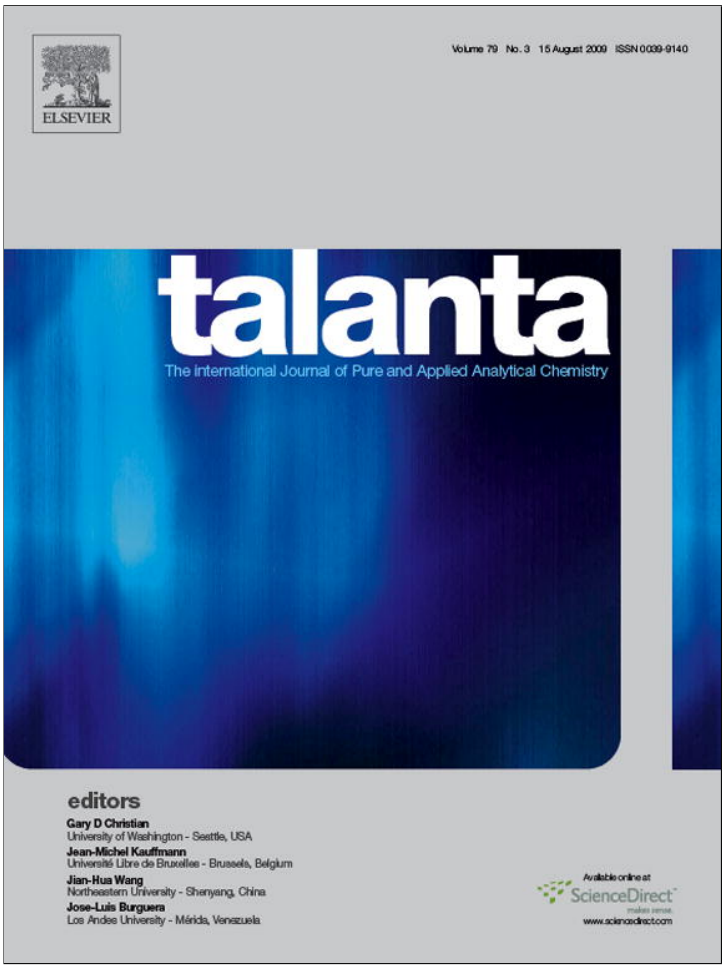

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Screen-printed immunosensor modified with carbon nanotubes in a continuous-flow system for the *Botrytis cinerea* determination in apple tissues

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ABSTRACT

Botrytis cinerea is a plant-pathogenic fungus that produces the disease known as grey mould in a wide variety of agriculturally important hosts in many countries. This paper describes the development of an immunosensor coupled to carbon-based screen-printed electrodes (SPCE) modified with multi-walled carbon nanotubes (CNTs), which show a rapid and sensitive determination of *B. cinerea* in apple tissues (Red-delicious) using a competitive immunoassay method.

Both the infected plant tissue sample and the *B. cinerea*-specific monoclonal antibody are allowed to react immunologically with the *B. cinerea* purified antigens immobilized on a rotating disk. Then, the bound antibodies are quantified by a horseradish peroxidise (HRP) enzyme labeled second antibodies specific to mouse IgG, using 4-tertbutylcatechol (4-TBC) as enzymatic mediators. The HRP, in the presence of hydrogen peroxide, catalyses the oxidation of 4-TBC to 4-tertbutyl o-benzoquinone. The electrochemical reduction back to 4-TBC is detected on SPCE-CNT at −0.15 V. The response current is inversely proportional to the amount of the *B. cinerea* antigens present in the fruit sample. The time consumed per assay was 30 min and the calculated detection limits for electrochemical method and the ELISA procedure are 0.02 and 10 μg mL^{-1}, respectively. Moreover the intra- and inter-assay coefficients of variation were below 7%. This electrochemical immunosensor promises to be usefully suited to the detection and quantification of *B. cinerea* in apparently healthy plant prior to the development of the symptoms.

1. Introduction

A wide range of plant species, including economically important crops such as vegetables, ornamentals, bulbs and fruits can be affected by grey mould caused by the fungal pathogen *Botrytis cinerea* [1,2].

