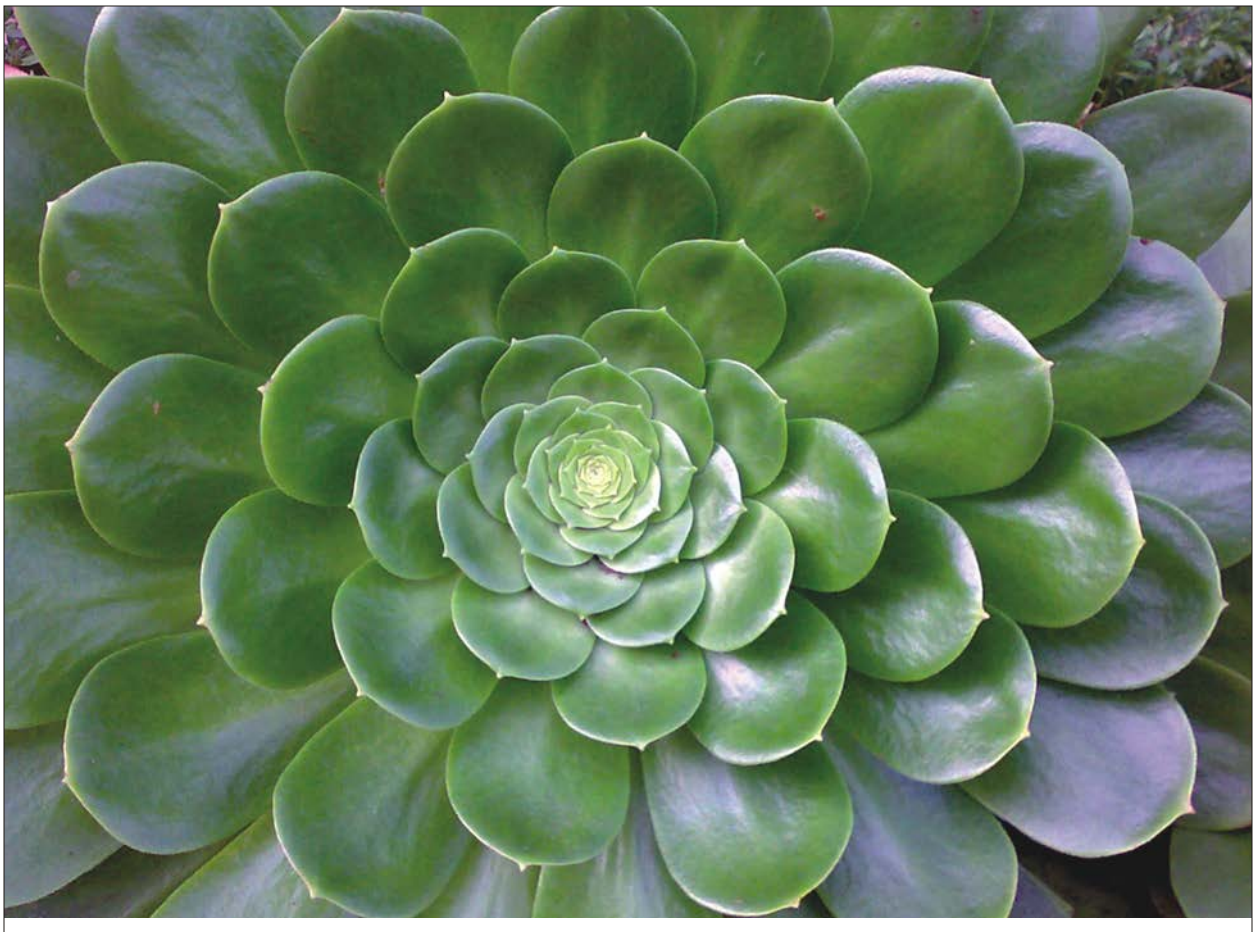

Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Journal of Plant Sciences

ISSN 1816-4951
DOI: 10.3923/jps.2016.69.74

Research Article
Catalase (CAT) and Ascorbate Peroxidase (APX) Genes Expression Level in Growth of Banana Plantlets (*Musa acuminata*) cv. Ambon Lumut Under Chromium Stress Condition

