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Microsensor *in vivo* monitoring of oxidative burst in oilseed rape (*Brassica napus* L.) leaves infected by *Sclerotinia sclerotiorum*

Qiao Xu[a,b], Sheng-Yi Liu[a], Qiu-Ju Zou[b], Xue-Lan Guo[a], Xu-Yan Dong[a], Pei-Wu Li[a], Dan-Yang Song[c], Hong Chen[a,d,*], Yuan-Di Zhao[b,**]

[a] *Institute of Oil Crops Research, Chinese Academy of Agricultural Sciences, Wuhan, Hubei 430062, PR China*
[b] *Key Laboratory of Biomedical Photonics of Ministry of Education - Wuhan National Laboratory for Optoelectronics - Hubei Bioinformatics and Molecular Imaging Key Laboratory, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, HuBei 430074, PR China*
[c] *College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, PR China*
[d] *Key Laboratory for Genetic Improvement of Oil Crops, Ministry of Agriculture, Wuhan, Hubei 430062, PR China*

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ABSTRACT

Oxidative burst is the rapid and transient production of large amounts of reactive oxygen species, including superoxide anion, hydrogen peroxide (H_2O_2), and hydroxyl radical. A rapid and simple technique was employed for *in vivo* detection of oxidative burst in oilseed rape (*Brassica napus* L.) leaves, using a modified electrode. Platinum (Pt) micro-particles were dispersed on a Pt electrode, coated with a poly (*o*-phenylenediamine) film. This exhibited high sensitivity, selectivity and stability in H_2O_2 detection. Amperometry was used to obtain satisfactory linear relationships between reductive current intensities and H_2O_2 concentrations at −0.1 V potential in different electrolytes. This electrode was used *in vivo* to detect oxidative burst in oilseed rape following fungal infection. Oxidative bursts induced by infection of the fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary exhibited notably different mechanisms between a susceptible and a resistant glucose oxidase-transgenic genotype.

1. Introduction

Plant disease resistance often depends on the plant recognizing pathogens early in the infection process. Recognition of pathogens triggers a range of inducible defense mechanisms believed to contribute to overall plant disease resistance. The striking release of reactive oxygen species (ROS), or 'oxidative burst', is correlated with a number of plant–pathogen interactions and may be important in disease resistance. A number of possible ROS roles have been proposed; direct killing of pathogens, involvement in structural changes in the cell wall, promotion of programmed cell death (PCD) in hypersensitive response (HR) and induction of defense gene expression. Hydrogen peroxide (H_2O_2) is generally the most versatile ROS in spots of necrotic tissue [1–4], so its detection in plant–pathogen interaction research is crucial.

Many methods have been employed for H_2O_2 measurement, such as UV–vis spectroscopy, chemi-luminescence and electrochemistry [5–7]. However, these methods are not suitable for oxidative burst detection in real-time *in vivo* and *in situ*. An accurate and reliable method for H_2O_2 determination using modified electrodes is of particular interest in many fields [8,9]. Modified electrodes are used widely in life sciences for simple, sensitive and direct detection. Furthermore, they can be used in real-time *in vivo* and *in situ* tests over extended periods [10].

Organic conductive polymers have been very useful in electrochemical analysis because of their unique conducting capability, mechanical properties and various promising applications [11]. Poly (*o*-phenylenediamine) (POPD), one of these conducting polymers, can be prepared by either chemical polymerization or electropolymerization of *o*-phenylenediamine (OPD). Generally a POPD film developing in an acidic solution is a ladder structure of phenazine units [12]. POPD can be used as electrode-modifying material in electrocatalysis, electrochemical display and electrochemical biosensors [13–15]. At the electroactive regions the POPD film is permselective to H_2O_2, while avoiding cation interference and electrode fouling [16].

* Corresponding author at: Institute of Oil Crops Research, Chinese Academy of Agricultural Sciences, Wuhan, Hubei 430062, PR China. Tel.: +86 27 86812943; fax: +86 27 86812862.
** Corresponding author at: Key Laboratory of Biomedical Photonics of Ministry of Education - Wuhan National Laboratory for Optoelectronics - Hubei Bioinformatics and Molecular Imaging Key Laboratory, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430074, PR China. Tel.: +86 27 87792235; fax: +86 27 87792202.
E-mail addresses: chenhong@oilcrops.cn (H. Chen), zydi@mail.hust.edu.cn (Y.-D. Zhao).

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Pt provides particular electrocatalytic oxidation activity to small organic molecules. Its activity is greatly reduced when the electroactive region is occupied by poison intermediate. Therefore, if metal micro-particles (such as Pt, Ru and Pd) are embedded in conducting polymer films, the noble metals will be conserved and catalyst poisoning reduced or avoided. Pt particles are a powerful catalyst for many electrode reactions. Yang and Wen developed Pt micro-particles deposited in a sulfonate–polyaniline film for the electrosorption of methanol and sorbitol [17]. Hepel studied the methanol oxidation at Pt nano-particles dispersed in a polypyrrole film [18].

In the present study, the modification of a POPD film was accomplished on a cylindrical Pt electrode with highly dispersed Pt micro-particles. The electrochemical behavior of H_2O_2 at this modified electrode was studied. The electrode was then used in H_2O_2 detection in oilseed rape tissue microenvironments, following invasion and infection by fungal pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary. Oxidative bursts induced by pathogen infection exhibited different defense mechanisms in susceptible and resistant oilseed rape genotypes.

