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AXENIC CULTURE OF THALASSIA TESTUDINUM BANKS EX KÖNIG (HYDROCHARITACEAE)[1]

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ABSTRACT

Cultures of *Thalassia testudinum* were established and maintained in the absence of other detectable organisms. Axenic cultures were initiated using surface sterilized seeds which were aseptically dissected from surface sterilized fruits. Seedlings were cultured in 75-ml (25-mm × 200-mm) culture tubes containing 30 ml of rooting substrate and 40 ml of chemically defined seawater media. Seedlings and culture media were analyzed for microbial contamination after 42 days of culture utilizing standard marine bacterial fungal isolating procedures and by light and scanning electron microscopy. Axenic seagrass cultures allow physiological studies such as nutrient assimilation kinetics, rhizosphere and phyllosphere microbial interactions through mono- and poly-axenic seagrass-microbial cultures.

AT THE FIRST INTERNATIONAL SEAGRASS WORKSHOP held in Leiden, The Netherlands, axenic culture of seagrasses was recommended as a research program needing investigation (McRoy, 1973). Axenic cultures provide aseptic material to study plant physiological responses to physical, chemical and biological variables, without microbial interferences. This technique could also be utilized for investigations on seagrass-microbial interactions at the phyllosphere and rhizosphere level.

Axenic culture of terrestrial plants has commonly been utilized to investigate fundamental plant biology, and complex plant-microorganism associations (Reuzer, 1962; Hale, Lindsey and Hameed, 1973). Algae and several aquatic vascular plants have also been cultured axenically (Pringsheim and Pringsheim, 1962; Wiedman, Walne and Trainor, 1964; Forsberg, 1966; Wetzel and McGregor, 1968 and Brown, 1982). However, successful seagrass axenic culture has not been reported, although *Ruppia maritima* L. has been established from seed and grown in algae-free cultures, but axenicity was not determined (Seeliger, Cordazzo and Koch, 1984; Thursby, 1984). In addition, possible axenic protoplasts have been isolated from *Zostera marina* L. (Mazzella et al., 1981), and surface-sterilized seagrass leaf fragments have been used in marine mycoflora studies (Newell and Fell, 1982).

Axenic plant cultures can be established in two ways; by organ and tissue culture, or by surface sterilization of whole plants or seeds (Hale et al., 1973; Sweet and Bolton, 1979). Surface bacteria and fungi are prevalent on *Thalassia testudinum* Banks *ex* König and other seagrasses in natura (Meyers et al., 1965; Sieberth et al., 1974; Doohan and Newcomb, 1976; Newell and Fell, 1980, 1982 and Kuo, McComb and Cambridge, 1981). Non-deleterious surface sterilization of whole plants (short-shoots or ramets) is ineffective due to the morphological characteristics of the plant shoots (Moffler, unpubl.). Associated microflora quickly propagate on "surface sterilized" shoots in vitro and may subsequently interfere with physiological studies (Moffler, unpubl.).

McMillan (1980) stated seagrass axenic cultures should be feasible utilizing surface sterilized seeds produced under laboratory conditions. We recently developed an in vitro culture technique for *Thalassia* seedlings in an attempt to follow leaf and root growth (Durako and Moffler, 1981). However, the cultures developed bacterial and fungal infections even though sterile procedures were implemented and seedlings were processed by surface sterilization techniques. As with short-shoots, contamination resulted due to the nature of seed/seedling morphology which allows microorganisms to escape contact with the surface sterilant. Forsberg (1966) indicated short surface sterilization periods (2–5 min) were ineffective and we found longer surface sterilization periods (20–30 min) caused severe injury or mortality to seedlings. Therefore, a modification of seedling surface sterilization techniques was indicated.

