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Convenient Assay of O_2^- Generated on Potato Tuber Tissue Slices Treated with Fungal Elicitor by Electron Spin Resonance – No Secondary Oxidative Burst Induction by H_2O_2 Treatment

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Since the discovery of generation of O_2^- in plant, many evidence for the oxidative burst (OXB) has been accumulated in various combinations of plant and pathogen or elicitor systems. O_2^- generating system responsible for the OXB was coupled with oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in microsomal fraction isolated from sliced aged potato tuber slices which were treated by hyphal wall components elicitor from *Phytophthora infestans* (HWC). We developed new assay method for quantitative measurement of oxygen radical O_2^- by using electron spin resonance (ESR) analysis during elicitor-induced OXB on the surface of plant tissues. The ESR analysis using an O_2^- trapper, Tiron (1,2-dihydroxy-3,5-benzenedisulfonic acid), provided a convenient assay for detecting only O_2^- during elicitor-induced OXB producing various active oxygen species (AOS) on plant tissue surface. Tiron was oxidized to Tiron semiquinon radical by O_2^-. Quantity of the radical signal was measured by specific spectra on ESR spectroscopy. The level of O_2^- was high in from surface of potato tuber tissue treated with hyphal cell wall elicitor (HWC) from *Phytophthora infestans*. There was no secondary OXB induction by H_2O_2 treatment in plant.

Keywords : ESR, H_2O_2, HWC elicitor, O_2^-, Oxidative burst

A generation of O_2^- occurred in various plants infected pathogen or treated with elicitors. The generation of O_2^- was proposed to play a key role in induction of several plant defense reactions (Doke et al., 1996; Jabs et al., 1997; Mehdy, 1994). O_2^- generating system responsible for the OXB (Oxidative burst) was coupled with oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in microsomal fraction isolated from sliced aged potato tuber slices (Doke and Miura, 1995). This activity of NADPH oxidation increased in microsomal fraction isolated from potato tubers infected an avirulent race of *Phytophthora infestans* (Doke, 1985). The O_2^- generating NADPH oxidase was postulated to present in plasma-membrane fraction of potato tissues and was depended on Ca^{++} signaling (Doke and Miura, 1995; Doke, 1985; Miura et al., 1995). The NADPH oxidase activity was also found in microsomal fraction isolated from tobacco mosaic virus (TMV)-infected leaves (Doke and Ohashi, 1988). The OXB system in plants seemed to be similar to that depending on the NADPH oxidase which was established in the OXB pathway of phagocytes in mammals (Rossi, 1986).

Since a rapid induction of AOS generation (OXB) was found in plant tissues which were attacked by incompatible pathogens or treated with an elicitor of induced resistance (Doke, 1983a, b), various methods for measurement of OXB in plants have been developed. In the original experiments, quantitative analysis of generation of O_2^- from plant tissues or protoplasts was carried out by spectrophotometrical measurement of cytocrome *c* or nitro blue-tetrazolium (NBT) reducing activity at 550 and 580 nm, respectively (Auh and Murphy, 1995; Doke, 1983a, b). These reductions were confirmed to be due to O_2^- by monitoring of inhibition in the presence of SOD.

It is well known that electron spin resonance (ESR) is also available for quantitative analysis of AOS generation from plant cells by using appropriate radical trappers in *in vitro* system (Mayak et al., 1983; Qiu et al., 1994). ESR system carried out quantitative analysis of that by using electron spin signal by reaction with radical trapper. 1,2-dihydroxy-3,5-benzenedisulfonic acid (Tiron) which was one of a radical trapper against O_2^-, is stabilized. The radical trapper oxidized by O_2^-, become Tiron semiquinon radical. The radical signal was quantitatively detectable by measuring specific spectra on ESR spectroscopy. As this method had been used for measurement of generation of O_2^- in *in vitro* system. Successful assay of O_2^- generated outside of plant tissues may be expected when a Tiron solution applied onto elicitor-treated plant tissues is

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subjected to ESR analysis.

This method has been usefully used for measurement of generation of O_2^- in *in vitro* system, and reminds those of development for measurement in *in vivo* system. In this paper, we described newly developed method for quantitative measurement of OXB on plant tissue surfaces *in vivo* on the basis of ESR analysis of Tiron semiquinon radical.

H_2O_2 induced when plant was treated with elicitor activated Ca^{++} channel and protein phosphorylation for generated a systemic signal to induce systemic oxidative burst for induction of systemic acquired resistance (SAR) (Park et al., 1998). Therefore H_2O_2 generated during OXB induced secondary OXB through NADPH oxidase pathway via Ca^{++} signaling. The signal may be OXB chain reaction or some new signal generated by H_2O_2. It is a possibility that endogenous H_2O_2 produced during OXB in elicitor-treated plant may induce secondary OXB for systemic signaling. We investigated the possibility using the ESR assay system in potato tuber tissue.

