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Original scientific paper

An EPR spin-probe and spin-trap study of the free radicals produced by plant plasma membranes

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Abstract: Plant plasma membranes are known to produce superoxide radicals, while the production of hydroxyl radical is thought to occur only in the cell wall. In this work it was demonstrated using combined spin-trap and spin-probe EPR spectroscopic techniques, that plant plasma membranes do produce superoxide and hydroxyl radicals but by kinetically different mechanisms. The results show that superoxide and hydroxyl radicals can be detected by DMPO spin-trap and that the mechanisms and location of their production can be differentiated using the reduction of spin-probes Tempone and 7-DS. It was shown that the mechanism of production of oxygen reactive species is NADH dependent and diphenylene iodonium inhibited. The kinetics of the reduction of Tempone, combined with scavengers or the absence of NADH indicates that hydroxyl radicals are produced by a mechanism independent of that of superoxide production. It was shown that a combination of the spin-probe and spin-trap technique can be used in free radical studies of biological systems, with a number of advantages inherent to them.

Keywords: maize plasma membrane, EPR, spin-trap, spin-probe, superoxide, hydroxyl radical.

INTRODUCTION

Oxygen free radicals have been shown to be produced by a multitude of cellular mechanisms in various organelles and enzymatic reactions.[1] In plants besides intracellular, plasma membrane and apoplastic sources of reactive oxygen species (ROS) have been shown to exist and be involved in phenomena such as the oxidative burst, considered to play a role in signaling, stress response, growth and development.[2,3] The production of extracellular activated ROS in plants may be mediated by the activity of enzymes located in the plasma membrane or in the apoplast.

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Plant plasma membranes are known to produce superoxide radicals, while the production of hydroxyl radical, previously detected in complex plant tissues, is thought to occur in the cell wall, as was recently demonstrated.[4] Studies of purified plasma membranes have demonstrated the presence of NAD(P)H-superoxide synthase activity in cells of numerous plants.[5–7] On the other hand, the involvement of plasma membrane peroxidase activity in ROS generation has also been demonstrated.[8] Hence, it has not been unequivocally determined whether superoxide is produced extracellulary by membrane bound superoxide synthase or cell wall peroxidase(s). To the best of our knowledge, there are no reports, showing the production of $^{\cdot}OH$ radicals by isolated plant plasma membranes.

The detection of short-lived free radicals is a difficult task, even in pure free radical generating chemical systems. It is more cumbersome in biological systems, since these regularly produce more than one free radical species and their presence results in some methodological limitations. For example, the frequently used superoxide probe lucigenin should not be used, since it has been shown that it itself can generate $^{\cdot}O_2^-$ in the presence of flavoprotein reductases or other enzymatic reducing systems.[9] Other assays of $^{\cdot}OH$ and $^{\cdot}O_2^-$ radicals, such as nitroblue tetrazolium reduction and cytochrome *c* reduction, suffer from the disadvantages of questionable specificity and low sensitivity.[10] Due to this, the use of the EPR technique of spin-trapping has an illustrious history in detecting $^{\cdot}OH$ and $^{\cdot}O_2^-$ in biology and chemistry. Although frequently used in investigations of free radical production in animal systems, the spin-trapping technique has seldom been used in plant systems. To date only three studies have been performed.[4,11,12] one of them on isolated membranes in which a spin-trap sensitive to only $^{\cdot}O_2^-$ radicals was used. Thus, we used the frequently employed DMPO spin-trap to investigate the free radicals produced by isolated plant cell plasma membranes. The EPR spectra of the DMPO/OOH and DMPO/OH adducts have different hyperfine splitting constants, which provide a means to distinguish between the spin-trapping of $^{\cdot}OH$ and $^{\cdot}O_2^-$ free radicals.[13] However, analysis of the kinetics and mechanisms is hampered by the well known problem associated with the reduction of once trapped adducts by free radicals freshly released from the membrane[14] and transformation of the OOH adduct to the OH adduct.[15] To overcome these obstacles, nitroxide spin-probes were used instead of spin-traps. These probes are themselves free radicals and their reaction with the free radicals produced by the membranes reduces them to hydroxylamines, which can be readily detected from the diminished intensity of their EPR spectra. The reduction of nitroxides in biological systems has been studied in detail,[16–18] but these studies were mostly aimed at elucidating the site of radical production within cells and not to distinguish the different types of free radicals. Combining the two approaches, it was shown in this study that it is possible to differentiate between the oxygen radical species released by the membrane and to determine the kinetics and mechanisms involved.

