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Exogenous IAA differentially affects growth, oxidative stress and antioxidants system in Cd stressed *Trigonella foenum-graecum* L. seedlings: Toxicity alleviation by up-regulation of ascorbate-glutathione cycle

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ABSTRACT

In the present study, effect of exogenous indole-3-acetic acid at their different levels (i.e. low; IAA_L, 10 μM and high; IAA_H, 100 μM) were studied on growth, oxidative stress biomarkers and antioxidant enzymes (SOD, POD, CAT and GST), and metabolites (AsA and GSH) as well as enzymes (APX, GR and DHAR) of ascorbate-glutathione cycle in *Trigonella foenum-graecum* L. seedlings grown under cadmium (Cd_1, 3 mg Cd kg^{-1} soil and Cd_2, 9 mg Cd kg^{-1} soil) stress. Cadmium (Cd) at both doses caused reduction in growth which was correlated with enhanced lipid peroxidation and damage to membrane as a result of excess accumulation of $O_2^{\bullet -}$ and H_2O_2. Cd also enhanced the oxidation of AsA and GSH to DHA and GSSG, respectively which give a clear sign of oxidative stress, despite of accelerated activity of enzymatic antioxidants: SOD, CAT, POD, GST as well as APX, DHAR (except in Cd_2 stress) and GR. Exogenous application of IAA_L resulted further rise in the activities of these enzymes, and maintained the redox status (> ratios: AsA/DHA and GSH/GSSG) of cells. The maintained redox status of cells under IAA_L treatment declined the level of ROS in Cd_1 and Cd_2 treated seedlings thereby alleviated the Cd toxicity and this effect was more pronounced under Cd_1 stress. Contrary to this, exogenous IAA_H suppressed the activity of DHAR and GR and disturbed the redox status (< ratios: AsA/DHA and GSH/GSSG) of cells, hence excess accumulation of ROS further aggravated the Cd induced damage. Thus, overall results suggest that IAA at low (IAA_L) and high (IAA_H) doses affected the Cd toxicity differently by regulating the ascorbate-glutathione cycle as well as activity of other antioxidants in *Trigonella* seedlings.

1. Introduction

In recent years, enhanced human activities have created several serious problems such as heavy metals pollution, pesticide toxicity, nutrient imbalance, etc. in the crop field. The concentration of heavy metal in biosphere (air, water and soil) has increased considerably that deteriorates health of humans, animals and plants. Cadmium (Cd), one of the toxic heavy metal is released into the environment by traffic, metal-working industries, mining and by the excessive use of phosphate fertilizers in agricultural field (Nriagu and Pacyna, 1988). Its high concentration can influence several vital metabolic activities in living organism including plants and human. Cd accumulation in plants causes reduction in photosynthesis, diminishes water and nutrient uptake (Sanita'di Toppi and Gabbrielli, 1999), and at higher concentration it also leads to some visible symptoms of injury such as chlorosis, growth inhibition, browning of root tips and finally death (Kahle, 1993). Cd is not a redox metal; however, its toxicity is mediated by excess formation of reactive oxygen species (ROS) under stress, through electron transport processes in mitochondria and chloroplast (Heyno et al., 2008). These ROS initiates a variety of auto oxidative chain reactions, takes place on membrane unsaturated fatty acids, producing lipid hydroperoxides and thereby cascade of reactions ultimately leading to destruction of organelles and macromolecules (Shaw et al., 2004). Free radicals and lipid peroxidation are mostly considered as major contributors of leaf senescence (Chang and Kao, 1998). The excess level of ROS are continuously regulated by several antioxidant enzymes such as superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E. C. 1.11.1.6),

Abbreviations: APX, ascorbate peroxidase; AsA, reduced ascorbate, CAT, catalase; Cd, cadmium; DHA, oxidized form of ascorbate (dehydroascorbate); DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized form of glutathione; GST, glutathione-*S*-transeferase; IAA, indole-3-acetic acid; MDA, malondialdehyde; POD, guaiacol peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase

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peroxidases (POD, E.C. 1.11.1.7) and glutathione-*S*-transeferase (GST, EC 2.5.1.18), and a complex antioxidant system—the ascorbate–glutathione cycle (Zhang and Kirkham, 1996) along with the various other endogenous antioxidants such as cysteine and thiols (Foyer et al., 2001). SOD is considered as first line of defence as it dismutase $O_2^{\bullet -}$, a more frequently formed ROS into O_2 and H_2O_2. Though H_2O_2 is less toxic than $O_2^{\bullet -}$ but its mobility across the membrane as well as greater stability than $O_2^{\bullet -}$ makes it more toxic at the cellular level, as it generates the most toxic $^{\bullet}OH$ during Fenton reaction in presence of Cu, Fe, Cr, etc. H_2O_2 is mainly detoxified by the enzymes CAT, POD and APX (Mittler, 2002). Ascorbate–glutathione cycle, a major H_2O_2 scavenging pathway mainly operates in chloroplast but also found in mitochondria, peroxisome and cytosol (Zhang and Kirkham, 1996). This cycle detoxifies H_2O_2 by involving APX enzyme and ascorbate as electron donor. Besides this, it also includes enzymatic antioxidants: DHAR, MDHAR and GR and non-enzymatic antioxidant: glutathione, and NADPH which acts as electron donor (Asada, 1999; Foyer and Noctor, 2011). Moreover, ascorbate and glutathione are associated with the cellular redox balance and the ratios of AsA/DHA and GSH/GSSG may function as signals for the regulation of antioxidant mechanisms (Mittler, 2002). Detoxification reactions must involve right balance between the formation and detoxification of ROS. However, under severe stress the balance between the generation and detoxification of ROS is disturbed that lead to the oxidative stress, and one of the consequent effects of such oxidative stress is disturbances in the regulation of plant growth regulators.

