Research Article

Phytochemical Analysis

Received: 15 May 2009; Revised: 10 August 2009; Accepted: 16 September 2009 Published online in Wiley Interscience: 11 November 2009

(www.interscience.wiley.com) DOI 10.1002/pca.1178

In Vivo Monitor Oxidative Burst Induced by Cd^{2+} Stress for the Oilseed Rape (Brassica napus L.) Based on Electrochemical Microbiosensor

Qiao Xu,[a] Fang Wei,[b] Zhan Wang,[a] Qin Yang,[a] Yuan-Di Zhao[a]* and Hong Chen[b]*

ABSTRACT:

Introduction – Since the mechanism of Cd^{2+} stress for plants is not clear, an *in vivo* method to monitor Cd^{2+} stress for plants is necessary. However, oxidative burst (OB) is a signal messenger in the process of Cd^{2+} stress for plants.

Objective – To establish an electrochemical method with poly-*o*-phenylenediamine and Pt microparticle modified Pt electrode (POPD–Pt-MP–Pt) as a microbiosensor for the *in vivo* detection of oxidative burst induced by Cd^{2+} stress in oilseed rape (*Brassica napus L.*).

Methodology – The optimal fabrication of POPD–Pt-MP–Pt biosensor was achieved. Electrochemical signal was collected by amperometry.

Results – After oilseed rape was exposed to 84.9 mM $CdCl_2$ stress, three oxidative bursts were observed in oilseed rape by amperometry at 3.3 h, 8.4 h and 13.2 h, respectively. However, there was no obvious signal observed in the controlled assay.

Conclusion – This contribution presents the *in vivo* monitoring of the OB process induced by Cd^{2+} stress in oilseed rape by POPD–Pt-MP–Pt microbiosensor in real-time. The novel electrochemical microbiosensor not only facilitates the real-time study in plant self-defence response to the adverse environment such as Cd^{2+} stress, but also provides an effective tool for probing the self-defence mechanism in plants. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: electrochemical microbiosensor; oilseed rape; oxidative burst; Cd^{2+} stress

Introduction

Cd is an environmental pollutant and toxin that is distributed worldwide, as well as being a serious toxin for plant physiological development. Cadmium ion (Cd^{2+}) is one of the forms of cadmium that exists in solution. Cd^{2+} is most likely to enter the root of plant from cortex tissue and arrives at the xylem to chelate with ligands such as organic acid and chelating peptide in the root (Heiss *et al.*, 2003). In morphology, Cd^{2+} can stunt plant growth, leading to chlorosis and decreased yield. In physiology, it can affect photosynthesis and nutrient transpiration in plants. At the cellular level, Cd^{2+} can result in the dysfunction of cell membrane, oxidative stress and even DNA damage. The oxidative stress mainly induces lipid peroxidation and oxidative burst (OB), and consequently inhibits the activity of antioxidant enzyme. With the accumulation of Cd^{2+} in the plant, the concentration of malondialdehyde (MDA) increases, which can reflect the status of free radicals in plant. The higher the concentration of MDA, the more free radicals such as ·OH are produced. In a study of the toxicity procession of heavy metal, Luna *et al.* (1994) proved that heavy metal stress for oat leaves was generated by oxygen free radials, which are reactive oxygen species (ROS), through a controlled assay in which free radical scavenger (Tiron benzoic acid and mannan) was added.

ROS is a metabolic product in processes of metabolization such as photosynthesis, breathing and nitrogen fixation in plant. With the help of ROS scavengers, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and peroxidase (POD), the concentration of ROS in plants is balanced dynamically and kept at a harmless level under normal growth conditions. However, in stress situations, plants have complex

* Correspondence to: Yuan-Di Zhao, Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics-Huazhong University of Science and Technology, Wuhan, Hubei 430074, People's Republic of China. E-mail: zydi@mail.hust.edu.cn

Hong Chen, Institute of Oil Crops Research, Chinese Academy of Agricultural Sciences, The Key Lab of Oil Crops Biology, Ministry of Agriculture, Wuhan, Hubei 430062, People's Republic of China. E-mail: chenhong@oilcrops.cn

[a] Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics-Huazhong University of Science and Technology, Wuhan, Hubei 430074, People's Republic of China

