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Characterisation of Different Polyclonal Antisera to Quantify *Herbaspirillum* spp. in Elephant Grass (*Pennisetum purpureum* Schun.)

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Abstract

Immunological techniques can be applied to detect micro-organisms in different ecosystems. To follow the fate of *Herbaspirillum seropedicae* strain Z67 and *H. rubrisubalbicans* strain M4 in the rhizosphere of elephant grass, *Pennisetum purpureum* Schun. var. Capim Cana D'África, polyclonal antisera were raised against both strains. Both antisera were purified with protein-A, followed by a primary characterisation for cross-reactivity. To reduce cross-reactivity for both sera, affinity purification was used for improvement. After that, both sera could be considered as species specific. The detection limit was 10^5 cells ml^{-1} for the anti *H. rubrisubalbicans* serum and 10^6 cells ml^{-1} for the anti *H. seropedicae* serum. It was shown that two months after inoculation, both strains could be detected in the rhizoplane of elephant grass in high densities. The estimated numbers by ELISA were 10^7 cells g^{-1} fresh weight.

Keywords: *Herbaspirillum*, diazotrophic bacteria, polyclonal antisera, *Pennisetum purpureum*

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1. Introduction

Herbaspirillum seropedicae is a nitrogen fixing bacterium which belongs to the β-Proteobacteria and was originally found to colonise leaves, stems and roots of several grasses (Baldani et al., 1986). Some years later, a group of *Pseudomonas rubrisubalbicans* isolates were compared to this species and then transferred to the *Herbaspirillum* genera. This group possesses several strains that were isolated from sugar cane and considered as a pathogen which causes mottled stripe disease in susceptible cane varieties (Baldani et al., 1996). The traditional way to quantify and isolate these organisms involves the maceration of plant tissue, dilution in saline solution and inoculation into the semi-solid N-free medium JNFb (Olivares et al., 1996). This procedure is time consuming and requires subsequent confirmation of bacterial identity. In the case of *Herbaspirillum* this may be accomplished by subjecting the isolates to tests on two different C-substrates (acetyl-glucosamine and meso-erithrytol to differentiate between the two *Herbaspirillum* species (Olivares et al., 1996) or by using specific oligonucleotide probes (Kirchhof et al., 1997). The use of immunological methods can overcome these problems and bacteria within plant tissues can be easily quantified and identified.

Immunological detection methods are based on the ability of antibodies to recognise specific three-dimensional structures (e.g. parts of proteins or polysaccharides) of biological molecules. These techniques play an important role as diagnostic tools in medicine and food technology (Kerr et al., 1992). In soil microbiology, these techniques are becoming increasingly important for the tracking of specific micro-organisms and for community analysis (Schloter et al., 1995). Using whole bacterial cells for immunisation, polyclonal antisera consist of a mixture of different antibodies synthesised in response to various antigenic determinants. To use these antisera as a probe for microbiological detection in plant/soil material it is essential to characterise the cross-reactivity and to improve, if necessary, the quality of the antisera by specific purification techniques. In contrast to the monoclonal antibodies, the production of polyclonal antisera is much easier and less time consuming. Immunological methods can be applied in a broader range of subjects.

Immunological methods have been used quite often in the field of plant microbe interaction, mainly to determine plant pathogenic bacteria on the root surface (Rodrigues et al., 1985). Other examples can be cited were plant growth promoting bacteria (PGPR-bacteria) were detected. Schloter et al. (1992) used serological techniques to detect specific *Azospirillum* strains in the rhizosphere of different plant cultivars or by Li and MacRae (1992) who quantified the natural population of *A. diazotrophicus* in sugar cane plants grown in Australia. For a review, see Schloter et al. (1995).

In this paper we describe the characterisation of two polyclonal antisera for

detection and quantification of *Herbaspirillum seropedicae* and *H. rubrisubalbicans* in germinated stem cuttings of elephant grass (Pennisetum purpureum) cv. Capim Cana D'África.

