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Microfluidic Immunosensor with Micromagnetic Beads Coupled to Carbon-Based Screen-Printed Electrodes (SPCEs) for Determination of *Botrytis cinerea* in Tissue of Fruits

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A wide range of plant species, including economically important crops such as vegetables, ornamentals, bulbs, and fundamentally fruits, can be affected by gray mold caused by the fungal pathogen *Botrytis cinerea*. This paper describes the development of a microfluidic immunosensor with micromagnetic beads (MMBs) coupled to carbon-based screen-printed electrodes (SPCEs) for the rapid and sensitive quantification of *B. cinerea* in apple (Red Delicious), table grape (pink Moscatel), and pear (William's) tissues. The detection of *B. cinerea* was carried out using a competitive immunoassay method based on the use of purified *B. cinerea* antigens immobilized on 3-aminopropyl-modified MMBs. The total assay time was 40 min, and the calculated detection limit was 0.008 μg mL^{-1}. Moreover, the intra- and interassay coefficients of variation were below 7%. The developed method allowed detects *B. cinerea* even in asymptomatic fruits and promises to be particularly useful for application in the agricultural industry.

KEYWORDS: Enzyme immunoassays; *Botrytis cinerea*; micromagnetic beads; horseradish peroxidase; microfluidic system; flow injection analysis

INTRODUCTION

Botrytis cinerea is a phytopathogenic fungus responsible for the disease known as gray mold, which causes substantial losses of fruits at postharvest. Fruit infections often remain dormant until the fruit ripens, when symptoms of the disease appear (*1*).

The control of this fungus is difficult, because this pathogen has a wide host range and multiple mechanisms of attack; moreover, it can infect all of the plant's organs at most of developmental stages (*2*). The gray mold disease is mostly controlled with fungicides, but the high amount of fungicide required to achieve satisfactory disease control may lead to different problems such as the presence of fungicide residues in fruits and the consequent pressures of consumers for the reduction of fungicides in the food chain and the environment (*3*, *4*). Moreover, the fungus is genetically variable and has developed strains resistant to many of the chemicals introduced in the past 20 years (*5*). For these reasons, there is an increased interest in the development of sensitive and reliable methods for the determination of the fungus in fruit tissues for disease control. Classical methods such as isolation on selective media are useful but subject to limitations, that is, many pathogens are masked by overgrowth of faster growing fungi. Quantitative nucleic acid-based methods have been developed (*6*), but these methods are expensive and difficult to perform as routine assays.

The detection of the fungus prior the occurrence of the rot requires the development of rapid and easy-to-use detection methods. To date, some methods based on enzyme-linked immunosorbent assay (ELISA) for the detection of the fungus have been reported (*7*). Another alternative is electrochemical immunosensors, which in recent years have been an attractive subject for food analysis (*8*, *9*). Miniaturized homemade screen-printed electrodes (SPCEs) have been designed as disposable transducers based on a three-electrode configuration (*10*, *11*).

In a previous work we have developed a screen-printed immunosensor modified with carbon nanotubes in a continuous-flow system for the determination of *B. cinerea* in apple tissues, in which the purified *B. cinerea* antigens were immobilized in a rotating disk on 3-aminopropyl-modified controlled-pore glass (APCPG) (*12*). This device was successfully used in a competitive assay for the determination of *B. cinerea* in apples.

In the present work we propose the incorporation of a SPCE-based immunosensor into a microfluidic system because microfluidic devices offer many potential advantages including reduced reagent consumption, smaller analysis volumes, faster analysis times, and increased instrument portability (*13*−*17*). Moreover, we employ micromagnetic beads (MMBs), particularly suitable for integration in microfluidic devices as solid support (*18*), for the immobilization of purified *B. cinerea* antigens, with the aim of increasing the sensitivity of the method.

