

SHORT TECHNICAL NOTE

Direct identification, and localization of *Azospirillum* in the rhizosphere of wheat using fluorescence-labelled monoclonal antibodies and confocal scanning laser microscopy

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Key words. *Azospirillum*, monoclonal antibodies, confocal scanning laser microscopy.

Summary

Azospirillum is a soil bacterium with plant growth-promoting potential. Strain-specific monoclonal antibodies (mAbs) which bind to different antigenic determinants of the bacterial cell surface with high specificity for *A. brasilense* Sp7 were used to monitor this bacterium in the rhizosphere of wheat plants. The mAbs were marked directly with the fluorochromes fluorescein (FITC) or tetramethylrhodamine (TRITC). With the labelled mAbs and confocal scanning laser microscopy, an *in situ* identification and localization of this bacterium in root segments was possible.

Introduction

The inoculation of plant roots with bacteria of the genus *Azospirillum* can result in a significant change in various growth parameters of the plant (Kapulnik *et al.*, 1985). It is reported that *Azospirillum* has a plant growth-promoting potential, which produces enhanced nitrogen uptake in the inoculated plants (Baldani *et al.*, 1983) and phytohormone production in the rhizosphere (Hartmann *et al.*, 1983). *Azospirillum* can colonize roots externally (in the mucigel layer; Bashan & Levanony, 1989) or internally (in the intercellular space; Patriquin & Döbereiner, 1978). However, the mechanisms of the plant bacteria interaction leading to plant growth promotion are not fully elucidated. A specific *in situ* localization of particular *Azospirillum* strains is necessary to reach this goal.

In our laboratory we use strain-specific monoclonal antibodies (mAbs) for *A. brasilense* Sp7 to identify, localize

and quantify inoculated *Azospirillum* in the rhizosphere. The strain-specific mAbs were classified into two subclasses according to their different antigenic determinants (Schlotter *et al.*, 1992a). One class of mAbs is bound to an 85-kDa outer membrane protein, while the second type recognizes a certain exopolysaccharide of the bacterial cell surface (M. Schlotter, unpublished observations).

With enzyme-coupled mAbs against the membrane protein and a sensitive immunoassay quantification of *A. brasilense* Sp7 in a soil extract, it was possible to detect 100 bacteria/ml (Schlotter *et al.*, 1992b). Using fluorescent markers conjugated to the mAbs and epifluorescence microscopy a specific cell identification is possible (Bohlool & Schmidt, 1980). However in samples with high autofluorescence – like roots or soil with high clay or humic content – problems exist concerning the use of epifluorescence microscopy. Therefore the application of confocal scanning laser microscopy was investigated.

Materials and methods

Bacterial strains

The bacterial strain *Azospirillum brasilense* Sp7 (DSM 1690) was obtained from the German collection of microorganisms (DSM) and grown in Luria broth (LB) at 33°C.

Monoclonal antibodies

The monoclonal antibodies (mAbs) were generated and characterized as described by Schlotter *et al.* (1992a).