MATERIALS AND METHODS—*Collection of plant material*—*Thalassia testudinum* fruits

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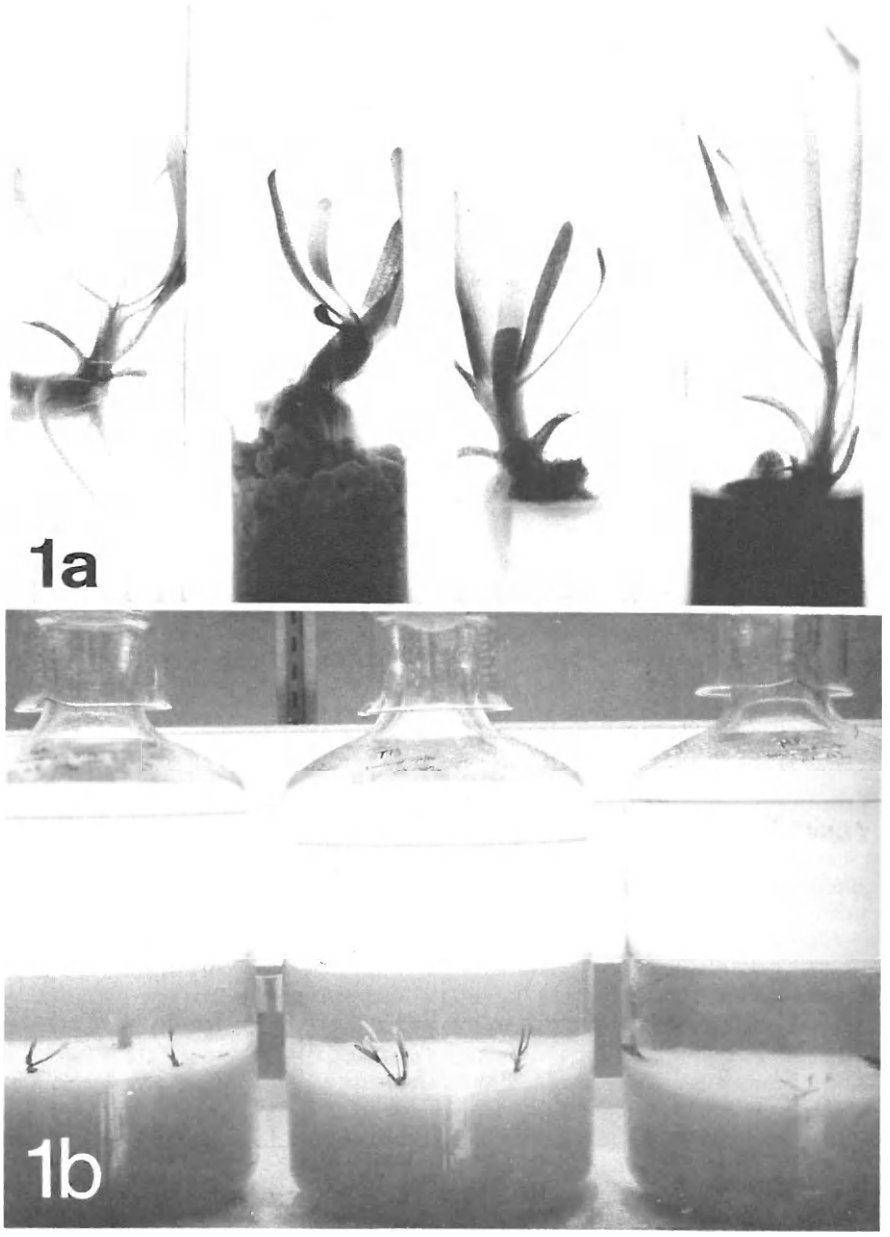

Fig. 1. *Thalassia testudinum* A. Axenic seedling cultures from left to right: without rooting substrate, aragonite, marine agar and marine agar with charcoal. ×0.88. B. Seedlings growing on marine agar in 12-l glass carboys. ×0.17.

were collected from shoreline drift material. Only healthy-appearing, green, turgid, indehisced fruits were collected. Fruits were placed in an ice chest between seawater moistened paper towels at an approximate temperature of 4 C. Fruits were transported back to the laboratory and processed in less than 36 hr from collection. Transporting fruit at cold temperature prevents fruit dehiscence, but cold exposure should be less than 48 hr since seedling viability is reduced with longer exposure (Moffler and Durako, unpubl.).