MATERIALS AND METHODS

Reagents and Solutions. All reagents used were of analytical reagent grade. The monoclonal antibody BC-12.CA4 and secondary antibody−enzyme conjugate (anti-mouse polyvalent immunoglobulins peroxidase conjugate) were obtained from *ADGEN* Diagnostics (Auchincruive,

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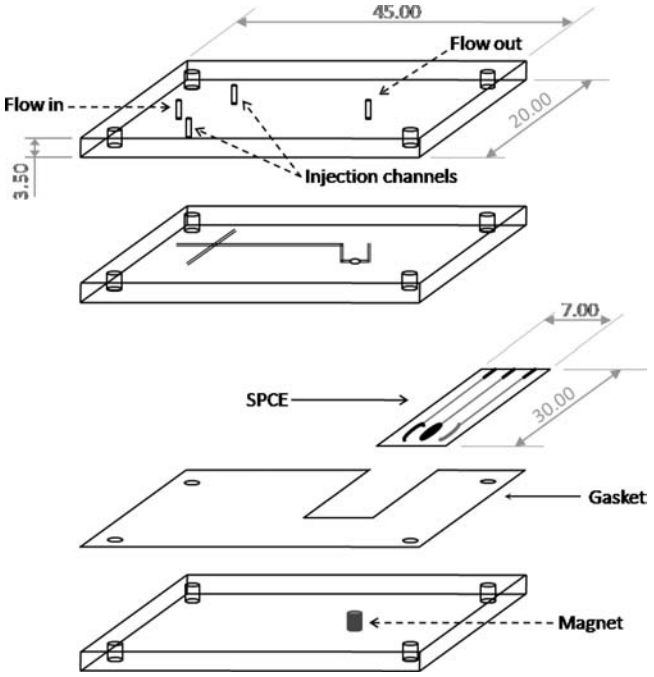

Figure 1. Schematic representation of the microfluidic immunosensor. All measurements are given in millimeters.

Scotland) and Sigma Chemical (St. Louis, MO) respectively. Glutaraldehyde (25% aqueous solution) and hydrogen peroxide (H_2O_2) were purchased from Merck (Darmstadt, Germany). The micromagnetic beads, amino functionalized (53572), were purchased by Fluka (Buchs, Switzerland). Bovine serum albumin (BSA) and 4-*tert*-butylcatechol were purchased from Sigma Chemical. All other reagents employed were of analytical grade and were used without further purification. Aqueous solutions were prepared using purified water from a Milli-Q system.

The SAPS ELISA kit for *Botrytis* was purchased from SAPS Offices, Cambridge University Botanic Garden, Cambridge, U.K., and was used in accordance with the manufacturer's instructions (*19*).

Flow-through Reactor/Detector Unit. The main body of the sensor was made of Plexiglas. **Figure 1** illustrates the design of the flow-through chamber containing the microfluidic immunosensor. All solutions and reagents were conditioned to 37 °C before the experiment, using a laboratory water bath (Vicking Mason Ii, Vicking SRL, Argentina).

Amperometric detection was performed using the BAS LC-4C, and the BAS 100 B (electrochemical analyzer Bioanalytical System, West Lafayette, IN) was used for cyclic voltammetric analysis. The electrochemical behavior of enzyme substrate 4-*tert*-butylcatechol (4-TBC) was examined by cyclic voltammetry at the SPCEs. A cyclic voltammetric study of 1 mM in an aqueous solution containing 0.1 M phosphate−citrate buffer, pH 5.0, was performed by scanning the potential from −400 to 750 mV versus Ag/AgCl. The cyclic voltammogram showed a well-defined anodic peak and a corresponding cathodic peak, which corresponds to the transformation of 4-TBC to 4-*tert*-butyl *o*-benzoquinone (4-TBBQ) and vice versa in a quasi-reversible two-electron process. A peak current ratio ($I_{p,a}/I_{p,c}$) of nearly unity, particularly during the recycling of potential, can be considered a criterion for the stability of 4-TBBQ produced at the surface of electrode under experimental conditions (data not shown). The potential applied to the detection was −0.15 V versus Ag/AgCl, pseudoreference electrode. At this potential, a catalytic current was well established. Baby Bee syringe pumps (Bioanalytical System) were used for pumping, introducing the sample, and stopping the flow.