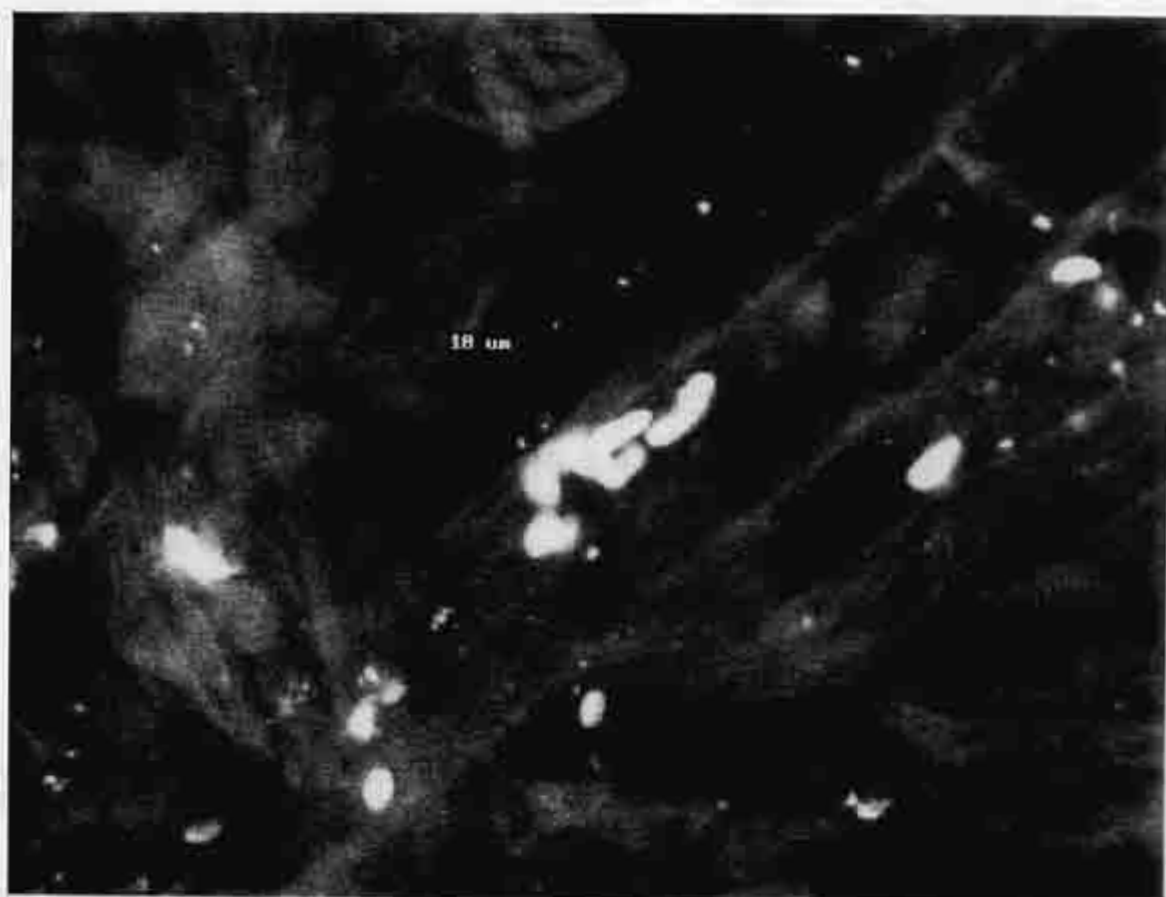


Fig. 1. An *xy* scan picture ($\times 1000$) of bacteria labelled with FITC mAbs on a 10-week-old wheat root surface using a single-channel confocal scanning laser microscope. The fluorescence was excited with an Ar laser at 488 nm and detected with a long-pass filter at 515 nm.

Coupling of the antibodies with fluorochromes. The antibodies were coupled either with the fluorochromes fluorescein isothiocyanate (FITC) (Sigma, Germany) or with tetramethyl rhodamine isothiocyanate (TRITC) (Sigma, Germany) according to Goding (1978).

Plant inoculation

Rhizotrons ($30 \times 15 \times 2$ cm) were filled with soil (high clay content). One wheat seedling was planted in each rhizotron. At the time of planting the plants were inoculated with 10^8 cells of an overnight culture of *A. brasilense* Sp7. The rhizotrons were moistened every 2 days with 3 ml water. After 10 weeks the rhizotrons were opened and the roots were carefully removed from the soil.

In situ immunostaining technique

For the immunostaining experiments a strain-specific mAb for *A. brasilense* Sp7 was used, which binds to cell-surface polysaccharides (Schlotter *et al.*, 1992a).

The staining of the root segments was performed in petri

dishes according to Bohlool & Schmidt (1980) and Harlow & Lane (1988). To prevent fading of the fluorochromes an antifading reagent containing 100 mg paraphenylenediamine in 10 ml phosphate-buffered saline (pH 9) and 90 ml glycerin was used.

Confocal scanning laser microscopy

A Carl Zeiss LSM 310 was used to record optical sections. The instrument was equipped with an Ar-ion laser (488 nm) and a HeNe-laser (543 nm). Objective lenses 40x/1.3, 63x/1.4 and 100x/1.4 were used. The instrument was capable of recording two fluorescence channels simultaneously. A three-dimensional software package enabled root and bacteria to be displayed at different angles.

