ocean®, Aquarium Systems, Mentor, Ohio) of 17 ppt, 1% sucrose (w/v) and 3 mg L^{-1} of the cytokinin 2iP (6[γ,γ-dimethylallylamino]-purine). The pH of the culture medium was 5.7. The cultures were contained in 473 ml clear polypropylene culture vessels (Better Plastics, Kissimmee, Florida) grown under a light fluence of approximately 60 μM m sec $^{-1}$. After sufficient cultures were grown, they were then transferred to a rooting medium of 17 ppt artificial seawater with 0.232 g L^{-1} $NaCO_3$, pH 7.0. After roots developed, the plants were transferred to 10 × 10 cm peat pots filled with a 50% top soil and 50% beach sand mix. The potted plants were placed in an outdoor tank with flowing seawater. Initially the plants were covered with netting for 2 weeks to allow acclimation to full sunlight. The plants grew for a total period of six weeks in the peat pots. Those plants used for the staple experiment were taken directly from the rooting medium.

Cultures used for Experiment 2 were grown in half strength Murashige and Skoog medium, Instant Ocean of 5 ppt, 1% sucrose (w/v) and 3 mg L^{-1} of 2iP (BIRD *et al.*, 1993). The pH, sucrose and cytokinin concentration and culture vessels were the same as in the first experiment. After *in vitro* multiplication for six weeks, plants were transferred directly into 10 × 10 cm peat pots for *ex vitro* rooting and grown in the aquaculture system for six weeks, including a two week period with shading. At the time of transplanting, we noticed that some of these plants were flowering.

Transplanting

Plants for Experiment 1 were transferred from Wilmington to Beaufort, NC, on August 2, 1991. At the site laboratory, one hundred plants of at least five nodes in length were attached to metal staples using floral twist ties. We then planted four field sites with plants growing in peat pots and those attached to metal staples: Middle Marsh 1, Middle Marsh 2 in the Rachel Carson Estuarine Reserve (National Oceanographic and Atmospheric Administration), a dredge island adjacent to Harker's Island and Pritrich Pond next to the NOAA laboratory. Each site had two 4 m^2 plots, one with peat pots and the other with plants on staples. There were 25 plantings (planting units or PU's) in each plot, placed on 0.5 m spaced centers. Prior to planting, any small amounts of other seagrasses in the plots were removed by hand. No *R. maritima* was found in the plots prior to planting. The sides of the peat pot PU's were peeled down by hand prior to placing them in the substrate.

For Experiment 2, the plants were transferred to Beaufort on June 30, 1992. Sites were planted at Middle Marsh 1 and 2 and Harker's Island. Each site had two 4 m^2 plots subdivided into four 1 m plots. Planting units were again placed on 0.5 m centers, and PU's for each spot were selected haphazardly. Again, prior to planting, any small amounts of other seagrasses were removed by hand.

Time zero measurements for all sites in Experiment 1 included determination of the number of PU's per site, number of shoots in three randomly chosen PU's (peat pot or staple unit), and area coverage by those three PU's. Plots were revisited after 1 month (on September 5, 1991) when the percent survival of the transplanting units and the changes in the numbers of shoots per plants were determined. Measurements of transplanting success at the end of 11 months (on June 26, 1992) for Experiment 1 included percent bottom covered and number of shoots m^{-2}. For percent cover, a 1 m^2 grid divided into 16 equal quadrats was laid down on each 1 m^2 sector of the 4 m^2 plots. The number of quadrats which contained seagrasses and the species were noted for determination of coverage.

At the end of 23 months for Experiment 1 (on June 30, 1993), the plots for Experiment 1 were revisited. The percent cover was determined for each plot as described previously. An adjacent, unplanted comparison area of 4 m^2 was also counted at a distance of 0.5 m from the experimental plots at both Middle Marsh sites.

