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COLONIZATION OF RICE ROOTS BY DIAZOTROPH BACTERIA

G. Diem, M. Rougier, I. Hamad-Fares, J.P. Balandreau & Y.R. Dommergues

Abstract

Colonization of roots of rice grown in agar, in sand and in soil, by diazotroph bacteria was studied by direct observations (scanning and transmission electron microscopy) and by an indirect method (assessment of location of diazotrophs along the roots through cultural methods together with Acetylene Reduction Activity – ARA – tests).

Since basal root segments appeared to show significant ARA and to be inhabited by living diazotrophs, these microorganisms were thought to be protected against deleterious effects from the outside by barriers which are discussed.

Introduction

A profuse and durable colonization of roots by diazotroph bacteria is a prerequisite for the establishment of a beneficial association between these bacteria and the host plant. Unfortunately many strains of diazotrophs (e.g. *Azotobacter*) known as efficient N_2-fixing microorganisms are not true rhizosphere bacteria (Brown, 1974). They are unable to thrive in the rhizosphere and their decline in the root zone is probably the major cause of the failure of inoculations with diazotrophs. But recently a tight plant – diazotroph association (*Spirillum lipoferum – Digitaria decumbens* cv *transvala*) was described by Döbereiner & Day (1976). In this association *S. lipoferum* appeared to be localized in the cells of the cortex.

The studies presented here are related to the root colonization of rice grown in the field or in gnotobiotic conditions. In this paper diazotroph bacteria which are assumed to be located in the root cortex are designated as endorhizospheric bacteria. Such bacteria can only be retrieved by crushing previously surface-sterilized roots.

Material and methods

Field-grown rice

The rice variety SE 302 G was grown in a very sandy soil (Sol beige from Sefa, Senegal). When the plant was nearing the flowering stage, rice roots were carefully removed from the soil and separated into two series, each of them being submitted to the following treatments:

Surface sterilization. Roots were washed several times in water in order to remove all soil particles. Then they were surface-sterilized by dipping for 1 ½ h into a chloramine T (Merck) 1% (w:v) water solution (Gautheret, 1959). Finally the roots were profusely washed in sterile distilled water and cut into 3–5 mm segments. Segments from basal (A, 0–5 cm from base), mid (B, 5–15 cm from base) and apical (C, more than 15 cm from base) regions of the root system were examined separately in order to determine the spatial

distribution of diazotrophs. Segments originating from each region were divided into two sub-series. Each segment from the first sub-series was placed in a vial containing 5 ml of the soft agar medium for diazotrophs (Döbereiner & Day, 1976) slightly modified by adding 1% glucose to permit diazotrophs other than *Spirillum* spp. to grow. Acetylene reducing activity (ARA) was measured with 8-day-old enrichment cultures obtained from these root segments. Segments from the second sub-series were weighed and crushed in sterile sand in order to obtain dilution suspensions which were used for the enumeration of diazotrophs (Villemin *et al.*, 1975). Bacterial numbers are expressed on a root dry weight basis; water content of rice roots was assumed to be ca. 80% of wet weight (Marechal, personal communication).

Root washing. The procedure was similar to the technique of Harley & Waid (1955). Roots were vigorously shaken by hand for several minutes in 50 ml sterile water. Between each shaking period the water was discarded. The process was repeated 20 times. Water from the last washing was retained for an enumeration according to the Villemin *et al.* (1975) procedure in order to check the efficiency of the washings. Washed roots were then cut into basal (A), mid (B) and apical (C) pieces which were subsequently divided into 3 – 5 mm segments for enumeration of diazotrophs and ARA determination as described previously.

Rice seedlings grown in the laboratory

The rice variety was IR8. Two types of seedlings were used: (1) 7 days old seedlings originating from sterile seeds were grown in Petri dishes on an agar mineral medium. When seeds were placed on the agar medium, inoculation was performed with suspensions of *Beijerinckia* isolate BC (*Beijerinckia* from Camargue alluvial soil) and *Enterobacter cloacae*. Most observations regarded colonization of the root cap. (2) 30 days old seedlings grown in sand watered with a mineral solution and placed in a sterile growth chamber. As previously, inoculation was performed at the time of seedling. *Beijerinckia* isolate BC was used for inoculation. Details of the technique have already been reported by Hamad-Fares (1976). The object of the observations was to elucidate the colonization of root hairs and root base. One part of the roots was cut into basal (B), mid (B) and apical (C) pieces for enumeration of diazotrophs and ARA determinations. The other parts of the roots were prepared for scanning (SEM) and transmission (TEM) microscopy as described by Hamad-Fares (1976).