B. cinerea is a ubiquitous pathogen, present often as latent infections, which can in some cases can produce a development symptomatic infections in the plant. The latency between infection and the appearance of visible symptoms can be long and variable in the case of *B. cinerea*. Consequently, an apparently healthy plant can deteriorate suddenly due to the development of latent or quiescent infection into visible disease [3].

Gray mould is difficult to be controlled satisfactorily with fungicides because the fungus is genetically variable and some strains has developed resistance to many of the chemicals introduced in the last 20 years [4]. In addition, there are also pressures from consumers to reduce pesticides in the food chain [5,6]. For these reasons, there is an increased interest in alternatives to fungicides for disease detection and control. To prevent the indiscriminate use of fungicides, a sensitive and reliable methods to determinate the fungus in plant tissue become crucial.

Detection and quantification of specific fungus in plant tissues in the presence of other fungus are difficult. Classical methods such as isolation on selective media are useful but they have limitations, i.e., many pathogens are masked by overgrowth of faster growing fungi. Quantitative nucleic acid-based methods have been developed [3], but these methods are expensive and not easy to perform routinely. Immunological methods provide a promising alternative. The fungus was detected by enzyme-linked immunosorbent assay (ELISA) [7–9]. But unfortunately, these techniques require highly qualified personnel, consume a lot of time, or need sophisticated instrumentation. Therefore, development of a new method with high sensitivity and specificity for direct detection of *B. cinerea* is highly desirable.

Heterogeneous enzyme immunoassays, coupled with flow injection (FI) system and electrochemical detection, represent a powerful analytical tool for the determination of low levels of many analytes such as antibodies, hormones, drugs, tumor markers, and viruses [10].

Electrochemical biosensors based on screen-printed-single-use electrodes are in tune with the requirements of in situ screen-

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ing devices and all the equipment needed for the electrochemical analysis is portable. Also, they have the major performance characteristics of biosensors, the minimum sample preparation, the simplicity of the apparatus, obtaining of fast results, the cost effectiveness, small and becoming miniaturized with new technologies [11]. However, in recent years, analyses with electrochemical detection constitute a methodology used extensively [12–15]. The advantages of this approach include the speed, accuracy, and precision with which many electrochemical measurements can be made.

Carbon nanotubes (CNTs) are a novel type of carbon material and can be considered as the result of folding graphite layers into carbon cylinders. The CNTs have generated great interest in future applications based on their field emission and electronic transport properties [16,17], their high mechanical strength and their chemical properties [18].

This work has been focused on their electrocatalytic behaviours toward the oxidation of biomolecules and their performance has been found to be much superior to those of other carbon electrodes in terms of reaction rate, reversibility and detection limit [19]. In view of the excellent properties of carbon nanotubes, they have been used to modify different electrodes for preparation of carbon nanotube nanoelectrode arrays and electrochemical sensing of chemical and biological species [20].

The advantages of carbon-based screen-printed electrodes (SPCEs), such as simple and low-cost fabrication and conveniently practical application in the detection of biomolecules, have been extensively illustrated [21–24]. The uses of CNTs for the preparation of CNT-modified screen-printed electrodes have been reported previously [25–28].

The objective of the current study was the development of an immunosensor coupled SPCEs modified with multi-walled-CNTs (SPCE-CNT) for rapid and sensitive determination of *B. cinerea* in plant tissues.

The detection of *B. cinerea* was carried out using a competitive immunoassay method based on the use of purified *B. cinerea* antigens that are immobilized on a rotating disk. The *B. cinerea* purified antigens or plant tissue sample (prepared in buffer PBS) and the *B. cinerea*-specific monoclonal antibody (BC-12.CA4) are allowed to react immunologically with the immobilized antigens, and the bound antibodies are quantified by a horseradish peroxidase (HRP) enzyme labeled second antibodies specific to mouse IgG, using 4-tertbutylcatechol (4-TBC) as enzymatic mediators. HRP in the presence of hydrogen peroxide (H_2O_2) catalyses the oxidation of 4-TBC to 4-tertbutyl o-benzoquinone (4-TBBQ) [29]. The electrochemical reduction back to 4-TBC is detected on SPCE-CNT at −0.15 V. The response current obtained from the product of enzymatic reaction is proportional to the activity of the enzyme and consequently, to the amount of antibodies bound to the surface of the immunosensor of interest, but the response current is inversely proportional to the amount of the *B. cinerea* antigens present in the fruit sample.

