Environmental Role of Nitrogen-fixing
Blue-green Algae and Asymbiotic Bacteria
Ecol. Bull. (Stockholm) 26: 312–318 (1978).

THE USE OF THE FLUORESCENT-ANTIBODY TECHNIQUE TO STUDY THE BEHAVIOUR OF A *BEIJERINCKIA* ISOLATE IN THE RHIZOSPHERE AND SPERMOSPHERE OF RICE

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Abstract

A fluorescein isothiocyanate labeled antiserum (FA) against an isolate of *Beijerinckia* sp. was prepared. This FA was found to be highly specific as shown by negative reaction when used for staining 6 species of *Azotobacter*, 4 species of *Beijerinckia* and 44 unidentified bacteria isolated from a spectrum of soils and rhizospheres. FA tagging allowed the growth assessment of the bacterium in the rice spermosphere and rhizosphere despite the presence of the whole soil microbial population. The colonization of different zones of these habitats by *Beijerinckia* sp. was studied.

Introduction

During a survey carried out at the Centre de Pédologie Biologique (Vandoeuvre, France) a bacterium of the genus *Beijerinckia* was isolated from a rice field soil in Camargue (Balandreau, 1975). This isolate was capable of active N_2 fixation when associated with rice in gnotobiotic culture (Hamad-Fares *et al.*, 1977) but no increased N_2 fixation was found when inoculated to rice grown in non-sterile soil as compared to non-inoculated soil (Hamad-Fares, 1976).

Evidence for extensive growth of the bacterium designated here as isolate BC (*Beijerinckia* from Camargue) in the rhizosphere of axenic rice seedlings was reported (Balandreau, 1975; Hamad-Fares, 1976). But as this organism is susceptible to soil bacteriostasis (Diem *et al.*, 1977) it is interesting to determine whether the rice rhizosphere effect can overcome soil inhibitors.

The fluorescent-antibody technique offers a means for direct observation of the behaviour of a specific bacterium in its natural environmental. Capabilities and limitations of the technique have been discussed by Schmidt (1973). This paper reports some aspects of the growth of isolate BC around germinating seeds and near the roots during the early growth of rice as examined with fluorescent-antibody (FA) methods.

Material and methods

FA preparation and specificity determination

Fluorescent-antibodies against BC were obtained as reported in an earlier paper (Diem *et al.*, 1977). Smears of pure cultures originating from six species of *Azotobacter*, 4 species of *Beijerinckia* and 44 unidentified rhizosphere bacteria were stained with FA to evaluate the specificity of conjugates. Preparations were viewed

on a Zeiss Universal microscope equipped for reflected fluorescence with Osram HB 200 mercury lamp Fl 500 reflector, BG 38 and FITC (KP 500) exciter filter and a number 50 barrier filter.

Behaviour of isolate BC in the spermosphere

Camargue soil (1.2 % C, 0.15 % N, pH 7.8, silty clay) diluted with sterile fine sand (2:1 w/w) was inoculated with a suspension of BC to provide 2×10^6 cells g^{-1} of soil and sufficient water was added to saturation. Seeds of rice (variety Cesariot) were coated with a mud prepared with this inoculated soil and then buried in the same soil. This method of inoculation was used to ensure a homogeneous distribution of the inoculum around the seeds. After 2 and 4 days incubation at 35°C triplicate sets of 20 germinating seeds were removed. Each seed was aseptically cut with a sterile razor into 3 parts: radicle, zone of root emergence and remaining zones of the seed. Groups of these parts were then separately shaken for a few minutes with 10 ml of a sterile 0.2 % solution of sodium hexametaphosphate. After appropriate dilution, 10 µl of the final suspension were deposited onto slides following the method of Trolldenier (1973). After drying and before staining with FA conjugates (Schmidt *et al.*, 1968), smears were treated with gelatine-rhodamine isothiocyanate conjugate to suppress nonspecific adsorption of FA (Bohlool & Schmidt, 1968). Under ultra-violet light, fluorescing cells of BC were counted and the bacterial content per g of soil was then calculated taking into account the size of microscopic field and the rate of soil dilution.

Direct observation of the growth of BC in the spermosphere was performed by placing rice seeds on a surface-smoothed soil which was previously moistened and lightly packed in a Petridish. A thin layer of water agar bearing BC cells (50×10^3 cells $\cdot$ cm^{-2}) was then firmly pressed onto the seeds and the surrounding soil. The Petri dish was incubated at 35°C in vertical position so that the coleoptile and emerging radicle of rice remained at the soil surface contacting the agar film. After 4 days incubation, the soil and the agar film were allowed to dry at approximately 45°C, the agar film was then removed, deposited onto slides and stained with FA conjugates as described above.