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Abstract

Background: Chromium (Cr) plays a role in the production of Reactive Oxygen Species (ROS) that cause oxidative stress. Oxidative stress is a condition when the amount of ROS, such as H_2O_2, O_2^- and OH^- (as a by products in a number of metabolic reactions in the cell organelles) exceeds the amount of antioxidants produced. Antioxidants can be either enzyme or non-enzyme. Antioxidant enzymes are such as catalase and ascorbate peroxidase. In the field, it was found that bananas cv. ambon lumut that can be grown in Cr polluted areas. In this study, it was observed that the level expression of catalase (CAT) and ascorbate peroxidase (APX) genes on banana plantlets cv. ambon lumut to determine the mechanisms of banana plants under Cr stress conditions. **Materials and Methods:** Banana plantlets were grown *in vitro* on MS medium with the treatment of Cr at 0, 50, 100, 200 and 400 ppm concentrations and maintained for 6 weeks. Growth parameters were analyzed by ANOVA at significance level (α) of 0.05. Gene expression level can be undertaken with qRT-PCR analysis approach. **Results:** The growth rate of banana plantlets decreased with increasing Cr concentrations in the growth mediums, especially at 200 and 400 ppm. The level of CAT and APX genes expression were observed in the roots and shoots of banana plantlets. At the root, CAT and APX genes are expressed in all treatment conditions, except in the treatment of 50 ppm. At the shoot, only CAT gene was expressed higher than the control and the highest level of CAT gene expression occurred in the treatment of 200 ppm. The level of CAT and APX genes expression reached the highest level on the treatment of 200 ppm. The level of gene expression of CAT reached more than 9 times compared to the control and APX more than three times. **Conclusion:** (1) The growth rate of banana plantlets decreased with increasing Cr concentrations in the growth mediums and (2) The level of CAT and APX gene expression in plantlets under Cr stress condition were higher than the control. Gene expression level in the roots is higher than the shoots.

Key words: Gene expression level, catalase, ascorbate peroxidase, growth, *Musa acuminata*, chromium stress

Received: May 16, 2016 Accepted: June 05, 2016 Published: August 15, 2016

Citation: Lida Amalia, Taufikurahman and Sri Nanan B. Widiyanto, 2016. Catalase (CAT) and ascorbate peroxidase (APX) genes expression level in growth of banana plantlets (*Musa acuminata*) cv. ambon lumut under chromium stress condition. J. Plant Sci., 11: 69-74.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Increasing industrial development have a positive impact for the community to fulfill the various needs of life and the availability of jobs, but it can also have a negative impact because the waste often cause environmental problems. The continued development of the industry will lead to an increase in pollution of water sources derived from industrial waste discharged into waters without prior treatment. This situation adversely affected if the contaminated water used for irrigation on agricultural land and a source of water that is used by the people (Hussain *et al*., 2006).

Chromium into the plant body can interfere with important metabolic processes, such as photosynthesis and respiration. The metabolic reactions produced a variety of products, namely Reactive Oxygen Species (ROS), such as H_2O_2, O_2^- and OH^-. If Cr concentration increase in the plant body, the production of ROS that would cause oxidative stress will also increase, which conduct to the damage in structure and function of cell membranes, DNA damage, gene mutations, protein oxidation, lipid peroxidation and cell death. Finally, the oxidative stress can inhibit the growth and lower yield (Panda and Choudhury, 2005; Hossain *et al*., 2012), such as study in *Vigna mungo* (Hussain *et al*., 2006) and in *Sorghum bicolor* L. (Revathi *et al*., 2011).

Oxidative stress is caused by increase in Reactive Oxygen Species (ROS), such as H_2O_2, O_2^- and OH^- (as a side product in a number of metabolic reactions in the cell organelles) exceeds the amount of antioxidants produced. Antioxidants can be either enzyme or non-enzyme. Antioxidant enzymes are catalase, glutathione reductase, ascorbate peroxidase and superoxide dismutase. Antioxidant non-enzymes are such as proline, ascorbic acid, glutathione, carotenoids and flavonoids (Azmat *et al*., 2009).