All pH measurements were made with an Orion Expandable ion analyzer (model EA 940, Orion Research, Cambridge, MA) equipped with a glass combination electrode (Orion Research).

Preparation of the *B. cinerea* Antigens. The purified *B. cinerea* antigens were prepared following the same procedure as our previous work (*12*).

B. cinerea Pers.:Fr (BNM 0527) was used in this study. The strain is deposited in the National Bank of Microorganisms (WDCM938) of the Facultad de Agronomia, Universidad de Buenos Aires (FAUBA). The isolates were maintained on potato dextrose agar (PDA) at 4 °C.

To induce the mycelial production, *B. cinerea* was grown on PDA for 8−12 days at 21 ± 2 °C. After this incubation period, the mycelium was removed, frozen in liquid nitrogen, blended in a Waring blender, and freeze-dried to obtain a fine powder. Then, the fine powder was suspended in 0.01 M phosphate-buffered saline (PBS, pH 7.2) and centrifuged at 1000*g* for 10 min. The supernatant, which contained the antigen, was stored in 0.01 M PBS, pH 7.2, at −20 °C between uses. In this study, the units of antigen were expressed as *Botrytis* antigen units (B-AgU), which were equivalent to μg mL^{-1} PBS extracts of freeze-dried fungal mycelium (*7*).

To induce the conidial production, *B. cinerea* was grown on PDA at 21 ± 2 °C. Immediately, when the mycelium appeared, the cultures were kept at 15 °C. After a week, spores were harvested and suspended in 10 mL of sterile 0.01 M PBS, pH 7.2, containing 0.05% (v/v) Tween 80. Finally, the concentration of spore suspension was determined with a Neubauer chamber and adjusted with PBS to 1×10^5 spores mL^{-1}. This spore suspension was used to infect the fruit samples.

Preparation of the SPCEs. An electrode pretreatment was carried out before each voltammetric experiment to oxidize the graphite impurities and to obtain a more hydrophilic surface, with the aim of improving the sensitivity and reproducibility of the results. The graphite electrode surface was pretreated by applying a potential of +1.6 V (versus Ag-SPE) for 120 s and of +1.8 V (versus Ag-SPE) for 60 s in 5 mL of 0.25 M acetate buffer, containing 10 mM KCl, pH 4.75, under stirred conditions. Then, the electrodes were washed using 0.01 M PBS, pH 7.2, and stored in the same buffer at 4 °C until used (*20*, *21*).

Immobilization of Purified Antigen of *B. cinerea* on Magnetic Beads. Purified antigens of *B. cinerea* were immobilized on MMBs modified with amino groups in an Eppendorf tube (*22*). One hundred microliters of MMBs amino functionalized were washed with 1.0 mL of PBS buffer, pH 7.2, three times. The pellet was suspended in 1.0 mL of an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 M sodium carbonate buffer, pH 10) with continuous mixing for 2 h at room temperature. After three washings with PBS buffer, pH 7.2, to remove the excess glutaraldehyde, 400 μL of antigen preparation (10 μg mL^{-1} 0.01 M PBS, pH 7.2) was coupled to the residual aldehyde groups with continuous mixing for 12 h at 4 °C. The immobilized antigen preparation was finally washed with PBS, pH 7.2, and suspended in 200 μL of the same buffer at 5 °C. Preparations of immobilized antigen were perfectly stable for at least 1 month.

Preparation of Extracts of Fruit Samples. The preparation of extracts of fruit samples was carried out according to the procedure described in our previous paper (*12*).