2. Material and Methods

Production and tests of the polyclonal antisera

The antibodies (*As*) were obtained by immunisation of 6-month-old, female New Zealand rabbits. Strains used for immunisation were the type strains of *H. seropedicae* BR 11175 (Z67) (*As-Hs*) and *H. rubrisubalbicans* BR 11192 (M4) (*As-Hr*) from the Embrapa-Agrobiologia diazotroph collection. Intact cells used for immunisation were grown overnight in Dygs media (Rodrigues Neto et al., 1986), harvested at OD_{560} 1.0, centrifuged at 3,500 x g, the supernatant discarded and resuspended in 2 ml of 0.85% NaCl after inactivation by 45 min treatment using U.V. light for 2 h to kill the bacteria. The immunisation was performed subcutaneously using 100 mg of fresh bacterial cells and introduced at eight different locations of the rabbits body at day 0, 14, 21, 28, 35 and 42. At day 47 sera were obtained by bleeding of the rabbits.

Purification of the antisera

The sera were purified using a protein-A column (Biorad, Germany) to extract the IgG fraction by the method described by Schloter et al. (1997). To obtain higher yields of purified antisera the elution buffer was modified: For IgG_{2a} and IgG_{2b} glycine buffer (100 mM) pH 4.0 was used, for IgG_1 and IgG_3 the pH of the buffer was reduced to pH 3.0.

For further reduction of cross-reactivity, affinity purification technique was used as described by Schloter et al. (1997). The bacteria selected for adsorption were *H. seropedicae* Z78 and *H. rubrisubalbicans* HRC51. The strains were grown in 500 ml of Dygs media for 24 h. The cells were centrifuged and the supernatant discarded. The pellets were resuspended in a volume of PBS to achieve the OD_{560} 20. The cells were inactivated by using sodium azide treatment (0.002%) for 5 min. Five ml of the bacterial suspension were used and incubated with 500 µl of the corresponding antiserum for 2 h at room temperature. Finally the bacterial suspension was centrifuged and the pellet discarded.

Characterisation of the antisera

All immunoassays were performed with 96-well PVC microtitre plates

(Integra Bioscience, Germany) according to Schloter et al. (1997) using three independent replicate microtitre plates for each assay and 4-fold repeat of each single value in one single plate.

To determine the titre for both purified antisera which gave the highest signal/noise ratio, several dilutions of *As-Hs* and *As-Hr* and a constant number of target and non-target cells (OD_{560} 0.6) were used.

To determine the maximum sensitivity of *As-Hs* and *As-Hr*, *H. seropedicae* Z67 and *H. rubrisubalbicans* M4 cells where diluted from 10^8 (OD_{560} 1.0) to 10^3 and the ELISA protocol performed. To calculate the exact bacterial densities 20 µl of each dilution was counted using the microcolony procedure.

To test cross-reaction several strains of different species were used (Table 1). Strains used in this study were inoculated in liquid Dyg's media overnight at 30°C centrifuged at 10,000 x g for 5 min, and resuspended in PBS buffer. The OD_{560} was adjusted to 1.0.