The assays were used for the detection and quantification of the fungus in infected apple fruits (Red Delicious) tissues before and after symptom induction, and does not require highly skilled technicians or expensive and dedicated equipment.

2. Materials and methods

2.1. 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 (Scotland, UK) and Sigma Chemical (St. Louis, MO, USA), respectively. Glutaraldehyde (25% aqueous solution) and H_2O_2 were purchased from Merck, Darmstadt. 3-Aminopropyl-modified controlled pore glass, 1400 Å mean pore diameter and 24 m^2 mg^{-1} surface area, was from Electro Nucleonics (Fairfield, NJ) and contained 48.2 μmol g^{-1} of amino groups. 4-Tert-butylcatechol was purchased from Sigma Chemical Co., St. Louis and all other reagents employed were of analytical grade and were used without further purifications. 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, England and was used in accordance with manufacture instructions [30].

2.2. Flow-through reactor/detector unit

The main body of the cell was made of Plexiglas. Fig. 1 illustrates the design of the Flow-through chamber containing the rotating disk and the detector system with a final cell volume of 20 μL. The SPCE is on the top of the rotating reactor. The rotating reactor is a disk of Plexiglas into which a miniature magnetic stirring bar has been embedded. Rotation of the lower reactor was effected with a laboratory magnetic stirrer with control of temperature (Metrohm AG, Herisau, Switzerland) and controlled with a variable transformer with an output between 0 and 250 V and maximum amperage of 7.5 A (Waritrans, Argentina). 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 potential applied to the CNT-GCE detection was −0.15 V versus Ag/AgCl, 3.0 mol L^{-1} NaCl reference electrode (BAS RE-6), and a Pt wire was the counter electrode. At this potential, a catalytic current was well established.

A pump (Wilson Minipuls 3 peristaltic pump, Gilson Electronics, Middleton, WI, USA) and a Baby Bee Syringe Pump (Bioanalytical System, West Lafayette, IN) were used for pumping, introducing the sample, and stopping the flow.

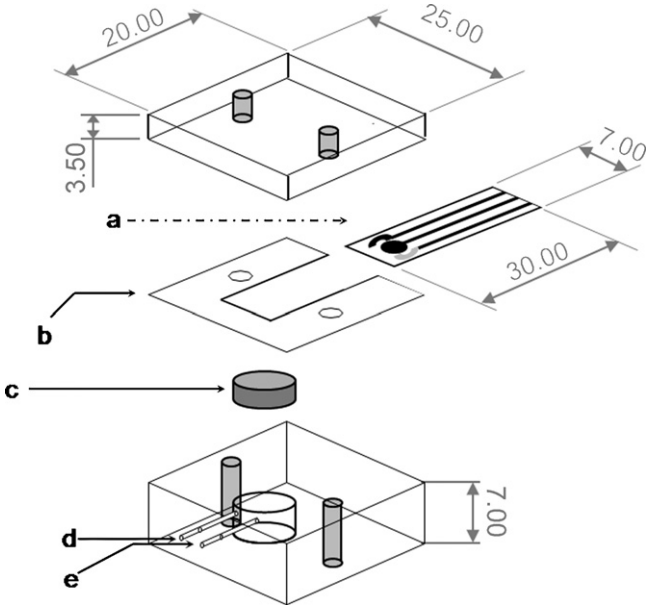

Fig. 1. Schematic representation of components in the bioreactor flow cell. (a) SPCE-CNT, (b) Gasket: Teflon, thickness 0.3 mm, (c) rotating disk, (d) flow out, and (e) flow in. All measurements are given in millimeters.

