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# Exogenous foliar application of fulvic acid alleviate cadmium toxicity in lettuce (*Lactuca sativa* L.)

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ABSTRACT

It was reported that fulvic acid (FA) has a positive effect on enhancing plant tolerance to various environmental stresses, including salinity stress and drought stress and so on. However, there is little study regarding the effects of FA on plants in response to heavy metal stress. Hence, the objective of this study was to investigate the potential effects of fulvic acid (FA) on cadmium (Cd) toxicity alleviation in lettuce seedlings. Our results showed that application of 0.5 g/L FA significantly mitigate Cd-induced toxic symptoms in lettuce seedlings. Cd stress triggered plant growth inhibition, photosynthetic pigment reduction, destruction of the photosynthesis apparatus, reactive oxygen species (ROS) accumulation, and nutrient elemental imbalance. We observed that FA promoted the growth in lettuce under Cd stress, mainly reflected in those alterations that the increase of biomass, chlorophyll content and photosynthesis capacity and reduction of the Cd content and lipid peroxidation in plant tissue. Foliar spraying of FA significantly alleviated these detrimental symptoms and facilitated nutrient element translocation from root to shoot, particularly the absorption of elements involved in photosynthesis, including iron (Fe), zinc (Zn), and manganese (Mn). In summary, foliar application of FA conferred Cd toxicity tolerance to lettuce by increasing ROS-scavenging capacity, inhibiting Cd uptake and the transport of elemental nutrients to shoots, which in turn protected the photosynthetic apparatus and promoted plant growth.

## 1. Introduction

In recent years, heavy metal pollution has attracted increasing attention worldwide, due to its release into the environment through anthropogenic activities such as mining, waste water for irrigation, sewage sludge application, excessive application of phosphate fertilizers, herbicides and pesticides to farmland, and vehicular and industrial activities (Rizwan et al., 2017). Among heavy metals, cadmium (Cd) is a highly toxic, non-essential element for humans, animals, and plants. It is easily taken up by plant roots and transported to shoots (Saidi et al., 2014), Cd toxicity causes various morphological, physiological, biochemical, and ultrastructural changes in plants. Its main symptoms manifested growth inhibition of shoots and roots, leaf chlorosis, browning of root tips and so on (Zhang et al., 2015). A large amount of toxic effects of Cd on metabolism have been reported, such as imbalance of nutritional elements, reduction of chlorophyll biosynthesis, enzymes inhibition, disruption of basic metabolic functioning, including photosynthesis, respiration, and nucleic acid production (Erdal and Turk, 2016; Ali et al., 2013; Santos et al., 2018). Moreover, the burst out of reactive oxygen in plants with Cd stress, which caused oxidative stress and responsible for damage to mitochondria and chloroplast ultrastructure, as well as cell membrane structure (Ali et al., 2013). Various methods have been reported to reduce Cd accumulation in plants, including exogenous application of materials such as plant growth regulators, elemental nutrients, and amino acids; the application of these substances could be an effective strategy to reduce Cd uptake by vegetables destined for human consumption (Parvaiz et al., 2016; Wang et al., 2016; Meng et al., 2009). However, few studies have been conducted on the effects of foliar application on plants exposed to Cd.

Humic substances are natural organic compounds comprising organic acids derived from the decomposition of plant and animal residues under the action of microorganisms and geochemistry (Morales et al., 2012). Humic substance can be divided into humin, humic acid, and fulvic acid (FA) according to its solubility at different pH (Suh et al., 2014). Among these substances, fulvic acid (FA) has a relatively low molecular weight and contains a high amount of oxygen-rich and carbon-poor functional groups (Weng et al., 2006). FA confers benefits to plants by enhancing drought resistance, improving nutrient uptake, stabilizing soil pH, and reducing fertilizer leaching (Suh et al., 2014). Adani et al. (1998) revealed that soil application of humic acid could stimulate the growth of tomato plants grown by hydroponics, and

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promote the nutrients uptake, especially Fe. Similar results were found that total yield was raised in tomato by foliar and soil application of humic acid (Yildirim, 2007). In many cases, humic acid is regarded as a plant growth regulator, which has the similar effect with auxins, but we do not confirm whether it contain auxin-like substances or not (Nardi et al., 2002). The explicit physiological mechanism that fulvic acid (FA) enhanced plant resistance against abiotic stresses is seldom known. Current report has exhibited that soil and foliar applied FA has beneficial effects on seedling growth, seed germination, and root weight of wheat plants (Katkat et al., 2009). Tang et al. (2014) reported that FA could also be applied to remove heavy metals from aqueous media. Ali et al. (2015) explored the effects of foliar FA application on wheat plants exposed to chromium (Cr) and found that FA alleviated Cr toxicity by increasing photosynthetic pigments, reducing Cr uptake, and improving antioxidant activities in wheat. However, few studies have examined the effect of FA application to soil to enhance Cd stress tolerance in lettuce. (Haghighi et al., 2013, Horuz et al., 2015). Therefore, this study was conducted to illuminate the effects of foliar application of fulvic acid on lettuce under Cd stress.

Lettuce (*Lactuca sativa* L.) is a common leafy vegetable that is cultivated commercially and in home gardens worldwide (Konstantopoulou et al., 2010; Matraszek et al., 2016). Lettuce is rich in a variety of vitamins, dietary fibers, low in calories as well as a source of carotenoids, which is beneficial for human health. In addition, it contains many nutrient minerals, such as Ca, Fe, Zn, Se etc., which is essential for human body to maintain normal function by serving as electrolytes (Matraszek et al., 2016). Lettuce is valued for its high nutritional value and is often used in salads as a fresh vegetable. It has a high capacity for Cd accumulation from soil without showing visible symptoms of metal toxicity, which poses a potential risk to human health (Cobb et al., 2000).

