Environmental Pollution 157 (2009) 2737–2742

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Influence of earthworm mucus and amino acids on tomato seedling growth and cadmium accumulation

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Earthworm mucus increased tomato seedlings growth and Cd accumulation through increasing chlorophyll content, antioxidative enzyme activities, and essential microelement accumulation.

ARTICLE INFO

Article history:
Received 22 December 2008
Received in revised form
22 April 2009
Accepted 24 April 2009

Keywords:
Metaphire guillemi
Lycopersicon esculentum
Chlorophyll
Antioxidative enzyme
Hydroponic

ABSTRACT

The effects on the growth of tomato seedlings and cadmium accumulation of earthworm mucus and a solution of amino acids matching those in earthworm mucus was studied through a hydroponic experiment. The experiment included four treatments: 5 mg Cd L^{-1} (CC), 5 mg Cd L^{-1} + 100 mL L^{-1} earthworm mucus (CE), 5 mg Cd L^{-1} + 100 mL L^{-1} amino acids solution (CA) and the control (CK). Results showed that, compared with CC treatment, either earthworm mucus or amino acids significantly increased tomato seedling growth and Cd accumulation but the increase was much higher in the CE treatment compared with the CA treatment. This may be due to earthworm mucus and amino acids significantly increasing the chlorophyll content, antioxidative enzyme activities, and essential microelement uptake and transport in the tomato seedlings. The much greater increase in the effect of earthworm mucus compared with amino acid treatments may be due to IAA-like substances in earthworm mucus.

1. Introduction

Cadmium (Cd) is not an essential element for plants, but can be readily taken up and accumulated by plants (Dong et al., 2006). It poses a serious health risk to humans by accumulation through the food chain (Kirkham, 2006). For plants, Cd induces various symptoms of phytotoxicity, such as chlorosis, biomass reduction, reduction in root elongation and even death (Dariusz et al., 2005). For humans, Cd causes organ damage (kidney, liver and heart) and other diseases (for example, pulmonary emphysema and the notorious Itai-Itai disease; Yeung and Hsu, 2005).

Although phytoextraction is regarded as a promising and environmentally friendly technology by removing metals from contaminated soils and concentrating them in the harvestable parts, its efficiency is restricted by lower bioavailability of metals to plants in contaminated soils (Salt et al., 1998). At present, research on increasing the efficiency of phytoextraction has been evolving in two main directions: (1) the use of hyperaccumulating plant species and (2) the use of high biomass plant species with induced accumulation of metals (Meers et al., 2004). Earthworms are ubiquitous invertebrate species living in soils and arguably the most important components of the soil biota in terms of maintenance of soil structure and fertility (Edwards, 2004). Many studies reported that in metal contaminated soils, earthworm activities not only enhanced plant growth, but also increased metal uptake and accumulation into plants (Ma et al., 2003; Wang et al., 2006). These results suggest that earthworms have potential applications in combination with induced high biomass plant species for phytoextracting metals from contaminated soils. Thus, a more comprehensive understanding of the mechanisms and causes of earthworm enhancement of metal accumulation in plants is beginning to emerge.

Earthworms release a mass of complicated glutinous mucus from their body wall, an action that plays a crucial role in earthworm feeding, osmoregulation, defense and reproduction activities (Heredia et al., 2007). Our previous study reported that earthworm (*Metaphire guillemi*) mucus not only enhanced tomato (*Lycopersicon esculentum*, Hezuo903) seedling growth and Cd tolerance, but also increased Cd accumulation in plants (Zhang et al., in press). However, how earthworm mucus enhances plant growth under Cd stress and increases Cd accumulation in plants is still unclear. Amino acids are one of the major components of earthworm mucus (Cortez and Bouché, 1987) and may have some function in metal uptake and transport in plants (Salt et al., 1995; Zhou et al., 2007). Zhou et al. (2007) found that exogenous cysteine significantly increased the dry weight of maize seedlings and significantly

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doi:10.1016/j.envpol.2009.04.027

increased Cu uptake and transport from maize seedling roots to shoots under 5 μM Cu in hydroponic solution. However, there is little information available about the effects of amino acids from earthworm mucus on plant growth and metal accumulation.