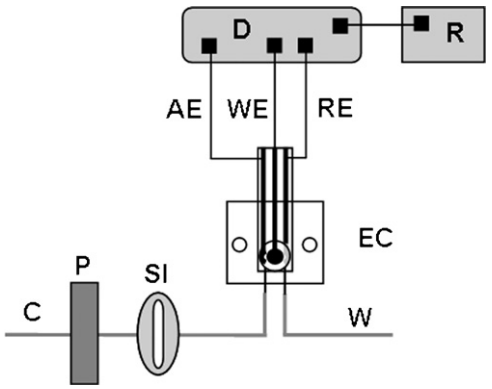

Fig. 2. Block diagram of the continuous-flow system and detection arrangement. P: pump (Gilson Minipuls 3 peristaltic pump, Gilson Electronics, Inc. Middleton, WI). C: carrier buffer line. SI: sample injection. W: waste line. EC: cell containing the rotating disk and GSPE. WE: SPGE-CNT. RE: pseudo-reference electrode. AE: auxiliary electrode. D: BAS LC-4C potentiostat (Bioanalytical Systems, West Lafayette, IN, USA). R: recorder.

Fig. 2 illustrates schematically the components of the single-line continuous-flow setup. The pump tubing was Tygon (Fisher Accu Rated, 0.25 mm i.d., Fisher Scientific, Pittsburgh, PA, USA), and the remaining tubing used was Teflon (0.25 mm i.d. from Cole–Parmer, Chicago, IL, USA).

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

2.3. Preparation of the B. cinerea antigens

A *B. cinerea* strain was recovered from apple tissues and it was used in this study. The isolates were maintained on potato dextrose agar (PDA) at 4 °C. For micelial production, the *B. cinerea* were grown on PDA, for 8–12 days at 21 ± 2 °C. After that, the mycelial mat was removed; snap frozen in liquid nitrogen, blended in a warring blendor and freeze dried to obtain a fine powder and resuspended in 0.01 mol L^{-1} PBS, pH 7.2. The antigen preparation was centrifuged at 1000 × *g* for 10 min, and the supernatant was stored in the 0.01 mol L^{-1} PBS (pH 7.2), at −20 °C between uses.

For conidial production, *B. cinerea* were grown on PDA at 21 ± 2 °C. When the mycelium appeared, cultures were kept at 15 °C for inducing sporulation. After a week, spores were harvested and suspended in 10 mL of sterile the 0.01 mol L^{-1} PBS (pH 7.2) containing 0.05% (v/v) Tween 80. The concentration of spore suspension was determined with a Neubauer chamber and adjusted with PBS to 1×10^5 spores mL^{-1}.

2.4. Preparation of the SPCE-CNT

An electrode pretreatment was carried out before each voltammetric experiment in order to oxidize the graphite impurities and to obtain a more hydrophilic surface [31], with the aim of improving the sensitivity and reproducibility of the results.

The graphite electrode surface is pretreated applying a potential +1.6 V (versus Ag-SPE) for 120 s and +1.8 V (versus Ag-SPE) for 60 s in 5 mL of 0.25 mol L^{-1} acetate buffer, containing 0.01 mol L^{-1} KCl (pH 4.75), under stirred conditions. Then, the electrodes were washed using 0.01 mol L^{-1} PBS, pH 7.2 and stored in the same buffer at 4 °C.

One milligram of MWCNT was dispersed with the aid of ultrasonic stirring for 45 min in methanol/water (50:50, v/v) in an aqueous 0.1% Nafion solution. A 5 μL aliquot of this dispersion was dropped on the screen-printed graphite working electrode surface and then the solvent was evaporated under an infrared heat lamp [32].

2.5. Immunosensor preparation

The rotating disk reactor (bottom part) was prepared by immobilizing *B. cinerea* antigens on 3-aminopropyl-modified controlled-pore glass (APCPG). The APCPG, smoothly spread on one side of a double-coated tape affixed to the disk surface, was allowed to react with an aqueous solution of 5% (w/w) glutaraldehyde at pH 10.00 (0.20 mol L^{-1} carbonate) for 2 h at room temperature. After washing with purified water and 0.10 mol L^{-1} phosphate buffer of pH 7.00, 25 μL of antigens preparation (10 μg mL^{-1} 0.01 mol L^{-1} PBS, pH 7.2) was coupled to the residual aldehyde groups overnight at 5 °C. The immobilized antibodies preparation was finally washed with phosphate buffer (pH 7.00) and stored in the same buffer at 5 °C between uses. The immobilized antigen preparations were perfectly stable for at least 1 month.

