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Short communication

Aluminum stress increases carbon-centered radicals in soybean roots

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ABSTRACT

The formation of radical species was examined in roots of soybean seedlings exposed to aluminum (Al). Electron spin resonance (ESR) spectra of root homogenates with the spin-trapping reagent 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) indicated the presence of carbon-centered radicals in plants not exposed to Al. Plants exposed to 50 μM Al showed a similar spectrum, with increased signal intensity. These radicals were likely produced through a H-atom abstraction reaction by hydroxyl ($^{\bullet}OH$) radicals, the synthesis of which was initiated by the formation of superoxide ($O_2^{\bullet-}$) anions. The increased production of the carbon-centered radicals may be responsible for the lipid peroxidation in Al-treated roots.

Introduction

Aluminum (Al) toxicity limits the growth of higher plants in acidic mineral soils. The earliest morphological symptom of Al toxicity is reduced root elongation, resulting in a poor root system, followed by inhibition of shoot growth, due to limited nutrient uptake (Marschner, 1995).

Several lines of evidence indicate that ionized Al induces oxidative stress in plant roots, although it does not catalyze redox reactions, because of the stable trivalent state of the ion. Enhanced lipid peroxidation was found in the root tips of soybean plants grown in Al-containing medium (Cakmak and Horst, 1991). Genes related to oxidative stress are induced in Al-treated roots (Richards et al., 1998), and the amelioration of Al toxicity has been observed in transgenic *Arabidopsis thaliana* over-expressing oxidative stress-related genes (Ezaki et al., 2000). Furthermore, the generation of reactive oxygen species (ROS) has been detected in Al-exposed roots, but the reasons for this production have not been elucidated. Yamamoto et al. (2002) suggested that reduced electron flow in the mitochondrial membrane, due to Al, was responsible for enhanced ROS production (presumably superoxide [$O_2^{\bullet-}$] anions). Although several lines of evidence have shown that Al induces $O_2^{\bullet-}$ anion production and lipid peroxidation in plant roots, the mechanisms that connect these findings are not understood. We found that carbon-centered radical species formed in Al-treated plant roots using the electron spin resonance (ESR) spin-trapping method and discuss the contribution of these radicals in lipid peroxidation.

Abbreviations: DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide; ESR, electron spin resonance; ROS, reactive oxygen species.

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Materials and methods

Chemicals

5-Diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was purchased from Tokyo Chemical Industry (Tokyo, Japan).

Plant growth

Seeds of soybean (*Glycine max* [L.] Merr.) were soaked for 2 days in distilled water under dark conditions and germinated on absorbent cotton. Seedlings were grown on an aerating incubation medium containing 1 mM $CaCl_2$. The roots were exposed to 50 μM $AlCl_3$ to examine the effects of Al on radical production. The pH of the solutions was adjusted to 4.4 with 0.1 M HCl and 0.1 M NaOH.

Staining with Schiff's reagent

Soybean roots exposed to 50 μM $AlCl_3$ were washed with distilled water and soaked in Schiff's reagent for 30 min. The roots were then rinsed three times with 0.5% (w/v) Na_2SO_3 in 0.05 M HCl for 3 min each. The stained roots were observed under an SZX9 stereomicroscope (Olympus, Tokyo, Japan).

Radical trapping with DEPMPO

The radicals present in the soybean roots were trapped with DEPMPO after homogenization. The roots of soybean seedlings incubated in the presence of $AlCl_3$ were dissected 40–50 mm from the tip. Two or three segments (approximately 0.15 g total wet weight) were homogenized with a tapered tissue grinder in 100 μL of 200 mM phosphate buffer (pH 7.4) for 2 min. Immediately after

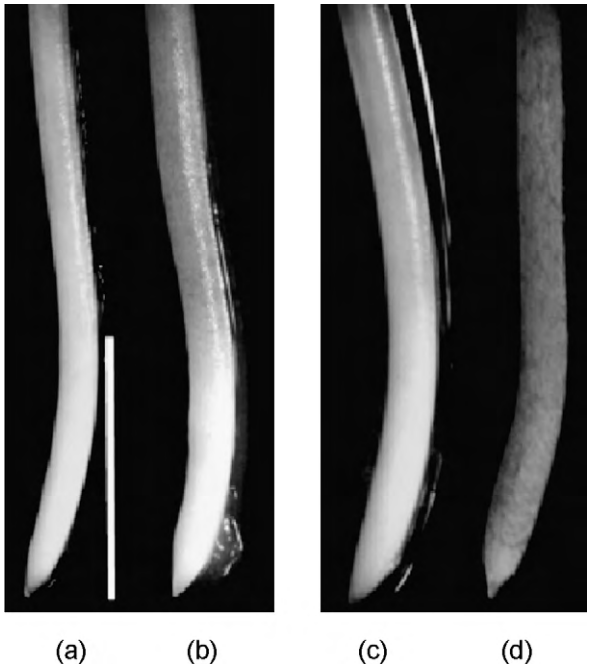

Fig. 1. Lipid peroxidation as observed using Schiff's reagent. Soybean seedlings exposed to 50 μM Al for 6 (b) or 24 h (d) were stained with Schiff's reagent. The roots of plants grown without Al for 6 (a) or 24 h (c) were similarly stained. Scale bar indicates 5 mm.

homogenization, the spin-trapping reagent DEPMPO (9 mM) was added to the homogenate, and an aliquot was sampled using a 100-μL capillary suction tube (Microcaps, Drummond Scientific Co., Broomall, PA, USA), after which one end was sealed with XX-VCS35 paste (Terumo, Tokyo, Japan). The capillary tube was then placed in an ESR tube.