At the end of 12 months for Experiment 2 (on

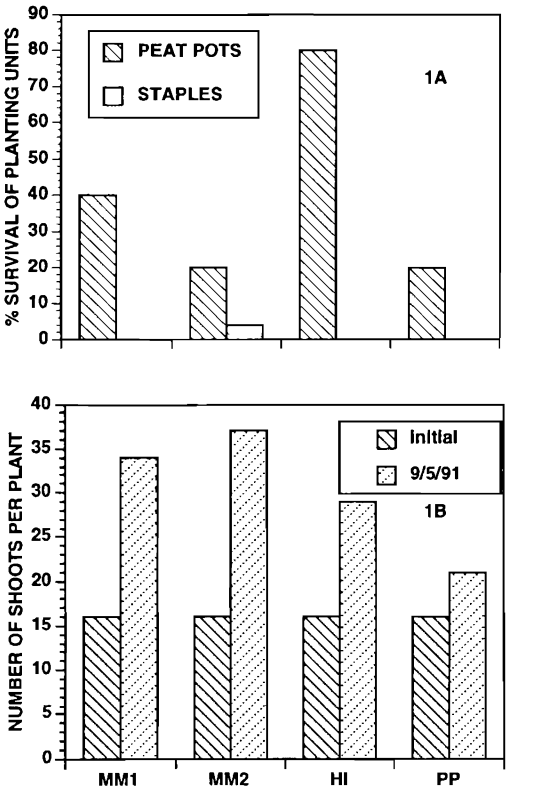

Figure 1. (A.) Percent survival of *in vitro* propagated *Ruppia maritima* transplanted by attachment to staples or as plants grown in peat pots. N = 25 planting units for each planting method at each of 4 sites. (B.) Increase in shoot numbers per planting unit after one month compared to the initial shoot numbers for the peat pot planting units at the 4 sites. MM1 and MM2 are sites at Middle Marsh 1 and 2, respectively. HI is Harker's Island and PP is Pritrich Pond.

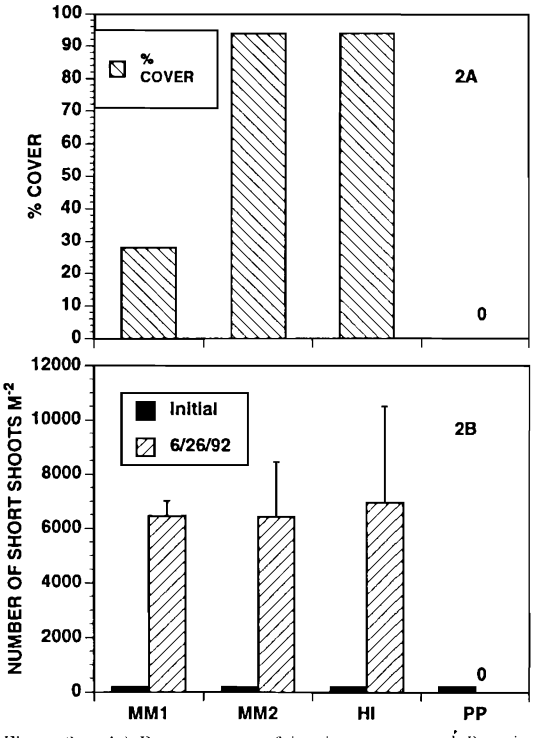

Figure 2. (A.) Percent cover of *in vitro* propagated *Ruppia maritima* at 4 sites for the first experiment 11 months after transplanting in the field. (B.) Number of short shoots m at the beginning of Experiment 1 (August 2, 1991) compared to the number of short shoots 11 months later at four sites (June 26, 1992). MM1 and MM2 are sites at Middle Marsh 1 and 2, respectively, HI is Harker's Island and PP is Pritrich Pond.

June 30, 1993), the two plots at each of three sites were visited. The percent cover was determined for each plot as described for Experiment 1. We used the same data from adjacent, unplanted areas taken for Experiment 1 at 23 months (described in the preceding paragraph) for comparisons with the data from Experiment 2.

RESULTS

Experiment 1

After one month there were observable differences in the percentage of potted plant units *versus* stapled plant units. Survival of peat pot PU's ranged from 20–80% at the four sites (Figure 1A). Survival of stapled PU's was extremely low, with only one of the four sites having any surviving stapled PU's (4% at Middle Marsh 2). No stapled PU's were found at the other three sites. The number of shoots per plant increased over this one month period in all the peat pot PU's (Figure 1B).