From the analysis of transcriptome data gene ontology banana (*Musa acuminata*) cv. Barangan who gets salinity, it is known that genes are expressed in response to stress oxidative (ROS), among others, the gene CAT (catalase) and ascorbate peroxidase (APX) (Widiyanto *et al*., 2013).

This study aimed to evaluate the level of gene expression of CAT and APX genes in banana plantlets (*Musa acuminata*) cv. ambon lumut in Cr stress condition. Therefore, it was measured gene expression levels of CAT and APX in banana plantlets cv. ambon lumut originating from Cr contaminated areas (Sukaregang Garut). It was suggested that cultivars can develop detoxification and Cr stress tolerance mechanisms. The cultivars able to prevent damage caused by ROS was assume, which can be developed to produce Cr stress-resistant seedlings.

MATERIALS AND METHODS

Banana plantlets (*Musa acuminata*) cv. ambon lumut (AAA group) *in vitro* culture: The plantlets obtained through the stages of initiation and multiplication of shoot apex culture banana plants from Cr contaminated land. The entire stage is carried out in the Laboratory of Transformation and Micro propagation SITH ITB. The Cr concentration in the growing medium, i.e., 0, 50, 100, 200 and 400 ppm. Concentration selection is based on the concentrations commonly found in soil contaminated with Cr (Diwan *et al*., 2010). The Cr source is used $K_2Cr_2O_7$ and is added to the basic MS (Murashige and Skoog) medium. Each treatment was repeated 7 times, so that the number of research units is $5 \times 7 = 35$ banana plantlets in a culture bottle. All cultures are maintained at room temperature incubation at 25-28°C in the TL 1000 Lux light irradiation for 12 h day^{-1}. Maintenance is carried out for 6 weeks (Fig. 1).

Fig. 1: Plantlet of banana (*Musa acuminata*) cv. ambon lumut *in vitro* culture

Growth parameters: The growth rate is determined based on the formula of Fitter and Hay (1994). It was calculated as follows :

$$\text{Growth rate} = \frac{\Delta W}{\Delta t} = \frac{W_2 - W_1}{t_2 - t_1}$$

where, W_2 is the fresh weight of plantlets at t_2 (g), W_1 is the weight of fresh plantlets at t_1 (g), t_2 is the final observations and t_1 is the initial observations. The growth rate is expressed in g week^{-1}.

Gene expression levels of CAT and APX: This is done followed steps:

- Isolation of total RNA by the method of CTAB-$LiCl_2$ (Chang *et al*., 1993) were modified. Plant tissue was grinded using a mortar with the addition of liquid nitrogen. The RNA extraction is done by using the CTAB extraction buffer (CTAB 2% (w/v), 2 M NaCl, 200 mM EDTA, 100 mM tris-HCl (pH 8.0), PVP 2.5% (w/v), β-mercaptoethanol 2% (w/v) and spermidine 0.5 g L^{-1}). Total RNA extraction is done by adding a buffer CI (Chloroform: Isoamyl alcohol (24: 1, v/v)) and 3 M $LiCl_2$. The RNA quality was confirmed using a spectrophotometer to see the value of Optical Density (OD) at wave lengths of 230, 260 and 280 nm. Only RNA samples with a ratio of OD260/280 above 1.6 and the ratio of OD260/230 above 1.8 were analyzed further
- For qRT-PCR analysis, the sequence of CAT gene and APX gene derived from the research results Widiyanto *et al.* (2013). Based on the sequence of CAT and APX gene sequences, primer design is done by using the Primer 3 program (http://bioinfo.ut.ee/primer3-0.4.0), with the following results:

Gene name	Primer sequence
CAT	Left primer: GTCGTTCCGATCTGGTTGTT
	Right primer: GATCTCGGCAAGAAAAGCAC
APX	Left primer: CCACGTGGATTGAAGTTGTG
	Right primer: TTGCGGAGGACTAGATCGTT
18S rRNA	Left primer: AATTGTTGGTCTTCAACGAGGAA
	Right primer: AAAGGGCAGGGACGTAGTCAA