Behaviour of isolate BC in the rhizosphere

The study was carried out using 14 days old rice seedlings and the establishment of isolate BC in the rice rhizosphere was examined by direct observation. The method used was derived from the buried-slide technique (Jackson, 1960) with which Brown (1973) studied bacterial growth in the rhizosphere.

A suspension of BC cells was uniformly spread over the surface of a thin layer water agar in a plastic Petri dish giving a density of ca. 50×10^3 cells $\cdot$ cm^{-2}. After absorption of the inoculum by the water agar, a 3 mm thick layer of Camargue soil was deposited onto the agar. The soil was then moistened to saturation by about 3 ml of water. One germinating seed of rice was buried at a point near the edge of the Petri dish. The rice seedling subsequently grew through a hole made in the lid of the dish. The dish was placed at an angle of 45° so that developing roots grew down in contact with the agar layer. After 2 weeks incubation in a growth cabinet (illumination 30,000 lux, 12 h illumination per day, temperature 20 – 25°C, humidity 70 – 80 %), the soil was allowed to dry. It was then removed by gentle tapping. The whole system of roots was embedded in the dried agar pellicle which was easily pulled off the bottom of the Petri dish. Pieces of agar pellicle together with embedded roots were then deposited onto slides to be stained with FA conjugates. Photomicrographs were taken with Ektachrome (Kodak) high speed film using 2 second exposures.

Results and discussion

FA specificity

The conjugate did not cross-react with any of 54 bacteria tested, including species of *Azotobacter* and *Beijerinckia* (Table 1). More detailed discussions about this FA specificity were reported by Diem *et al.* (1977). Further attempts to stain smears issued from non-inoculated soils and from the same soils inoculated with *Beijerinckia* only showed fluorescing cells in the latter. Thus, it was assumed that FA against isolate BC was sufficiently sensitive and specific to permit extensive study of the bacterium in non-sterile soils.

Table 1. Immunofluorescence reaction of *Beijerinckia* isolate BC conjugates with species of *Azotobacter* and *Beijerinckia*.

Microorganisms tested	Fluorescence reaction
A. chroococcum (2 isolates)	Negative
A. vinelandii (2 isolates)	Negative
A. agilis	Negative
A. insignis	Negative
A. macrocytogenes	Negative
A. beijerinckia	Negative
B. mobilis	Negative
B. indica	Negative
B. lacticogenes	Negative
B. fluminensis	Negative

Behaviour of BC in the rice spermosphere

The growth of BC in different regions around germinating rice seeds as evaluated by the cell count technique is illustrated in Fig. 1. The bacterial population was found to be readily stimulated by a spermosphere effect. Initially, bacterial growth near the emergence point seemed to be lower than in the other areas of the seed but no differences were noted after 4 days incubation. By this time, the rice radicle appeared to be free from BC colonization. As the radicle had just developed, it may be assumed that the exudation of available nutrients from the radicle was lower than that of the germinating seed and the contact with BC was not long enough to allow the growth of this bacterium in competition with the indigenous microflora.

Direct examination showed high density of a BC population in the vicinity of the seeds (Fig. 2). In another work, Diem & Villemin (unpublished) found that N_2 fixation

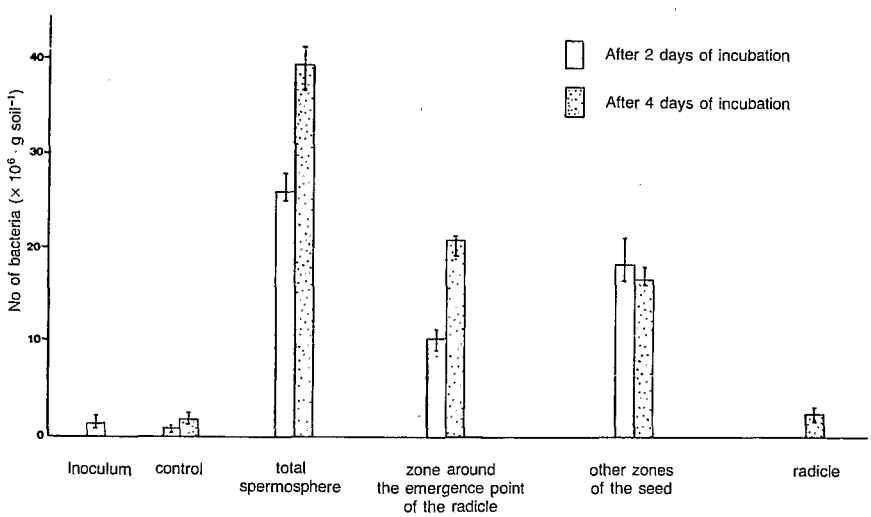


Figure 1. Growth of *Beijerinckia* isolate BC in the spermosphere of rice assessed by the cell count method (Trolldenier, 1973).