The peat pot PU's were evaluated after 11 months. None of the transplants had survived at the Pritrich pond site (Figure 2A and B). At the other three sites, *Ruppia maritima* in the peat pot PU's had begun to coalesce and areal coverage was surveyed on a whole plot basis. The total coverage by *R. maritima* in the three plots with *R. maritima* ranged from 28 to 94% of the planting area. (Figure 2A). The number of short shoots m $^{-2}$ increased from 200 at the time of planting to a range of 5,400 to 6,800 (Figure 2B). There were only sporadic short shoots of *Zostera marina* present in the planted plots.

After 23 months, the plots at Middle Marsh 1 and 2 showed a decline in *Ruppia maritima* cover (Figure 2A compared to Figures 3A and B). The percent cover of *R. maritima* in the experimental plots was less than that of the nearby comparison plot. We noticed an increase in the seagrass *Zostera marina* in the experimental plots so we measured its percent cover. The percent cover by *Z. marina* L. in these experimental plots was similar to that in the comparison plot (Figure 3A and B). The experimental plots at the dredge island near Harker's Island had no seagrasses. These sites were more exposed, and were subject to a long (*ca.* 3 km) wave fetch and high winds (sustained speeds of 110 km hr $^{-1}$, gusts of 180 km hr $^{-1}$) during the severe winter storm that struck the U.S. east coast in March of 1993.

Experiment 2

The three sites were inspected 12 months after planting. The percent cover of *Ruppia maritima* in the two experimental plots at Middle Marsh 1 (Figure 3A) varied from 12 to 43%, and in Middle Marsh 2, it varied from 80 to 99% (Figure 3B). At the Middle Marsh 1 site, the percent cover of *R. maritima* was similar to the comparison plot; but at the Middle Marsh 2 site, the planted plots showed a higher percent cover than the comparison site. There was slightly greater percent cover of *Zostera marina* than *R. maritima* at Middle Marsh 1, but no noticeable differences between the two seagrass species at Middle Marsh 2. The percent cover of *Z. marina* within the experimental plot was similar to that of the comparison plot at both sites (Figure 3). As in Experiment 1, the experimental plots at the dredge island near Harker's Island had no seagrasses.

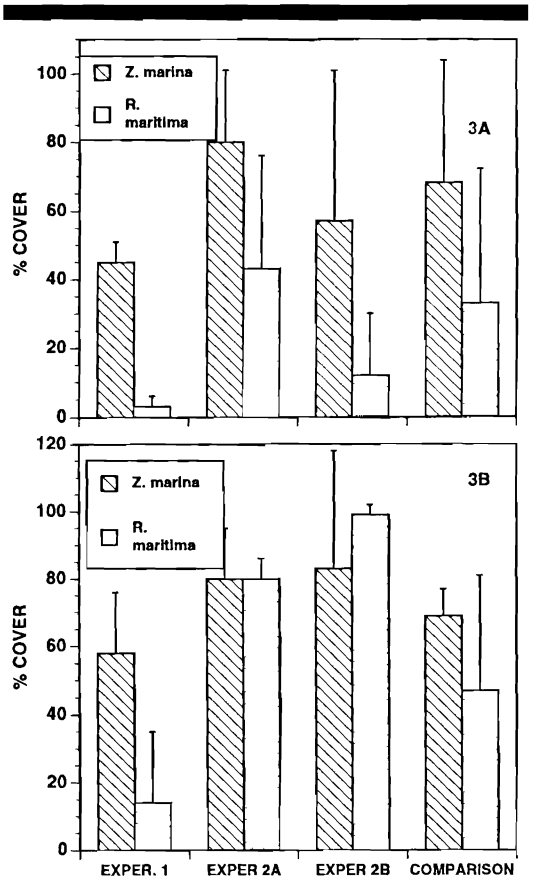

Figure 3. (A.) The percent cover of *Ruppia maritima* and *Zostera marina* in the experimental plots at Middle Marsh Site 1 for Experiment 1 after 23 months and Experiment 2 after 12 months. There were two plots for Experiment 2 labeled 2A and 2B. Percent cover of a nearby comparison plot was also determined. The means were determined from using the four subsamples taken from these plots and the lines denote the standard deviations of these means. (B.) The percent cover of *Ruppia maritima* and *Zostera marina* in the experimental plots at Middle Marsh Site 2 for Experiment 1 after 23 months and Experiment 2 after 12 months. There were two plots for Experiment 2 labeled 2A and 2B. Percent cover of a nearby comparison plot was also determined. The means were determined from using the four subsamples and the lines denote the standard deviations of these means.