[b] Institute of Oil Crops Research, Chinese Academy of Agricultural Sciences, The Key Lab of Oil Crops Biology, Ministry of Agriculture, Wuhan, Hubei 430062, People's Republic of China

detoxification systems to defend against invasion and stress from adverse environmental conditions. Once stress occurs, the balance is disturbed. Then, ROS, mainly $O_2^{\cdot-}$ and H_2O_2 (Apostol *et al.*, 1989), are rapidly overproduced to form OB, which is the most important and fastest detoxification action in the plant. However, since overproduction of H_2O_2 will harm the plant, an ROS-scavenging system removes the excess and maintains the ROS at the normal level. Accordingly, ROS play an important role in anti-stress signal transduction, membrane lipid oxidation, cell wall reinforcement and defence actions (Poli *et al.*, 2004).

Until now, the toxicity of Cd^{2+} stress to plants is far from well understood (Groppa *et al.*, 2008), and the present results are mainly obtained *in vitro*. In conventional methods, typically, different defending enzymes are collected at different time intervals to determine the degree of stress caused by Cd^{2+} and the antioxdative mechanism investigated (Molina *et al.*, 2008; Smeets *et al.*, 2008). However, these *in vitro* analytical methods may result in the decrease of enzyme activity. Moreover, they usually involve complex operation procedures and need expensive kits. Therefore, it is of great importance to develop a simple, cheap and *in vivo* diagnostic tool for monitoring Cd^{2+} stress in plants in real time. Oxidative burst will occur when the plant is stressed. If the concentration variations of ROS can be monitored *in vivo*, the corresponding defence reaction to Cd^{2+} stress can be monitored in real time as well. Thus, the mechanisms of the defence reaction induced by Cd^{2+} stress and the effects of Cd^{2+} stress on plant physiology can be thoroughly studied.

Although many methods have been developed to monitor the ROS in plant, such as fluorescence (Lindfor and Ivaska, 2002), spectrophotometry (Jeffers *et al.*, 2007) and luminescence (Dahlberg *et al.*, 2007), none of them satisfy the requirement for *in vivo* and real-time detection. Because of its attractive features, such as simplicity and sensitivity (Zhao *et al.*, 1998; Zhao *et al.*, 2003), electrochemical methods based on the detection of H_2O_2, which is a major component of ROS, have drawn intensive attention (Dai *et al.*, 2009). Further, the electrochemical method is suitable for *in vivo* assay, which makes it unique compared with the traditional methods for ROS detection. If the concentration variation of H_2O_2 in plants could be monitored in real time and *in vivo* by electrochemical method, the study of the defence mechanisms of plants against Cd^{2+} stress could go further.

In recent years, conductive polymers have attracted great interest due to their special chemical and physical properties. Electrochemical properties have been systematically studied for various conductive polymers, especially for polyaniline, polyacetylene and polypyrole (Prigodin *et al.*, 2008). Poly-*o*-phenylenediamine (POPD) is a ladder-shaped construction polymer with a repeat unit of phenazine (Craig and O'Neill, 2006) and has been widely applied in electrocatalysis, electrochemical display, biosensor and electrode modification material (Coutanceau *et al.*, 2006). The POPD film has excellent permselectivity due to the existence of positive charge. Hence, most neutral molecular and anionic ions can easily pass through such a film and reach the surface of the electrode while most cationic ions cannot. This feature allows POPD-modified electrodes to prevent interference from fouling and interference ions, which is essential for analytical applications (Gao *et al.*, 1994).

In the vast majority of *in vivo* measurements, a carbon fibre microelectrode is the most commonly used electrode (Santos *et al.*, 2008; Du *et al.*, 2008). However, this kind of electrode is unsuitable for *in vivo* measurement in plants because of its high absorbability and poor ductility. On the other hand, a Pt electrode, with its good metal ductility and electrocatalytic activity, has been utilized as a platform for the electrodeposition of catalytic metal microparticles such as Pt, Ru and Pd, together with conducting polymer, for detection of small molecules such as methanol and glycerol (Grace and Pandian, 2006; Wang *et al.*, 2009). Therefore, in this paper, a POPD and Pt microparticle (MP) modified Pt electrode was prepared to detect H_2O_2 in plants induced by Cd^{2+} stress.

