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Avidin–Biotin Complex Incorporation into Enzyme-Linked Immunosorbent Assay (ABELISA) for Improving the Detection of *Azospirillum brasilense* Cd

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Abstract. Incorporation of the avidin–biotin complex into either indirect or competitive enzyme-linked immunosorbent assays significantly improved the quantitative detection and enumeration of the rhizosphere bacteria *Azospirillum brasilense* Cd both in culture and in the roots. The improvement was within the range of 10^4 to 5×10^7 colony forming units/ml or g root. Frozen samples yielded higher detection values within the range of 10^5–10^6 colony forming units/ml or g root.

Azospirillum species may be used to inoculate various plant species in order to improve plant productivity [1, 18]. A reliable method is essential for identification and enumeration of the applied strain for evaluation of its colonization ability in the rhizosphere. Two such approaches have been used: semi-selective media that allow the development of several different *Azospirillum* strains [2, 12, 19, 21] and immuno detection such as fluorescent antibody technique [22], and, recently, enzyme-linked immunosorbent assay (ELISA) [14]. Although both immuno detection procedures are capable of identifying a particular strain, the enumeration of low or medium levels of bacteria (10^4–10^6 CFU/ml) is frequently difficult owing to low intensity of the color produced by the enzymatic reaction, which is in many determinations only slightly higher than the control background.

The avidin–biotin complex is used in several immunoassay procedures commonly used in clinical medicine [8, 15, 20, 23, 26, 27]. Avidin, a glycoprotein abundantly available from egg white, has an extremely high affinity for biotin. Biotin molecules can be coupled to antibodies (biotinylation), and avidin can be covalently conjugated with various enzymes. Biotin bound to an antibody is still available for interaction with avidin. Since avidin has four binding sites for biotin and since multiple biotin molecules can be bound to a certain antibody molecule, the biotinylated antibody can bind to more than one avidin molecule. These two fundamental properties of the complex indicate a potential for amplification of antigen detection when incorporated into immunoassays [8, 23, 26].

The aims of the present study were to enhance the detection level of *A. brasilense* Cd in the ELISA procedure by incorporation of the avidin–biotin complex to increase the color intensity produced by the entire procedure, thus facilitating low- and medium-level detection of *A. brasilense* Cd and to utilize this immuno tool in rhizosphere studies.

Materials and Methods

Bacteria. *Azospirillum brasilense* Cd (ATCC 29710) was used.

Culture conditions, bacterial counts, antisera production, IgG purification, antibody specificity, and indirect and competition enzyme-linked immunosorbent assay (ELISA) were performed as previously described in detail [2, 13, 14].

***A. brasilense* Cd detection by avidin–biotin complex incorporated into ELISA.** The entire procedure is described in Fig. 1. Specific anti-*A. brasilense* Cd IgG were elicited in white rabbits by the procedure described previously in detail [14]. Biotinylated goat–antirabbit antibodies and alkaline phosphatase-labeled avidin were obtained from Bioyeda Co. (Rehovot, Israel) and used at a concentration of 1 : 1000. Disodium paranitrophenyl phosphate (0.1–0.4 mg/ml) served as a substrate for the enzymatic reaction in the ELISA and ABELISA procedures.

Inoculation of wheat seedlings with *A. brasilense* Cd. Seeds were disinfected in 1% NaOCl for 2 min, rinsed thoroughly in tap water, allowed to absorb for 3 h, and then transferred aseptically