The plant growth regulators are known for reducing negative impacts of different abiotic stresses (Agami and Mohamed, 2013; Singh and Prasad, 2014). Auxins are plant growth regulators involved in mediating a number of essential plant growth and developmental processes, such as cell elongation, induction of root growth, and flower and fruit development (Kukavica et al., 2007). Indole-3-acetic acid (IAA) is considered as main auxin in plants that stimulates growth through cell elongation and lateral root formation, which probably support more absorption of minerals, and it also act as signaling molecule under stress (Mansfield and McAinsh, 1995). It is known for a long time that ROS may result in direct oxidation of IAA (Beffa et al., 1990). Therefore, in presence of metals, the generation of ROS may affect the auxin physiology that consequently leads to reduction in plant biomass.

Among different kinds of plants *Trigonella foenum-graecum* L. (Fenugreek) is one of the widely grown vegetable, and particularly India is the major producer of fenugreek. It is consumed as spice, leafy vegetable and serves as medicine for carminative, analgesic, anti-inflammatory, gastric troubles, diabetes and leucorrhea (Bashri et al., 2013). As metal contamination is one of the prominent problems of both developing as well as developed countries, so production of such an important plant in metal contaminated areas and particularly in catchment area of cities may decreased. Thus, the goal of this study is to understand the Cd toxicity and its regulation by exogenous auxin (IAA at low and high doses) with special focus on the involvement of ascorbate-glutathione cycle as well as other enzymatic and non-enzymatic antioxidant in *Trigonella foenum-graecum* L.

2. Materials and methods

2.1. Plant material and growth conditions

Healthy seeds of *Trigonella foenum-graecum* L. var. Antara were sterilized with sodium hypochlorite solution (2%, v/v) for 15 min, and after repeated washing with distilled water, they were soaked for 4 h. Later wet seeds wrapped in muslin cloth and placed in dark at 25 ± 2 °C for germination. After 24 h the germinated seeds were sown in plastic pots containing 150 g sandy loam soil and farmyard manure (9:1, w/w). Selected physical and chemical characteristics of the soils are shown in Table 1. Soil pH and electrical conductivity (EC) were measured in deionised water (soil: solution ratio, 1:5); organic carbon was measured by dry combustion. Total N content was determined by the Kjeldahl method (Nelson and Sommers, 1973). Phosphorus was extracted by nitric–perchloric acid digestion and measured using the colorimetric method (Jackson, 1973) and the K content was assayed using a flame photometer (Model 130, Systronic, India). The background Cd in the soils was determined using an aqua regia (1:3 fresh mixture of concentrated HNO_3 and HCl) digestion. To select the doses of Cd for experimental analysis, screening experiments were performed, where test plant grown under different levels (1, 3, 6, 9, 12 and 15 mg kg^{-1} soil) of Cd (as $CdCl_2$) and showed insignificant effect at 1 mg $kg^{-1}$and thereafter it declined the growth by 8, 12, 14, 21% and 29% (on dry weight basis), respectively. On the basis of this experiment two doses of Cd i.e. Cd_1:3 mg Cd kg^{-1} soil (within permissible limit, Awasthi, 2000) and Cd_2:9 mg Cd kg^{-1} soil (above permissible limit) were selected and mixed with soil before sowing of germinated seeds. The pots were placed in open field under 900 ± 100 μmol m^{-2} sec^{-1} photosynthetic active radiation, 65 ± 5% humidity, 20 ± 5 °C temperature. The seedlings were regularly irrigated with distilled water and after 15 days of growth the two doses i.e. low (IAA_L, 10 μM) and high (IAA_H, 100 μM) of IAA were exogenously applied. Indole acetic acid stock solution (400 μM, 100 ml) was prepared by dissolving 7 mg of IAA in 1 ml of ethanol and finally made it 100 ml with the addition of double distilled water and 10 and 100 μM of IAA was prepared by dilution of stock solution of IAA, and added 0.1% Tween 20 as surfactant. The experimental design includes nine combinations i.e. control (without treatment), Cd_1, Cd_2, IAA_L, Cd_1+IAA_L, Cd_2+IAA_L, IAA_H, Cd_1+IAA_H, Cd_2 $+AA_H$. The different parameters were analyzed after 15 days of IAA treatment. Whole seedlings were used for the estimation of Cd content and growth while oxidative stress and antioxidants were estimated in the fresh leaves of the seedlings.

Table 1
Physical and chemical properties of the experimental soil used in this study.

Properties	
Physical:	
Texture	Sandy loam
Sand (%)	69.50
Silt (%)	18.50
Clay (%)	12.00
Chemical:	
pH	7.81
Electrical Conductivity (dSm^{-1})	0.21
Organic carbon (%)	0.52
N (g Kg^{-1})	1.24
P (mg Kg^{-1})	12.25
K (mg Kg^{-1})	389.25
Cd concentration (mg kg^{-1})	nd

2.2. Estimation of Cd content

Cd content was estimated by the method of Allen et al. (1986) in dried plant samples (100 mg) which was digested in tri-acid mixture (HNO_3, H_2SO_4 and $HClO_4$ in 5:1:1 ratio, v/v) at 80 °C until a transparent solution was obtained. Thereafter, the digested sample was filtered using Whatman no. 42 filter paper and Cd content in the filtrate was estimated by atomic absorption spectrometer (iCE 3000 Series, Model 3500 AAS, Thermo scientific,