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Nancy B. Elliott
D. Craig Edwards
and
Paul J. Godfrey

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Production Editors
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Cover Photo: Dr. Lynn Margulis, Symposium Keynote Speaker, describes the structure and ecology of living stromatolites. Some, visible as grayish mounds near her feet, line the shore of Storrs Lake whereas others occur farther out in deep water. (See paper by D. C. Edwards, this volume).

Back Cover Photo: Group photo of the 6th Symposium participants and speakers.

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THE KLEBSIELLA-HALODULE SYMBIOSIS: DISTRIBUTION AND PHYSIOLOGY OF THE ENDOPHYTE

Garriet W. Smith
Department of Biology
University of South Carolina at Aiken
Aiken, SC 29801

ABSTRACT

The seagrass *Halodule wrightii* has, for some time, been known as a pioneering species. This species, along with *Syringodium filliforme*, is the major pioneering seagrasses in Caribbean coastal environments. *H. wrightii* is able to establish productive stands in nutrient-poor sediments and oligotrophic waters when other species tend to fail. Metabolic activity and nitrogen fixation studies on *H. wrightii* root-rhizomes showed that activity could not be eliminated by surface-sterilization. Light and electron microscopy revealed that root-rhizome segments contained bacteria located primarily in intercellular spaces in cortical tissue. These bacteria were isolated and identified as a *Klebsiella* species. Pure cultures were used to prepare fluorescent antibody tags for the isolate. These tags showed that the endophytic relationship was widespread in root-rhizome tissue. Oxygen, pH, and salinity ranges were determined for endophytic growth and nitrogenase activity. Carbon source utilization patterns indicated that this specific relationship extends from the US east coast to the east coast of Central America.

INTRODUCTION

Specific symbiotic associations between bacteria and plants have been known for a long time. These associations range from pathogenic to beneficial. Among the many beneficial associations, there exist a wide range of specificity between the bacterium and the plant. For example, a number of bacteria occur as epiphytes, on either leaves or roots, that provide usable nutrients for the plant by transforming phosphorus (Wehner and Smith, 1994) or fixing nitrogen (Smith, 1987). On the other hand, some interactions between bacteria and plants have become obligate, involving the lateral transfer of sometimes rather large segments of DNA (Margulis, 1991). Other examples of plant-bacterial interactions fall between these extremes.

As with all plants, seagrasses exhibit a wide range of interactions with their microbiota (Craven and Hayasaka, 1982; Morgan and Smith, 1992; Smith and Hayasaka, 1982a; Smith et al., 1984; Wong and Smith, 1994). With the exception of chloroplasts and mitochondria, the most specific relationship between a seagrass and a bacterium is that of the seagrass *Halodule wrightii* and a *Klebsiella* sp., a nitrogen-fixing endosymbiot. This report summarizes what is known about this relationship and reports findings on the distribution of the relationship at various locations along the east coast of the U.S. and in the Caribbean.

ISOLATION AND CHARACTERIZATION

The presence of a putative endosymbiotic organism was first indicated during studies of overall microbial metabolism associated with *H. wrightii* roots (Smith and Hayasaka, 1986). Tetrazolium-linked dehydrogenase assays were conducted on root segments to determine the level of microbial activity in the rhizoplane. A subsample of the root segments were surface sterilized in a hypochlorite solution to remove the rhizoplane microbial community. Microscopic observations indicated that the community was eliminated, but dehydrogenase activity, although less than nontreated samples, was relatively high (Table 1).

Root segments from the hypochlorite treatments were viewed under the microscope. The distribution of reduced tetrazolium (formazan) within the root tissue was not uniform, but concentrated in 'metabolic hot spots' between, and sometimes within, cortical

Table 1. Tetrazolium-linked dehydrogenase activity associated with *H. wrightii* root segments.