- Confirmation of the gene expression quantity was performed by qRT-PCR approach. First performed on all samples cDNA synthesis using thermo scientific revert aid first strand cDNA synthesis Kit. The reaction was performed by adding reaction mix (5X reaction buffer, ribolock RNase inhibitor (20 U μL^{-1}), 10 mM dNTP mix dan revert aid M-Mul V RT (200 U μL^{-1})) (Thermo scientific protocol). Proceed to the RT-PCR using SYBR green and cDNA markers that have been synthesized. Optimization of PCR reactions performed with annealing temperature confirm each sample and qRT-PCR primer using the thermo scientific maxima SYBR green qRT-PCR (Thermo scientific protocol). Reaction cycle was 50°C for 2 min, 95°C for 10 min, 95°C for 15 sec, reading 60°C for 30 sec, 72°C for 30 sec, reading the reaction cycle is repeated 40 times that at the start of the first reading, ended with a 4°C for 10 min. Primary housekeeping genes was used 18S rRNA gene primer. The qRT-PCR results (Ct) were analyzed using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistics analysis: Growth parameters were analyzed by looking homogeneity with Bartlett's test. If the result is homogeneous, the analysis was continued with analysis of variance (ANOVA) at significance level (α) of 0.05. Statistical processing is done with SPSS 18.

RESULTS

Chromium stress treatment influence on the growth rate of banana plantlets. The increase in the chromium concentration can reduced growth rates. Figure 2 and 3 show that the growth rate of banana planlets decreased, especially at concentrations of 200 and 400 ppm.

Visually in Fig. 3 can be seen that the height of plantlets decreased with increasing concentrations of Cr in the growth medium. The color of the leaves on the plantlets are grown in a medium with a concentration of 400 ppm aesthetically more yellow.

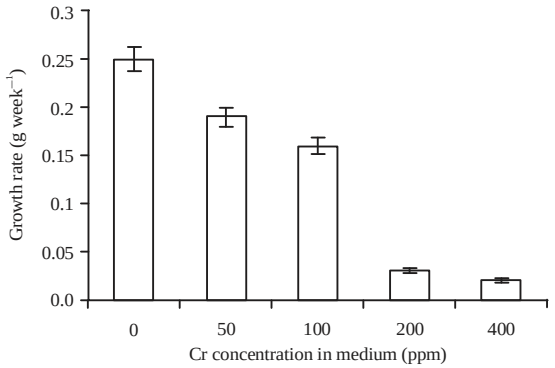

Fig. 2: Growth rate of banana plantlets (*Musa acuminata*) cv. ambon lumut (Significantly different with significant $<\alpha = 0.05$)

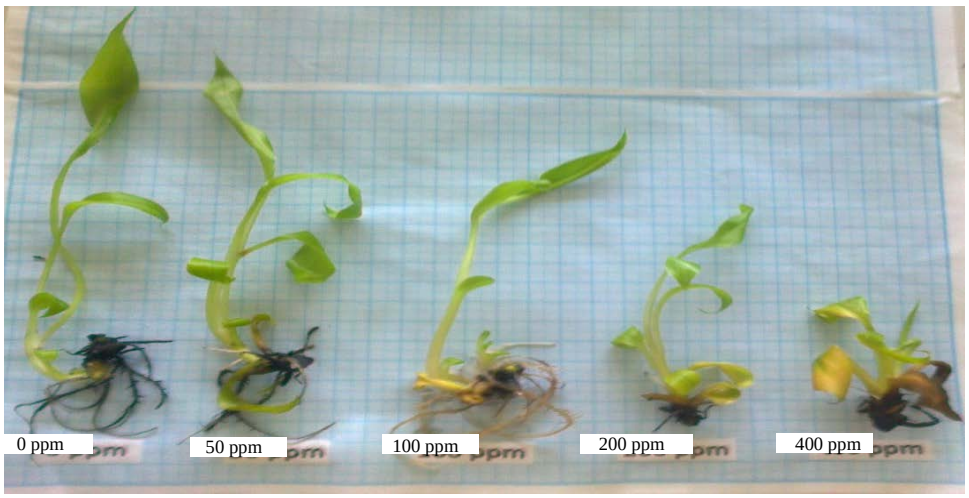

Fig. 3: Comparison the growth of banana plantlets (*Musa acuminata*) cv. ambon lumut, 6 weeks after treatment