2.6. Amperometric analysis of B. cinerea in apple fruits tissues samples

This method was applied in the determination of *B. cinerea* in 24 commercials apple fruits tissues samples and in the detection of *B. cinerea* in control infected plant tissues.

The control apples were infected using a spore suspension of 1×10^5 spores mL^{-1}. Fruit were wounded (3 mm × 3mm × 3 mm) with a punch, and 20 μL of the spore suspension were pipetted into a wound. The apples were kept at 25 °C and evaluations of rot incidence and lesion diameters were made along 10 days. Ten fruit per the assays were used with three wounds each one. Each experiment was repeated three times.

Infected and uninfected apples tissues were ground to a fine powder in liquid N_2. The PBS extracts were prepared from powdered apples tissues extracts by adding 0.1 g plant tissue into 0.9 mL PBS and vortexed for 1 min to ensure homogeneous suspensions before using for the immunological assay.

The unspecific binding (on the surface of the rotating disc) was blocked by 5 min treatment at 37 °C with 3% skim milk in a 0.01 mol L^{-1} PBS, pH 7.2. The apples PBS extracts were diluted twofold with a solution of the monoclonal antibody IgG mouse anti-*B. cinerea* at a concentration of 15 μg mL^{-1} (0.01 mol L^{-1} PBS, pH 7.2) and then 25 μL of this solution were injected into the PBS carrier stream at a flow rate of 100 μL min^{-1} and incubated 10 min at 25 °C with stirring.

The immunosensor was washed with 0.01 mol L^{-1} PBS, pH 7.2. After washing, 25 μL of the anti-mouse IgG-HRP conjugate (dilution of 1/2000 in 0.01 mol L^{-1} PBS, pH 7.2) was injected and incubated 10 min at 25 °C with stirring. The immunosensor was then washed free of any traces of unbound enzyme conjugate with 0.01 mol L^{-1} PBS (pH 7.2). Finally the substrate solution (25 μL of a 0.1 mol L^{-1} phosphate–citrate buffer, pH 5.0 containing 1.0×10^{-3} mol L^{-1} H_2O_2 and 1.0×10^{-3} mol L^{-1} 4-TBC) was injected into the carrier stream and the enzymatic product was detected by amperometric flow injection analysis. For the next analysis the immunoreactor was conditioned by injection of desorption buffer (0.1 mol L^{-1} glycine–HCl, pH 2) for 2 min and then washed with 0.01 mol L^{-1} in PBS, pH 7.2. The SPCE-CNTs were reused for five consecutive determination.

A standard curve for the amperometric procedure was produced by following our protocol with a series of purified antigens that covered the relevant range of antigen concentration (0–100 μg mL^{-1}). When not in use, the immunosensor was stored in 0.01 mol L^{-1} PBS (pH 7.2) containing sodium azide (0.01%) at 4 °C.

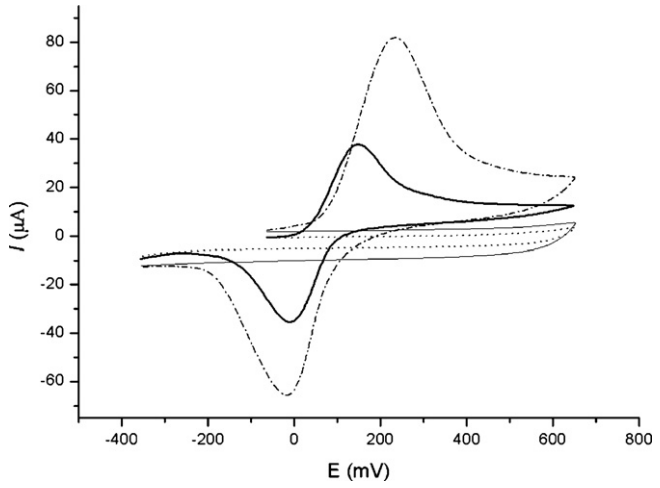

Fig. 3. Cyclic voltammograms at a CNT-SPCE (-·-·-) and at a bare GCE (—) for 1.0×10^{-3} mol L^{-1} 4-TBC in aqueous solution containing 0.1 mol L^{-1} phosphate–citrate buffer (pH 5.0). The background voltammograms of CNT-SPCE (···) and a bare GCE (—) was made in aqueous solution containing 0.1 M phosphate–citrate buffer (pH 5.0). Scan rate: 0.1 V s^{-1}, $T = (25 \pm 1)$ °C.