EXPERIMENTAL

Plasma membrane isolation

Inbred line VA35 of maize (*Zea mays* L.) was used. The seeds, germinated 3 d on water, were grown for 14 d on a modified Knopp solution, the concentrations of NO_3^- and NH_4^+ being 10.9 and 7.2 mM. The plants were grown in a controlled environment under a 12 h light/dark regime at 22/18 °C and relative humidity of 70 %. The plasma membranes were isolated as described previously.[7] The cut roots were ground in cold grinding buffer (250 mM sucrose; 3 mM EDTA; 50 mM Tris-HCl, pH 7.5; 1 mM DTT; and 10 % w/v glycerol) with a chilled mortar and pestle. The microsomal fraction obtained from the homogenate (10 min at 12,000 g and 30 min at 100,000 g) was washed and suspended in phase buffer (5 mM K-phosphate buffer, pH 7.8; 330 mM sucrose; 3 mM KCl) in order to purify the plasma membranes by three steps of phase partitioning in a two-phase system (6.5 % (w/w) dextran T 500, 6.5 % (w/v) polyethylene glycol 335, 330 mM sucrose, 3 mM KCl, 5 mM K_2HPO_4, pH 7.8). The final upper phase was diluted (2 mM Tris-HCl, pH 7.5, 250 mM sucrose) and centrifuged. The resulting pellet, containing purified plasma membranes, was resuspended in the same buffer to give a final concentration of 3–4 mg of protein per ml and stored at –60 °C. The results presented were obtained from 4 independent membrane isolations.

Sample preparation

The spin-trap DMPO (5,5-dimethyl-1-pyrroline *N*-oxide) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). It was purified by a standard procedure.[19] DPI (diphenylene iodonium) was purchased from Sigma and NADH was purchased from BDH; only freshly prepared solution was used to avoid auto-oxidation. Authentic DMPO/OH and DMPO/OOH spin adducts were generated by hydroxyl and superoxide generating systems, respectively. The hydroxyl radicals were generated in a Fenton reaction system consisting of 0.5 mM H_2O_2 and 75 μM Fe^{2+}, the stock solution of $FeCl_2$ being prepurged with N_2 to ensure that only Fe^{2+} was present in the reaction system. Superoxide radicals were generated in a hypoxanthine/xanthine-oxidase system (HX/XO) con-