Results and discussion

Conventional epifluorescence microscopy

For studying microbial populations in soil and rhizosphere, the sensitivity of standard epifluorescence methods is

Fig. 2. An *xy* scan picture ($\times 1000$) of bacteria labelled with TRITC mAbs on a 10-week-old wheat root surface using a two-channel confocal scanning laser microscope. The fluorescence of the mAbs was excited with an HeNe laser at 543 nm and detected with a long-pass filter at 590 nm. The autofluorescence of the root was excited with an Ar laser at 488 nm and detected with a long-pass filter at 515 nm.



limited, because autofluorescence problems do occur *in situ*. The optical properties of the fluorescence markers used are of central importance. Using TRITC or Texas Red at an excitation wavelength of 584 nm and an emission wavelength of 612 nm, autofluorescence due to the soil and root material was present, but within an acceptable range. Localization and identification of micro-organisms under these conditions was possible to some extent (not shown). In contrast, using an excitation wavelength of 485 nm and an emission wavelength of 538 nm (recommended for FITC), root and soil components showed very high autofluorescence. In mature roots and in soils with a high content of organic matter bacteria could hardly be identified under these conditions (not shown).

Confocal scanning laser microscopy

Confocal laser scanning microscopy solved many of these problems. The technique of optical sectioning with a confocal scanning laser microscope removes the out-of-focus fluorescence from the roots, leading to a sharp, clear picture. In these optical sections the bacteria can be detected very easily.

Using only a single exciting laser wavelength (488 nm) it was possible to stimulate autofluorescence of root material and specific fluorescence of the FITC-labelled bacteria at the same wavelength. Figure 1 shows an *xy* scan picture of

bacteria labelled with FITC-mAbs on a 10-week-old wheat root surface. It was possible to localize marked *A. brasilense* Sp7 cells on the root surface. Using bacteria marked with TRITC mAbs and excitation with green light, the background signal was so weak that the root could not be localized (not shown).

A two-laser system provided the opportunity to split autofluorescence and fluorescence of the labelled bacteria. Figure 2 shows an *xy* scan picture of bacteria labelled with TRITC mAbs on the surface of a 10-week-old wheat root. The root fluorescence was excited with the Ar-laser line at 488 nm and detected with a 515-nm long-pass filter. For the TRITC label we used excitation with the green HeNe laser line at 543 nm and a 590-nm long-pass filter for detection. It was also possible to excite both signals simultaneously and to split the emissions for simultaneous recording at two channels.

With a two-laser system it was not only possible to localize bacteria on the root surface, a *z*-scan gave the additional opportunity to determine the exact position of the bacteria in the depth of the mucigel layer. Figure 3 shows a *z*-scan of 30 μm in 0.5- μm steps of bacteria labelled with TRITC mAbs on the surface of a 10-week-old wheat root. It could be shown that *A. brasilense* Sp7 did not penetrate into the root interior, but colonized the root in the mucigel layer. This is in accordance with results of other groups (Bashan & Levanony, 1989).