DISCUSSION

Seagrass restoration practices incorporate a large number of techniques and approaches. Transplant studies with *Zostera marina*, *Halodule wrightii* Ascherson, and *Syringodium filiforme* Kützing have shown that the metal staple method of securing the seagrasses to the sediment works well in most cases (Fonseca *et al.*, 1985, 1987). The poor success we had with using *Ruppia maritima* held by staples may be a reflection of its morphology in culture. These plants have thinner rhizomes than field collected plants. The thin rhizomes could have possibly abraded against the metal staple. Stout and Heck (1991) reported success using the staple technique with field collected *R. maritima*. In retrospect, a field collected *R. maritima* staple control should have been included in this experiment for comparative purposes.

Both of these experiments suggest that the *in vitro* cultured *R. maritima* grew when transplanted to the field. There was a marked increase in shoot number m $^{-2}$ in the first experiment, as well

as areal coverage. In Experiment 1, three of these sites showed *R. maritima* coverage after 11 months. The fourth site, Pritrich Pond, is an area characterized by greater water turbidity and more boat traffic. It may be unsuitable for seagrass habitat.

In Experiment 2, one of the Middle March sites showed an increase in percent cover of the experimental plots in relationship to the comparison plot. These comparison plots may have had *R. maritima* plants recruited from seeds (possibly even those produced by the transplants themselves). The data suggest that the transplants did better in Experiment 1 than in Experiment 2 as there was more percent cover for the first year at all three sites. Factors that contributed to lower areal cover between the two experiments might also be responsible for the decline in cover of the experimental plots from Experiment 1 when measured at 11 *versus* 23 months. Such factors might be storm effects or an apparently successful recruitment of *Zostera marina* into the experimental plots. A large number of physical factors such as temperature and salinity also affect the growth and distribution of *R. maritima* (DUNTON, 1990; LAZAR and DAWES, 1991). Because we did not carry out an extensive monitoring study, it is impossible to suggest what factors led to these differences. Certainly, the severe winter storm of 1993 impacted the experiments at the dredge island near Harker's Island as it did several natural beds (FONSECA, *personal observations*). This site was exposed on several sides. The Middle Marsh sites were protected by marsh and oyster bar on four sides, and were probably less impacted by the winter storm.

In Experiment 1, there was little observable *Zostera marina* recruitment into the experimental plots after 11 months. When these plots and those of Experiment 2 were examined in June of 1993, coverage of *Z. marina* was as great or greater than *R. maritima*. Possibly, *Z. marina* might have competitively displaced *R. maritima*. This increase in *Z. marina* might be due to a period of successful seed dispersal and recruitment. The differences in recruitment between the two years suggests high variability in seedling germination and recruitment for this species as reported elsewhere (CHURCHILL, 1983).

These data suggest that *in vitro* propagated *Ruppia maritima* represents a viable source of planting material for habitat restoration. Thus, we are encouraged in our efforts to similarly propagate other seagrass species (*e.g.*, *Zostera marina* and *Thalassia testudinum* Banks *ex* König). There did not appear to be any differences between the two *in vitro* propagation media on the success of the transplants. The latter method requires less artificial sea salts and results in faster growth (BIRD *et al.*, 1993). The second experiment also suggested successful transplantation occurred with plants that were rooted *ex vitro*. This procedure saves the time and cost of *in vitro* rooting procedures. However, *in vitro* rooting might be beneficial if transplanting methods can be developed which do not require the use of peat pots.

