A simple and robust micro-sensor-based technique for *in vivo* detection of oxidative burst elicited by *Sclerotinia sclerotiorum* and active substances in oilseed rape

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Abstract

The oxidative burst is an early event in plant disease defence responses and was characterized by a rapid and transient production of large amounts of reactive oxygen species (ROS), including superoxide anion O^{2}-, hydrogen peroxide (H_2O_2), and possibly hydroxyl radical (OH^*). Major drawbacks of current detection technique in vitro are their low specificity and sensitivity. Most oxidative burst is highly reactive and in a short period and therefore hard to be detected in complex biological matrices. Detection of oxidative burst in living organisms carries a significant analytical challenge.

In this work, it was demonstrated that a robust and simple technique based on electrochemically modified micro-sensor was adopted for in vivo detection of oxidative burst in oilseed rape. The modification of poly(o-phenylenediamine) (PoPD) film with highly dispersed platinum micro-particles on Pt wire electrode increased the electrocatalytic reduction of hydrogen peroxide. The PoPD modified Pt electrode exhibited high sensitivity, selectivity and stability in hydrogen peroxide detection. Chronoamperometry was used to obtain satisfactory linearity connection between reduction current and concentration of hydrogen peroxide at the potential of -0.1 V. The correlation coefficient is calculated to be 0.9939 in the range of 20 × 10-6 – 20 × 10^{-3} mol/L with a detection limit of 10 × 10-6 mol/L in 1mM NaCl electrolyte. The micro-sensor prepared as above was used to detect oxidative burst in vivo in oil-seed rape against fungal pathogen (*Sclerotinia sclerotiorum*) and stressed by elicitor active substances, such as methyl jasmonate (MeJA), salicylic acid (SA), benro(l,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH). It has been found that the oxygen bursts induced by different inducers exhibit multifarious characteristics between susceptible cultivar and glucose oxidase (GO)-transgenic oilseed rape.

Key words: Oilseed rape, Brassica napus, oxidative burst, Micro-sensor, genetically modified

Introduction

Oxidative burst, a rapid and transient production of reactive oxygen species (ROS), including superoxide anion O_2^-, hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^*), may mediate protection against physical stress and pathogens and physical stress by a variety of machanisms.(Ann, et al., 1997) Although a number of ways to detected ROS such as UV-vis spectroscopy, chemiluminescence, and electrochemistry have been proposed(Coutanceau, et al., 2000; Wu, et al., 1996; Gao, et al., 1994). However, these methods were not suitable for oxidative burst detection in real time in vivo and in situ. Modified electrodes were used widely in life science for providing simple, sensitive and direct detection. Furthermore, it also can be used in real-time, *in vivo*, *in situ* test for a long time (Teruhisa, et al., 1996). Furthermore an accurate and reliable method for H_2O_2 determination by modified electrode was of particular interest in many fields (Tu, et al., 2005; Johnson, et al., 1994)

In this work, the modification of poly (*o*-phenylenediamine) (POPD) film was accomplished on plantinum (Pt) cylinder electrode with highly dispersed Pt micro-particles. The electrochemical behavior of H_2O_2 at this modified electrode was studied. After that, this electrode was used in H_2O_2 detection in oilseed rape tissue microenvironment when it was stressed by elicitor active substances, such as methyl jasmonate (MeJA), salicylic acid (SA), benro(l,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH).

Experiment section

Instruments and Reagents

CHI660 software-controlled electrochemistry workstation (CHI660, Shanghai CH Instruments, Inc.). *O*-phenylenediamine, kalium chloroplatinate, hydrogen peroxide, and all chemical reagents were analytical grade and purchased from Sinopharm. *O*-phenylenediamine was used after three-recrystallization from ethanol. Solutions were prepared from these reagents and thrice distilled water.

Plant materials

The oilseed rape (*Brassica napus L.*) genotype used in this study was winter type cultivar, 84039, which was moderately susceptible to S. sclerotiorum. Glucose oxidase (GO)-transgenic oilseed rape, GO16, had been proven as high resistance type to S. sclerotiorum.

Pretreatments and modification of the electrode

In the electrochemical experiments, an Ag/AgCl electrode was used as a reference electrode and a Pt wire electrode as an auxiliary electrode. The Pt/Pt/POPD electrode was prepared using a two-stage procedure. In the first stage, Pt micro-particles were deposited on the Pt electrode's surface, and then, POPD film was deposited on the Pt micro-particles modified Pt electrode.

The Pt micro-particles modification could be accomplished by cycling the Pt electrode between -0.2 V and 1.0 V in an electrolyte containing 0.5 M H_2SO_4 and 2 mM K_2PtCl_6. Then cycle the Pt/Pt electrode from -0.2 to 1.2 V in an electrolyte containing 0.5 M H_2SO_4 and 50 mM o-phenylenediamine. After that a Pt micro-particles dispersed Pt electrode modified with poly (o-phenylenediamine) film was obtained (Pt/Pt/POPD electrode). The responses of this electrode to the H_2O_2 concentration in different electrolytes were studied and finer linear relations were obtained.