3. Results and discussion

3.1. Electrochemical study of 4-TBC with the SPCE-CNT

Fig. 3 shows the comparison of cyclic voltammograms obtained at a SPCE-CNT and at an unmodified SPCE for 1 mM solution of 4-tertbutylcatechol (4-TBC) in an aqueous solution containing 0.10 mol L^{-1} phosphate buffer pH 7.0 as supporting electrolyte. As it can be seen, 4-TBC exhibit well-defined one anodic and a corresponding cathodic peak versus Ag/AgCl at the CNT-GCE, which corresponds to the transformation of 4-TBC to 4-TBBQ and vice-versa within a quasi-reversible two-electron process, whose peak currents are considerably larger than those obtained at the bare GCE. The modification of the electrode surface with CNTs produced an enhancement of the electrochemical response for this compound.

The improved amperometric response at the CNT-modified electrode is attributed to the increased surface area upon electrode modification [33]. Other relevant advantages of the amperometric detection at CNTs-GCE are the high repeatability and very low and stable background currents of the measurements achieved over the whole potential range [33]. The ability of carbon nanotubes to circumvent electrode surface fouling during amperometric sensing has been recognized for molecules such as NADH [34] and can be attributed to the unique spatial structure of CNTs.

The effect of the concentration of CNT on the surface of GCE was studied in range of 0.1–3 mg of CNTs, which were dispersed in 1 mL of methanol/water (50:50 v/v) in an aqueous 0.1% Nafion solution. A 10 μL aliquot of this dispersion was dropped on the graphite working electrode surface and then the solvent was evaporated under an infrared heat lamp. A significant increase of electric signal was observed between 0.5 and 1 mg mL^{-1}. Insignificant differences were obtained for greater concentrations. For convenience 1 mg mL^{-1} of CNT was used to electrode modifications.

3.2. Optimum conditions for the immune reactions and the determination of enzymatic products

It has been shown that the theoretical framework developed for static ELISA system cannot be applied to describe the kinetics of antibody–antigen interactions occurring in a continuous-flow immunoassay [35]. The buffer flow reduces the limitations of diffusion as observed in static ELISA systems [36], the controlled pore glass increase the area for antigens immobilization about three orders of magnitude [37] and a high sensitivity can be attained by a rotating bioreactor and continuous-flow/stopped-flow/continuous-flow processing [38].

The proposed method manifolds follow the EIA principles, but instead of using a microtiter plate, the reagents and washing buffers were pumped consecutively through the immunosensor containing immobilized antibodies, coupled to electrochemically detection for the determination of *B. cinerea*. The HRP enzyme-labeled second antibody specific to mouse IgG was used as conjugate and the product of the enzymatic indicator reaction was measured using electrochemical detection.

The implementation of continuous-flow/stopped-flow programming and the location of two facing independent reactors (Fig. 1) permits: (a) utilization of relatively low immunoreactants loading conditions, (b) instantaneous operation under high initial rate conditions, (c) easy detection of accumulated products, and (d) reduction of apparent Michaelis–Menten constant, K'_M.

A more complete reagent homogenization is achieved [39], because the cell works as a mixing chamber by facilitating the arrival of immunoreactants, the arrival of enzymatic substrate at the active sites and the release of products from the same sites. The net result is high values of current.

The effect of the rotation rate was evaluated in a range of 60–300 rpm. A significant increase of electric signal was observed between 60 and 180 rpm. Insignificant differences were obtained for greater rotation velocities. For convenience a rotation rate of 180 rpm was used to evaluate other parameters. If the rotating disk in the cell is devoid of rotation, the response is lower because diffusional reactions are too slow to be observed in the time scale of electrochemical analysis. If a rotation of 180 rpm is imposed on the rotating disc at the bottom of the cell, the signal is dramatically enlarged.