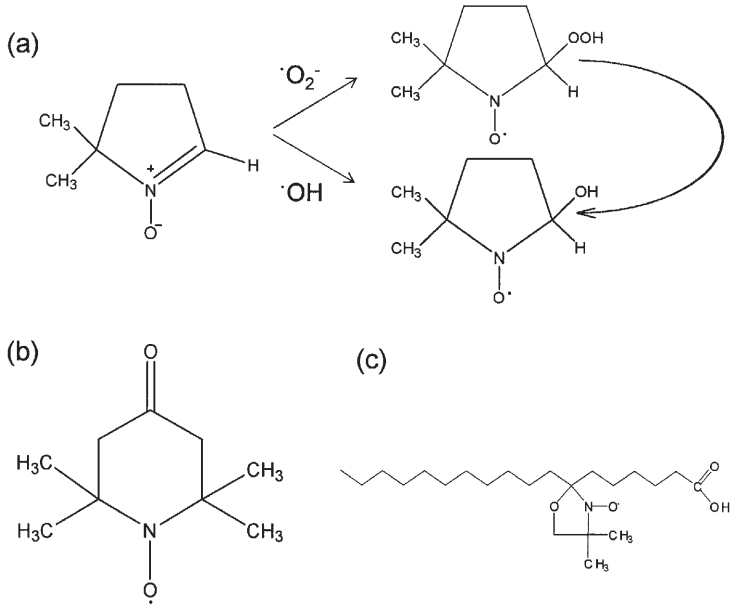

Fig. 1. (a) Formation of hydroxyl and superoxide adducts of DMPO. (b,c) Structures of Tempone and 7-DS, respectively.

sisting of 0.4 M hypoxanthine and 0.4 i.u/ml of xanthine oxidase (Sigma) dissolved in a 50 mM HEPES buffer.[20]

A standard membrane sample preparation consisted of 2.75 mg protein/ml of membranes, 3.3 mM NADH, in 50 mM HEPES buffer, pH 7.5. The concentration of DMPO was 240 mM in all experiments. All preparations for the spin-trapping experiments, except the Fenton system, included 1 mM of the chelating agent DETAPAC (diethylenetriamine pentaacetic acid) to ensure that trace metal impurities (especially iron) were removed. Nitroxide spin-probes (Fig. 1b, c) Tempone (2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-one) and 7-DS (2-(5-carboxypentyl)-2-undecyl-4,4-dimethyloxazolidine-3-oxyl) were purchased from Molecular Probes, (Junction City, OR, USA).

EPR spectroscopy

The EPR spectra were recorded at room temperature using a Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) using the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW; scan range, 200 G. The spectra were recorded and analyzed using EW software (Scientific Software). Spectral simulations were performed using WINEPR Simfonia (Bruker Analytische Messtechnik GmbH).

RESULTS AND DISCUSSION

Figure 2a shows the EPR specrtum of the DMPO adduct obtained in the radical generating Fenton system; it is typical for the DMPO/OH adduct.[21] Figure 2b shows that the half-life of the trapped DMPO/OH adduct is rather long, which is consistent with its reported half-life of about 2 hours.[22]

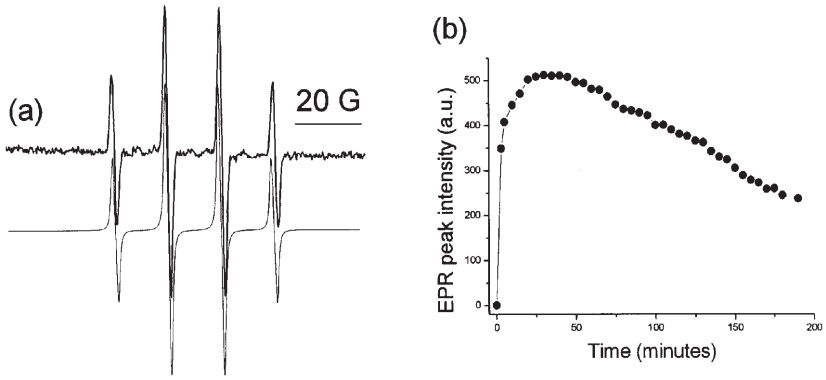

Fig. 2. (a) EPR spectrum of the DMPO/OH adduct obtained by performing the Fenton reaction in the presence of DMPO; reaction time = 60 min. The dashed line is a computer simulation of the spectrum using the following parameters: a_N = 14.9 gauss, a_H = 14.9 gauss.[21] (b) The time course of the DMPO/OH formation and disappearance in the Fenton system. The relative intensity was obtained by measuring the peak-to-peak line height of the adduct.