A Fenton reaction system was used to generate $^{\bullet}$OH. Hydrogen peroxide (H_2O_2; 10 mM) was mixed with $FeSO_4$ (10 mM), and the $^{\bullet}$OH produced was trapped with DEPMPO (9 mM). Just after trapping, ESR spectra were produced in a similar manner to that for the root homogenates.

Table 1
Hyperfine coupling constants of DEPMPO adducts.

Radical	Hyperfine coupling constants (mT)		
	a^H	a^N	a^P
$^{\bullet}$OH	1.36	1.38	4.76
Unknown	2.15	1.48	4.60

ESR measurement

ESR spectra were recorded at room temperature using a JES-FA 100 spectrometer (JEOL, Akishima, Japan) with the X-band adjusted to the following settings: modulation frequency, 100 kHz; modulation amplitude, 0.2 mT; scanning field, 320 ± 15 mT; and microwave power, 0.998 mW. Spectral simulations were performed using IsoSimu/FA ver. 2.1.0 (JEOL).

Results and discussion

In the roots of soybean plants exposed to 50 μM Al and treated with Schiff's reagent, staining was observed more than 5 mm from the root tip after 6 h, and the stain continued to spread throughout the roots for 24 h (Fig. 1). This is in contrast to the control roots, which were only faintly stained by Schiff's reagent. As Schiff's reagent reacts with malondialdehyde to form a colorimetric product (Dahle et al., 1962), staining indicates lipid peroxidation because malonedialdehyde is a degradation product of lipid peroxidation. Thus, Al-induced oxidative stress was detectable as early as 6 h after treatment.

The induction of oxidative stress by Al in plant roots has been widely reported, although the metal itself does not catalyze redox reactions, since it occurs only as a trivalent cation. The enhancement of lipid peroxidation was evident in root tips of soybean grown in Al-containing medium (Cakmak and Horst, 1991), and Al-induced lipid peroxidation in pea roots was identified as an early injury (Yamamoto et al., 2001). Furthermore, Al-induced oxidative stress can be indirectly recognized as the emergence of defense mechanisms against stress. Al-exposed soybean roots showed increased activity of superoxide dismutase and peroxidase in crude extracts of the roots (Cakmak and Horst, 1991). Of the

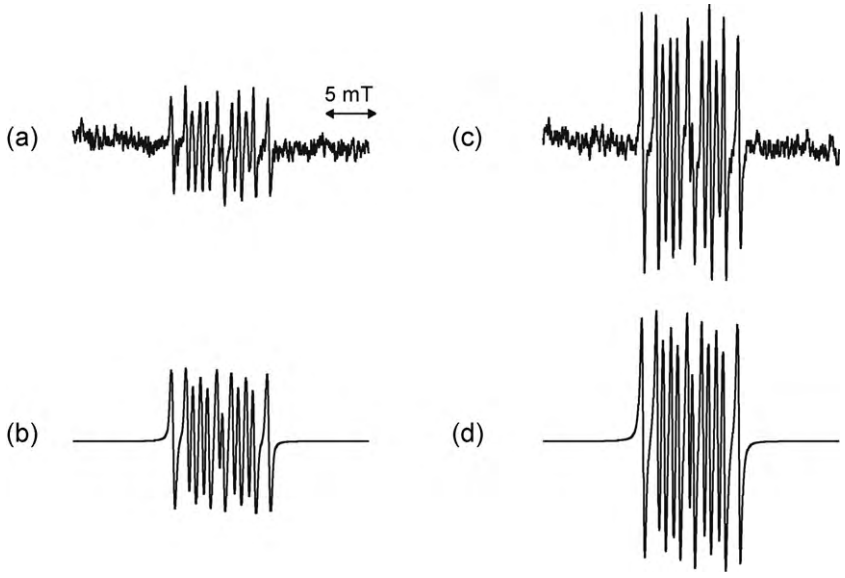

Fig. 2. ESR spectra of root homogenates with or without Al exposure. Root homogenates with (c) or without (a) exposure to 50 μM Al for 24 h had 9 mM DEPMPO added and were then subjected to ESR measurements. Spectral simulation was undertaken with hyperfine coupling constants of a^N = 1.48, a^H = 2.15, and a^P = 4.60 mT for root homogenates prepared from Al-free (b) or Al-exposed (d) plants.