In a first step, the fruit samples were infected using a spore suspension (1×10^{-5} spores mL^{-1}). Apples, pears, and table grapes were wounded using a punch. The wound size of apples and pears was 3 mm × 3 mm × 3 mm, whereas the one of table grapes was 1 mm × 1 mm × 1 mm. After that, 20 μL of the spore suspension was put into each wound. Then, the fruits were kept at 25 °C and evaluations of rot incidence and lesion diameters were made over 10 days. Ten fruits for the assays were used with three wounds each. Each experiment was repeated three times.

In the second step, fruit tissues infected and uninfected were removed and were ground to a fine powder in liquid N_2.

Finally, the extracts of fruit samples were prepared by adding 0.1 g of powdered fruit tissue into 0.9 mL of PBS and vortexed for 1 min to obtain a homogeneous suspension.

Determination of *B. cinerea* by Amperometric Analysis. Table 1 summarizes the complete analytical procedure required for the determination of *B. cinerea* antigens.

First, the modified MMBs were put in 1 mL of desorption buffer (0.1 M citrate buffer, pH 2.00), and the mixture was stirred at room temperature for 2 min. After that, they were rinsed with 0.01 M PBS, pH 7.2.

The nonspecific binding was blocked by 10 min of treatment at 37 °C with 1.5% bovine serum albumin (BSA) in 0.01 M PBS, pH 7.2, and finally washed with 0.01 M PBS buffer (pH 7.2) and stored in 200 μL of the same buffer.

Then, 20 μL of modified MMBs was mixed with 25 μL of fruit PBS extracts and 25 μL of the monoclonal antibody IgG mouse anti-*B. cinerea* (15 μg mL^{-1} in 0.01 M PBS, pH 7.2) and shaken for 10 min at 37 °C.

Table 1. Sequences Required for the Quantitative Determination of *B. cinerea*

sequence	condition	time
preparation of extracts of fruit samples	powdered fruit tissues (with liquid N_2) + 0.01 M PBS, pH 7.2	10 min
pretreatment of samples	*B. cinerea* antigen-coated microbeads + antibody (15 μg mL^{-1}) + extract of fruit sample	10 min
washing buffer	PBS buffer, pH 7.2	3 min
injection of magnetic beads	20 μL of modified magnetic beads at 5 μL min^{-1}	4 min
washing buffer	flow rate = 5 μL min^{-1} (PBS, pH 7.2)	3 min
enzyme conjugated	25 μL of antibody−HRP conjugated at 5 μL min^{-1}	5 min
washing buffer	flow rate = 5 μL min^{-1} (PBS, pH 7.2)	3 min
substrate	(5 μL of 0.1 M phosphate−citrate buffer, pH 5.0, containing 1 mM H_2O_2 and 1 mM 4-TBC)	1 min
signal analysis	LC-4C amperometric detector, −0.15 V	1 min

B. cinerea present in the sample was allowed to compete by the anti-*B. cinerea*-specific monoclonal antibody (BC-12.CA4) with the immobilized purified *B. cinerea* antigens on 3-aminopropyl-modified MMBs.

The MMBs were recovered using an external magnet and washed three times with 0.01 M PBS buffer, pH 7.2, to remove the excess of sample, and they were suspended in 200 μL of 0.01 M PBS, pH 7.2. The microfluidic device was prepared by injection of 20 μL of MMBs in the flow system by a micropump at a flow rate of 5 μL min^{-1} for 4 min. A permanent magnet was used to attract the beads at a specific area of the channel, near the screen-printed electrode. The magnet was not moved during the experiment to keep the beads in the channel, and they were not carried away by the continuing flow. The carrier buffer was 0.01 M PBS, pH 7.2.