Axenic culture establishment—Fruit processing and culture establishment were performed using sterile techniques at room temperature under a laminar flow hood employing HEPA (high-efficiency particulate filter for particles larger than 0.3 m) air filtration. Fruits were surface-sterilized for 10 min in 5% clorox

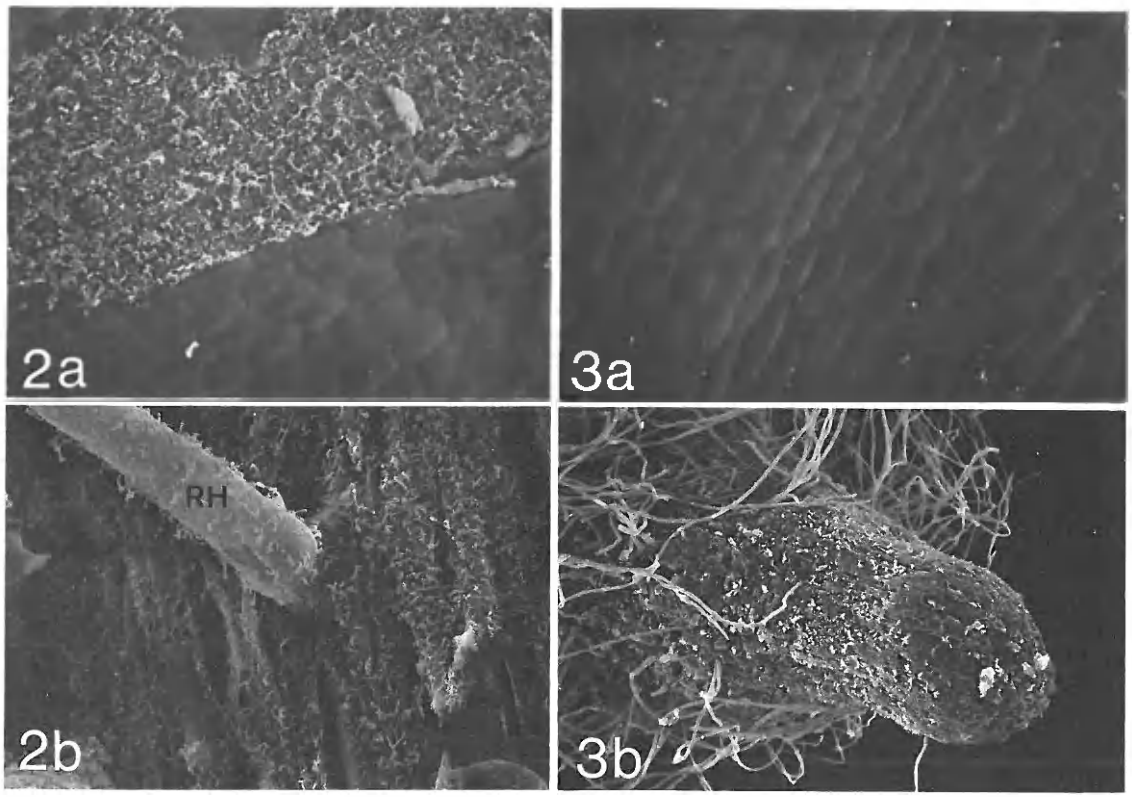

Fig. 2, 3. **2.** *Thalassia testudinum* A. Leaf surface with bacterial colony. ×335. B. Root surface and root hair (RH) portion with bacterial infestation. ×670. **3.** *Thalassia testudinum* A. Leaf surface without microbial contamination, ×335. B. Root tip and root hairs without microbial contamination. ×34.

(i.e., 0.264% v/v sodium hypochlorite) in sterile seawater (32‰) at pH 8.0. Following surface sterilization, fruits were rinsed three times in sterile seawater (32‰) and placed in a holding dish containing sterile seawater with 200 units/ml of penicillin-streptomycin. Fruits remained in the holding solution for approximately 5–10 min.

