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Hydrogen-rich water alleviates aluminum-induced inhibition of root elongation in alfalfa via decreasing nitric oxide production

Meng Chen [a], Weiti Cui [a], Kaikai Zhu [a], Yanjie Xie [a], Chunhua Zhang [b], Wenbiao Shen [a,*]

[a] College of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China
[b] Laboratory Center of Life Sciences, Nanjing Agricultural University, Nanjing 210095, China

HIGHLIGHTS

- HRW can alleviate Al-induced alfalfa seedling growth inhibition.
- Al uptake in alfalfa seedling roots was decreased by HRW.
- HRW improves Al-induced inhibition of root elongation by reducing NO production.

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ABSTRACT

One of the earliest and distinct symptoms of aluminum (Al) toxicity is the inhibition of root elongation. Although hydrogen gas (H_2) is recently described as an important bio-regulator in plants, whether and how H_2 regulates Al-induced inhibition of root elongation is largely unknown. To address these gaps, hydrogen-rich water (HRW) was used to investigate a physiological role of H_2 and its possible molecular mechanism. Individual or simultaneous (in particular) exposure of alfalfa seedlings to Al, or a fresh but not old nitric oxide (NO)-releasing compound sodium nitroprusside (SNP), not only increased NO production, but also led to a significant inhibition of root elongation. Above responses were differentially alleviated by pretreatment with 50% saturation of HRW. The addition of HRW also alleviated the appearance of Al toxicity symptoms, including the improvement of seedling growth and less accumulation of Al. Subsequent results revealed that the removal of NO by the NO scavenger, similar to HRW, could decrease NO production and alleviate Al- or SNP-induced inhibition of root growth. Thus, we proposed that HRW alleviated Al-induced inhibition of alfalfa root elongation by decreasing NO production. Such findings may be applicable to enhance crop yield and improve stress tolerance.

1. Introduction

An overwhelming number of studies suggest that the most easily recognized symptom of aluminum (Al) toxicity is the inhibition of root growth, being regarded as a widely accepted measurement of Al toxicity in plant kingdoms [1,2]. Evidence further suggests that hormones, and the second messengers or modulators, such as ethylene and auxin [3–5], salicylic acid [6], carbon monoxide (CO) [7], and nitric oxide (NO) [8–13], are involved in Al-induced inhibition of root elongation. Among these, corresponding physiological roles and molecular mechanism of NO in plant tolerance against Al stress remain controversial. For example, previous results showed that exogenous NO-releasing compound (sodium nitroprusside, SNP) could aggravate Al-induced inhibition of root elongation in rice bean plants by affecting cell wall and plasma membrane properties [14]. However, these results are indeed contrast with the positive role of NO observed in *Hibiscus moscheutos* [9], rice [12,15], rye and wheat [13]. Therefore, the research on the role of NO in Al stress is just beginning [16].

It is well accepted that the complex responses regulated by above hormones, signaling molecules and regulators are most likely to be achieved by a combinational signaling process [2,17,18]. Additionally, whether there are some other novel singling molecule(s) involved in the regulation of root elongation upon Al stress remains to be deciphered.

A number of clinical trials have recently reported that hydrogen gas (H_2) is a therapeutic antioxidant and signaling molecule [19–22]. The production of H_2 by bacteria, green algae and higher

Abbreviations: Al, aluminum; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-FM DA, 4-amino-5-methylamino-2′7′-difluorofluorescein diacetate; EPR, electron paramagnetic resonance; H_2, hydrogen gas; HRW, hydrogen-rich water; ICP-OES, inductively coupled plasma-optical emission spectrometer; NO, nitric oxide; NOS, nitric oxide synthase; NR, nitrate reductase; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ROS, reactive oxygen species; SNP, sodium nitroprusside.

* Corresponding author. Tel.: +86 25 84399032; fax: +86 25 84396542.
E-mail addresses: wbshenh@njau.edu.cn, 13851857655@139.com (W. Shen).

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plants has been reported previously [23,24]. Similar to the beneficial roles in animals [25–28], H_2 has been emerged as an important bio-regulator in plants that was increased under abiotic stresses and modulates several physiological processes, including the regulation of plant responses against salinity and drought stress in rice or Arabidopsis [29–31], and paraquat exposure in alfalfa [32]. It has also been suggested to be involved in the response to cadmium (Cd) stress. For example, Cui et al. [33] reported that exogenously applied hydrogen-rich water (HRW), which was previously used to characterize H_2 physiological roles and possible signaling transduction pathway in animals [26,34,35], ameliorated Cd toxicity and its-induced oxidative damage in alfalfa seedling roots.

However, to the best of our knowledge, the impact of H_2 on Al toxicity has never been addressed in direct experiments. Therefore, the aim of this study was test whether and how H_2 modulates Al-induced inhibition of root elongation in plants. The results could extend our knowledge of H_2 in plants and is also vital for both fundamental and applied plant biology.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless stated otherwise. The chemicals used for treatments were: sodium nitroprusside (SNP, 100 μM; a well-known NO-releasing compound) [36]; 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO, 20 μM; a scavenger of NO) [37,38]; tungstate (100 μM; a NR inhibitor); 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO, 20 μM; another scavenger of NO) [38] purchased from TCI company; $AlCl_3$ was provided from Shanghai Medical Instrument, Co., Ltd., China National Medicine (Group), Shanghai, China. The concentrations used in this study were determined in pilot experiments from which maximal induced responses were obtained.