Results and Discussion

Interpretation of ARA determinations and enumeration of diazotrophs led to two types of similar conclusions as far as spatial distribution of microorganisms in the rhizosphere is concerned (Tables 1 and 2). First, the basal zone appeared to be generally more susceptible to the colonization of diazotroph bacteria. Second, 20 repeated washings never resulted in the elimination of diazotroph bacteria, suggesting that such microorganisms were tightly fixed to the roots. Third, surface sterilization never killed all the diazotroph bacteria situated in the basal zone, indicating that in such microhabitats some kind of protection occurred. Fissures abundant in the old root cortex were assumed to act as protecting sites. The preferential colonization of the root base was confirmed recently by Fetiarison & Sain-Macary (personal communication) for maize and *Pennisetum polystachion*.

SEM pictures showed that the apex of rice roots grown in agar were profusely colonized by *Beijerinckia* isolate BC or by *Enterobacter cloacae* (Figs. 1, 2 and 3). SEM and TEM pictures revealed that these diazotroph bacteria appeared often to be embedded in the mucigel which is abundant in this zone (Figs. 4, 5 and 6). In contrast, root apical zones of rice grown in nonsterile soil were not colonized by BC cells (Diem *et al.*, 1977). Such a discrepancy between soil-grown and axenically grown seedlings may be due to the fact that in the former system the bacterial growth is slower than in the latter one, hence a poor colonization of the root tip.

Whatever variations may be observed, the surface sterilization always suggests that diazotroph bacteria situated in the basal zone are fairly well protected against deleterious

Table 1. Acetylene reduction activity (ARA) of enrichment cultures obtained from rice root segments after surface sterilization or successive washings and incubation in malate-glucose agar. (Field grown rice from Senegal.)

Root zone	Treatment	ARA test	Root segments (%) showing an ARA positive	low	negative
Basal	S	1	9	14	77
(A)	S	2	27	18	55
	W	1	100	0	0
Mid	S	1	0	25	75
(B)	S	2	18	18	64
	W	1	100	0	0
Apical	S	1	0	10	90
(C)	S	2	10	19	71
	W	1	100	0	0

S: after surface sterilization. W: after 20 washings.

ARA test 1: Test made with 8-day-old cultures. C_2H_2 was injected at inoculation time.
2: Second lecture made 7 days after the first one.

ARA positive: >14 nmoles C_2H_4 per root segment.
low: 30 nmoles − 14 nmoles C_2H_4 per root segment.
negative: <30 nmoles C_2H_4 per root segment.

Table 2. Most probable number of diazotroph bacteria estimated from rice roots after surface sterilization or successive washings. (Field grown rice from Senegal.)

Root zone	After surface sterilization	After 20 washings
Basal (A)	2240	120000
Mid (B)	0	10000
Apical (C)	not studied	40000

Results are expressed by the most probable number of bacteria g^{-1} of roots as assessed by C_2H_2 reduction of enrichment cultures according to the procedure of Villemin *et al.* (1975).

effects from the outside. Since bacteria located on the root apex surface are less protected against the lethal action of Chloramine T than bacteria located at the root base (Table 3) the protection by the superficial mucigel is assumed to be less efficient than the protection occurring at the base of the root, which is presumably due to barriers e.g. remnants of dead cells or crevices as suggested by Mosse (1975). SEM pictures of the basal zone suggest that such a protection may also result from the localization of some bacterial cells or microcolonies inside the root between the cortex cells (Fig. 7) or possibly inside the large lacunas occurring in the rice root (Fig. 8). TEM data are still lacking and should be necessary to confirm this hypothesis.

Sloughing off of cortical cells in old parts of the root could be a result of an abrasive action of sand grains and microbial attack (Old & Nicolson, 1975). Therefore, in our experiment with rice grown in a very sandy soil, the epidermis of the roots might have