Materials and methods

Plant material. Tubers of potato plant, cv. Rishiri (an interspecific hybrid between *Solanum tuberosum* L. and *S. demissum* L., carrying the R_1 resistance gene to *P. infestans* (Mont.) de Barry) were used. The cultivar was susceptible to race 1.2.3.4 and resistance to race 0 of the fungus. This cultivar was reported to carry cells, which were hypersensitively reactive to the incompatible races, or hyphal wall component (HWC) by 90% among tissue cells (Doke and Tomiyama, 1980). Potato was harvested on late September in Hokkaido and on early July in Nagoya, respectively, and then stored at 4°C until used.

Preparation of potato tissue slices. To collect sample solution containing Tiron radical for measurement of O_2^- during OXB by ESR, a well (9 mm in diameter, 8 mm depth) was made on the surface of tuber slice (10 mm thick) by a drill, rinsed with distilled water and incubated for 18 h at 20°C in the dark (Fig. 1).

Preparation of HWC elicitor. HWC elicitor from mycelia of *P. infestans* was prepared according to the method of Doke and Tomiyama (1980).

Measurement of O_2^- by ESR. Amount of O_2^- was determined as Tiron radical signal by using ESR (ESR 850, Jeol Ltd., Japan). An aliquot of Tiron solution (final 50 mM), which was applied in a well of tuber tissue for definite times, was placed in a 100 µl capillary quarts tube, and then the capillary quartz tube was inserted in the microwave cavity (Fig. 1). Relative quantity of O_2^- signal was calculated on the base of a function of standard signal which was obtained from Mn. ESR parameters used were as followed: microwave crowave power, 10 mW; microwave frequency, 9.35 GHz; modulation amplitude, 0.1 mT; and time constant, 0.1 sec.

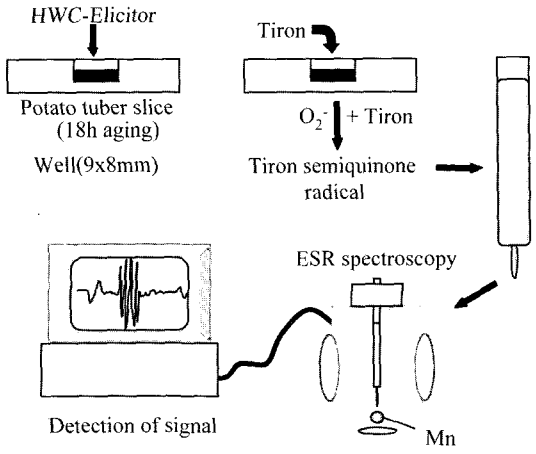

Fig. 1. Diagrammatic illustration of sampling system for ESR analysis. To collect sample solution, a well (9 mm in diameter, 8 mm depth) was made on the surface of tuber slice (10 mm thick) by a drill. An aliquot of Tiron solution (50 mM), which had been applied in the well of tuber tissue, was placed in a 100 µl capillary quartz tube for subjecting to ESR analysis.

Results

Measurement of O_2^- during OXB by ESR. The generation of O_2^- in HWC-stimulated OXB on the tuber tissue was determined by measuring Tiron semiquinon radical with ESR. The spectrum of Tiron semiquinon radical signal was intensively gained from Tiron solution, which was applied on the tuber slices, treated with HWC (Fig. 2a). The signal was greatly reduced when HWC was applied with DPI at 100 µM (Fig. 2b). The signal was scarcely observed in samples from Tris-buffer treated surface (Fig. 2c). Amplitude of the spectrum of Tiron semiquinon radical signal from HWC-treated tissue surface was the highest in the samples at 2-5 min after application of 50 mM Tiron on the HWC-treated tissues (Fig. 3A). At sample collection time of 2 min, relative intensity of the Tiron semiquinon radical from HWC-treated tissue surface was linearly dependent on the concentrations of Tiron applied from 5 to 50 mM (Fig. 3B). Under these conditions of 2 min reaction time and concentration of Tiron at 50 mM, amplitude of the spectrum of Tiron semiquinon radical signal was determined at different concentrations of HWC at 0, 0.1, 0.5 or 1.0 mg/ml. The amplitude of signals was dependent on concentrations of HWC treated at 0 to