In this study, we have developed a POPD–Pt-MP–Pt electrochemical microbiosensor, with the advantages of microsize and good ductility, for the detection of the OB process induced by Cd^{2+} stress in oilseed rape. This novel tool could provide reliable information on the timing of the plant's response to Cd^{2+} stress, which represents a simple technique in probing the defence and the OB mechanism of plant against Cd^{2+} stress.

Experimental

Instruments and reagents

The instrument used was a CHI660 software-controlled electrochemistry workstation (Shanghai CH Instruments Inc., China) and an S-3000N scanning electron microscope (SEM) (Hitachi, Japan). Metal content was measured by AA-300 flame atomic absorption spectrometry (AAS) (Perkin Elmer, USA). Kalium chloroplatinate (K_2PtCl_6), *o*-phenylenediamine (OPD), H_2O_2 and all chemical reagents were of analytical grade and purchased from Sinopharm. OPD was used after three recrystallizations from ethanol. Solutions were prepared from these reagents and two distilled water.

Plant materials

The oilseed rape (*Brassica napus* L.) used in the study was provided by Institute of Oil Crops Research, Chinese Academy of Agricultural Sciences. The oilseed rapes (at the age of 28–30 days, around 15 cm tall) grew in the glasshouse at 15–21°C and leaves at the four-true-leaf stage were used.

In determining accumulation of Cd^{2+} by AAS, 300 mg leaf and leafstalk were digested in a mixture of HNO_3 and $HClO_4$ (4 : 1 mL) until dryness at 100°C.

Preparation and modification of the POPD–Pt-MP–Pt electrode

Pt-electrode with diameter of 0.1 mm and length of 5.1 mm was used. One terminal of the Pt wire of diameter 0.1 mm and length 10 mm, was connected with a silver wire of length 5 cm. The other terminal of silver was connected with brass wire. Then the 'whole' wire was sealed in a glass tube with 0.3 cm diameter and the other terminal of Pt and brass wires exposed.

A three-electrode system was used. The POPD–Pt-MP–Pt electrode was the working electrode, Ag–AgCl was the reference electrode and Pt was the counter electrode. For the preparation of POPD–Pt-MP–Pt electrode, Pt-MP was first deposited on the bare Pt electrode by immersing the Pt electrode in the solution of 2 mM K_2PtCl_6 and 0.5 M H_2SO_4, scanned from –0.2 to 1.0 V with 30 cycles; then, Pt-MP modified Pt was deposited in the solution of 50 mM OPD and 0.5 M H_2SO_4 and scanned for 30 cycles in the range –0.2 to 1.2 V to obtain a POPD–Pt-MP–Pt electrode.

In vivo monitoring OB in the oilseed rape

POPD–Pt-MP–Pt electrode was inserted into the end of leafstalk close to mainstalk, and then Pt counter electrode was inserted into the leafstalk 2 cm distance from POPD–Pt-MP–Pt electrode, consequently inserting Ag–AgCl into leafstalk with 2 cm distance away from the Pt counter electrode. A 10 mL ali quot of 1.946 mg/mL Cd^{2+} was added to the soil of the oilseed rape. Amperometry was used to measure the change of H_2O_2 after plant was stressed by Cd^{2+} in 70 h. Meanwhile, 10 mL water was given to

the control and the same experiments were carried out as well. All the processes were carried out in room temperature.

Results and Discussion

SEM characterization of Pt, Pt-MP–Pt and POPD–Pt-MP–Pt microelectrode

SEM was employed to characterize the surface morphology of the Pt, Pt-MP–Pt and POPD–Pt-MP–Pt microelectrodes. As shown in Fig. 1A, the surface of the bare Pt electrode is relatively smooth (Fig. 1A). However, Fig. 1B shows that some particles formed on electrode, with the diameter of 1–3 μm. It might be Pt microparticles grown on the surface of the bare Pt electrode in K_2PtCl_6 solution. Furthermore, in Fig. 1C, a compact and porous film was formed on the Pt electrode, which is generated by polymerization of OPD on Pt-MP–Pt electrode.