Figure 3 shows the EPR spectra of the DMPO spin adducts obtained in the HX/XO radical generating system. Although the HX/XO system is supposed to produce only $^{\cdot}O_2^-$ radicals, it is obvious that the EPR signal does not originate only from the DMPO/OOH adduct, but also from the DMPO/OH adduct. The signal of the DMPO/OOH adduct decreases with time whereas that of the DMPO/OH adduct increases, which, of course, does not imply that HX/XO system produces $^{\cdot}OH$ radicals, but shows the well known OOH/OH adduct transformation[15] depicted in Fig. 1a. It

is difficult to assess the kinetics of the transformation and the kinetics of natural decay of the individual species directly from the peak heights since the EPR spectra of the two adducts have a different number of lines. Double integration of EPR spectra can provide information on the total amount of trapped radicals, but the signal-to-noise ratio prevents an accurate deconvolution of the individual components of spectra. Therefore spectral simulation was used to assess the relative amounts of adducts and the curve in Fig. 3d shows that the transformation is rather rapid.

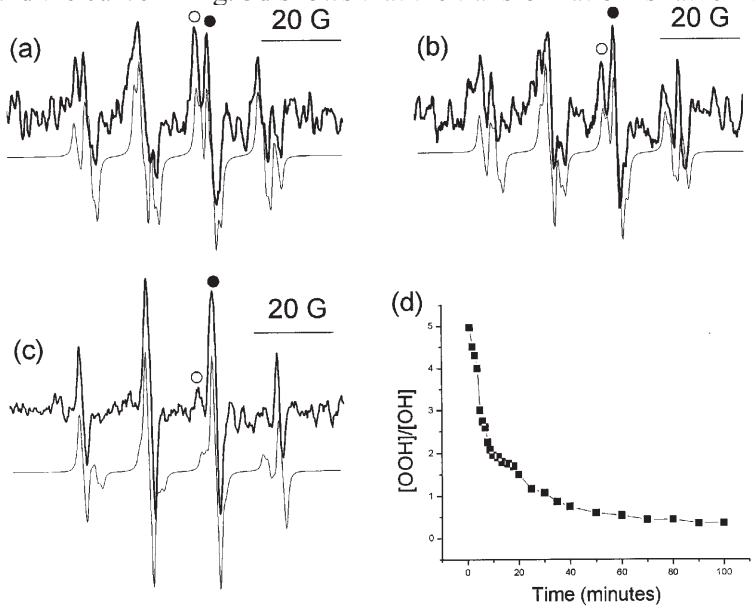

Fig. 3. EPR spectra of DMPO adducts generated in the HX/XO reaction system in the presence of DMPO at t = 2 min (a); t = 20 min (b); t = 35 min (c). The dashed lines are computer simulations of the spectra using a_N = 14.9 gauss, a_H = 14.9 gauss for the OH adduct and a_N = 13.83 gauss, $a_{H\beta}$ = 11.19, $a_{H\gamma}$ = 1.15 gauss for the OOH adduct[21] using the ratio of OOH/OH adducts equal to 78: 22 (a), 67: 33 (b), and 33: 67 (c). (d) Time course of the DMPO/OOH-to-DPMO/OH ratio produced in the HX/XO system. The relative intensity was obtained from spectral simulation of these adducts (see Figs. 3a–c).

The spectra of the DMPO adducts in the presence and absence of plasma membranes are show in Figure 4a and 4b, respectively. It is obvious that a negligible amount of free radicals is present in the system without plasma membranes (Fig. 4b). In the presence of plasma membranes, the spectrum includes lines from both the DMPO/OOH and DMPO/OH adducts. Their individual amounts and their ratio remained the same over a 60 min time period (Fig. 4c). The data in Fig. 4c imply some kind of steady state, however, the system is a non-steady state one and the analysis of this feature is fairly complex. The almost constant concentration of the DMPO/OOH adduct is a consequence of the equilibrium achieved by its production and disappearance through transformation into DMPO/OH and through its natural decay. The DMPO/OH adduct can arise from two independent sources: *i*)