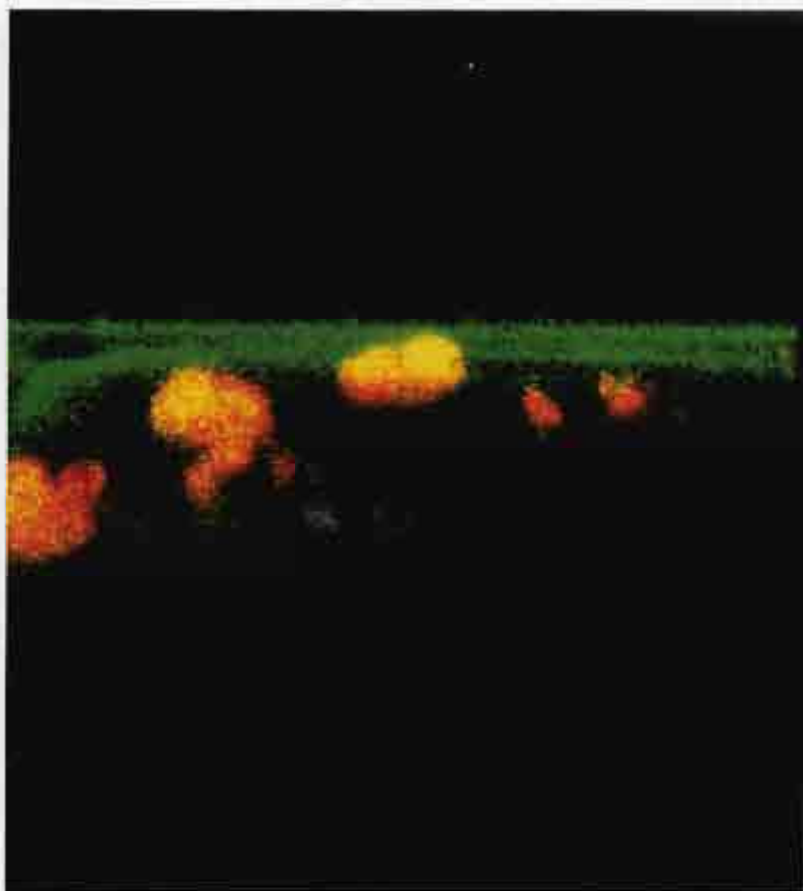


Fig. 3. A *z* scan picture ($\times 1000$) of bacteria labelled with TRITC mAbs on a 10-week-old wheat root surface using a two-channel confocal scanning laser microscope (depth of $30\ \mu\text{m}$ in $0.5\text{-}\mu\text{m}$ steps). The fluorescence of the mAbs was excited with an HeNe laser at $543\ \text{nm}$ and detected with a long-pass filter at $590\ \text{nm}$. The autofluorescence of the root was excited with an Ar laser at $488\ \text{nm}$ and detected with a long-pass filter at $515\ \text{nm}$.

A two-laser system also provided the ability to scan in the *xyz*-direction. In smaller roots or root hairs this technique may enable us to quantify the mAb-labelled bacteria. Figure 4 shows an *xyz* scan of a root hair ($30\ \mu\text{m}$ in $1\text{-}\mu\text{m}$ steps) of bacteria labelled with TRITC mAbs.

Experiments to use double-marked bacteria in confocal scanning laser microscopy are in progress. Two differently labelled mAbs of different specificity or a combination of

labelling with mAbs and gene probes like oligonucleotides, directed against 23S-rRNA (Kirchhof & Hartmann, 1992), may be applied simultaneously.

Acknowledgment

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

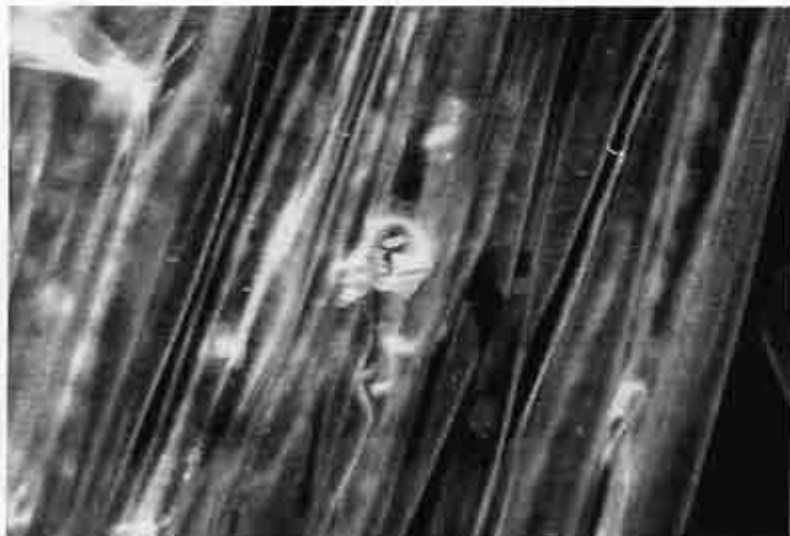


Fig. 4. An *xyz* scan picture ($\times 1000$) of bacteria labelled with TRITC mAbs on a 10-week-old wheat root surface using a two-channel confocal scanning laser microscope (*z* scan: depth of $30\ \mu\text{m}$ in $1\text{-}\mu\text{m}$ steps). The fluorescence of the mAbs was excited with an HeNe laser at $543\ \text{nm}$ and detected with a long-pass filter at $590\ \text{nm}$. The autofluorescence of the root was excited with an Ar laser at $488\ \text{nm}$ and detected with a long-pass filter at $515\ \text{nm}$.

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