TREATMENT	DEHYDROGENASE ACTIVITY (STD. DEV.)
NONE	3.73 (0.32)
HYPOCHLORITE	1.07 (0.06)
TETRACYCLINE	.32 (0.11)
TETRACYCLINE + HYPOCHLORITE	.37 (0.01)
AZIDE	.21 (0.02)
AZIDE + HYPOCHLORITE	.14 (0.02)

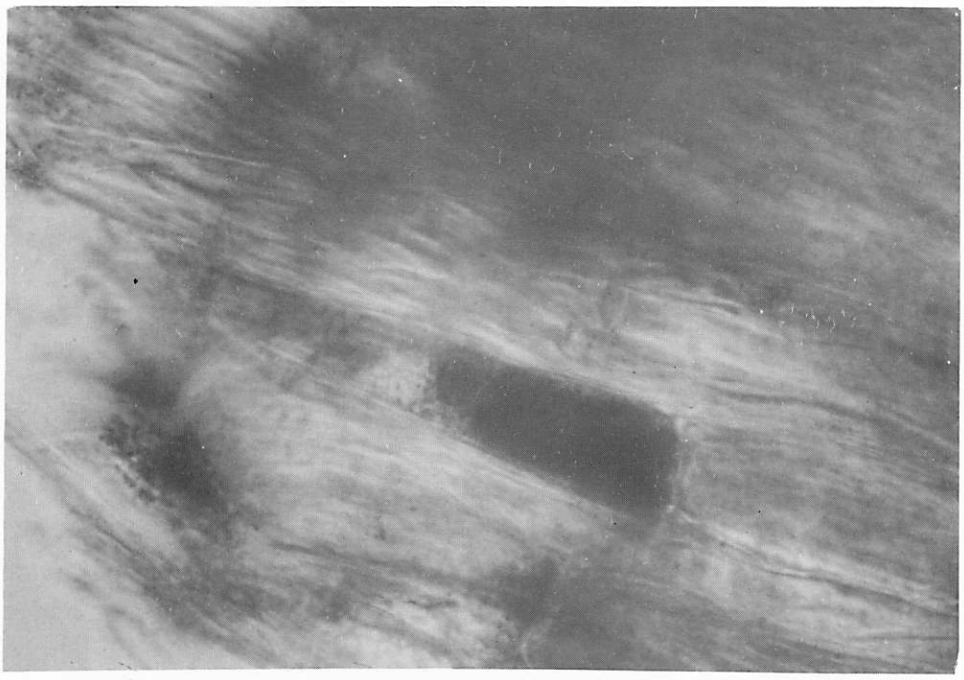

Figure 1. Photomicrograph of tetrazolium reduction in a *Halodule wrightii* root segment magnified over 1500X. Note root cortical cell filled with metabolising (as indicated by dark colored formazan particules) bacteria.

cells (Figure 1).

Slices were aseptically made through these 'hot spots' and the cut ends were placed on the surface of glycerol artificial seawater plates (GASW, previously chosen for its' nonspecificity). In every case, a single bacterial type grew from the cut surfaces (Smith and Hayasaka, 1982). These bacteria were exposed to a number of standard biochemical tests which placed them in the genus *Klebsiella*, most closely to *K. pneumoniae* type 4B. To confirm that these isolates were the ones responsible for the root dehydrogenase activity, antibodies were prepared against them by inoculating rabbits, harvesting the serum, removing nonspecific antibodies by cross-adsorption, and exposing the antibodies to thin sections of plastic embedded 'hot spots' of *H. wrightii* root sections. These root sections were then exposed to anti-rabbit fluorescein-conjugated goat immunoglobulin and viewed under an epifluorescent microscope (Schmidt and Hayasaka, 1985). Areas of fluorescence were