by the natural microflora as measured by acetylene reduction activity (ARA) was readily induced in the rice spermosphere in agreement with the observations of Dommergues *et al.* (1973). However, the presence of added BC cells did not enhance ARA in the rice spermosphere as compared with non-inoculated soil. Additional experiments showed that C_2H_4 originated from acetylene reduction and not from seed germination processes. Thus, there was evidence that N_2 fixation could occur in the spermosphere owing to high amounts of available exudates in this zone.

Behaviour of BC in the rice rhizosphere

In the seedling stage of rice, the colonization of roots by this isolate was poor although an increase of its population could be observed in the surroundings of roots (Figs. 3 & 4). A few colonies were observed on the root surface but no colonization occurred in the root tip region. These results were consistent with the findings of Rovira & Campbell (1974) who showed that the number of bacteria on soil-grown roots of wheat, particularly when young, was quite low. According to Rovira *et al.* (1974) only a small proportion (4 – 10%) of the root surface is occupied by bacteria.

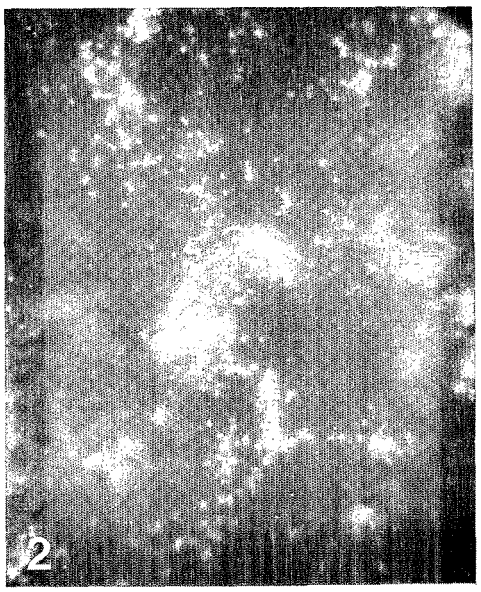
Sparse colonies of BC could occur on root hairs (Fig. 5) but generally root hairs of rice seedlings did not seem to be preferential zones of colonization. Working on sugar beet seedlings, Gyurko (1968) showed profuse development of soil bacteria along root hairs; but such a observation was never made in our experiment. In contrast to oats, Rovira (1956) already noted the consistent absence of bacteria on the root hairs of tomato.

Many studies have reported that the root tips of soil-grown plants were consistently free from microorganisms. This phenomenon might be explained by the fact that bacterial colonization depends on its growth rate on the host. Because of soil bacteriostasis, the growth rate of bacteria in natural conditions is undoubtedly slower than the rate of root growth so that colonies of bacteria only occur behind root tips. A similar assumption was made by Darbyshire & Greaves (1971) to explain the scarcity of amoebae on the youngest parts of pea roots.

Direct observations showed that isolate BC was not regularly stimulated in the rhizosphere of rice seedlings. Some areas were densely covered by bacteria in contrast with the others where BC colonies were sparse. Environmental conditions controlling bacterial growth in the root vicinity are thought to vary widely in space since exudation sites or sloughing sites are not evenly distributed and since soil is a heterogeneous medium. Marked stimulation of bacterial growth around seeds suggests that the spermosphere effect is much stronger than the rhizosphere effect. Exudates of rice roots were able to stimulate isolate BC but its reduced growth might be due to the following factors:

First, the low exudation rate at the seedling stage of rice; second, the low temperature (25°C) used in the experiment (optimal temperature for BC growth being ca. 35°C); third, presence of antagonistic or competing microflora in Camargue soil: actinomycetes and sulfate-reducing bacteria as shown in field observations. Symptoms of sulfate-reduction along rice roots could also be obtained in the laboratory even with 5 weeks old cultures (Fig. 6; Dommergues & Mangenot, 1970).