Detection of H_2O_2 in solution

In order to detect H_2O_2, cycle voltammetry (CV) was employed to measure the 50 mmol/L H_2O_2 dissolved in 1 mmol/L NaCl solution at Pt, Pt-MP–Pt, POPD–Pt and POPD–Pt-MP–Pt electrodes. The results showed that the reduction current of H_2O_2 at POPD–Pt–Pt electrode was the largest one among these electrodes. This might be because of the microsize of Pt-MP and the porous structure of the POPD film, both of which increased the effective area of the electrode and consequently improved its electrocatalytic activity toward H_2O_2. According to the results above, amperometry was used to demonstrate the unique capability of POPD–Pt-MP–Pt for the detection of H_2O_2 (Fig. 2) by continuously adding H_2O_2 into the solution of 1 mmol/L NaCl. It was observed that the concentration of H_2O_2 was linear to the currents in the range 1.2×10^{-5} to 1.2×10^{-3} M, and the linear relationship was $I = 2.55 + 0.0034c$ ($R = 0.990$), with the detection limitation 4.0×10^{-6} M. The interference of ascorbic acid, which usually exists in plants, was investigated for the selectivity of the POPD–Pt-MP–Pt electrode by amperometry in coexistence with H_2O_2. In the solution of 1 mmol/L NaCl containing 4.9×10^{-5} M H_2O_2, there was little interference from 10× ascorbic acid, which demonstrated that the POPD–Pt-MP–Pt electrode is a promising material for the detection of H_2O_2.

Detection of H_2O_2 in oilseed rape by amperometry *in vivo*

During the whole *in vivo* experiment process, oilseed rape absorbed water from soil and transferred water to the leaf, which guaranteed that the working electrode, reference electrode and counter electrode could all work in the solution condition, as Fig. 3 shows. Meanwhile, ROS generated from cells in leaves could be dispersed by diffusion, passive transport and active transport, and finally detected by the working electrode. Therefore, the POPD–Pt-MP–Pt electrode can be applied for the measurement of OB induced by Cd^{2+} stress in living oilseed rape *in vivo*.

As H_2O_2, the main form of ROS in plants, can be sensitively detected on the POPD–Pt-MP–Pt electrode by amperometry around –0.2 V (Di *et al.*, 2007), –0.2 V was selected as the working potential. Figure 4a shows the change of ROS in the leafstalk as the control, indicating that there is no obvious change of ROS under normal condition. In normal conditions, ROS in plants is kept at a balanced level with the help of a ROS-scavenging system. However, after Cd^{2+} stress occurs, abnormal current will respond to this Cd^{2+} stress, in another word, the 'balance' is disturbed by Cd^{2+} stress and this change can be monitored by the POPD–Pt-MP–Pt electrode (Fig. 4b). The abnormal current response might be caused by the rapid release of reactive oxygen species, which is are called an 'oxidative burst'. For the first OB, as shown in Fig. 4b, its high point appeared around 3.3 h after Cd^{2+}