The aim of the present study was to investigate the basic mechanism of earthworm mucus increasing metal accumulation in plants through a hydroponic experiment. The objectives were (1) to compare the effects of earthworm (*M. guillemi*) mucus and amino acid solution on tomato (*L. esculentum*, Hezuo903) seedling growth and Cd accumulation, and (2) to investigate the potential effects on the chlorophyll content, antioxidative enzyme activity and four essential microelement concentrations in plants.

2. Materials and methods

2.1. Earthworm mucus collection

Earthworms (*M. guillemi*) were collected by hand from a vegetable garden in Rudong county, Jiangsu province, China, and transferred without delay to the laboratory. *M. guillemi* is a common species of burrowing earthworm in China known to be resistant to metals (Ma et al., 2003). All earthworms were adult with full clitellate. The initial fresh weights were 3.00–4.50 g. The worms were kept in the laboratory for 7 days in native soils (in darkness, 15 °C) before the experiment. Adult earthworms were rinsed several times with deionized water, dried with tissue paper, and then kept on moist filter paper in Petri dishes (2 worms per dish) in an incubation box (in darkness, 22 °C) for 48 h to void their gut contents. To avoid coprophagy, the filter papers were changed twice daily. At the end of the voiding period, the earthworms were rinsed again with deionized water, dried with tissue paper, transferred into a 500 mL beaker (60 individuals, about 200 g fresh weight per beaker) and uniformly sprinkled with 10 g quartz sand (<0.2 mm). After incubation in an incubation box (in darkness, 22 °C) for 4 h, the beaker, earthworms and quartz sand were washed 5 times with 290 mL deionized water. The washing water was filtered, diluted to 300 mL with deionized water and stored at −20 °C. The major chemical properties of earthworm mucus are shown in Table 1.

2.2. Determination of amino acids in earthworm mucus

The amino acid composition of earthworm mucus was determined referring to the National Standard of PR China of Determination of Amino Acids in Feeds (GB/T 18246 – 2000). Briefly, 5 mL of earthworm mucus was mixed with 10 mL of 1% trichloroacetic acid solution, and the mixture centrifuged at 10 733 *g* for 15 min. The supernatants were filtered through a 0.45 μm Millipore filter and determined by Hitachi L-8900 automatic amino acid analyzer. The amino acid composition of earthworm mucus is as follows (nmol mL^{-1}): leucine 311.4, glycine 207.7, valine 200.4, isoleucine 133.2, lysine 120.8, threonine 113.3, serine 105.4, phenylalanine 104.0, proline 92.6, histidine 67.9, tyrosine 63.6, methionine 52.9, arginine 51.8, cysteine 5.8.

2.3. Plants and experiment design

The experiment was carried out in October 2008 at the Laboratory of Soil Ecology, Nanjing Agricultural University. Selected seeds of tomato (*L. esculentum*, Hezuo 903), which is widely cultivated in southeastern China (Dong et al., 2006), were submerged in deionized water for 4 h, sterilized with 0.5% K_2MnO_4 for 30 min, rinsed several times with deionized water, and then germinated on moist filter paper in Petri dishes in an incubation box, at 25 °C and 60% relative humidity. Seedlings were cultivated hydroponically in a greenhouse, with natural light (sunshine duration is 12 h per day) and temperature of 25/18 °C (average during day and night, respectively) and humidity of 50–60%, to a height of 3 cm. Seedlings were transplanted to a 6 l plastic container which contained 5.5 l of hydroponic solution and were covered with a polyethylene plate with six evenly spaced holes. One plant was fixed in each hole, such that the plant roots were completely immersed in hydroponic solution. These seedlings were grown in half-strength hydroponic solution for the first 5 days, and then in full-strength hydroponic solution. The chemical composition of the hydroponic solution was as following: KNO_3 1.25 mM, $Ca(NO_3)_2$ 1.25 mM, $MgSO_4$ 0.5 mM, KH_2PO_4 0.25 mM, H_3BO_3 12.5 μM, $MnCl_2$ 2.25 μM, Fe-EDTA 5 μM, $ZnSO_4$ 0.5 μM, $(NH_4)_6Mo_7O_{24}$ 0.025 μM, $CuSO_4$ 0.075 μM (Wei et al., 2007). The hydroponic solution was prepared with deionized water, and its pH was adjusted to 5.8 ± 0.1 with a pH meter (PHS-3C) and 1 M HNO_3. The hydroponic solution was replaced every 5 days and continuously aerated by a pump.