Immediately, 25 μL of the anti-mouse IgG−HRP (peroxidase) conjugate (dilution of 1500-fold in 0.01 M PBS, pH 7.2) was injected into the PBS carrier stream at a flow rate of 5 μL min^{-1} for 5 min. The flow line of the immunosensor was washed with 0.1 M sodium citrate buffer, pH 5, 5 μL of substrate solution (1 mM H_2O_2 and 1 mM 4-TBC in 0.1 M citrate buffer, pH 5) was injected into the carrier stream at 5 μL min^{-1} for 1 min, and the enzymatic product (P) was measured on the surface of a screen-printed electrode at −0.15 V.

Amperometric measurements were performed at −0.15 V at room temperature in 0.1 M phosphate−citrate buffer, pH 5, and the resulting cathodic current was displayed on the $x-y$ digital recorder. The stock solution of 4-TBC was prepared freshly before the experiment and stored in the dark for the duration of the experiment. When not in use, the immunosensor was stored in 0.01 M PBS, pH 7.2, containing sodium azide (0.01%) at 4 °C.

RESULTS AND DISCUSSION

A microfluidic immunosensor coupled to SPCEs with electrochemical detection for the rapid and sensitive quantification of *B. cinerea* has been developed. The immunosensor was applied to determine the fungus in different kinds of fruits such as apple (Red Delicious), table grape (pink Moscatel), and pear (William's), before and after the occurrence of rot symptoms. The immunosensor was based on a competitive reaction by the specific monoclonal antibody (BC-12.CA4) between the free antigen (present in the fruit tissue sample or as purified antigen) and the purified *B. cinerea* antigen immobilized on 3-aminopropyl-modified MMBs.

As explained, MMBs were injected into microchannels and were manipulated for an external removable magnet. Then, the bound antibodies were quantified by using a second antibody specific to mouse IgG labeled with horseradish peroxidase (HRP). 4-TBC was used as enzymatic mediators. When hydrogen peroxide (H_2O_2) is present, HRP catalyzes the oxidation of 4-TBC to 4-TBBQ (*23*). The electrochemical reduction back to 4-TBC was detected on SPCEs at −0.15 V. The response current was inversely proportional to the amount of *B. cinerea* antigens present in the fruit sample.

Prior to application of the immunosensor to the determination of *B. cinerea*, the following series of steps were carried out

Optimization of the Immune Reactions and Determination of Enzymatic Products. The optimal flow rate was determined by analyzing an antigen preparation of 25 μg mL^{-1} *B. cinerea* at different flow rates and evaluating the current generated during the immune reaction. As shown in **Figure 2**, flow rates from 1 to 5 μL min^{-1} had little effect on the anti-mouse IgG−HRP conjugate and the immunocomplex formed on the MMBs (antigen−antibody reaction), whereas when the flow rate exceeded 7.5 μL min^{-1}, the signal was dramatically reduced. Therefore, a flow rate of 5 μL min^{-1} was used for injections of reagents and washing buffer.

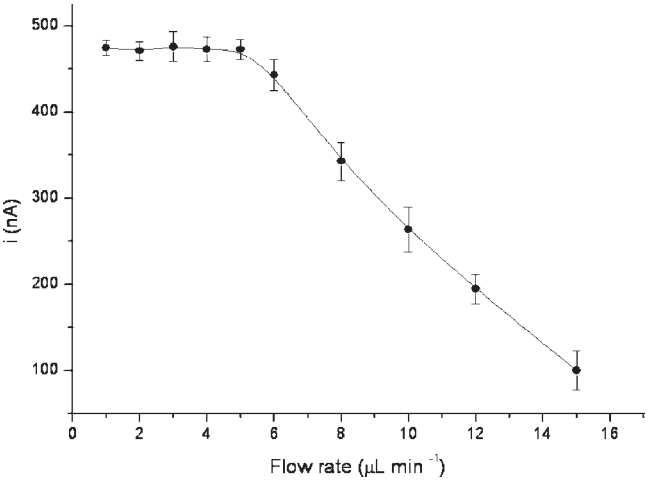

Figure 2. Effect of flow rate analyzing a 25 μg mL^{-1} *B. cinerea* standard at different flow rates from 1 to 15 μL min^{-1}. The error values represent the standard deviation.