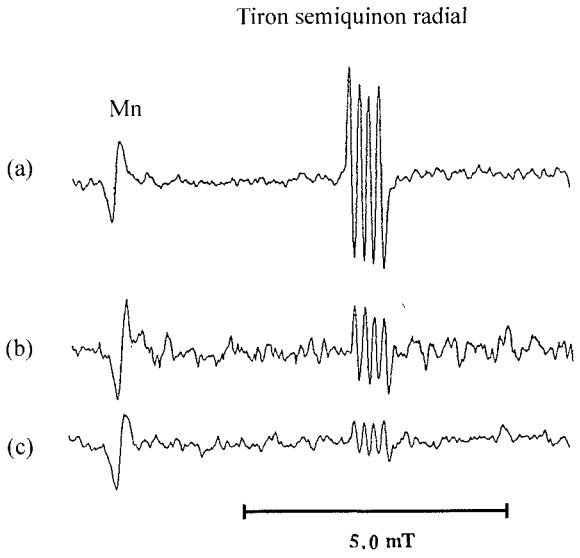

Fig. 2. ESR spectrum of the Tiron radical in samples obtained from Tiron solution (50 mM) applied for 2 min on the HWC-treated surface of potato tuber tissues. Time after treatment with HWC solution (1 mg/ml) (a), HWC solution containing DPI (50 μm) (b) or Tris-buffer (10 mM, pH 7.4) (c). ESR parameters used were as followed: microwave crowave power, 10 mW; microwave frequency, 9.35 GHz; modulation amplitude, 0.1 mT; and time constant, 0.1 sec.

1.0 mg/ml when determined at 60 min after HWC treatment (Fig. 3C). Intensity of detectable O_2^- signal from HWC-treated tissue increased with time until 60 min after treatment with HWC (Fig. 3D).

No secondary OXB by H_2O_2 in plant tissue. H_2O_2 treatment of tissues together with various inhibitors of elicitor-stimulated local OXB inhibited the induction of the sub-systemic OXB (Park et al., 1998). These results suggested a possibility that local OXB was induced through a secondary activation of NADPH oxidase by application of H_2O_2. Therefore, generation of O_2^- in tissue surface applied with H_2O_2 (1 mM) was determined by measuring O_2^- signal trapped by Tiron with ESR system. O_2^- measured by Tron radical semiquinon signal was not detected in tissues applied with H_2O_2 while it was significantly high in HWC-treated tissues (Fig. 4).

Discussion

Several methods have been developed to detect O_2^- in biological system (Mayak et al., 1983; Qiu et al., 1994). Elicitor-treated plant tissue was shown to reduce extracellular cytocrome *c* or NBT (Doke, 1983a; Doke, 1983b; Auh and Murphy, 1995). These reductions were inhibited by SOD, suggesting that O_2^- is released out of plant tissues treated with elicitor. In the present study, it was examined

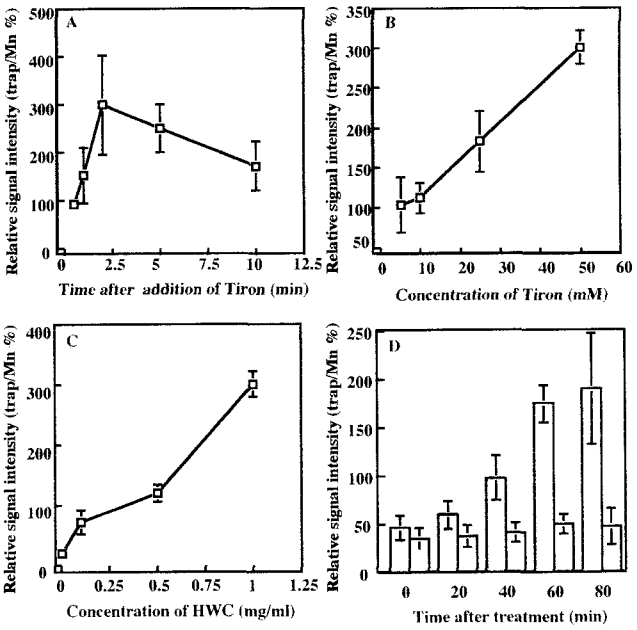

Fig. 3. Conditions to measure O_2^- during OXB by using ESR system. (A) Effect of timing of sampling after addition of Tiron solution (50 mM) on intensity of ESR signal of generated O_2^-. Tiron was applied in a well of potato tuber tissues from 1 h after treatment with HWC solution (1 mg/ml). (B) Effect of concentration of applied Tiron on intensity of ESR signal of O_2^- from tissue treated with HWC. Samples were taken 2 min after application of Tiron on a well of potato tuber tissues 1 h after treatment with HWC solution (1 mg/ml). (C) Intensity of ESR signal O_2^- from a well of potato tuber tissues treated with HWC at different concentrations. Samples were taken 2 min after the addition of Tiron (50 mM) at 1 h after treatment with HWC solution. (D) Time course measurement of ESR signal of O_2^- generated on the surface of potato tuber slices of treatment with HWC. Solid column: treated with HWC, Shaded column: treated with buffer only. Each value represents the mean of three experiments with the S.D.. Experimental conditions are the same as for Fig. 1.

