Environmental and Experimental Botany 122 (2016) 1–9

Contents lists available at ScienceDirect

Environmental and Experimental Botany

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

Plant steroidal hormone epibrassinolide regulate – Heavy metal stress tolerance in *Oryza sativa* L. by modulating antioxidant defense expression

Priyanka Sharma[a,*], Anil Kumar[b], Renu Bhardwaj[c]

[a] Department of Biotechnology, Savitribai Phule Pune University, Ganeshkhind, Pune 411007, Maharashtra, India
[b] Serum Institute of India Ltd., Hadapsar, Pune 411028, Maharashtra, India
[c] Department of Botanical & Environmental Sciences, Guru Nanak Dev University, Amritsar 143005, Punjab, India

ARTICLE INFO

Article history:
Received 18 May 2015
Received in revised form 31 July 2015
Accepted 22 August 2015
Available online 28 August 2015

Keywords:
Antioxidative enzymes
Brassinosteroids
Chromium metal
Gene expression
Rice (*Oryza sativa*)

ABSTRACT

The present research paper is aimed to study the effect of 24-epibrassinolide (EBL) on growth of seedling, metal uptake, stress markers, antioxidative enzyme activity and gene expression in chromium (Cr) stressed rice seedlings variety Ratna. The exogenous application of EBL caused a significant reduction in Cr accumulation and improved the growth of seedlings. However, the results also showed that antioxidative enzymes activity and mRNA expression of these genes (*Mn-SOD, Cu/Zn-SOD, Cat A, Cat B, APX* and *GR*) significantly increased in seedlings treated with Cr ions under the influence of EBL. Taken together, our results demonstrate that exogenous application of EBL is more effective to ameliorate the Cr metal stress by up-regulating the activity of antioxidative enzymes.

1. Introduction

Plants are frequently exposed to various environmental factors, which constitute their macro and microenvironment. Any deviation in these factors from the optimum level is harmful and ultimately leads to stress in plants (Kumar et al., 2008; Parvaiz et al., 2008). Stress may be caused due to various abiotic and biotic factors. Chromium is the seventh most abundant metal in the earth's crust (Katz and Salem, 1994) and important contaminated released into the atmosphere due to its huge industrial use (Nriagu and Neiboer, 1988). Cr phytotoxicity can result in inhibition of plant growth and their physiological activities (Panda et al., 2003). A common consequence of abiotic and biotic stresses are the effect on cell signaling cascades and cellular responses, like activation of stress proteins, up-regulation of antioxidant enzymes and accumulation of compatible solutes (Triantaphylides and Havaux, 2009). These stresses can further aggravate the production of reactive oxygen species (ROS) which are capable of causing oxidative damage. The various antioxidants and antioxidant enzymes scavenge ROS and act as defence system pathway (Dat et al., 2000).

Another approach of defence to plants includes certain secondary metabolites and some plant growth regulators like ABA, Ethylene, Auxin, Jasmonic acid and plant steroids. Brassinosteroids (BRs), show high activity even at very low concentrations. However, the physiological functions of BRs in plants are not yet fully understood. Recent research has been explored for their promising role in reducing the effects of environmental stresses (Kagale et al., 2007; Arora et al., 2008). Exogenous application of BRs improved the antioxidant system by regulating the activities of antioxidant enzymes and antioxidants to provide protection to the plants under stress conditions (Ozdemir et al., 2004; Hayat et al., 2007; Sharma and Bhardwaj, 2007; Arora et al., 2008). Genetic and biochemical analysis has led to the identification of BRs receptor, some key signaling elements and gene expression that is responsive to BR. Although numerous reports have confirmed the potential of plant hormones to synergistically improve crop performance under environmental stress conditions. Although much has been learned about their roles in plant development, the mechanisms by which BRs control plant stress responses and regulate stress-responsive gene expression are not fully known.With the concern of Cr metal phytotoxicity and stress protective role of BRs, this study was designed to evaluate their influence on metal ion uptake, oxidative stress protection, antioxidative enzyme activities and gene expression in *Oryza sativa* seedlings.

Abbreviations: APX, ascorbate peroxidase; BRs, brassinosteroids; CAT, catalase; EBL, 24-epibrassinolide; EDTA, ethylenediaminetetraacetic acid; GR, glutathione reductase; ROS, reactive oxygen species; PMSF, phenylmethylsulfonyl fluoride; PVP, polyvinylpyrrolidone; SOD, superoxide dismutase.

* Corresponding author at: Department of Biotechnology, Savitribai Phule Pune University, Pune, Ganeshkhind-411007, Maharashtra, India. Tel.: +91 20 25694952; fax: +91 2025691821.
E-mail address: priya_agn@yahoo.com (P. Sharma).

http://dx.doi.org/10.1016/j.envexpbot.2015.08.005
0098-8472/

Rice (*O. sativa* L.) is one of the major food crops in many countries. In India, rice cultivated on large scale in Karnataka, Maharashtra, Andhra Pradesh and West Bengal. Cultivation of rice is mostly depended on groundwater, particularly in the dry season. But groundwater of most states has been highly contaminated with toxic heavy metals especially with Cr. Thus there is a possibility of induction of Cr in rice, cultivated with contaminated irrigation water and soil. As per Environmental Protection Agency (EPA) priority pollutants chromium is major toxic contaminants present in the environment. Chromium is released into soil as a waste product of several metal finishing, tanning, petroleum refining industries, etc. (Barnhart, 1997). Considering the fact that groundwater contains chromium as a major contaminant, which transfer to cereal food, our aim was to minimize the accumulation of Cr metal ion and ameliorate the Cr toxicity in *O. sativa* under the effect of 24-epibrassinolide, a potent steroidal hormone.