The volume of MMBs injected after the pretreatment sample procedure was studied in the range of 5−25 μL. Sensitivity was almost tripled in the range from 5 to 20 μL. Over 20 μL of MMB dilutions, the central channel was obstructed.

Finally, a sample volume of 20 μL was used to evaluate other parameters. The rate of enzymatic response under flow conditions was studied in the pH range of 4−7 and reached a maximum at pH 5.0. The pH value used was 5.0 in phosphate−citrate buffer. The effect of varying the 4-TBC concentration from 0.1 to 5 mM on the enzymatic response was evaluated. The optimum 4-TBC concentration determined, 1 mM, was then used.

Preparation of Antigens and Samples. The procedure of sample preparation included a previous treatment using liquid nitrogen. In preliminary tests this step was not taken into account, and the resulting signal was very low. This can be explained by taking into account the monoclonal antibody, BC-12.CA4, which recognizes an antigen, possibly a glycoprotein, with the antigenic binding site on L-rhamnose. The antigen is expressed from the first appearance of the germ tube during germination and can be observed by immunofluorescence along the entire length and tip of the germ tube but not on conidia. It appears that these antigenic sites are exposed in high quantities when the sample is treated with liquid nitrogen (*7*).

Tests in which the fruit samples were infected using different spore suspensions of *B. cinerea* were also made: 1×10^4, 1×10^5, and 1×10^6 spores mL^{-1}, respectively (data not shown). The currents measured after 4 days of incubation (25 °C) did not show differences, because the method cannot detect spores, only detect germ tubes in the precise moment they appear, and the quantity of germinated spores does not always depend of the quantity of spores inoculated.

Quantitative Determination of *B. cinerea*. A standard curve for the amperometric procedure was produced following our protocol with a series of purified antigens that covered the relevant range of 0−80 $\mu g\ mL^{-1}$. The linear regression equation was $i = 698.22 - 7.916C_{B.\ cinerea}$, with the linear regression coefficient $r = 0.998$ and a detection limit (DL) of 0.008 $\mu g\ mL^{-1}$. The DL was considered to be the concentration that gives a signal 3 times the standard deviation (SD) of the blank.

The coefficient of variation (CV) for the determination of 25 $\mu g\ mL^{-1}$ *B. cinerea* was below 4.5% (six replicates).

The total time required for the proposed assay was approximately 40 min.

The precision of the electrochemical assay was checked with a control solution at 5, 25, and 75 $\mu g\ mL^{-1}$ *B. cinerea* purified antigen concentrations and with three extracts of fruit samples obtained at 4, 7, and 10 days of incubation (25 °C), respectively. The within-assay precision was tested with five measurements in the same run for each control and for each extract of fruit sample. These series of analyses were repeated for three consecutive days to estimate the between-assay precision. The results obtained are presented in **Table 2**. The *B. cinerea* assay showed good precision; the CV within-assay values were < 4.5%, and the between-assay values were < 7%.

Table 2. Within-Assay Precision (Five Measurements in the Same Run for Each Control and for Each Extract of Fruit Sample) and Between-Assay Precision (Five Measurements for Each Control and for Each Extract of Fruit Sample, Repeated for Three Consecutive Days)

	within-assay		between-assay	
	mean	CV %	mean	CV %
control[a]				
5 $\mu g\ mL^{-1}$	5.09	2.54	5.42	5.47
25 $\mu g\ mL^{-1}$	25.14	1.90	25.31	6.51
75 $\mu g\ mL^{-1}$	74.71	4.31	75.83	4.85
sample[b]				
S_1	12.58	2.97	12.94	5.58
S_2	43.77	3.11	43.95	6.13
S_3	71.86	4.01	72.78	4.78

[a] *B. cinerea* antigen. [b] Extract of fruit sample S_1 obtained at 4 days of incubation, S_2 obtained at 7 days of incubation, and S_3 obtained at 10 days of incubation. The incubation was realized at 25 °C.