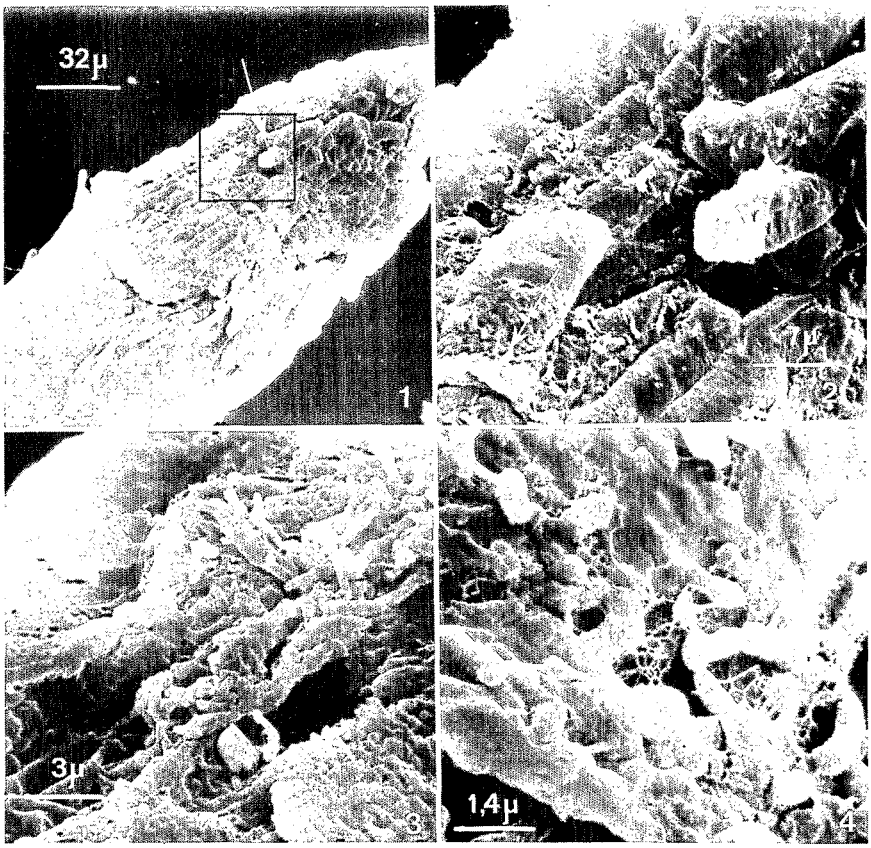

Figure 1. Scanning electron micrograph of root apex of rice seedling grown in sterile mineral agar (SAM) medium and colonized by *Beijerinckia* isolate BC. Note high growth of bacteria on epidermal cells (arrow). The outlined area is enlarged in Fig. 2.

Figure 2. Enlarged area from Fig. 1. Scanning electron micrograph of *Beijerinckia* isolate BC cells growing on root apex of rice seedling in SAM medium. Arrow indicates bacterial cells embedded in apex mucigel.

Figure 3. Scanning electron micrograph of *Enterobacter cloacae* growing on root apex of rice seedling in SAM medium.

Figure 4. Scanning electron micrograph of isolate BC cells embedded in mucigel of root apex of rice seedling in SAM medium.

been damaged, especially in the basal zone. Thus, we can assume that the internal colonization of soil-grown roots by diazotroph bacteria might be possible at the basal zone where a variety of entries occur at the onset of decomposition of the root epidermis and cortex. This hypothesis is supported by electron micrographs obtained from roots of rice grown in African soil showing an important internal colonization of the cortex belonging to old parts of the root. Cortical cells devoid of cytoplasm appeared to enclose various types of soil debris associated with bacteria between their remnant cell walls (Figs. 9 and 10).

Using TEM to study the distribution of the total bacterial microflora in the wheat