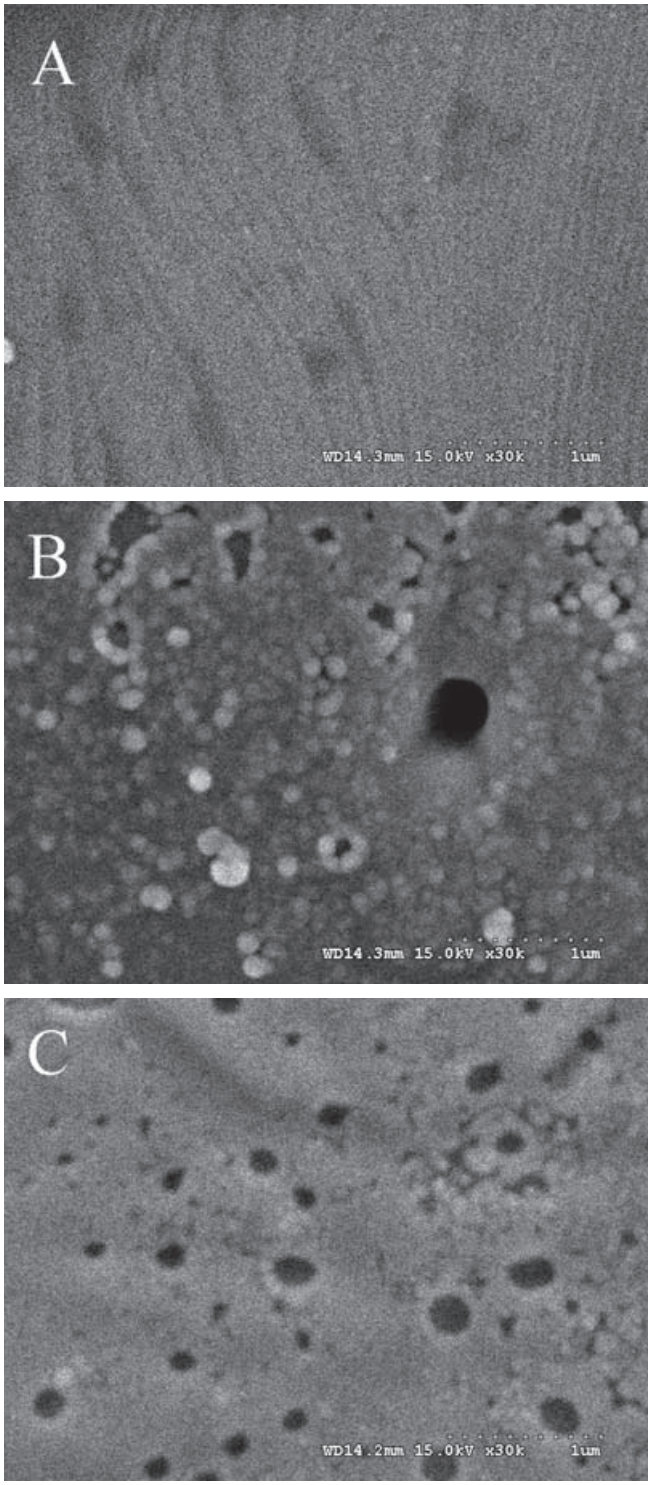

Figure 1. Scanning electron micrograph (SEM) images of bare Pt (A), Pt-MP–Pt (B) and POPD–Pt-MP–Pt (C).

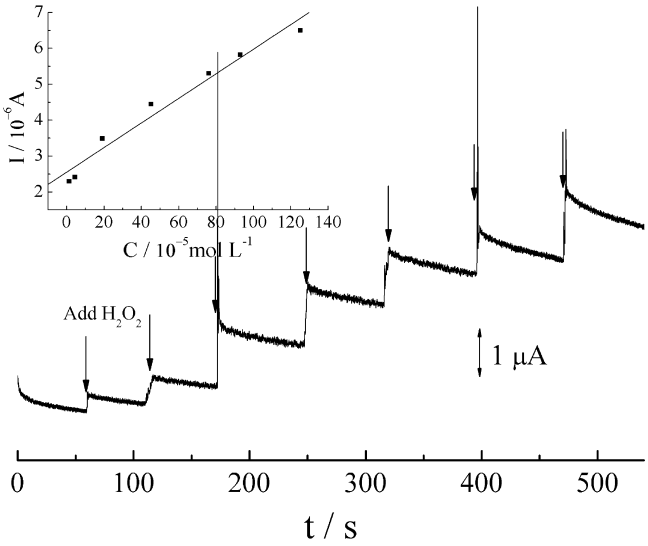

Figure 2. Amperometric response curve of POPD–Pt-MP–Pt electrode upon successive additions of H_2O_2 to 8 mL 1 mmol/L NaCl at −0.2 V. Insert: calibration curve for H_2O_2 determination.

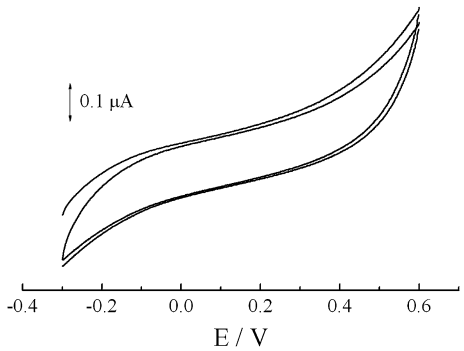

Figure 3. CV of POPD–Pt-MP–Pt electrode *in vivo*.