In favourable areas, isolate BC seemed to multiply profusely within the interstices of soil particles (Fig. 7). Similar observations were reported by Rovira & Campbell (1974) who considered the large numbers of different microhabitats in the rhizosphere to



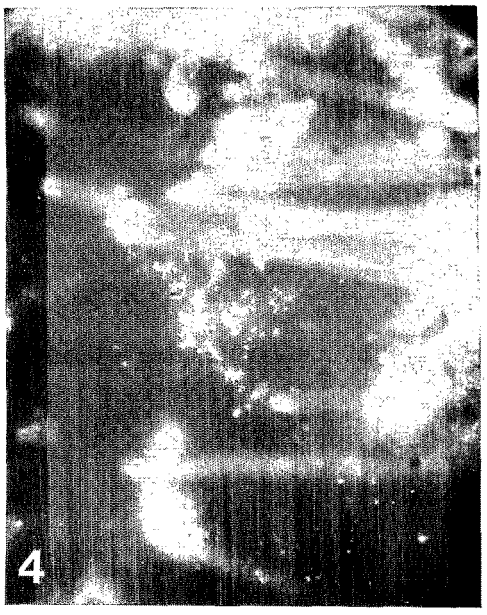


Figure 2. Direct observation of the population of *Beijerinckia* isolate BC, with Fluorescent-Antibody (FA) technique, in the surroundings of germinating rice seed after 4 days incubation.

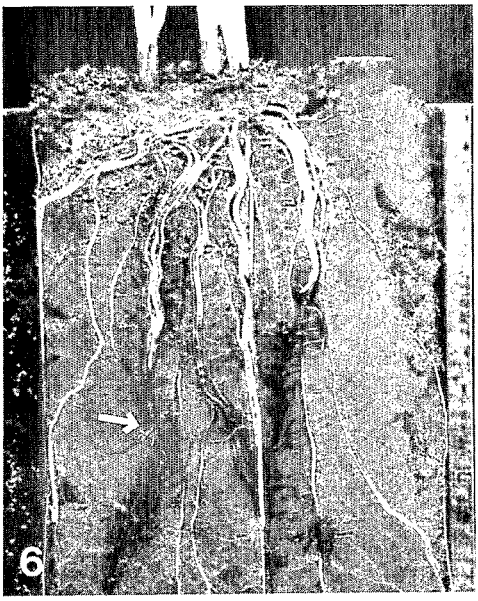
Figures 3–7. Direct observation of the behaviour of isolate BC, with FA technique, in the rhizosphere of rice grown in soil and under laboratory conditions. One cm in each photomicrograph represents 35.5 µm.

Figure 3. Fluorescing cells of isolate BC growing near the main root of rice seedling. Note the autofluorescence of the rice root.

Figure 4. Fluorescing colonies of isolate BC in the zone of root hairs of rice seedling.

present problems in observing the root microflora.

Some difficulties were encountered in this application of the FA technique due to autofluorescence of the rice roots. Treatments with gelatine-rhodamine did not adequately control this problem. According to Bhatnagar (personal communication) this difficulty might be resolved by using methylene blue as a counterstain. Modifications in the staining technique to reduce the autofluorescence of rice roots are being explored.



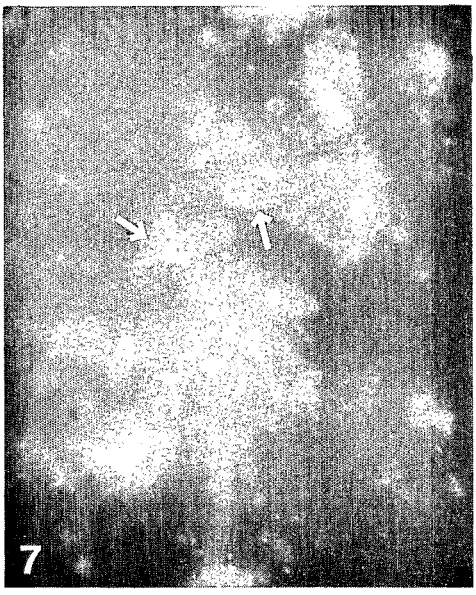


Figure 5. Sparse colonies of isolate BC on root hair surface.

Figure 6. Symptoms of sulfate-reduction along roots of 5 weeks old rice grown in a soil box under laboratory conditions.

Figure 7. Fluorescing cells of isolate BC adhering to soil particles (arrow).

Acknowledgements

The authors are indebted to Prof. Gadal for the use of the culture cabinet. H.G.D. is grateful to Prof. F. Mangenot for advice and valuable discussions.

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DISCUSSION

Y. Okon: Can you see many N_2-fixing bacteria when you take your rice plants from the field, by using the FA technique?

H. G. Diem: By using the FA technique, you can easily see the homologous bacteria if the population is high. In the case of *Beijerinckia* from Camargue I have difficulties to detect the bacterium in natural soil because its population is very low.

D. G. Patriquin: Have you looked for *Spirillum lipoferum* in the system?

H. G. Diem: Fluorescent-antibodies against *Spirillum lipoferum* and other bacteria resembling *Spirillum lipoferum* have just been prepared in the laboratory. They will be used soon to detect these organisms in natural habitats.