Thalassia testudinum appears to have viviparous seeds with germination occurring prior to fruit dehiscence (Moffler, unpubl.), hence, the term seedling is used for material dissected from fruits. Seedlings were aseptically dissected from the surface-sterilized fruits and in turn surface sterilized in 70% v/v ethanol in seawater (32‰) for 30 sec. Seedlings were rinsed three times in sterile seawater and placed in a holding dish containing sterile seawater (32‰) with 200 units/ml of penicillin-streptomycin for 10 min.

Individual seedlings were placed in sterile 75-ml (25-mm × 200-mm) glass culture tubes and cultured under four different treatments with four replicates of each treatment. Three of the treatments were comprised of 40 ml of liquid media over a semi-solid rooting substrate. Sterile synthetic seawater medium consisting of Gates and Wilson's (1960) NH-15 synthetic seawater medium with modifications presented by Walker (1982) at a pH of 8.0 was introduced over 30 cm^3 of three separate sterile solid substrates: aragonite (crushed shell-coral rock mixture); marine agar; and marine agar + powdered charcoal (carbon decolorizing neutral, 20 g/l). Following autoclaving, 30 ml of sterile marine agar was poured into the culture tubes and allowed to gel, after which the sterile liquid seawater medium was added. The aragonite was obtained locally and autoclaved. A fourth treatment was 70 ml of sterile synthetic seawater medium without a solid subtrate. The agar medium was made with 8 g of purified agar per liter of NH-15. The liquid seawater media was exchanged at monthly intervals.

Cultures were maintained in a positive pressure "clean room" employing UV-sterilized and HEPA-filtered air. Culture tubes were placed in stands on shelving in front of a light bank of four 48-in Vitalite power groove fluorescent lamps with a PAR photonflux density of 690 $ES^{-1}\,m^{-2}$ (Fig. 1A). Cultures were grown