was added, followed by the second peak around 8.4 h and the third around 13.2 h. Each peak lasted for at least 4 h, which indicates that oxidative burst occurred. By the equation $Q = I \times t$, the corresponding amount of H_2O_2 under Cd^{2+} stress for the three OBs was estimated to be 6.2×10^{-10}, 3.9×10^{-10} and 3.1×10^{-10} mol, respectively. After the third OB, ROS in plant gradually recovered to a new balance, which might be attributed to the ROS-scavenging system consisting of both non-enzyme and enzyme scavenging systems (Roosens *et al.*, 2003). In addition, no further OB in the plants was observed over the following 60 h. Non-enzyme scavenging systems mainly include ascorbate, glutathione, vitamin E, mannitol and flavonoids, in which ROS induced by Cd^{2+} stress can be scavenged by the ascorbate–glutathione cycle (Jimenez *et al.*, 1997). The enzyme scavenging system includes SOD, CAT, POD, ascorbate peroxidase (APX) and glutathione peroxidase (GPX), in which SOD can convert $O_2^{\cdot-}$ to H_2O_2; consequently, H_2O_2 can be detoxified to H_2O by other peroxidases such as POD, APX and GPX (Venancio *et al.*, 2002). The oxidative burst was measured in eight oilseed rapes in the experiments. The results showed that all those plants had three oxidative bursts in the condition of Cd^{2+} stress. The results also showed that every plant in Cd^{2+} stress appeared to have a different peak

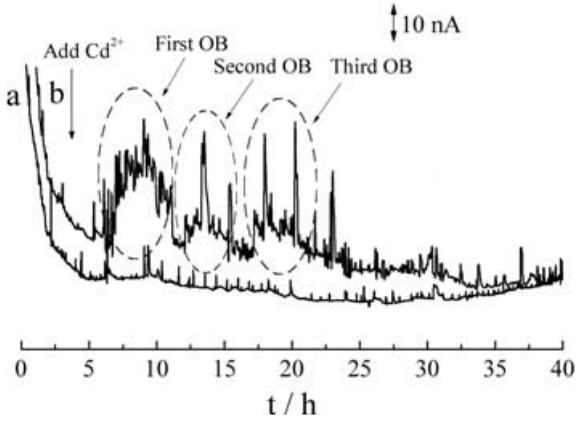

Figure 4. *In vivo* amperometric *i–t* curves without (a) and with (b) Cd^{2+} stress on POPD–Pt-MP–Pt electrode, applied potential −0.2 V.

shape, indicating that reproducibility of burst time and intensity of the peaks was not good. It is assumed that the difference might be result from several factors, such as the diversity of plant or the unavoidable difference in insertion of working electrode.

Verification existence of Cd^{2+} in oilseed rape by AAS after *in vivo* assay

AAS was also employed to determine the concentration of Cd^{2+} in the dry leafstalk of the plant before and after Cd^{2+} stress. Corresponding to the absorbance values, the concentration of Cd^{2+} in the plant leafstalk was deduced to be 2.53 μM. In other words, 0.15 mg Cd^{2+} was contained in 1 g dry leafstalk of oilseed rape. Meanwhile, concentration of Cd^{2+} in the control plant was still at the same undetectable level.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant no. 90717101, 30670553). Dr. Hong Chen is grateful to the Director Foundation of Institute of Oil Crops Research, Chinese Academy of Agricultural Sciences. We also thank the Analytical and Testing Center (Huazhong University of Science and Technology) for the help in SEM measurement.

References

Apostol I, Heinstein PF, Low PS. 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: role in de-fense and signal transduction. *Plant Physiol* **90**: 109–116.

Coutanceau C, Croissant MJ, Napporn T, Lamy C. 2000. Electrocatalytic reduction of dioxygen at platinum particles dispersed in a polyaniline film. *Electrochim Acta* **4**: 579–588

Craig JD, O'Neill RD. 2003. Comparison of simple aromatic amines for electrosynthesis of permselective polymers in biosensor fabrication. *Analyst* **128**: 905–911.

Dahlberg M, Dahlgren C, Hellstrand K, Movitz C. 2008. A new chemiluminescence paradox: Peptides derived from the N-terminal domain of annexin AI inhibits isoluminol enhanced chemiluminescence. *Luminescence* **23**: 139–143.

Dai Z, Xiao Y, Yu XZ, Mai ZB, Zhao XJ, Zou XY. 2009. Direct electrochemistry of myoglobin based on ionic liquid-clay composite films. *Biosens Bioelectron* **24**: 1629–1634.

Di J, Peng S, Shen C, Gao Y, Tu Y. 2007. One-step method embedding superoxide dismutase and gold nanoparticles in silica sol–gel network

in the presence of cysteine for construction of third-generation biosensor. *Biosens Bioelectron* **23**: 682–687.