Table 1. Origin and morphology of the strains used for cross-reaction testing

Strains	Species	Origin of the isolate	Plant origin	Plant part
Pal 3	*A. diazotrophicus*	Alagoas, BR	Sugar cane	Roots
Pal 5	*A. diazotrophicus*	Alagoas, BR	Sugar cane	Roots
Br 17	*A. lipoferum*	Rio de Janeiro, BR	Maize	Roots
75 B	*Herbaspirillum* (like)	Rio de Janeiro, BR	*P. purpureum*	Roots
BA 13	*Herbaspirillum* (like)	Rio de Janeiro, BR	Banana	Leaves
BA 12	*Herbaspirillum* (like)	Rio de Janeiro, BR	Banana	Roots
AB7	*Herbaspirillum* (like)	Rio de Janeiro, BR	Pineapple	Leaves
Z 67	*H. seropedicae*	Rio de Janeiro, BR	Rice	Roots
Z 176	*H. seropedicae*	Rio de Janeiro, BR	Maize	Roots
Z 152	*H. seropedicae*	Rio de Janeiro, BR	Maize	Roots
HCC 100	*H. seropedicae*	Rio de Janeiro, BR	Sugar cane	Stems
HAWAI	*H. seropedicae*	Hawaii	Sugar cane	Roots
HRC 54	*H. seropedicae*	Rio de Janeiro, BR	Sugar cane	Roots
Z 78	*H. seropedicae*	Rio de Janeiro, BR	Sorghum	Roots
M4	*H. rubrisubalbicans*	USA	Sugar cane	Leaves
HCC 103	*H. rubrisubalbicans*	Rio de Janeiro, BR	Sugar cane	Stems
HPD 1	*H. rubrisubalbicans*	Rio de Janeiro, BR	*Digitaria insularis*	Roots
HCC 101	*H. seropedicae*	Rio de Janeiro, BR	Sugar cane	Stems
HRC 51	*H. rubrisubalbicans*	Rio de Janeiro, BR	Sugar cane	Roots
HPD 5	*H. seropedicae*	Rio de Janeiro, BR	Weed plant	roots
B 4362	*H. rubrisubalbicans*	Rio de Janeiro, BR	Sugar cane	Leaves
HSR 1	*H. seropedicae*	Rio de Janeiro, BR	Sorghum	Roots
HSR 4	*H. seropedicae*	Rio de Janeiro, BR	Sorghum	Roots
M1	*H. rubrisubalbicans*	Mauritius	Sugar cane	Leaves

Plant material

Cut stems of *Pennisetum purpureum* Schum var. Capim Cana D'África were collected from the Experimental Station of Embrapa-Agrobiologia. Individual nodes having 5–7 cm (including part of the internode material) were coinoculated by immersion in a bacterial overnight suspension of *H. seropedicae* Z67 and *H. rubrisubalbicans* M4, respectively, *H. seropedicae* Z67, *H. rubrisubalbicans* M4 and *A. diazotrophicus* Pal3 during 60 min. As control, plants were immersed in water for the same period. The inocula were grown in 125 ml Erlenmeyer flasks containing Dygs medium for 48 h at 30°C in a rotary shaker. These stem pieces were planted in sterile vermiculite + sand (2:1) supplemented with Hoagland's solution without N (Hoagland and Arnon, 1951). At 70 days after planting (DAP) the plants were harvested and plant material of each treatment was used for the ELISA quantification. From each treatment 5 plants were independently investigated.

Quantification for H. seropedicae and H. rubrisubalbicans in plant material

Roots were washed in tap water and separated into two fractions of 1 g each. One fraction was immersed in Cloramine T 1% for 5 min for surface sterilisation and the other was maintained in distilled water for 1 h. After the sterilisation, plant material was washed in phosphate saline buffer (PBS) and transferred to sterile water (1 h). All plant material was macerated in 9 ml of PBS. Finally 0.2 g of poly-ethylene-glycol PEG 6000 (Sigma, Germany) and 0.2 g of Chelex 100 (Sigma, Germany) to eliminate organic and ionic bonds was added. The material was incubated one hour at 4°C filtered through Whatman paper No1 and passed through a 5 µm filter (Milipor, Germany) to eliminate plant debris, followed by a centrifugation- and resuspension step in 1 ml PBS.

To inactivate the endogenous peroxidase enzyme present in the plant tissue glutaraldehyde fixation was used (4% solution in phosphate buffer 50 mM). The solution was mixed with 500 µl of plant suspension and incubated for 2 hr at room temperature.

After this step the ELISA protocol was used with 3 replicates for each treatment. To increase the detection signal, the biotin-streptavidin system (Amersham, Germany) (Schloter et al., 1992) was used. As a control treatment, the pre-immune serum in the presence of the plant extract was used. The cell numbers were determined using a standard curve of the target bacteria in dilutions from 10^8 to 10^3 cells ml^{-1}.

To obtain data, which could be used for further statistical analysis (Student Newman Keuls test), the whole extraction and ELISA procedure was repeated for each plant in 3 independent parallels.