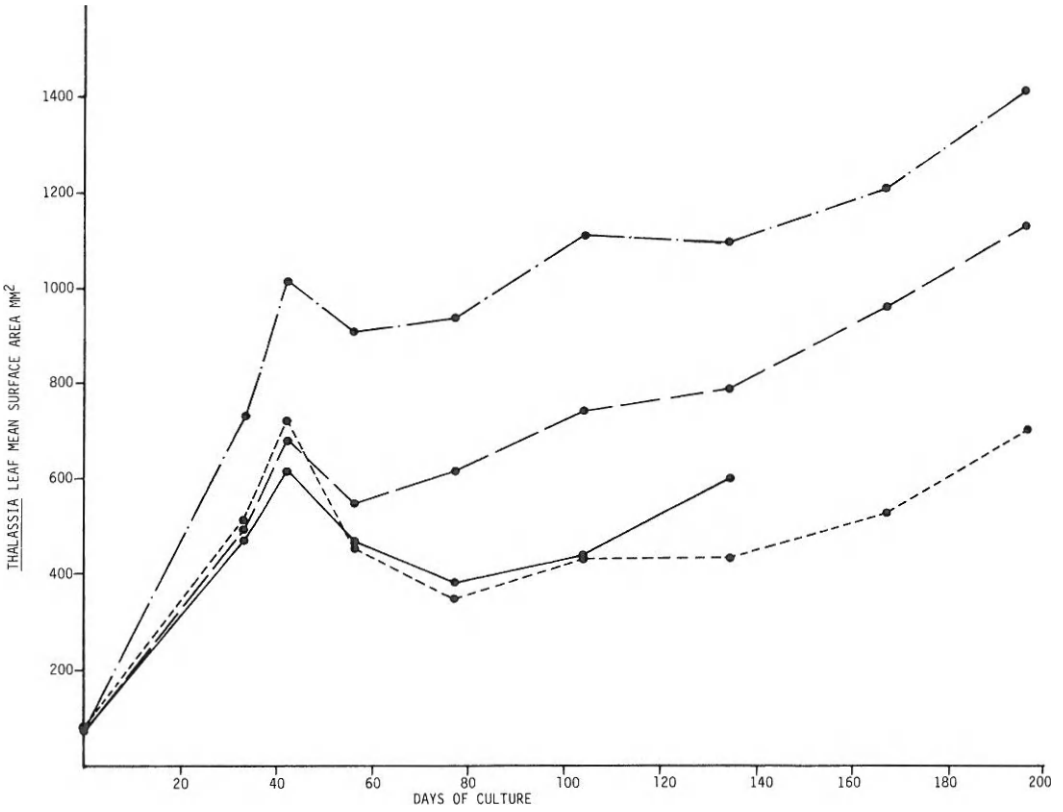

Fig. 4. *Thalassia testudinum* leaf mean surface area growth over time for seedlings growing on four different substrates: (——) no rooting substrate, (·····) marine agar, (— —) aragonite, (— · —) marine agar + charcoal.

on a 14:10 hr light:dark photoperiod at 27 C. In addition to tube cultures, Figure 1B shows *Thalassia* seedlings established in 12-l glass carboys allowing the study of rhizome and short-shoot production and growth.

Testing for axenicity—*Thalassia* cultures were tested for apparent axenicity after 42 days of culture. Possible marine bacteria and fungal contaminants were checked by standard inoculation and incubation (both broth and agar with artificial seawater base) into a peptone yeast extraction marine medium and a glycerin marine medium (Schlieper, 1972). In addition to inoculation with liquid media, aseptically removed *Thalassia* leaf sections were incubated on the microbial nutrient media. Microbial cultures were analyzed visually and by light microscopy after 7, 14 and 21 days for the presence of microbial colonies. Scanning electron microscopy (SEM) was also used for plant surface microbe detection on excised tissue segments. Tissue pieces underwent 5% glutaraldehyde-seawater fixation at 5 C under vacuum for 72 hr. Post-fixation utilized 1% OsO_4-seawater at 5 C under vacuum for 1 hr. Tissue was dehydrated by an ethanol series under vacuum and critical point dried. Tissue was coated with gold and observed on a Hitachi HHS-2R SEM.

Relative growth measurements—In order to avoid possible contamination, relative growth (leaf mean surface area) was estimated at approximately 14-day intervals using a metric ruler behind or alongside the culture tube (see Durako and Moffler, 1981). Therefore, these measurements only represent an estimate of relative growth.

RESULTS AND DISCUSSION—Utilizing the above fruit/seedling surface-sterilization procedure, we were able to establish axenic *Thalassia testudinum* cultures. Most cultures when tested for axenicity did not contain detectable microorganisms. However, several cultures did contain bacterial contamination as evidenced in Fig. 2A, B. The contaminated cultures were treated with 500 units/ml of penicillin-streptomycin and axenicity retested seven days after antibiotic treatment. Bacterial contamination was not detectable by microbial nutrient media