We have propagated *R. maritima* in the lab for more than four years with year-round growth. The use of *in vitro* cultured *R. maritima* can allow planting to take place throughout the year to take advantage of seasonal windows or periods. One planting strategy could include transplanting *R. maritima* out into the field several months before the onset of the local reproductive season. The occurrence of flowering plants from our cultures indicates that *in vitro* culture methods do not affect the plants' abilities to flower. This approach could lead to both vegetative spread of plants and to dispersal by seed.

The use of propagated seagrasses has great promise for not only slow growing seagrass species, but particularly for large scale planting projects. Small projects of the kind often associated with Clean Water Act permits can readily use naturally occurring seagrass beds with little impact to the donor bed. However, large scale restoration projects covering many hectares would likely seriously impact existing donor beds. Although large scale projects have been rare in the past, the advent of cost-effective propagation methods makes their consideration plausible. Research is required to determine the scale of restoration projects whose cost (both ecological and financial) would benefit from use of plants produced from different types of propagation techniques.

ACKNOWLEDGEMENTS

The authors wish to thank B. Cody, C. O'Hara and Paula Whitfield for their assistance with this project. This project was supported in part by the National Oceanographic and Atmospheric Administration Coastal Ocean Program (Grant #NA90AA-D-SG776) and North Carolina Sea Grant College Program under Grant #NA90AA-D-SG062. The views expressed herein are those of the authors and do not necessarily reflect the

views of NOAA or any of its sub-agencies. This is CMSR Contribution No. 103.

LITERATURE CITED

BIRD, K.T.; CODY, B.R.; JEWETT-SMITH, J., and KANE, M.E., 1993. Salinity effects on *Ruppia maritima* cultured *in vitro*. *Botanica Marina*, 36, 23–28.

CHURCHILL, A.C., 1983. Field studies on seed germination and seedling development in *Zostera marina* L. *Aquatic Botany*, 16, 21–29.

DUNTON, K.H., 1990. Production ecology of *Ruppia maritima* L.s.l. and *Halodule wrightii* Aschers, in two subtropical estuaries. *Journal of Experimental Marine Biology and Ecology*, 143, 147–164.

FONSECA, M.S.; KENWORTHY, W.J., and THAYER, G.W., 1987. Transplanting of the seagrasses *Halodule wrightii, Syringodium filiforme* and *Thalassia testudinum* for sediment stabilization and natural development in the southeast region of the United States. U.S. Army Corps of Engineers Final Report. Technical Report Number EL-87-8. U.S. Army Corps of Engineers, Washington, DC, 49p.

FONSECA, M.S.; KENWORTHY, W.J.; THAYER, G.W.; HELLER, D.Y., and CHEAP, K.M., 1985. Transplanting of the seagrass *Zostera marina* and *Halodule wrightii* for sediment stabilization and habitat development on the east coast of the United States. U.S. Army Corps of Engineers Final Report. Technical Report Number EL-85-9. U.S. Army Corps of Engineers, Washington, DC, 49p.

KIRKMAN, H., 1978. Growing *Zostera capricorni* Aschers. in tanks. *Aquatic Botany*, 4, 367–372.

KOCH, E. W. and DURAKO, M.J., 1991. *In vitro* studies of the submerged angiosperm *Ruppia maritima*: Auxin and cytokinin effects on plant growth and development. *Marine Biology*, 110, 1–6.

LAZAR, A.C. and DAWES, C.J., 1991. A seasonal study of the seagrass *Ruppia maritima* L. in Tampa Bay, Florida. Organic constituents and tolerances to salinity and temperature. *Botanica Marina*, 34, 256–269.

LEWIS, R.R. (ed.), 1982. *Creation and Restoration of Coastal Plant Communities*. Boca Raton, Florida: CRC Press, 219p.

PIERIK, R.L.M., 1987. *In Vitro Culture of Higher Plants*. Dordrecht, The Netherlands: Martin Nijhoff Publishers, 344p.

SHORT, F.T., 1985. Method for the culture of tropical seagrasses. *Aquatic Botany*, 22, 187–193.

STOUT, J.P. and HECK, K.L., JR., 1991. Reintroduction of oligohaline estuarine grassbeds: Techniques and functional ecology. Dauphin Island Sea Lab Technical Report No. 91-001., Dauphin Island Sea Lab, Dauphin Island, Alabama. 58p.