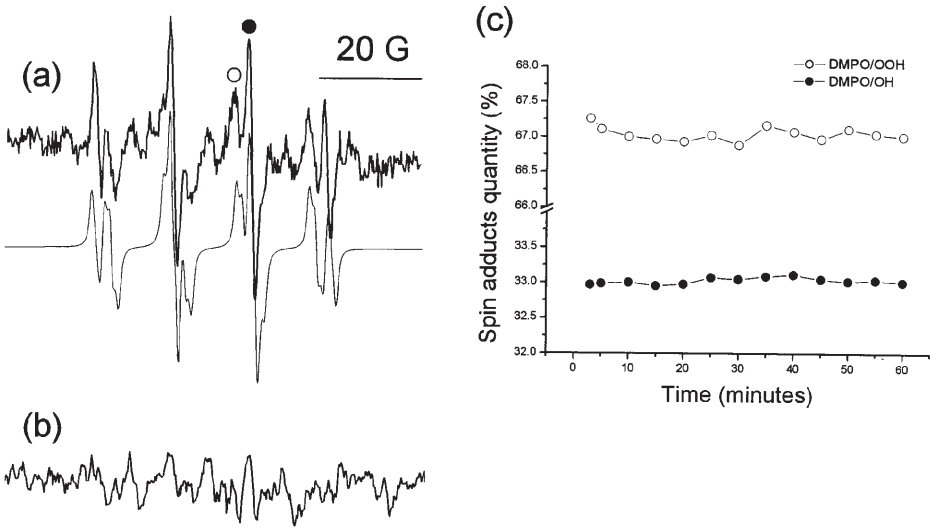

Fig. 4. EPR spectra of a standard plasma membrane preparation including 3.3 mM NADH and DMPO. (a) EPR spectrum of the trapped radicals obtained at t = 50 min. The dashed line is a computer simulation of the spectrum using the same parameters as in Figs. 1 and 2 and the ratio of OOH/OH adducts equals to 67:33. (b) The spectrum showing the trapped radicals under the same conditions as in (a), but in the absence of membranes. (c) Kinetics of DMPO/OOH and DMPO/OH adducts. The relative intensities were obtained from spectral simulation of the EPR spectra.

membrane production of $^{\cdot}OH$ radicals and *ii*) OOH/OH transformation. This should lead to a continuous increase in the amount of DMPO/OH adducts, since their natural decay is slow (Fig. 2b) compared to both processes leading to their production. The probable explanation for the absence of such an increase is the reduction of already trapped DMPO/OH adducts by $^{\cdot}O_2^-$ radicals.[22]

The results shown in Fig. 4 clearly demonstrate that it is feasible to use DMPO for detecting and distinguishing between the oxygen free radicals produced by plasma membranes. There is little doubt that the energized membranes are capable of producing $^{\cdot}O_2^-$ radicals,[3,7,11] but the problem remains whether there is an independent production of $^{\cdot}OH$ radicals. Our results would argue in favor of such an assumption; however it is virtually impossible to analyze the mechanisms of the production of radicals using the data shown in Fig. 4. For example, the analysis of the production of DMPO/OH adducts would require knowledge of the rate constants of at least three independent reactions. In addition, the detection of DMPO/OH adducts, apart from the OOH/OH transformation, does not necessarily imply authentic production of $^{\cdot}OH$ radicals by the plasma membranes, bacause significant levels of $^{\cdot}OH$ could be formed through the reaction of H_2O_2 with Fe^{2+}, which is generated by the reduction of Fe^{3+} by $^{\cdot}O_2^-$.[2,12] For this reason, in almost all studies, specific inhibitors and/or scavengers were employed to clarify the mechanism of free radical production.[3,4,7,12] However, the application of inhibitors further diminishes the sig-