3. Results and Discussion

Characterisation of the protein-A purified sera

After protein-A purification, both antisera *As-Hs(protA)* and *As-Hr(protA)* showed a high level of intragenetic cross-reactivity even when using the antiserum dilution, that showed the highest target/noise level. It was not possible to differentiate between both *Herbaspirillum* species (Fig. 1) indicating that both species are closely related and share a large number of antigenic determinants. A cross-reactivity of both sera with other bacteria was not visible. From the cross-reactivity data it can be concluded that at least 40% of the proteins and polysaccharides showing antigenic response from *H. seropedicae* and *H. rubrisubalbicans* are so closely related that they can not be distinguished by the protein-A purified antisera. Intraspecies homology of proteins and polysaccharides is for both *Herbaspirillum* species between 70% and 80%.

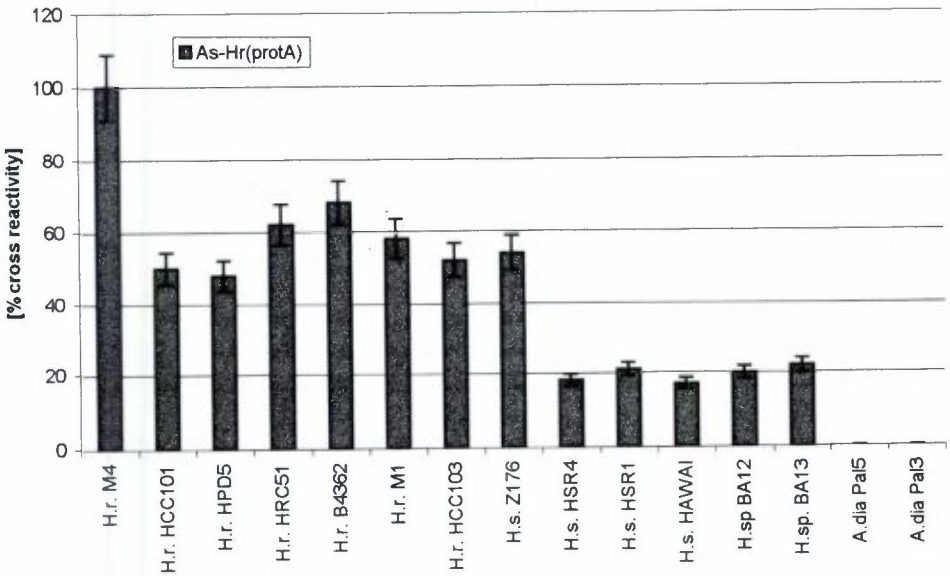
Characterisation of the affinity purified sera

Affinity purification was obtained by using *H. seropedicae* Z78 and *H. rubrisubalbicans* HRC51 respectively as adsorbant for selecting species specific antigens.