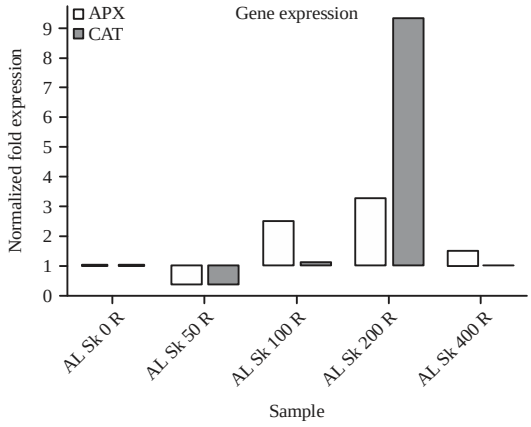

Fig. 4: Expression profiling of APX and CAT genes in roots of banana plantlets (*Musa acuminata*) cv. ambon lumut, 6 weeks after treatment

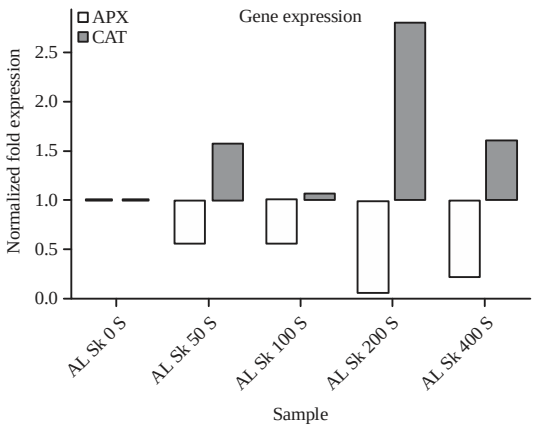

Fig. 5: Expression profiling of APX and CAT genes at the shoot of the banana plantlets (*Musa acuminata*) cv. ambon lumut, 6 weeks after treatment

Expression profiling of gene APX and CAT in the roots of banana plantlets (*Musa acuminata*) cv. ambon lumut after 6 weeks of treatment is shown in Fig. 4. In the banana plantlets with the treatment of 50 ppm, APX and CAT genes down-regulated experience, so it is lower than the control (0 ppm), whereas on other treatment seen these genes up-regulated experience. Especially in the treatment of 200 ppm, the level of gene expression of CAT reached more than 9 times compared to the control and APX gene expression level more than 3 times.

Gene expression profiling on the shoots was different from the roots. On the shoot, only CAT gene up-regulated in all treatments, while APX gene experience down regulated in all treatments. Figure 5 shows that, in the treatment of 200 ppm, the level of CAT gene expression is more than 2.5 times compared to the control.

Figure 6 showed a comparison of the level of CAT and APX genes expression in the roots and shoots. Both in the roots and in the shoots, the highest level of CAT gene expression at a concentration of 200 ppm, but at the roots higher than at the shoots.

DISCUSSION

Referring to the results presented in Fig. 2 and 3 that there has been a reduction in the growth rate of chromium treatment, this can be explained as follows. Reduction of weight is due to Cr affect various processes in the plant body, which is in the process of photosynthesis occurs inhibition of electron transfer, inactivation of Calvin cycle enzyme, reducing CO_2 fixation and chloroplasts disorganization (Shanker *et al*., 2005). With the disruption of this process, of course plant