Additionally, the old SNP solution was used as the negative control by maintaining the separated solution of SNP (100 μM) for at least 10 d in the light in a specific open tube to eliminate the entire NO [39,40].

2.2. Preparation of hydrogen-rich water (HRW)

Purified H_2 gas (99.99%, v/v) generated from a hydrogen gas generator (SHC-300, Saikesaisi Hydrogen Energy Co., Ltd., China) was bubbled into 1 L 0.5 mM $CaCl_2$ (pH 4.5) solution at a rate of 150 mL min^{-1} for 30 min. Then, the corresponding hydrogen-rich water (HRW) was rapidly diluted to the required saturations (1, 10 and 50%, [v/v]). In our experimental conditions, the H_2 concentration in freshly prepared HRW analyzed by gas chromatography [41] was 0.22 mM, and maintained at a relative constant level in 25 °C for at least 12 h.

2.3. Plant materials, growth condition and treatments, and determination of seedling growth

Commercially available alfalfa (*Medicago sativa* L. cv. Biaogan) seeds were surface-sterilized with 5% NaClO for 10 min, rinsed extensively in distilled water and germinated for 2 d at 25 °C in the darkness. Uniform seedlings were then chosen and transferred to the plastic chambers and cultured in nutrient medium (quarter-strength Hoagland's solution, pH 4.5) for 2 d and 0.5 mM $CaCl_2$ solution (pH 4.5) for another 1 d, in the illuminating incubator at 25 ± 1 °C, with a light intensity of 200 μmol m^{-2} s^{-1} and 14 h photoperiod.

Afterwards, the seedlings were incubated with 0.5 mM $CaCl_2$ (pH 4.5) solution with or without either 100 μM old or fresh SNP, 20 μM PTIO, 20 μM cPTIO, 100 μM tungstate, the indicated saturations of HRW, or varying concentrations of $AlCl_3$ alone or their combinations for 12 h or the indicated time points and followed by another 24 h or the indicated time points of incubation in 150 μM $AlCl_3$. The control solution (Con) contained 0.5 mM $CaCl_2$, pH 4.5. In our test, the pH for both nutrient medium and treatment solutions was adjusted to 4.5 by using HCl, and nutrient medium and $CaCl_2$ solution were renewed each day to maintain identical concentrations. After different treatments, alfalfa seedlings were photographed. Also, the root and shoot parts of seedlings were harvested, and fresh weight (FW) and dry weight (DW) were determined. Root elongation was measured with a ruler at the indicated times as well ($n > 40$).

2.4. Hematoxylin staining

Hematoxylin staining was prepared as previously described by Polle et al. [42]. The staining solution consists of 0.2% hematoxylin (Sigma) and 0.02% $NaIO_3$, dissolution of which was aided by adding a drop of 0.1N NaOH. After various treatments, the seedling roots were rinsed with distilled water and placed in approximately 50 mL of aerated hematoxylin staining solution for 50 min at room temperature. The roots were then placed in aerated distilled water for 10 min to remove excess stain, then observed under a light microscope (model Stemi 2000-C; Carl Zeiss, Germany) and photographed on color film (Powershot A620, Canon Photo Film, Japan).

2.5. Determination of Al content

Fresh seedlings were washed twice times with EDTA-Na_2 solution and rinsed briefly in de-ionized water after treatments. Afterwards, root tissues were cut into smaller pieces and oven-dried at 60 °C, then digested with HNO_3 using a Microwave Digestion System (Milestone Ethos T, Italy). The Al contents were determined using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Perkin Elmer Optima 2100DV).

2.6. NO detection by electron paramagnetic resonance (EPR)

After various treatments, 0.1 g of root samples was homogenized and then incubated in 0.3 mL of buffered solution (50 mM HEPES, 1 mM dithiothreitol, and 1 mM $MgCl_2$, pH 7.6) at 37 °C for 2 min. After centrifugation at 12,000 × *g* 4 °C for 2 min, the supernatant was added to 0.3 mL of freshly made $Fe^{2+}(DETC)_2$ solution (2 M $Na_2S_2O_4$, 3.3 mM DETC, 3.3 mM $FeSO_4$, 33 g L^{-1} bovine serum albumin) in low light conditions [43]. After incubation at room temperature for 2 min, 0.2 mL of ethyl acetate was added to the mixture, shaken for 5 min and centrifuged at 12,000 × *g* 4 °C for 5 min. The organic solvent layer was used to determine NO by using a Bruker A300 spectrometer (Bruker Instrument, Germany) under the following conditions: room temperature; microwave frequency, 9.85 GHz; microwave power, 63.49 mW; modulation frequency, 100 kHz.

2.7. Confocal determination of endogenous NO production

Using a fairly specific NO fluorescent probe 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) [44,45], the endogenous NO level of root tissues was detected by a TCS-SP2 confocal laser scanning microscopy (Leica Lasertechnik GmbH, Heidelberg, Germany; excitation at 488 nm, emission at 500–530 nm). Roots were collected at the indicated time points and loaded with 5 μM DAF-FM DA in 20 mM HEPES/NaOH buffer (pH 7.5) for 30 min, then washed three times for 5 min each and