After 15 days growth, similar sized tomato seedlings were selected and transplanted to 120 mL plastic pots containing 100 mL of hydroponic solution, and covered with a polyethylene plate. One plant was fixed to the middle of the polyethylene plate and the plant roots were completely immersed in hydroponic solution. Cadmium was prepared as an aqueous solution of cadmium chloride ($CdCl_2 \cdot 2.5H_2O$) and amino acid solution was prepared to match the composition of earthworm mucus. The major chemical properties of the amino acid solution are as follows: pH 6.61, dissolved organic carbon (DOC) 74.3 μg mL^{-1}, NH_4^+-N 1.9 μg mL^{-1}, NO_3^--N 3.0 μg mL^{-1}. The experiment included four treatments: 5 mg Cd L^{-1} (CC), 5 mg Cd L^{-1} + 100 mL L^{-1} earthworm mucus (CE), 5 mg Cd L^{-1} + 100 mL L^{-1} amino acids solution (CA) and just the hydroponic solution for the control (CK). After the addition of Cd, earthworm mucus and amino acid solution, the composition of basic nutrient elements was as described above and the pH of the hydroponic solution was adjusted to 5.8 ± 0.1 with 1 M HNO_3 solution as required. Each treatment was replicated 4 times and randomly arranged in the same greenhouse. The hydroponic solution, including Cd, earthworm mucus and amino acids, was replaced every other day, and the experiment duration was 6 days.

2.4. Determination of pigment concentrations

Pigment concentrations were determined according to Qiu et al. (2008). Briefly, fresh leaf samples (about 0.2 g) were mixed with ethanol and calcium carbonate and homogenized with a mortar and pestle. The homogenates were filtered and then diluted to 25 mL with ethanol. The absorbance was determined at 460, 649 and 665 nm. The concentrations of chlorophylls and carotenoids were calculated according to the formula of Lichtenthaler and Wellburn (1983).

2.5. Determination of anti-oxidative enzyme activities

Fresh samples (about 0.5 g) were washed with deionized water and ground with a mortar and pestle under chilled conditions in the homogenization buffer specific for each enzyme. The homogenate was centrifuged at 15 455 *g* for 20 min at 4 °C, and the supernatants were used for determination of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities according to Wu et al. (2003) and Qiu et al. (2008). Enzyme activity was calculated in terms of U g^{-1} fresh weight (U, enzyme activity unit).

Superoxide dismutase activity was determined using the photochemical nitro-blue tetrazolium (NBT) method. The reaction mixture in 3 mL total volume contained 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 75 μM NBT, 10 μM Na_2-EDTA, and 20 μM riboflavin. The photoreduction of NBT (formation of purple formazan) was measured at 560 nm, and an inhibition curve was made against different volumes of extract. Peroxidase activity was determined with guaiacol as the substrate in a total volume of 3 mL. The reaction mixture contained 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H_2O_2, and enzyme extract. Increasing absorbance due to oxidation of guaiacol was determined at 470 nm. Catalase activity was determined by monitoring the disappearance of H_2O_2 by determining the decrease in absorbance at 240 nm of a reaction mixture consisting of 25 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2 and enzyme extract (Wu et al., 2003; Qiu et al., 2008).

2.6. Cadmium and four microelements analysis

At harvest, all plants were thoroughly washed several times with deionized water and separated into roots, stems and leaves, and oven dried at 65 °C for 48 h to constant weight. Cadmium and Fe, Mn, Zn, and Cu concentrations were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES, Optima 2100 DV, Perkin Elmer) after digesting the samples with a HNO_3–$HClO_4$ (4:1, v/v) mixture (Wang et al., 2006).

2.7. Statistical analysis

All data are presented as the mean value ± standard error (SE). Analyses were performed using the SPSS statistical software package (version 16.0) and the means were separated by Duncan's new multiple range test (Wang et al., 2006).

Table 1
Chemical properties of earthworm mucus.

pH	Chemical composition (μg mL^{-1})					Amino acid (μmol mL^{-1})
	DOC	NH_4^+-N	NO_3^--N	Total P	Total K	
6.66	91.2	39.5	10.6	9.3	84.2	1.6

3. Results

3.1. Plant growth parameters

Cadmium significantly suppressed tomato seedling growth compared with the control (CK); the dry weight of plant roots, stems, and leaves was significantly decreased by 69.1%, 37.7% and 65.3%, respectively (Fig. 1). Compared with the 5 mg Cd L^{-1} treatment (CC), earthworm mucus significantly increased the dry weight