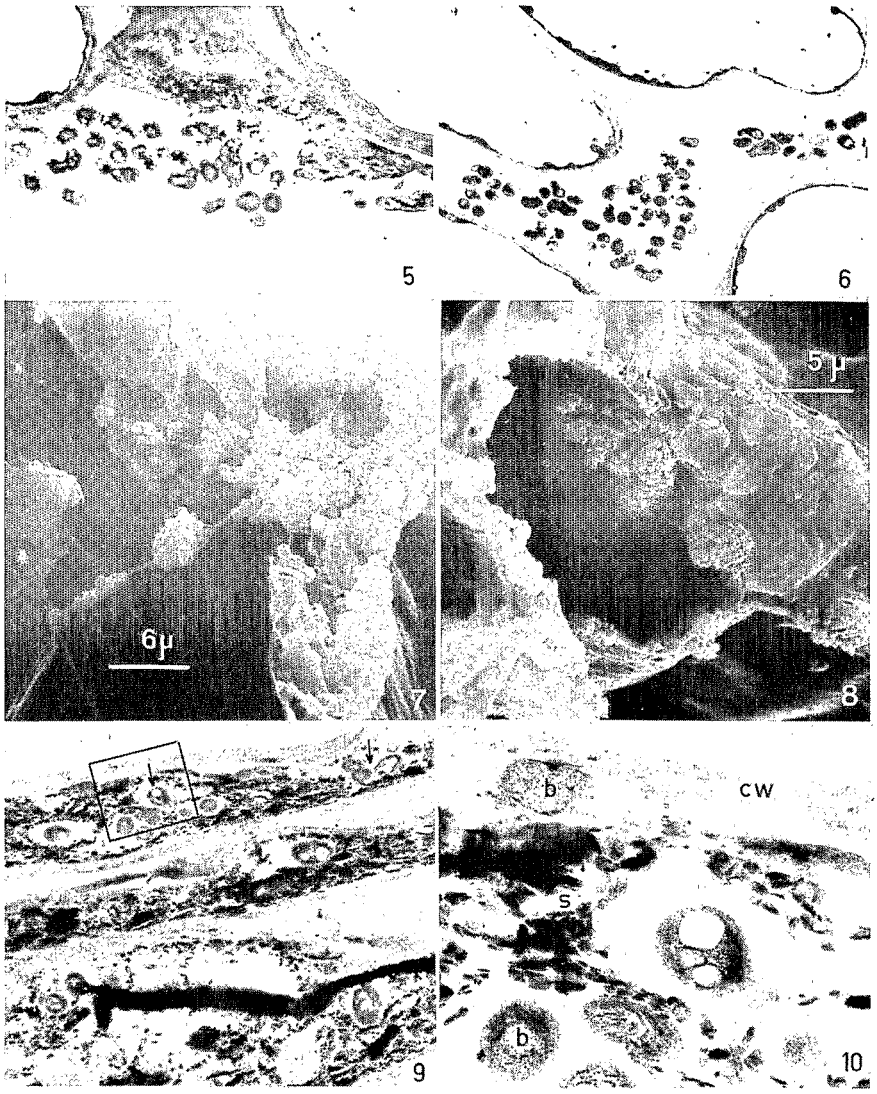

Figure 5. Transmission electron micrograph of a colony of isolate BC growing in mucigel surrounding apex of rice seedling in SAM medium.

Figure 6. Transmission electron micrograph of colonies of isolate BC growing in mucigel between apex cells of rice seedling in SAM medium.

Figure 7. Scanning electron micrograph of colonies of isolate BC between cortex cells inside rice roots (30 days old rice grown in sand with isolate BC inoculum).

Figure 8. Scanning electron micrograph of colonies of isolate BC in lacunas of rice roots (30 days old rice grown in sand with isolate BC inoculum).

Figure 9. Transmission electron micrograph of soil bacteria (arrow) embedded in soil debris between remnants of cortical cell walls of rice roots (Field grown rice from Senegal). The outlined area is enlarged in Fig. 10 (× 2900).

Figure 10. Enlarged area from Fig. 9. Transmission electron micrograph of soil bacteria associated with soil debris between remnants of cortical cell walls. Bacteria (b). Host cell walls (cw). Soil debris (s). (× 20000).

Table 3. Percentage of rice root segments from 30 days old rice seedlings grown with *Beijerinckia* isolate BC inoculum that exhibited bacterial growth or C_2H_2 reduction after surface sterilization and incubation in malate-glucose agar medium. (Sand grown rice in the laboratory.)

	Apical zone	Root hairs	Secondary roots	Basal zone
Beijerinckia growth	6.7	0.0	0.0	33.3
Positive ARA test	0.0	0.0	0.0	33.3

rhizosphere, Rovira (1973) and Foster & Rovira (1973) found that the colonization of the apex was sparse in contrast with the older basal portion of the root where the outer cells were distorted and invaded by bacteria. Studying the invasion of pea roots by *Acanthamoeba palestinensis*, Darbyshire & Greaves (1971) also found that the greatest concentrations of amoebae were located at the base of the tap root and older lateral roots.

Both SEM and TEM studies of Old & Nicolson (1975) showed that young roots, the surface of which is intact and covered with mucigel, were relatively free from bacterial colonization. When mucigel was absent and when epidermal cells of old roots were damaged, there was extensive microbial colonization and bacteria could penetrate into the root tissues. Thus, our results which are restricted to diazotroph bacteria appear to be consistent with the findings of other authors on the colonization of roots by the soil microflora in general.

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