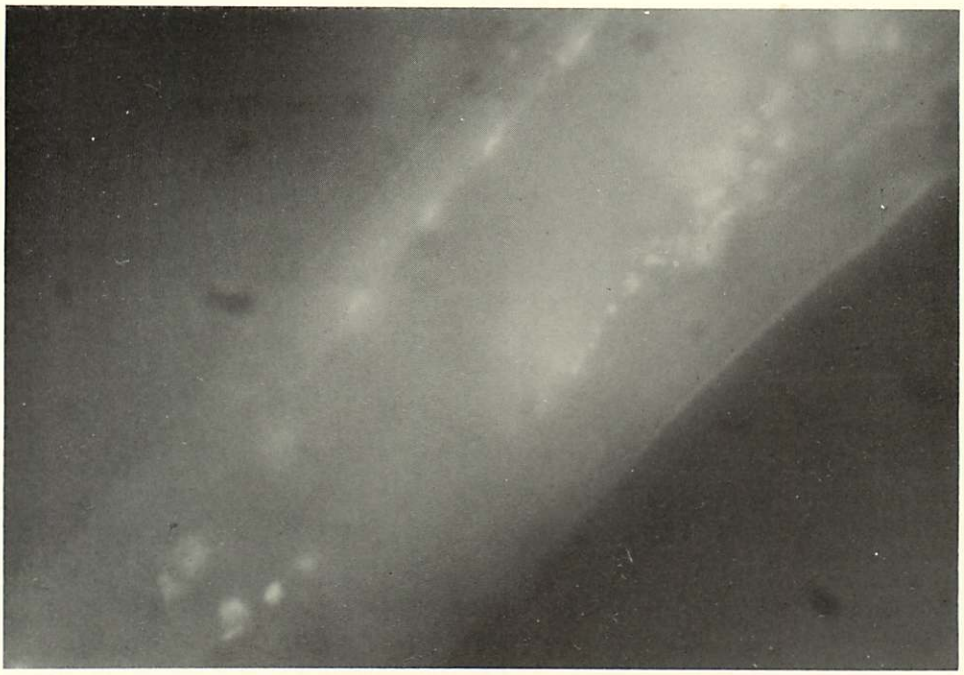

Figure 2. Fluorescent-antibody stained *H. wrightii* root segment showing the presence of the bacterial isolate KP2 (light areas). Magnification over 1500X.

observed that corresponded with dehydrogenase activty (Figure 2). The isolates, therefore, grown on the GASW medium from the slices through *H. wrightii* metabolic 'hot spots' and identified as *Klebsiella* were also the bacteria colonizing root cortical tissue. The isolate was designated KP2.

ENDOPHYTIC NITROGEN FIXATION

Klebsiella-like bacteria had previously been isolated from terrestrial plants (Aho *et al.*, 1974; Bagley *et al.*,1978; Haahatela *et al.*, 1981; Nelson *et al.*, 1976; Pedersen *et al.*;1978) where they were implicated in nitrogen fixation. Because washed cells of KP2 could also fix nitrogen (Smith and Hayasaka, 1982) and nitrogen fixation had been reported in association with *H. wrightii* (Patriquin and Knowles. 1972), the role of KP2 in this process was studied. Root segments subjected to the acetylene reduction technique (for the determination of nitrogen fixation) were found to be very active (Smith and Hayasaka, 1982). In addition, when these *H. wrightii* root segments were surface-sterilized, significant nitrogen fixing activity was observed. The amount of nitrogen fixed was temperature dependant, with endophytic activity contributing over thirty percent of the total (Figure 3).

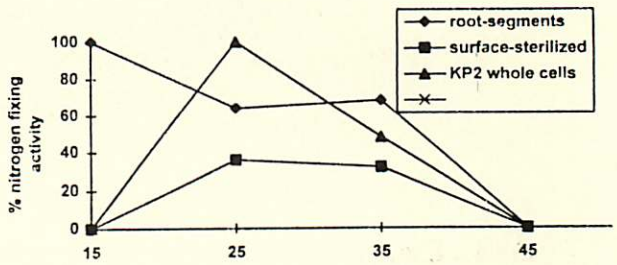

Figure 3. Effect of temperature on the percentage of nitrogen fixing activity.