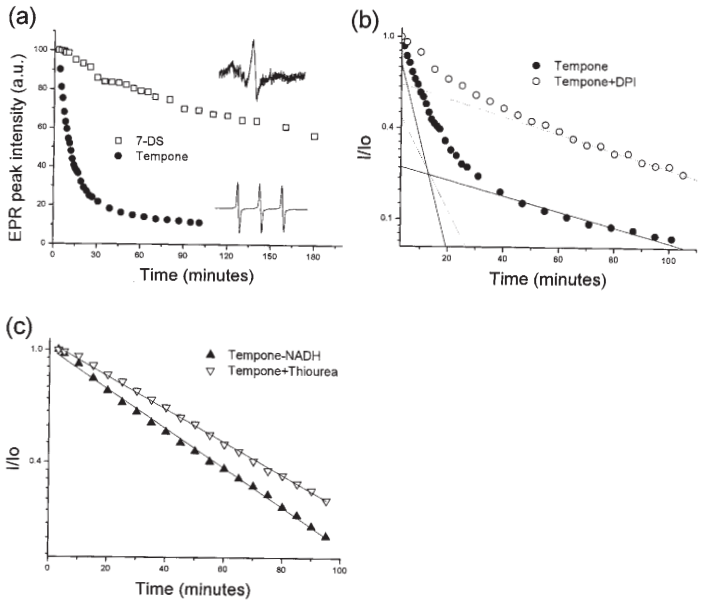

Fig. 5. Reduction of nitroxide spin labels in the presence of isolated membranes. (a) Reduction of Tempone and 7-DS nitroxides (37 μM) in a standard membrane preparation (7-DS-to-membrane lipid ratio: 1:100). (b) Kinetics of the reduction of Tempone in a standard membrane preparation and in the presence of 0.1 mM of DPI. The straight lines represent the deconvolution of the overall kinetics into two exponential components. (c) Kinetics of the reduction of Tempone in a standard membrane preparation in the presence of thiourea (1 mM) and in the absence of NADH.

nal-to-noise of the EPR spectra of trapped radicals, thus increasing the difficulties in spectral simulation and the hinderance of OOH/OH adduct transformation is not removed. Novel spin-traps DEPMPO and EMPO, the adducts of which are more stabile and have a slower OOH/OH adduct transformation[20,23] can be used instead of DMPO (work in progress) and alleviate problems associated with the quantitative assessment of trapped radicals. However, their adducts also are not immune to reduction by membrane produced free radicals and still represent a rather complex system for investigating of the production of free radical species.

These results imply that an additional approach has to be used. Spin probes appear to be capable of complementing the above-presented spin trapping studies. Spin probes are themselves free radicals, which can be reduced to hydroxylamines by membrane produced free radicals. Such an approach has several advantages: *i*) spin-probes can be reduced by both $^{\cdot}OH$ and $^{\cdot}O_2^-$,[24] but there are no processes analogous to OOH/OH adduct transformation, *ii*) spin-probes exhibit an intense EPR signal allowing quantitative analysis due to the high signal-to-noise ratio, *iii*) localization of production of free radicals is possible, since spin-probes located in or excluded from the membranes can be used.[16,17]

Figure 5a shows the very slow reduction of membrane-residing 7-DS relative

to the reduction of Tempone, which is known to reside almost exclusively outside the membranes. These results suggest that free radical are readily released from the membrane inducing rapid reduction of Tempone, and that only a minor portion of them remains in the membrane long enough to reduce 7-DS. Tempone can readily cross the membrane, however, the kinetics of reduction shown in Fig. 5a implies that the amount of Tempone reduced within the membrane is negligible as compared to the rate of reduction caused by membrane released radicals.

Figure 5b shows the complex kinetics of Tempone reduction, which can be deconvoluted into two componenets using double exponential decay. This implies that plasma membranes have a complex system of simultaneous production of both the superoxide and hydroxyl radical species. DPI decreases significantly the fast component (Table I) indicating that DPI is capable of inhibiting at least one of the sources of ROS production. In the literature, it is disputed as to whether DPI inhibits only the superoxide synthase in the plasma membranes, or whether it is also capable of inhibiting a peroxidatic mechanism of ROS production at higher DPI concentrations,[7,25] such as those used in the present experiments. Here, it is demonstrated that two of the possible sources of ROS can be kinetically differentiated with specific kinetic constants, which indicates that DPI primarily inhibits one ROS production mechanism, presumably by inhibiting superoxide synthase mediated production of $^{\cdot}O_2^-$ radicals.[10]