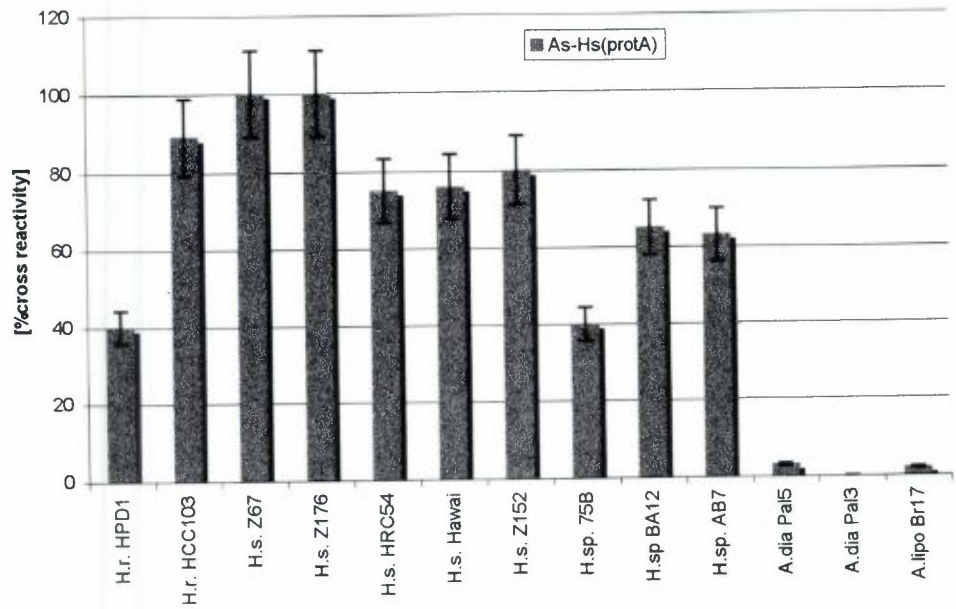
After purification of *As-Hs(protA)* with *H. seropedicae* Z78 the antiserum *(As-Hs(protA)-Z78)* showed almost strain specific properties for the immunogen *H. seropedicae* Z67 (Fig. 2). Almost no cross-reactivities were visible with other *H. seropedicae* or *H. rubrisubalbicans* species. The used dilution of the serum after affinity purification was very low, indicating a low number of antibodies in the serum. This shows clearly that although *H. seropedicae* Z78 and Z67 were isolated from different root material (Table 1), they share most antigenic determinants. A further characterisation of this serum using protein and polysaccharide extracts of *H. seropedicae* Z78 and Z67 could give more evidence on the number of shared antigens.

After purification of *As-Hs(protA)* with *H. rubrisubalbicans* HRC51 the antiserum *(As-Hs(protA)-HRC51)* showed almost species specific properties (Fig. 2). The cross-reactivities of *As-Hs(protA)-HRC51* with *H. rubrisubalbicans* strains was reduced below 10%. In between the species *H. seropedicae* the cross-reactivity values ranged from nearly 90–150%, indicating a certain amount of intraspecies variability of the shared antigens.

After purification of *As-Hr(protA)* with *H. rubrisubalbicans* HRC51 the antiserum *(As-Hr(protA)-HRC51)* cross-reactivities of *As-Hr (protA)-HRC51* were reduced compared to *As-Hr(protA)* with some strains of



(a)



(b)

Figure 1. Cross-reactivity of the antisera As-Hr(protA) (a) and As-Hs(protA) (b) with different strains using ELISA (% cross-reactivity compared to the strain used for immunisation). Error bars indicate standard deviations.