In separate experiments, both untreated and surface-sterilized root segments showed maximum rates when incubated at 35°C. Assays conducted on a seasonal basis at *in situ* temperatures also indicated that nitrogen fixation was highest during the warmer summer months in North Carolina for *H. wrightii* (Smith and Hayasaka, 1982). Interestingly, the temporate seagrass *Zostera marina*, which grows in dual beds with *H. wrightii*, showed *in situ* rates highest during

the cooler winter months (Smith and Hayasaka, 1982b). Seasonal rates of ammonification by the rhizoplane bacteria, however, were reversed for the two species (Smith *et al.* 1984). This indicates a tightly coupled recycling mechanism, regulated by temperature, may exist between these two species.

Additional studies showed that oxygen played a major role in determining the rate of nitrogen fixation by KP2. Assays conducted on root segments showed that under anaerobic conditions, nitrogen fixation was significantly reduced with untreated roots and eliminated with surface-sterilized roots (Smith and Hayasaka, 1982). However, assays conducted on whole KP2 cells showed significant increases in nitrogen fixation rates under anaerobic conditions (Davidson, 1988). KP2 cells may exist under anaerobic conditions inside root cortical cells, but the mechanism by which oxygen is excluded from these cells is unknown. Diffusion of oxgen into the root should be impeded by the establisment of an extensive mucoid rhizoplane population but some oxygen would be required for the plant to provide the endophyte with an energy source.

Nitrogen fixation assays of isolated root-rhizome segments have generally been conducted without an added carbon source, but whole cell assays have indicated that sucrose stimulated nitrogen fixation over a number of other carbon sources (Davidson, 1988).

DISTRIBUTION

The distribution of the symbiotic relationship between KP2 and *H. wrightii* has been investigated using assays designed to test if there is significant INT activiy with surface-sterilized (SS) root segments, nitrogen fixation associated with SS segments and morphological and biochemical charactistics of isolates from SS roots. Recently, carbon source utilization patterns of isolates from SS roots has been employed to determine the similarity of isolates obtained from *H. wrightii* SS roots. This technique determines which of 95 different carbon sources the isolate is able to metabolize. The data are then entered into a database and comparisons made among isolates obtained from different seagrass species at various locations. Results from these studies are given in Table 2.

SUMMARY

The symbiotic relationship between KP2 and *H. wrightii* appears to be relatively specific. The isolate has not been found in the roots of other seagrass species from the Atlantic, and other *Halodule* species from the Pacific have not been tested. The indication is that there may be specific complementary receptors between these two species, much like the relationship between *Rhizobium* species and specific legumes. If this is the case, KP2 may bind to the seagrass root and induce cell elongation (KP2 does convert tryptophan to indole acetic acid, a plant hormone responsable for cell elongation), thereby gaining entry into the cell. KP2 does show a positive chemotactic response toward seagrass amino acid root exudate (Wood and Hayasaka, 1981). Once in cortical tissue the bacteria may proliferate producing anaerobic conditions toward the

Table 2. Evidence of the KP2-*H. wrightii* symbiosis from various locations.

LOCATION	INT-REDUCTION	NITROGEN FIXATION	CSUP[a]
North Carolina	+	+	+
Florida (Key Biscane)	+	+	-
Jamaica	+	+	-
Barbados	+	+	-
Belize	+	+	+
Bahamas (San Salvador)	+	+	+

All assays were run on surface sterilized root tissue. [a]Carbon Source Utilization Patterns. The CSUP technique was not developed during the Florida, Jamaica and Barbados trips.

center of the microcolony, thus stimulating nitrogen fixation activity. The molecular basis of this relationship has not yet been investigated although the role of *H. wrightii* as a pioneering seagrass species has been established.

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