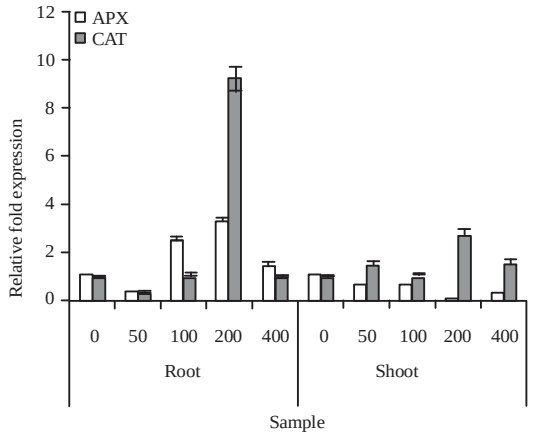

Fig. 6: Comparison of the APX and CAT genes expression level in roots and shoots of banana plantlets (*Musa acuminata*) cv. ambon lumut, 6 weeks after treatment

growth will also be affected. Likewise, treatment of various studies with Cr can be seen that all the distractions that arise, due to Cr influential in the production of Reactive Oxygen Species (ROS) that cause oxidative stress (Shanker *et al*., 2005; Panda and Choudhury, 2005; Subrahmanyam, 2008; Paolacci *et al*., 2009; Yildiz *et al*., 2012). The ROS react with a variety of fats, proteins, pigments, nucleic acids and cause lipid peroxidation, membrane damage and enzymes inactivity, thus affecting cell viability (Diwan *et al*., 2010). One important process is interrupted due to an increase in ROS is decreasing the rate of photosynthesis in various plant species. This is because the chloroplast is an important target of oxidative stress (Paolacci *et al*., 2009).

Meanwhile the results in Fig. 4 and 5 can be explained that level expression of APX and CAT genes were closely related to the production of final product, APX and CAT enzymes. From several studies that measure the activity of APX and CAT enzymes, APX enzyme activity increased by 33.3-109% at a concentration of 200-225 mM Cr compared to controls at the shoot of *Vigna radiata* (Diwan *et al*., 2010) and *Hordeum vulgare* (Yildiz *et al*., 2012). CAT enzyme activity was increased by 40.8% at a concentration of 225 mM (Yildiz *et al*., 2012).

When it compare the gene expression level of APX and CAT in Fig. 6, it can be see that both at the root and at the shoot, the highest level of CAT gene expression at a concentration of 200 ppm. But at the root higher than at the shoot, because the root is directly interact with the growing medium Cr contaminate, resulting in the accumulation of Cr generally is at the root. From the results of study on the *Leersia hexandra* Swartz, Cr accumulate in the root cell wall (Zhang *et al*., 2009; Liu *et al*., 2009). Oliveira (2012) also concluded that Cr mainly accumulated in roots, few were translocate to the leaves. It is put forward based on the study results in *Lolium perenne* that the roots accumulate Cr 10 times more than the leaves, study on some types of vegetables that can be known that the translocation of Cr running very slow and accumulation in root 100 times higher than the shoot, study on the bean which found that only 0.1% Cr accumulation, while at the root of 98% and the results of study on *Amaranthus viridis* and *Brassica oleracea* are known that Cr accumulated mainly in the roots. Likewise, the results of study Amalia *et al*. (2014) in *Musa acuminata* cv. ambon lumut, Cr content in the root of 15 times higher than in the shoots with 400 ppm Cr treatment. Thus, to overcome the increased production of ROS, antioxidant enzymes needed more, then the CAT gene expression is higher in the roots.

CONCLUSION

Based on the results of the study above we can be summarized as follows; (1) the growth rate of banana planlets decreased with increasing Cr concentrations in the growth mediums. (2) The level of CAT and APX genes expression were observed in the roots and shoots of banana plantlets. At the root, CAT and APX genes are expressed in all treatment conditions, except in the treatment of 50 ppm Cr. At the shoot, only CAT gene was expressed higher than the control and the highest level of CAT gene expression occurred in the treatment of 200 ppm. (3) The level of CAT and APX genes expression reached the highest level on the treatment of 200 ppm Cr concentration.

ACKNOWLEDGEMENT

This study was supported by grants Doctoral Programs of Directorate of Higher Education, Ministry of Research and Higher Education of the Republic of Indonesia. The authors thanks to Dr. Diky Setya Diningrat for technical supporting in the research.

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