TABLE I. Rate constants of Tempone reduction in plasma membrane system

Sample	Fast component k/s^{-1}	Slow component k/s^{-1}
Membranes with NADH	0.122	0.0085
Membranes with NADH and DPI	0.065	0.0096
Membranes without NADH		0.018
Membranes with NADH and thiourea		0.015

Figure 5c shows that the decay of the Tempone signal in the presence of the hydroxyl scavenging reagent thiourea, as well as in the absence of NADH in the system follows a first order decay, indicating the presence of a single radical in both cases. This validates our experimental approach, showing that the reduction of spin-probes can be used in studies of the production of free radicals in biological systems. In de-energized membranes (absence of NADH), species capable of reducing nitroxides are still present, showing that such membranes still produce ROS (most probably only the $^{\cdot}OH$ radical). The kinetic constants obtained (Table I) are not the same as those of the slow component obtained with standard energized membranes, as well as in the presence of $^{\cdot}OH$ quencher (thiourea), arguing in favour of the existence of a number of different mechanisms of the production of ROS in the plasma membranes producing $^{\cdot}O_2^-$ and $^{\cdot}OH$. Thus, the selecitve inhibition of one of them does not completely stop the production of such radical species.

CONCLUSIONS

The spin-trap technique, using DMPO enabled the detection of two oxygen radical species ($^{\cdot}O_2^-$ and $^{\cdot}OH$) produced by plant plasma membranes. However, due to the low signal-to-noise ratio and OOH/OH adduct transformation, inhibitors and/or free radical scavengers could not be employed and the mechanisms of production of the radicals were impossible to analyse. Reduction of spin-probes Tempone and 7-DS was used to complement previous studies. Using these spin-probes, it was shown that oxygen free radicals are quickly released from the membranes or more probably they are produced at the surface of the membranes. The complex kinetics of Tempone reduction showed that plasma membranes have a system for the simultaneous production of both superoxide and hydroxyl radicals. Also, the combination of spin-probe reduction with the application of various inhibitors showed that two independent sets of mechanisms are probably responsible for the production of these two radical species. While the production of $^{\cdot}O_2^-$ radicals has previously been shown to be possible by the plasma membranes in more or less appropriate experimental procedures, $^{\cdot}OH$ production has not been previously demonstrated. The fact that it is demonstrated here shows that the combination of the spin-probe and spin-trap technique is very effective for the study of free radical species produced in biological systems.

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ИЗВОД

ПРОУЧАВАЊЕ СЛОБОДНИХ РАДИКАЛА КОЈЕ ПРОИЗВОДЕ ПЛАЗМА МЕМБРАНЕ БИЉАКА МЕТОДОМ СПИНСКИХ ХВАТАЛА И ПРОБА

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До сада је показано да плазма мембране биљака производе супероксидне радикале, док се сматрало да се производња хидроксилних радикала одиграва само у ћелијском зиду. У овоме раду, употребом EPR спектроскопије методом спинских хватала и проба, показано је да плазма мембране производе и супероксидне и хидроксилне радикале и то путем кинетички различитих механизама. Наши резултати показују да се супероксидни и хидроксилни радикали могу детектовати помоћу DMPO спинског хватала и да механизми и локација производње радикала може бити одређена помоћу редукције спинских проба Темпона и 7-DS. Такође смо показали да је механизам производње кисеоничних радикалских врста NADH зависан, а инхибиран од стране DPI. Кинетика редукције Темпона комбинована са уклањачима слободних радикала или у одсуству NADH указује да се хидроксилни радикали производе независним механизмом у односу на производњу супероксида. Дакле, комбинована употреба техника спинских хватала и спинских проба показала се као веома корисна за праћење слободних радикала у биолошким системима.

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