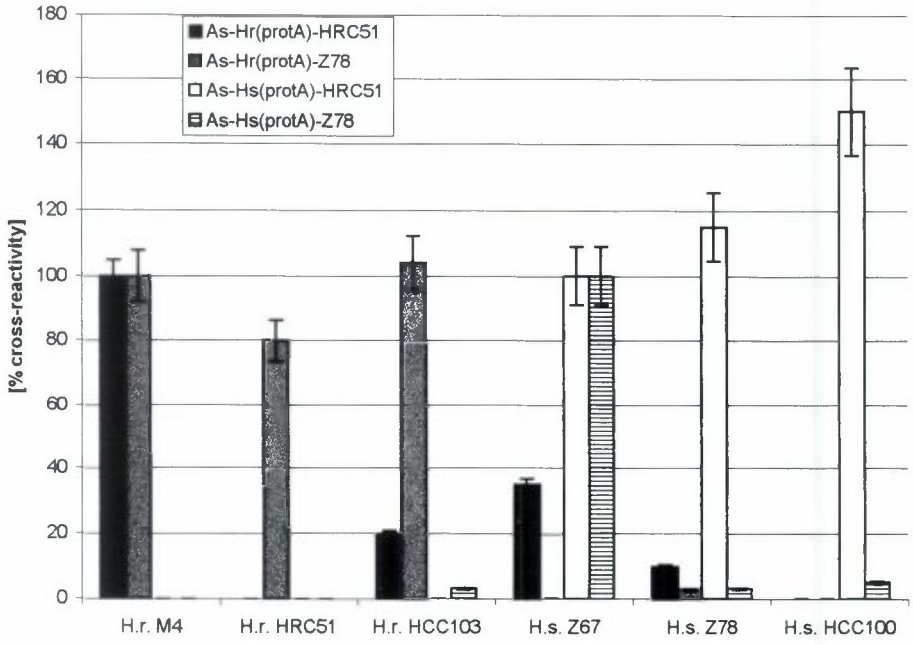


Figure 2. Cross-reactivity of the antisera As-Hr(protA)-Z78, As-Hr(protA)-HRC51, As-Hs(protA)-Z78 and As-Hs(protA)-HRC51 with different *Herbaspirillum* strains using ELISA (% cross-reactivity compared to the strain used for immunisation). Error bars indicate standard deviations.

H. rubrisubalbicans and *H. seropedicae*. Nevertheless a strict species or strains specificity of the serum was not visible (Fig. 2).

After purification of *As-Hr(protA)* with *H. seropedicae* Z78 the antiserum *(As-Hr(protA)-HRC51)* showed almost species specific properties (Fig. 2). The intraspecific variation of the signal strength was very low (between 80–100%), indicating that the homogeneity of antigens and their distribution are more conserved in *H. rubrisubalbicans* compared to *H. seropedicae*.

Validation of the antisera As-Hs(protA)-HRC51 and As-Hr(protA)-Z78

Using serial dilutions of the strains used for immunisation the detection limit of the purified sera *As-Hs(protA)-HRC51* and *As-Hr(protA)-Z78* could be determined. For *As-Hs(protA)-HRC51* the detection limit was 10^6 cells ml^{-1}, for *As-Hr(protA)-Z78* 10^5 cells ml^{-1} (Fig. 3). This level could be reduced by the chemiluminescence-ELISA (Schloter et al., 1992) for at least one decimal, but the expected numbers of *H. seropedicae* or *H. rubrisubalbicans* in plant tissue

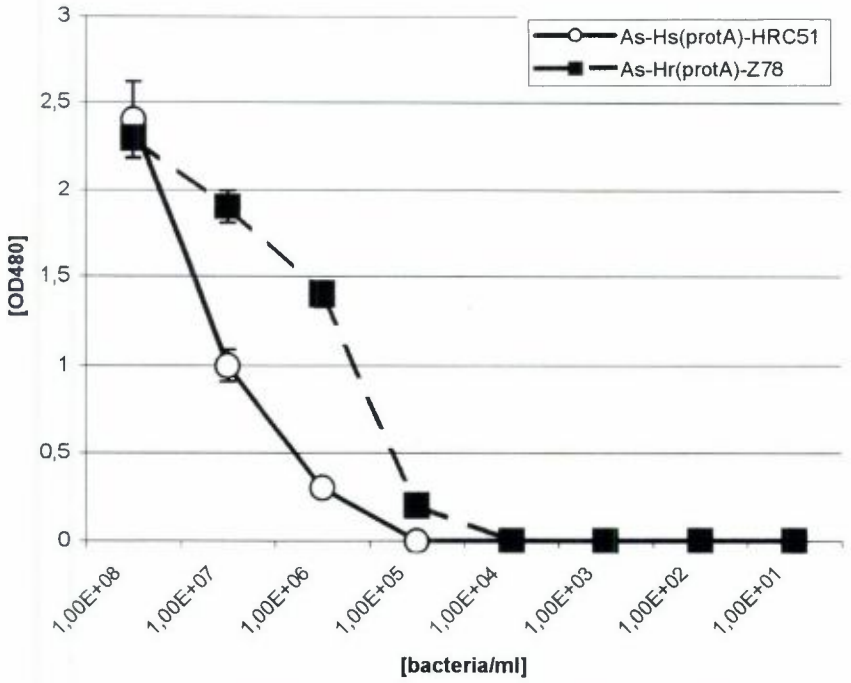


Figure 3. Validation of the antisera As-Hs(protA)-HRC51 and As-Hr(protA)-Z78 for quantification by ELISA using *H. seropedicae* Z67 as target for As-Hs(protA)-HRC51 and *H. rubrisubalbicans* M4 as target for As-Hr(protA)-Z78. Error bars indicate standard deviations.

are expected to be much larger, as it is assumed that large populations are necessary to contribute to the nitrogen accumulation via biological nitrogen fixation.