Du FY, Huang WH, Shi YX, Wang ZL, Cheng JK. 2008. Real-time monitoring of NO release from single cells using carbon fiber microdisk electrodes modified with single-walled carbon nanotubes. *Biosens Bioelectron* **24**: 415–421.

Gao ZQ, Bobacka J, Ivaska A. 1994. Electrochemical study of bilayer conducting polymers: polypyrrole/polyaniline system. *J Electroanal Chem* **364**: 127–133

Grace AN, Pandian K. 2006. Pt, Pt–Pd and Pt–Pd/Ru nanoparticles entrapped polyaniline electrodes—a potent electrocatalyst towards the oxidation of glycerol. *Electrochem Commun* **8**: 1340–1348.

Groppa MD, Rosales EP, Iannone MF, Benavides MP. 2008. Nitric oxide, polyamines and Cd-induced phytotoxicity in wheat roots. *Phytochemistry* **69**: 2609–2615.

Heiss S, Wachter A, Bogs J, Cobbett C, Rausch T. 2003. Phytochelatin synthase (PCS) protein is induced in Brassica juncea leaves after prolonged Cd exposure. *J Exp Bot* **54**: 1833–1839.

Jeffers P, Kerins S, Baker CJ, Kieran PM. 2007. Generation of reactive oxygen and antioxidant species by hydrodynamically stressed suspensions of *Morinda citrifolia*. *Biotech Prog* **23**: 138–145.

Jimenez A, Hernandez JA, del Rio LA, Sevilla F. 1997. Evidence for the presence of the ascorbate–glutathione cycle in mitochondria and peroxisomes of pea leaves. *Plant Physiol* **114**: 275–284.

Lindfor T, Ivaska A. 2002. pH sensitivity of polyaniline and its substituted derivatives. *J Electroanal Chem* **531**: 43–52.

Luna CM, Gonzalez CA, Tripp VS. 1994. Oxidative damage caused by on excess of copper in oat leaves. *Plant Cell Physiol* **35**: 11–15.

Molina AS, Nievas C, Chaca MVP, Garibotto F, González U, Marsa SM, Luna C, Giménez MS, Zirulnik F. 2008. Cadmium-induced oxidative damage and antioxidative defense mechanisms in *Vigna mungo L*. *Plant Growth Regul* **56**: 285–295.

Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. 2004. Oxidative stress and cell signalling. *Current Med Chem* **11**: 1163–1182.

Prigodin VN, Hsu FC, Park JH, Waldmann O, Epstein AJ. 2008. Electron–ion interaction in doped conducting polymers. *Phys Rev B* **78**: 035203.

Roosens N, Verbruggen N, Meerts P, Ximenez-Embun P, Smith JAC. 2003. Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of thlaspi caerulescens from western europe. *Plant Cell Environ* **26**: 1657–1672.

Santos RM, Lourenço CF, Piedade AP, Andrews R, Pomerleau F, Huettl P, Gerhardt GA, Laranjinha J, Barbosa RM. 2008. A comparative study of carbon fiber-based microelectrodes for the measurement of nitric oxide in brain tissue. *Biosens Bioelectron* **24**: 704–709.

Smeets K, Ruytinx J, Semanea B, Van Belleghemc F, Remansa T, Van Sanden S, Vangronsveld J, Cuypers A. 2008. Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environ Exp Bot* **63**: 1–8.

Venancio EC, Napporn WT, Motheo AJ. 2002. Electro-oxidation of glycerol on platinum dispersed in polyaniline matrices. *Electrochim Acta* **47**: 1495–1501.

Wang J, Qi X, Meng F, Ning Y, Chen SF, Pang D, Wen YF. 2009. Polyaniline nanofibers: inducing action of neodymium oxide and inhibiting effect on electrochemical degradation and modified platinum electrode application to the electrocatalytic oxidation of methanol. *J Phys Chem C* **113**: 1459–1465.

Zhao YD, Pang DW, Wang ZL, Cheng JK, Luo ZF, Feng CJ, Shen HY, Zhang XC. 1998. Electrochemical studies of antitumor drugs I. fundamental electrochemical characteristics of an iron (III) schiff base complex and its interaction with DNA. *Acta Chim Sin* **56**: 178–183.

Zhao YD, Zhang WD, Luo QM, Li SFY. 2003. The oxidation and reduction behavior of nitrite at carbon nanotube powder microelectrodes. *Microchem J* **75**: 189–198.