The correlations between the lesion diameters of the fruit samples and the amount of *B. cinerea* antigen detected by the proposed method from extracts fruit samples obtained at 4, 7, and 10 days of incubation (25 °C), respectively, are presented in **Table 3**. These results showed a correlation between the damage level and the amount of fungus present in the fruit samples. *B. cinerea* can be detected by our method even when fruit rot is not visible. The fungus was detected when it began to germinate (about 4 days after inoculation and incubation of the fruit samples).

The accuracy was tested with dilution and recovery tests. A dilution test was performed with a control solution of 80 $\mu g\ mL^{-1}$ *B. cinerea* purified antigens concentration in 0.01 M PBS, pH 7.2 (**Figure 3**).

The method developed was compared with a commercial spectrophotometric system SAPS-ELISA kit for the quantification of *B. cinerea* (*19*) in 30 commercial fruit samples. The analysis performed with the extracts of fruit obtained was realized between 4 and 7 days after inoculation and incubation at 25 °C of the fruit samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between the two methods

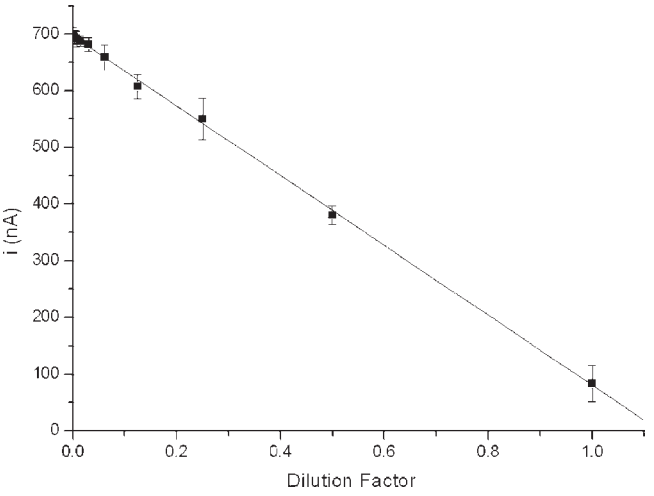

Figure 3. Dilution test results for control solution of 80 $\mu g\ mL^{-1}$ *B. cinerea* purified antigen concentrations with 0.01 M PBS, pH 7.2. Each value is based on five determinations. The error values represent the standard deviation.

Table 3. Correlation between the Lesion Diameters of the Fruit Samples and the Amount of *B. cinerea* Antigen Detected by the Proposed Method from Extracts of Fruit Samples Obtained at 4, 7, and 10 Days of Incubation, Respectively

fruit sample	days of incubation	lesion diameter (mm)	*B. cinerea* antigen detected ($\mu g\ mL^{-1}$)
apples (Red Delicious)	control	uninfected	not detected
	4	not visible	10.40
	7	20.19 ± 0.52	40.23
	10	50.14 ± 4.54	68.76
table grapes (pink Moscatel)	control	uninfected	not detected
	4	not visible	14.46
	7	3.72 ± 0.54	48.65
	10	5.32 ± 0.12	75.08
pears (William's)	control	uninfected	not detected
	4	not visible	12.72
	7	15.20 ± 1.27	42.02
	10	39.04 ± 1.74	71.02