The sample size was studied in the range 5–40 μL. Sensitivity is almost tripled in the range between 5 and 25 μL. Insignificant differences were obtained for greater sample size. A sample size of 25 μL was used to evaluate other parameters.

The rate of enzymatic response under stopped-flow conditions was studied in the pH range 4–7 and shows a maximum value of activity at pH 5. The pH value used was 5.00 in 0.1 mol L^{-1} phosphate–citrate buffer.

The effect of varying H_2O_2 concentration from 1.0×10^{-4} to 1.0×10^{-2} mol L^{-1}, for 1.0×10^{-3} mol L^{-1} 4-TBC solution and the effect of varying 4-TBC concentration from 1.5×10^{-4} to 2.5×10^{-2} mol L^{-1} for 1.0×10^{-3} mol L^{-1} H_2O_2 solution on the bioreactor response were evaluated. The optimum concentrations of H_2O_2 and 4-TBC were 1.0×10^{-3} mol L^{-1}, respectively. Those concentrations were then used.

3.3. Quantitative test for the detection of B. cinerea

Under the selected conditions described above, the electrochemical response of the enzymatic product is proportional to the activity of the enzyme and consequently is inversely proportional to the amount of the *B. cinerea* antigens present in the fruit sample.

A standard curve for the amperometric procedure was produced by following our protocol with a series of purified antigens that covered the relevant range of antigen concentration (0–100 μg mL^{-1}). The linear regression equation was $i = 526.03 + -4.78 \times C_{B.\ cinerea}$, with the linear regression coefficient $r = 0.996$. The coefficient of variation (CV) for the determination of 25 μg mL^{-1} *B. cinerea* was below 4% (six replicates). The detection limit (DL) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For the proposed method the DL was 0.02 μg mL^{-1}. The commercial spectrophotometric

Table 1
Within-assay precision (five measurements in the same run for each control serum) and between-assay precision (five measurements for each control, repeated for three consecutive days).

Control solution[a]	Within-assay		Between-assay	
	Mean	CV%	Mean	CV%
5	5.22	2.20	5.61	4.41
25	24.78	3.78	25.40	6.54
75	76.01	2.86	76.94	5.86

[a] ($\mu g\,mL^{-1}$) *B. cinerea* antigen.

system SAPS-ELISA Kit for Botrytis permit to make quantitative estimates of the fungal antigen present in a fruit sample within a 1 h practical session. The SAPS-ELISA Kit for Botrytis detecting in a linear manner, between 10 and 30 $\mu g\,mL^{-1}$ with a DL of 3.5 $\mu g\,mL^{-1}$ of *B. cinerea* purified antigens concentrations. This result shows that electrochemical detection was more sensitive than spectrophotometric method.

The precision of the electrochemical assay was checked with a control solution at 5, 25 and 75 $\mu g\,mL^{-1}$ *B. cinerea* purified antigens concentrations. The within-assay precision was tested with five measurements in the same run for each sample. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The results obtained are presented in Table 1. The *B. cinerea* assay showed good precision; the CV within-assay values were below 4% and the between-assay values were below 7%.

The accuracy was tested with dilution and recovery tests. A dilution test was performed with a control solution of 100 $\mu g\,mL^{-1}$ *B. cinerea* purified antigens concentrations with 0.01 mol L^{-1} PBS, pH 7.2 (Fig. 4).

The electrochemical system was compared with a commercial spectrophotometric system SAPS-ELISA Kit for Botrytis for the determination of *B. cinerea* in 24 commercials apple fruits tissues samples. Reproducibility assays were made using a repetitive standard ($n = 6$) of 25 $\mu g\,mL^{-1}$ *B. cinerea* (Table 2).