whether the elicitor-treated tuber tissue produces O_2^- by ESR measurement. ESR analysis of Tiron solution applied on elicitor-treated tissues showed a typical Tiron semiquinon radical signal derived from O_2^- (Fig. 2). Here, we carried out to measure generation of O_2^- in elicitor-treated tuber tissue by ESR system in *in vivo* system. The Tiron semiquinon radical signal formed by oxidization of Tiron by O_2^- exuded into the well from the HWC-treated potato tuber tissue was appeared as a narrow four line spectrum which was previously reported (Fig. 2). In this system, we determined application time and concentration of Tiron to measure O_2^- generation of HWC-treated tissue. Tiron radical signal was maximum in 2-5 min after application to the tissue (Fig. 3A). Amplitude of Tiron radical signal in HWC was dependent from10 to 50 mM Tiron. (Fig. 3B). In previously reported papers, 10 mM Tiron was used in their

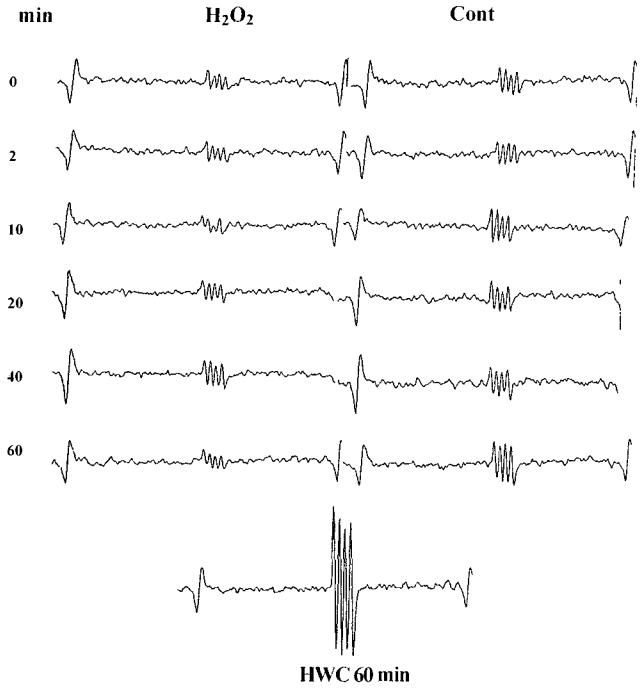

Fig. 4. Time course changes of ESR signal trapped by Tiron from the potato tuber tissues treated with H_2O_2 (1 mM). Experimental conditions are the same as for Fig. 1. As a reference, ESR spectra of a sample from tissue treated with HWC solution (1.0 mg/ml) at 60 min was indicated.

in vitro system (Mayak et al., 1983; Qiu et al., 1994). However, we thought that *in vivo* system may require high concentration of Tiron to generate clear signal than in vitro system does, because the Tiron radical formed by trapping O_2^- in plasmamembrane of tuber cell, may exude from out of cell wall.

To analyze the signal quantitatively, we compared amplitude of the Tiron semiquinon radical signal spectrum with that of Mn metal electron spin signal which is permanently stable. According to the result shown in this study O_2^- was generated in the elicitor-treated tissues, suggesting that OXB occurs in the elicitor treated tissues. Our previous data showed luminol mediated chemiluminescense (CL) induced by H_2O_2 during OXB was inhibited by catalase (Miura et al., 1995; Park et al., 1998). Considering the catalase-inhibitory CL during local OXB, O_2^- was initially generated, and then dismutated to H_2O_2 during OXB. ESR analysis using Tiron provided a convenient assay of O_2^- during OXB of plant tissue surface. The luminol-mediated CL (data not shown) and Tiron semiquinon radical were not detected in tissues treated elicitor in the presence of DPI (Fig. 1), an inhibitor of O_2^- generating NADPH oxidase (Hancock and Jones, 1987). Therefore, Tiron semiquinon radical are thought to be due to the activation of the NADPH of O_2^- in HWC-treated tuber tissue under above described condition. Therefore, ESR analysis of O_2^- during OXB using Tiron provided a convenient assay of O_2^- during OXB generating various AOS on plant tissue surfaces.

Inhibitors of the local OXB depending on NADPH oxidase as well as scavenger of H_2O_2 inhibited induction of the systemic OXB (Park et al., 1998). However, a secondary local OXB thruogh activation of NADPH oxidase was not induced by exogenous H_2O_2 (Fig. 4). H_2O_2-triggered systemic signal for induction was different from that for activation of NADPH oxidase. Some studies reported that cytosolic Ca^{++} transiently increased immediately after application of H_2O_2 and through Ca^{++} channel following each different stimulus (Price et al., 1994). These reports supported the assumption that H_2O_2 stimulate a secondary signaling different from signaling directly stimulated by elicitor. Thus, we proposed that H_2O_2 passably stimulated a novel secondary signaling system in tissues for the systemic OXB.

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