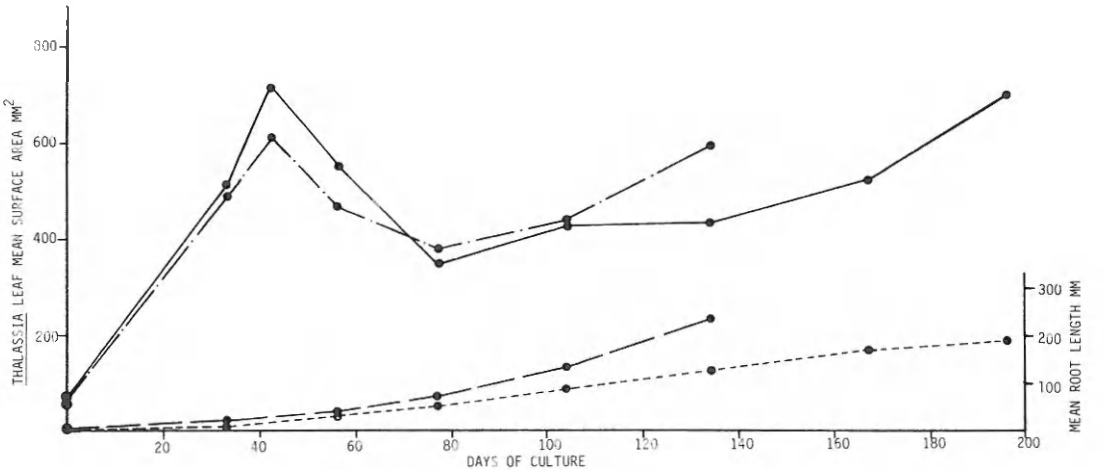

Fig. 5. *Thalassia testudinum* leaf mean surface area and mean root length growth over time for seedlings growing on two different substrates: (——) mean leaf area for seedlings on marine agar, (— · —) mean leaf area for seedlings growing without rooting substrate, (— —) mean root length for seedlings on marine agar, (-----) mean root length for no rooting substrate.

or SEM techniques after the second antibiotic treatment (Fig. 3A, B).

Thalassia seedling growth rates were highest in the tubes containing aragonite or charcoal as the rooting substrate (Fig. 4). These two substrates were more opaque than the semi-transparent marine agar and very transparent tubes without solid substrate. This may indicate that exposure of *Thalassia* seedling roots to light inhibits growth. Higher growth in the agar with charcoal media may reflect the removal by the charcoal of an inhibitory substance or metabolite from the media. Another possible explanation is that higher growth rates may reflect increased nutrient uptake, with the particulate matrix allowing for better ion exchange (Rosenfeld, 1979; Iizumi, Hattori and McRoy, 1982).

New leaves became visually evident at 14-day intervals and may be photosynthetic in vitro for 21–28 days or longer. Zieman (1968) and Patriquin (1973) observed that new foliage leaves developed at approximately 14–16-day intervals in situ, suggesting that the plastochron interval in *Thalassia* may be relatively constant with age. After 40 days of culture, green leaf surface area declined for approximately 20 days (Fig. 5). Leaf surface area gradually increased again. The decline in leaf surface area reflects older leaf senescence without the addition of new leaves. Concomitant with the decline of leaf surface area was the production and growth of roots, suggesting resources may temporarily be shunted from leaf growth to root production. This particular phenomenon needs further detailed investigation.

Axenic seagrass cultures will be important for delineating physiological characteristics while eliminating interference from epiflora. Research has been conducted under field conditions on various aspects of seagrass growth, productivity, nutrient uptake and transfer, and nitrogen fixation (Patriquin, 1972, 1973; Harlin, 1975, 1980; Zieman, 1975; Capone and Taylor, 1977; Capone et al., 1979; Schroeder and Thorhaug, 1980; Pulich, 1982; Smith, Hayasaka and Thayer, 1984). While these studies are important to our understanding of seagrass biology, it is also important to determine specific interactions of variables without the influence of field environmental pressures. Axenic culture specifically allows the study of variables without interference of the microbial epiflora so it will be especially important in nutrient assimilation kinetics investigations.

The culture technique may be used as a tool for investigating specific rhizosphere and phyllosphere microbial interactions through mono- and poly-axenic seagrass-microbial cultures (Capone and Taylor, 1977; Kuo et al., 1981; Reuzer, 1962 and Smith et al., 1984). Separation of the solid substrate from the overlying seawater with an ion and oxygen impermeable inert matrix could allow investigation of anaerobic microbial-seagrass interaction including root exudate effects (Wetzel, 1969; Kuo et al., 1981; Wood and Hayasaka, 1981 and Smith, Kozuchi and Hayasaka, 1982). Axenic culture will also provide an aseptic tissue source for tissue and cell culture studies. The axenic culture techniques presented in this paper achieve axenicity of seedlings collected from

the environment without significant mortality and should be applicable to other seagrass species.

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