3.4. Correlations with ELISA assay procedure

The electrochemical system was compared with a commercial spectrophotometric system for the quantification of *B. cinerea* in 24 commercials apple fruits tissues samples. The slopes obtained were reasonably close to 1, indicating a good correspondence between

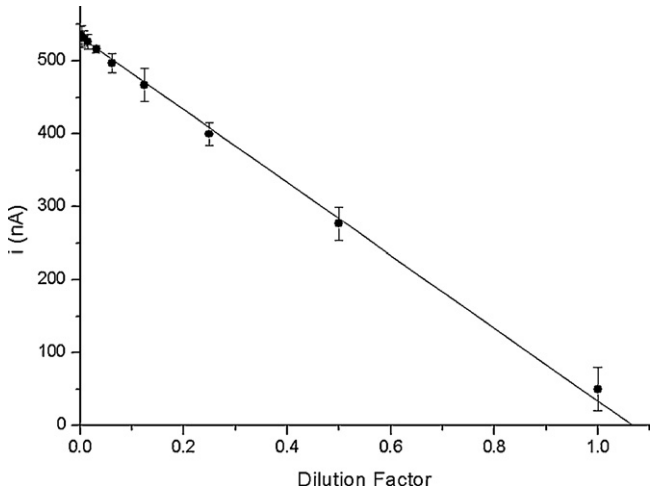

Fig. 4. Dilution test results for the control solution of 100 $\mu g\,mL^{-1}$ *B. cinerea* purified antigens concentrations with 0.01 mol L^{-1} PBS, pH 7.2. Each value of current is based on five determinations.

Table 2
Reproducibility assays using repetitive standards ($n = 6$) of 25 $\mu g\,mL^{-1}$ *B. cinerea* antigen concentration.

Standards of 25 $\mu g\,mL^{-1}$ *B. cinerea* antigen	Proposed method ($\mu g\,mL^{-1}$)	EIA ($\mu g\,mL^{-1}$)
1	25.11	24.31
2	25.57	25.57
3	24.82	24.44
4	25.34	25.54
5	25.79	25.98
6	24.27	26.47
$X \pm SD$[a]	25.15 ± 0.549	25.38 ± 0.853

[a] X ($\mu g\,mL^{-1}$), mean ± SD, standard deviation.

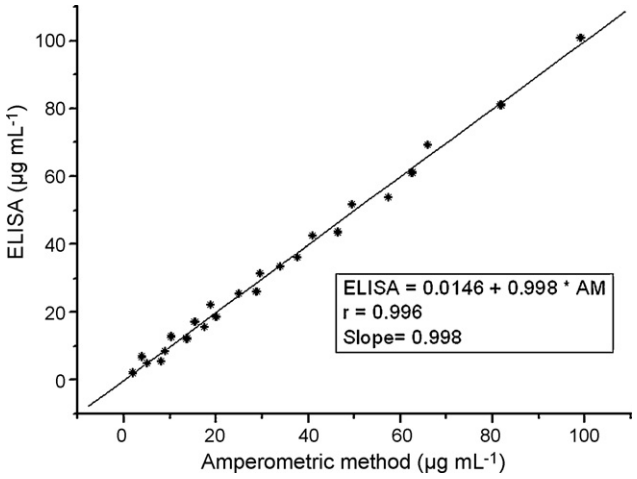

Fig. 5. Correlation between proposed method and commercial photometric assays.

the two methods (Fig. 5). Compared with the SAPS-ELISA Kit for Botrytis, our method shows large enhancement in sensitivity, low detection limit, speed and simplicity. These results suggest that the sensitivity reached for this procedure allow to determine very low level of *B. cinerea* antigens in apparently healthy plant that can deteriorate suddenly due to the development of latent or quiescent infection into visible disease.

4. Conclusions

In this work, a microfluidic immunosensor coupled with flow injection (FI) system for rapid, sensitive and selective quantification with very low levels of *B. cinerea* in commercials apple fruits tissues samples was developed using electrochemical detection.

The overall assay time (30 min) was shorter than the time reported for ELISA commercial test kits (60 min), with no reduction on the selectivity, being these important advantages. Also minimizes the waste of expensive reagents; shows physical and chemical stability, low background current, wide working potential range, and accuracy. Modification of SPCE with CNTs produced an enhancement of the electrochemical responses obtained for reduction of 4-tertbutyl o-benzoquinone to 4-TBC.

In conclusion, this method promises to be particularly useful in the analysis of symptomless plants, either to locate latent infections (avoiding conventional culturing techniques which are not only time-consuming but also not able to give a quantitative result) or to assess disease development before symptoms become visible.

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