Detection of H2O2 in leaf tissue of oilseed rape

The Pt/Pt/POPD electrode was used for measurements in real time *in vivo* and *in situ*. Elicitor active substances were deposited on oilseed rape leaves. Pt wire counter electrode was inserted into caulis, Ag/AgCl reference electrode was inserted into the leafstalk, where was 1 cm next to the nervation and along the veins 3 cm away from the edges of lamina. After that, a working PA electrode was inserted into the veins along the edges of lamina. The best spot to insert the electrode was 1 cm away from the spot at which elicitor active substances had been deposited.

Results and discussion

Response characteristic of Pt/Pt/POPD electrode

The electrochemical activity of the H_2O_2 on the Pt/Pt/POPD electrode was detected. At the Pt/Pt/POPD electrode, an obvious H_2O_2 reductive peak appeared at -0.1V (as shown in (Fig. 1). It could be explained like that, at the electroactive regions of POPD, the film has a permselective to H_2O_2, and to preventing from the cation interferences and electrode fouling by high molecular weight species. The small molecular H_2O_2 permeated through the POPD film and then was catalyzed by the Pt micro-particles, which leading to the increase of catalytic activity. So this Pt/Pt/POPD electrode showed great sensitivity and selectivity to the H_2O_2.

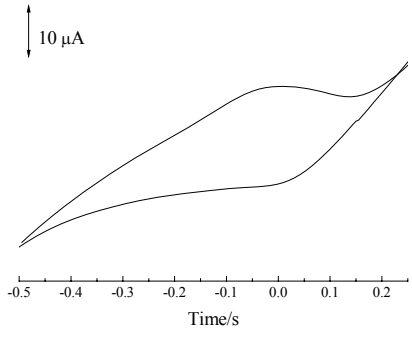

Fig. 1 Cyclic voltammetry of Pt/ Pt/ POPD electrode for the reduction of H_2O_2 in 1 mM NaCl with 50 mM H_2O_2. Scan rate: 100mV/s.

H_2O_2 concentration detection in 1mM electrolyte

H_2O_2 presented strong reductive capability at -0.1 V on Pt/Pt/POPD electrode. Chronoamperometry was used to detect the relationship between H_2O_2 concentration and reductive current intensity. The correlation coefficient is calculated to be 0.9939 in the range of $20 \times 10^{-6} – 20 \times 10^{-3}$ mol/L with a detection limit of 10×10^{-6} mol/L in 1mM NaCl electrolyte (as shown in Fig.2).

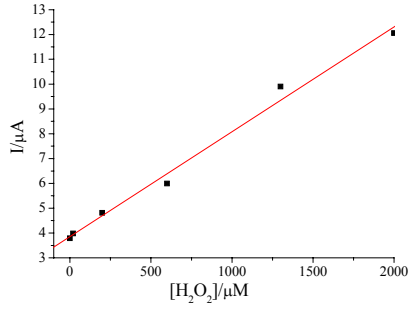

Fig. 2 The connection between reductive current intensity in 1mM NaCl and H_2O_2 concentration on Pt/ Pt/ POPD electrode. Constant potential: -0.1 V.

Monitoring of oxidative burst induced by elicitor active substances

Salicylic acid (SA) is a well-known inducer of plant systematic acquired resistance in plant–pathogen interaction. It accumulates at the infection position during pathogen attack, and then spreads to other parts of the plant (Zhao, et al., 2005). A pioneering study demonstrated that the application of exogenous SA or its derivatives induced synthesis of pathogenesis related resistance (White, et al., 1979). Jasmine acid and its related compounds have long been observed to be transducers of elicitor signals for the production of plant secondary metabolites.

It was known that in normal physiological condition, ROS concentration maintained low level both in susceptible cultivar and glucose oxidase (GO)-transgenic oilseed rape, because there were highly efficient scavenging mechanisms in plant cell to overcome ROS toxicity. But after induced by elicitor active substances, the oxygen bursts exhibited entirely different characteristic depend on elicitors and genotype of rapeseed as shown as in Fig 3, 4. These results were in accord with our previous work (Zou, et al., 2006).

Fig. 3.The oxygen bursts variation within the natural oilseed rape lamina induced by salicylic acid detected by using micro-sensor based on Pt/Pt/POPD electrode. Constant potential: -0.1 V. A: susceptible oilseed rape genotype, 84039; B: Glucose oxidase (GO)-transgenic oilseed rape, GO16

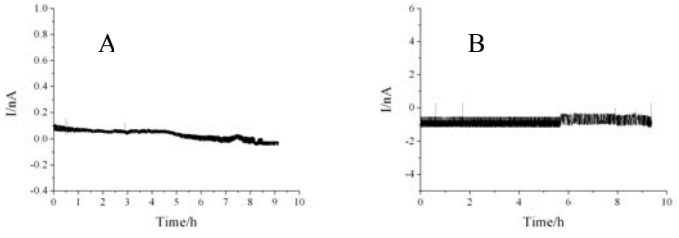

Fig. 4.The ROS variation within the natural oilseed rape lamina induced by jasmine acid detected by using micro-sensor based on Pt/Pt/POPD electrode. Constant potential: -0.1 V. A: susceptible oilseed rape genotype, 84039; B: Glucose oxidase (GO)-transgenic oilseed rape, GO16

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