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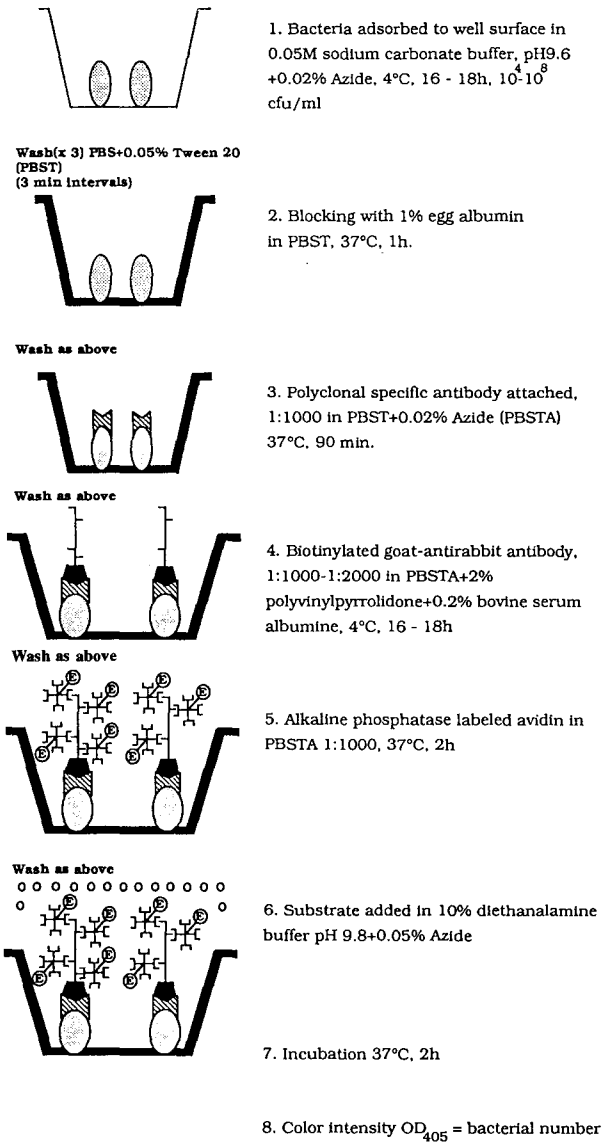

Fig. 1. Schematic representation of the avidin–biotin indirect-ELISA (ABELISA) procedure for the detection of *A. brasilense* Cd.

to large glass test tubes (200 ml) containing washed (distilled water) pure quartz sand (150 ml). The seedlings were grown for 6 days (two-leaf stage) at 22 ± 2°C in a growth chamber (model EF7H, Conviron, Controlled Environment Co., Canada) 10/14 h (day/night) at 130 $\mu E/m^2/s$ and then inoculated with 5 ml of 10^8 of *A. brasilense* Cd per 150 ml sand.

Bacterial determination in roots. Bacteria from inoculated and control plants were prepared from root homogenate as follows: unwashed roots were cut into pieces, 3–5 mm long, and were homogenized by a disperser (model x10/20, Yistral, Ballrechten-Dottingen, FRG) in 0.06 *M* potassium phosphate buffer, pH 7.0, in an ice bath. The slurry obtained was further homogenized by a fine glass homogenizer (Kontes, Vineland, New Jersey). After centrifugation at 12,000 *g* for 10 min, the pellet was dissolved in a minimum volume of 2–3 ml of buffer and used either for coating wells in microtiter plates or for competition-ELISA. Serial dilutions of the root pellet suspension in 0.1-ml portions were incubated at 30 ± 2°C for 48 h, and the number of colonies was determined.

Bacterial freezing. Washed *A. brasilense* Cd cells or root extracts were frozen by soaking flasks containing small amounts of suspensions into a bath containing dry ice in technical grade ethanol at −70°C. After freezing, the preparations were transferred to −20°C. Samples were thawed at room temperature before testing.

Experimental design. All experiments were randomly designed in a microtiter plate in triplicate with two to six wells as a single replicate. Experiments were repeated three to eight times each, and the results presented are the means of all experiments. Each ELISA plate contained internal controls, since the plates differ in their performances. Controls used in this study were preimmune sera, wells with the conjugates or substrate but without antibodies or antigens.

Results and Discussion

There has always been a need in plant rhizosphere studies to develop rapid, simple assay systems that can detect and enumerate minute numbers of rhizosphere bacteria, especially *Azospirillum* species, in a reproducible manner. The conventional selective media approach failed in this task. Whenever a particular *Azospirillum* strain was applied to the rhizosphere, its monitoring was unreliable as a result of a background of other unidentified rhizosphere bacteria capable of growing on the medium [2, 12]. Enzyme-linked immunosorbent assays (ELISA) gave a satisfactory answer to the problem of positively identifying specific phytopathogenic bacteria [25], rhizobia [16, 17], and *Azospirillum* strains [14], although the serology features of *Azospirillum* are poorly understood [9, 10, 13]. ELISA procedures were used for detection of a specific *Azospirillum* strain in the root system of several plant species [5–7] as well as in sand or soil [3, 4].