Measurements in plant material

Bacterial colonisation of plant material was quantified using the antisera *As-Hs(protA)-HRC51* and *As-Hr(protA)-Z78* (Table 2). Both *Herbaspirillum* strains (*H. seropedicae* Z67 and *H. rubrisubalbicans* M4) were able to colonise *P. purpureum* plants after coinoculation. Nevertheless the root parts, which were colonised were different for both strains. Whereas *H. seropedicae* Z67 was only able to colonise the rhizoplane, but in very high numbers (10^8 cells g^{-1} root fresh weight), *H. rubrisubalbicans* M4 was found in the outer parts of the roots in quite low numbers (10^7 cells g^{-1} root fresh weight), but was able to colonise also the root interior in the same magnitude. It seems that *H. seropedicae* Z67 is a more efficient coloniser of the rhizoplane and is able to outcompete

Table 2. Quantification of *H. seropedicae* Z67 and *H. rubrisubalbicans* M4 in roots of co-inoculated *Pennisetum purpureum* plants (double inoculation with *H. seropedicae* Z67 and *H. rubrisubalbicans* M4; triple inoculation with *H. seropedicae* Z67, *H. rubrisubalbicans* M4 and *A. diazotrophicus* Pal3) by the indirect ELISA technique. Serum As-Hs(protA)-HRC51 was used for quantification of *H. seropedicae* Z67; serum As-Hr(protA)Z78 was used for quantification of *H. rubrisubalbicans* M4. The standard deviation was below 10% in all cases (number of cells g fresh weigh^{-1}). Assuming normal distribution and homogeneous variances the mean values were tested by Student-Newman-Keuls-test (a;b,c indicating highly significant differences between treatments).

Inoculum	Control		Z 67 + M 4		Pal 3 + Z 67 + M4	
	Washed	Surface sterilised	Washed	Surface sterilised	Washed	Surface sterilised
H.s. Z 67	N.D. (c)	N.D. (c)	1×10^8 (a)	N.D. (c)	8×10^7 (a)	3×10^7 (b)
H.r. M 4	N.D. (c)	N.D. (c)	2×10^7 (b)	1×10^7 (b)	1.5×10^7 (b)	N.D. (c)

N.D. = not detected, below the detection limit.

H. rubrisubalbicans M4. In parts, that could not been colonised by *H. seropedicae* Z67 (inner root parts), *H. rubrisubalbicans* apparently found an ecological niche.

By coinoculation of both *Herbaspirillum* strains and an *A. diazotrophicus* strain, the colonisation of the outer root parts was not effected. In contrast triple inoculation resulted in a colonising of the root interior by *H. seropedicae*, which was not found by double inoculation of both *Herbaspirillum* strains (see above). This root interior colonising resulted in a displacement of *H. rubrisubalbicans*, which could not be detected in the root interior any more. These results underline the better colonising efficiency of *P. purpureum* of *H. seropedicae* Z67 by outcompeting *H. rubrisubalbicans*, in the situation of triple inoculation not only in the outer part of the roots but also in the inner parts. One reason for this better colonising efficiency might be the original source of both *Herbaspirillum* strains. Whereas *H. seropedicae* Z67 was isolated from the roots, *H. rubrisubalbicans* M4 was isolated from leaves. Nevertheless none of the strains was originally from *Pennisetum*. Another reason for the different colonisation pattern after triple inoculation might be a possible effect of the *A. diazotrophicus* strain, which has to be studied in the future in more detail.

In the natural non-inoculated control plants, neither *H. seropedicae* nor *H. rubrisubalbicans* were detected at densities higher than the detection limit of ELISA.

4. Conclusion

These results show that immunological methods provide a good tool to investigate plant microbe interactions. The technique might be used to substitute the time-consuming MPN method to quantify specific nitrogen-fixing bacteria in plant material.

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