2. Material and methods

2.1. Chemicals

Analytical grade chemicals were purchased from Fisher Scientific (UK) and Merck (Germany). High performance liquid chromatography (HPLC) grade chemicals and standards 24-epibrassinolide was obtained from Sigma–Aldrich (UK). Ultrapure water used was purified using the Milli-Q-plus filter system by Millipore (USA).

2.2. Plant material

The plant material for the present study was the seedlings of *O. sativa* var. Ratna. The certified seeds of *O. sativa* were procured from Agricultural College of Pune, Shivajinagar, Pune, Maharashtra, India.

2.3. Effect of EBL on growth, Cr ion uptake and antioxidant defense of *Oryza sativa* seedlings

The surface sterilized seeds of *O. sativa* var. Ratna were soaked for 12 h in different concentrations of EBL (0, 1 μM, 0.01 μM and 0.1 nM). The hormone treated seeds were sown in petridishes which contained 0.5 mM concentration of Cr ion. The 0.5 mM concentration of Cr was taken after calculating LD_{50} value. After 12 days of sowing, seedlings were harvested to analyze the growth of seedlings, metal uptake, stress indices, antioxidative enzyme (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR)) analysis by quantitative and qualitative methods. The gene expression level of antioxidative enzymes (*SOD, CAT, APX* and *GR)* was also studied. Observations were made on growth parameters viz. shoot length, root length, fresh and dry weight, relative water content (RWC) and Cr metal uptake.

2.3.1. Heavy metal analysis

The 12 days old seedlings of *O. sativa* were oven dried at 80 °C for 24 h. The dried samples of seedling were digested in digestion mixture containing H_2SO_4: HNO_3:$HClO_4$ (1:5:1) by following the method of Allen et al. (1976) with minor modifications. Digested and filtered samples were analyzed for Cr ion content by atomic absorption spectrophotometer (AAS) (Shimadzu).

The bioconcentration factor (BCF) was calculated as follows:

$$BCF = \frac{\text{Traceelementconcentrationinplanttissues (mg/kg) at harvest}}{\text{Initial concentration of the element in the external nutrientsolution (mg/l)}}$$

2.3.2. Impact of EBL on stress indices

Stress indices reflect the level and damages of oxidative stress caused by various abiotic factors. The various stress indices parameters like Malondialdehyde (Guo et al., 2006), H_2O_2 content level (Velikova et al., 2000), proline concentration (Bates et al., 1973) and ascorbic acid content (Cakmak and Marschner 1992) was studied as stress marker to evaluate the intensity of Cr-stress under the influence of EBL in rice seedlings.

2.3.3. Antioxidative enzyme activity assay

For enzyme activity assays, 12 days old seedlings were ground in liquid nitrogen and extraction was done using an extraction buffer (100 mM potassium phosphate buffer (pH 7.0), 1 mM PMSF, 1 mM EDTA and 1% PVP). The supernatant was used for determining the activities of various antioxidative enzymes by using UV–visible double beam spectrophotometer (Shimadzu). The activity of catalase was assayed as per the method of Aebi (1983). The unit activity of CAT was calculated using an extinction coefficient of $6.93 \times 10^{-3}\,mM^{-1}\,cm^{-1}$. The total SOD activity was estimated according to Kono (1978) method. The enzyme activity was calculated as the concentration of SOD required for inhibiting the reduction of NBT by 50% at 540 nm. Glutathione reductase activity was measured using the protocol given by Carlberg and Mannervik (1975). The activity was calculated using the extinction coefficient of $6.22\,mM^{-1}\,cm^{-1}$ to determine as the amount of enzyme required to oxidize 1 μM of NADPH $min^{-1}\,g^{-1}$ tissue. The activity of ascorbate peroxidase (EC 1.11.1.11) was determined as per method of Nakano and Asada (1981) following a decrease in absorbance at 290 nm.

2.3.4. Native PAGE and enzyme staining activity

Equal amount of extracted protein (40 μg for SOD, APX and GR; 10 μg for CAT) were electrophoresed in 10% (SOD, APX and GR) or 8% (CAT) polyacrylamide gels under non-denaturing conditions as described by Laemmli (1970), except that SDS was omitted. Isoforms of CAT enzymes were visualized by the method of Woodbury et al. (1971). The gels were suspended in a 0.03% H_2O_2 solution for 20 min in dark condition after electrophoresis. Thereafter gels stained in a solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride till yellow band appearance. To visualize SOD profile, gels were stained according to the method of Beauchamp and Fridovich (1971). This method is based on NBT reduction by photochemically generated superoxide from riboflavin and TEMED. To identify different isoforms of SOD, gels were pre-incubated with inhibitors (3 mM KCN and 5 mM H_2O_2).

Glutathione reductase isozymes were stained in a solution of 0.25 M Tris, pH 7.8, containing 0.24 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.4 mM NADPH, 0.34 mM 2,6-dichlorophenolindophenol**,** and 3.6 mM GSSG for 1 h in the dark, as described by Anderson et al. (1995). Duplicate gels were also stained in the absence of GSSG to distinguish GR from other nonspecific sources of reduction. Similarly APX isozymes were separated by native PAGE for 1.5 h at 4 °C at a constant current of 20 mA per gel. Ascorbate (2 mM) was included in the carrier buffer. The gels were pre-run for 30 min to allow ascorbate present in the carrier buffer to enter the gel prior to the application of samples, according to the method of Rao et al. (1996).