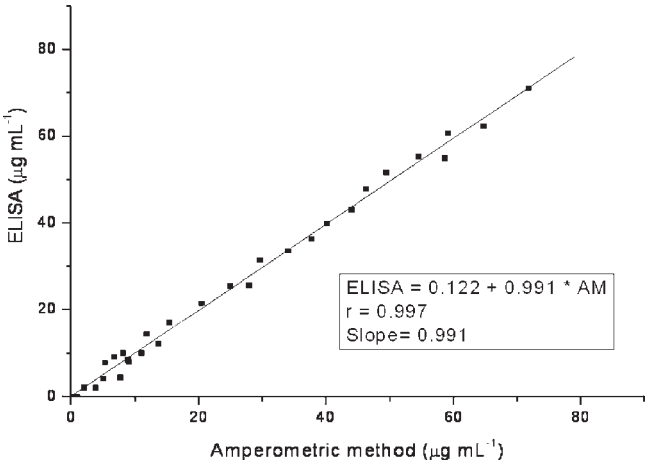

Figure 4. Correlation between proposed method and commercial photometric assays. The analysis performed with the extract of fruit obtained was realized between 4 and 7 days after inoculation and incubation at 25 °C of fruit samples.

(**Figure 4**). Compared with the SAPS-ELISA kit for *Botrytis* (*19*), our method shows great enhancement in sensitivity, low detection limit, speed, and simplicity. Reproducibility assays were made using a repetitive standard (n = 6) of 25 μg mL^{-1} *B. cinerea* (**Table 4**). The SAPS-ELISA kit for *Botrytis* (*19*) permits quantitative estimations of the fungal antigen present in a fruit sample within a 1 h practical session and detection in a linear manner, between 10 and 30 μg mL^{-1} of *B. cinerea* purified antigens concentrations with a DL of 3.5 μg mL^{-1}. This result shows that electrochemical detection was more sensitive than spectrophotometric method. MMBs used as solid supports for immunoassay reactions feature a large binding surface area per volume, and hence a large number of analyte molecules are bound within a small volume, allowing a sensitive detection (*24*, *25*)

In our previous work (*12*), the purified antigens of *B. cinerea* were immobilized on a rotating disk with 3-aminopropyl-modified controlled-pore glass (APCPG), and the biosensor was subjected to agitation to improve the immunoreaction and analytical signal.

In the present immunosensor, which was developed with a microfluidic system, all reactions and washing procedures were performed using a syringe pump. Thus, the present device has the potential to answer the need for inexpensive, sensitive, and portable automation. As mentioned, the purified antigens of *B. cinerea* were immobilized on modified MMBs, which increase significantly the immunoreactive surface area; as a consequence, the DL was lower (0.008 μg mL^{-1}) than that with our previous immunosensor (0.02 μg mL^{-1}) (*12*) by about 10 times. In the present work, the DL achieved allowed us to detect the presence of *B. cinerea* even if the fruits did not show visible rot. Moreover, in this immunosensor, once the MMBs were used, the external magnet was removed and the microfluidic system was washed. Then, the SPCEs were discarded, but the same microfluidic system was used for all of the work.

In conclusion, the integration of a microfluidic device with a syringe pump, based on the use of modified MMBs, with a screen-printed electrode to measure an electrical signal increased the capability to determine low levels of *B. cinerea* in asymptomatic fruits, with high sensitivity. The increase of reactive surface and the reduced diffusion distances of our device permitted a faster time of analysis (40 min) with no reduction in the selectivity. The waste of expensive reagents is also minimized, and the method shows physical and chemical stability, low background current, wide working potential range, and accuracy. The use of MMBs as versatile and efficient substrates for the immobilization of antigens of *B. cinerea* not only enhances the amount of antigens immobilized on the particle surface but also preserves the activity of the immobilized biomolecules.

Table 4. Reproducibility Assays Using Repetitive Standards (n = 6) of 25 μg mL^{-1} *B. cinerea* Antigen Concentration

standards of 25 μg mL^{-1} *B. cinerea* antigen	proposed method (μg mL^{-1})	ELISA (μg mL^{-1})
1	25.43	26.12
2	24.89	25.44
3	24.36	25.68
4	25.62	24.96
5	25.37	26.37
6	25.09	23.85
$X \pm$ SD[a]	25.12 $\pm$ 0.45	25.40 $\pm$ 0.91

[a] X (μg mL^{-1}), mean $\pm$ standard deviation.

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