An inherent disadvantage of all conventional ELISA procedures is that when very low bacterial populations are present in a sample ($\sim 10^4$ cfu/ml), the detectable levels are only slightly higher than background. Therefore, the reliability of quantitative results becomes questionable and needs verification by other microbiological methods. Since lowering ELISA detection levels ($<10^4$ cfu/ml) has not yet been successful for bacteria associated with plant material, we attempted to amplify the ELISA detection level at its lower marginal levels ($<10^6$ cfu/ml) by incorporating the avidin–biotin complex

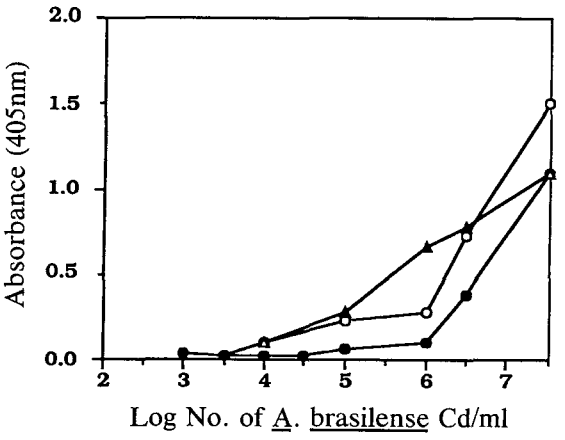

Fig. 2. Detection of *A. brasilense* Cd by the two ELISA procedures: ●, detection by indirect-ELISA; ○, detection by ABELISA of freshly prepared bacteria; ▲, detection by ABELISA of frozen bacteria.

into the ELISA basic procedures, i.e., indirect and competition ELISA. The realization that the avidin–biotin complex could be effectively employed to immunoassays was first proposed by Guesdon et al. [11], suggesting several methods for utilizing this complex in order to increase the sensitivity of ELISA.

Since biotin is a relatively small molecule, the labeling of antibodies with it hardly affects the physical characteristics or biological activity of biotinylated antibodies [26]. Non-reacted biotin can be easily separated from the biotinylated antibodies by a simple dialysis procedure [27]. Biotinylation of antibodies and production of avidin conjugates are relatively simple procedures and can be performed even on unpurified antibodies or on whole antisera. Once biotinylated, the antibodies can be stored for long periods at −20°C. Alkaline phosphatase-labeled avidin can be stored up to 1 year at 4°C. The preparations are very stable and can be obtained from commercial sources.

Comparison of several dilutions of *A. brasilense* Cd by both indirect-ELISA and by ABELISA revealed that color intensity that developed in the different reaction mixtures (directly reflecting the bacterial number in various samples [14]) is higher in ABELISA throughout the range of 10^4 to 5×10^7 cfu/ml. Freezing of the bacterial samples before testing further improved the detection level, mainly within the range of 10^5 to 10^6 cfu/ml (Fig. 2). The relative increase (by integration) in detection of series of bacterial concentrations by ABELISA revealed a 92% improvement compared with indirect-ELISA for fresh bacteria and an additional 38% increase when frozen bacteria were tested. Analyzing inoculated vs. noninoculated roots in competition-ABELISA showed only 8.5% inhibition by noninoculated roots compared with 89.2% inhibition by inoculated roots, corresponding to 5×10^7 cfu/g fresh weight of roots. Enumeration of *A. brasilense* Cd in roots (previously frozen) by indirect-ABELISA showed mean increase in absorbance (405 nm) from 0.016 (noninoculated roots) to 0.248 (inoculated roots), corresponding to 4×10^5 cfu/g fresh weight of roots. The values obtained for *A. brasilense* Cd detection by both ABELISA procedures were higher than the values commonly obtained by conventional indirect and competition ELISA procedures [14].

In the case of detecting different species of *Azospirillum*, the proposed procedure might have a disadvantage. It is known that *A. lipoferum* but not *A. brasilense* requires external biotin for growth [24]. Thus, biotin receptors, probably present in the bacterial cell, may attract biotin molecules. These biotin molecules may in turn interact with conjugated avidin and cause enhancement in detection values by ABELISA. These values will not be in accordance with the actual bacterial cells in the sample. This point should be considered when one performs ABELISA assays with *A. lipoferum*.

Our study demonstrated that biotinylated antibodies may provide a useful tool for identification and enumeration of *Azospirillum* spp. in the rhizosphere, since inoculated beneficial bacteria are often present in the plant rhizosphere at low concentrations. Immuno assays utilizing biotinylated antibodies and enzyme-labeled avidin should be further developed in order to improve their sensitivity to a level comparable to that reported for the human disease agent *Streptococcus pneumoniae* (10^3 organisms/ml) [27] as well as for the detection and enumeration of other bacterial species present in the rhizosphere. We propose that ABELISA has potential use in research laboratories concentrating on rhizosphere bacteria; the assays are simple, accurate, reproducible, inexpensive, and may be conducive to partial or complete automation as is commonly used in clinical ELISA procedures.

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