2. Experimental

2.1. Instruments and reagents

Instruments used were a CHI660 software-controlled electrochemistry workstation (Shanghai CH Instruments Inc.) and a S-3000N scanning electron microscope (SEM) (Hitachi). Kalium chloroplatinate, OPD, H_2O_2 and all chemical reagents were analytical grade and purchased from Sinopharm. OPD was used after three recrystallizations from ethanol. Solutions were prepared from these reagents and thrice distilled water.

2.2. Plant materials

Two oilseed rape (*Brassica napus* L.) genotypes were used in this study. One was a winter-type cultivar, 84039, moderately susceptible to *S. sclerotiorum*. The other was glucose oxidase (GO)-transgenic oilseed rape, GO016, with high resistance to *S. sclerotiorum*.

2.3. Pretreatments and modification of the electrode

The Pt wire electrode (diameter is 100 μm and length is 4.45 mm) was dipped into boiling HNO_3 solution for 3–5 min. It was then put into acetone and distilled water for ultrasonic abstersion for 5 min each. Afterward the electrode was thoroughly washed with thrice distilled water, single compartment cell with a three-electrode configuration was used for all the electrochemical experiments. An Ag/AgCl wire electrode (home made) was used as a reference electrode and a Pt wire electrode as an auxiliary electrode. $K_3Fe(CN)_6$ was used as a probe molecule for Pt electrode characterization and electrode surface area determined as $1.430 \pm 0.104\ mm^2$. The electrode possessed the electrochemistry characteristics of common electrode with normal size, the state characteristics of an ultra-microelectrode were not obtained.

Preparing the Pt/Pt/POPD electrode was a two-stage procedure. In the first stage, Pt micro-particles were deposited on the Pt-electrode surface, and in the second, a POPD film was deposited over the Pt micro-particles and Pt electrode.

The Pt micro-particles deposition was fabricated by cycling the Pt electrode between −0.2 and 1.0 V in an electrolyte containing 0.5 M H_2SO_4 and 2 mM K_2PtCl_6. SEM was employed for both Pt/Pt and Pt-electrode surface characterization.

The Pt/Pt electrode was cycled from −0.2 to 1.2 V in an electrolyte containing 0.5 M H_2SO_4 and 50 mM OPD, to produce a covering POPD film on the Pt/Pt electrode (i.e. a Pt/Pt/POPD electrode). The response of this electrode to H_2O_2 concentration in different electrolytes was studied and accurate relationships established.

2.4. Inoculation and incubation

The pathogen *S. sclerotiorum* was collected from infected stems of oilseed rape in Wuhan, China. Mycelium of *S. sclerotiorum* was grown on PDA plates at 25 °C. Inoculation was by mycelial discs (2 mm thick and 3 mm in diameter), excised from the edge of a 5-day-old culture and placed on the upper sides of leaves (one disk per leaf). After inoculation, the plants were covered with plastic bags sprayed inside with water to provide a saturated atmosphere at 25 ± 3 °C.

2.5. Detection of H_2O_2 in leaf tissue of oilseed rape

Inoculated leaves with similar shape were chosen; Pt wire counter electrode was inserted into main stalk, home made Ag/AgCl wire reference electrode was inserted into the leafstalk of the chosen leaves, where 3 cm away from the edges of leaves was. The Pt/Pt/POPD wire electrode was accurately inserted into the nervation inside the leaves, where is 1 cm left from the inoculated position. The electrodes were kept in same position for all experiments. Amperometry was used to detect H_2O_2 concentration variations. The same experiments were carried out on normal leaves without inoculation as the controls.

3. Results and discussion

3.1. Optimization of the electrode

Cyclic voltammetry [19], chronoamperometry [20], pulse amperometry [21] and constant potential [22] have been used to prepare electrodes modified with Pt micro-particles. Cyclic voltammetry is widely used for its convenient manipulation and the excellent film obtained. Then in this study, platinum microparticles were dispersed on electrode by this way. The surfaces before and after the modification were observed by SEM, the results are shown in Fig. 1. It could be seen that there were ∼1 μm microparticles dispersed on the Pt electrode (Fig. 1B) comparing with the Pt electrode (Fig. 1A), and the surface area had clearly increased.

The POPD film can be formed in acidic, alkaline or neutral solutions. However, an acidic solution is required for a uniform film with better activity. The film can be formed by constant potential [7] or cyclic voltammetry [23]. POPD films prepared by cyclic voltammetry are uniform, compact and insoluble in water, weak acid and weak alkali [24]. Then, POPD films were prepared by this way.

It was found that the thickness of the POPD film was controlled by the number of cycles at a specific OPD concentration. A whole POPD film could not be formed without enough cyclic voltammetry runs. However, the electrochemical response of the Pt/Pt/POPD electrode declined when the number of cycles exceeded 30. It is possible that excessively thick films inhibited the penetration of the electrochemical activity molecules, accordingly, 30 cycles was chosen.

3.2. Response characteristics of Pt/Pt/POPD electrode

H_2O_2 presented strong reductive capability at −0.1 V on Pt/Pt/POPD-electrode. Amperometry was used to study the relationship between H_2O_2 concentrations and reduction currents. The reduction response of Pt/Pt/POPD electrode to H_2O_2 in 1 mM NaCl