In this study, we use the method of foliar spraying to investigate the effects of FA on lettuce exposed to Cd and try to elucidate its possible mitigation mechanism for two reasons. Firstly, FA can chelate directly with Cd in solution (Tang et al., 2014). Meanwhile, foliar spraying is easy to achieve cultivation in the field. The objectives of this study were to examine (a) whether FA supply ameliorates Cd toxicity in lettuce, (b) the optimal concentration of FA to alleviate Cd toxicity, and (c) the possible mechanism of Cd toxicity defense by FA in lettuce.

## 2. Materials and methods

### 2.1. Plant material and growth conditions

Lettuce (*Lactuca sativa* L.) seeds, acquired from Jiangsu Agricultural Institutes (Nanjing Province, China) were germinated in a tray filled with vermiculite at 25 ℃ in a climate chamber. Uniformly sized seedlings were selected 5 days later and transplanted into plastic cups (three plants per cup) containing 400 mL a half-strength Hoagland nutrient solution (pH 6.0). The selected seedlings were incubated in the climate chamber at a temperature of 25/20 ℃ (day/night), with a light intensity of 600 $\mu mol\ m^{-2}\ s^{-1}$ and a 12-h photoperiod.

### 2.2. Experimental design

Firstly, we have studied which FA dose works better to improve Cd damage by these indicators: the plant growth, chlorophyll content, P.I., photosynthesis, electrolyte leakage and reduction of Cd accumulation. After 10 days of growth, the seedlings in three leaf stage were treated with 20 μM Cd ($CdCl_2 \cdot 2.5\ H_2O$) in hydroponics, Meanwhile, the seedlings exposed to Cd were sprayed with 0, 0.1, 0.3, 0.5, 1.0, 2.0 g/L fulvic acid (FA) in the morning every day. FA (> 90%) was purchased from Bio Aladdin (Shanghai, china). Seedling without Cd and fulvic acid treatment were used as the control (Con), All treatments we described as (1) Con, (2) Cd, (3) Cd + 0.1FA, (4) Cd + 0.3FA, (5) Cd + 0.5FA, (6) Cd + 1.0FA, (7) Cd + 2.0FA.

Secondly, we have chosen an optimum FA concentration (0.5 g/L) and seen how it influences in Chlorophyll a fluorescence, photosynthetic pigments and gas exchange parameters, oxidative stress, Antioxidant enzymes and nutrient element uptake. Based on preliminary trials, four treatments respectively were Control, 20 μM Cd, Control + FA, Cd + FA. The solutions were renewed every other day. Each treatment was designed with three replicate randomly, and each replicate contain three plants. After treatment 2 weeks later, seedlings were harvested to make the further analysis.

### 2.3. Plant biomass measurements and chlorophyll content measurements

Fifteen days after treatment, lettuce seedlings were harvested and photographed. After that, samples were separated into leaves and roots, which fresh weights were determined immediately with electronic balance. Dry weights were estimated after drying samples in an oven at 80 °C until biomass became constant. Chlorophyll content was measured with the help of SPAD-502 chlorophyll meter. Photosynthetic pigment was determined with spectrophotometry, which extracted with 95% ethanol. The details of experimentation depend on the method of Knudson et al. (1977).

### 2.4. Chlorophyll fluorescence parameter measurements and JIP-test analysis

Leaves were adapted in the dark at least 30 min before measurements. Chlorophyll fluorescence parameters were measured using the Handy Plant Efficiency Analyzer (Plant Efficiency Analyzer; Hansatech, UK). The measured light source is a red light with a wavelength of 650 nm and a light intensity of 3000 $\mu mol\ m^{-2}\ s^{-1}$ provided by three light-emitting diodes, which continue to record 1 s.

An OJIP curve was plotted to normalize the fluorescence data to relative variable fluorescence data, using the following equation: $Vt = (Ft - Fo)/(FM - Fo)$, where Vt is the relative variable fluorescence at time t, Fo is the initial fluorescence, Ft is the fluorescence at time t, and FM is the maximum fluorescence. JIP test parameters (transient fluorescence steps O, J, I, and P) were calculated according to the JIP-test algorithm described by Strasser et al. (2004). The analyzed parameters are described in Supplementary Table 1.

### 2.5. Gas exchange parameter measurements

The second fully expanded leaves from the top of plants were used to estimate the net photosynthetic rate (Pn), stomatal conductance (Gs) and transpiration rate (E) using a portable photosynthesis system (Li-COR 6400, Lincoln, NE, USA). The experiment was performed on sunny day from 9:00–11:30 a.m.

### 2.6. Determination of lipid peroxidation, hydrogen peroxide ($H_2O_2$), Superoxide radical ($O_2^{\cdot -}$) and eletctrolyte leakage (EL)

The level of lipid peroxidation in leave and roots were estimated by quantifying the content of the thiobarbituric acid reactive substance (TBARS), which determined by the thiobarbituric acid (TBA) based on the method of Hodges et al. (1999) with minor modifications (Zhao et al., 2017).

$H_2O_2$ content was measured depending on the description of Zhao et al. (2017). $O_2^{\cdot -}$ was measured by monitoring nitrite formation from hydroxylamine in the presence of $O_2^{\cdot -}$, according to the method of Jabs et al. (1996) with some modifications.

The measurement of EL in leaves was according to the method of Bajji et al. (2002). Fresh leaves (0.1 g) were cut into the same size leaf discs of the same size and put into a tube containing 10 mL of deionized water. The initial electrical conductivity (EC1) was measured after the tubes were placed at 25 °C for 4 h. Then, the tubes were put into boiling water bath for 30 min. The second electrical conductivity (EC2) was