Studies on the induction of oxidative stress by 4-*tert*-octylphenol in *Ceratophyllum demersum*

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對特辛基酚誘導金魚藻氧化逆境之研究

Studies on the induction of oxidative stress by 4-*tert*-octylphenol in *Ceratophyllum demersum*

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Studies on the induction of oxidative stress by 4-*tert*-octylphenol in *Ceratophyllum demersum*

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摘要

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論文摘要內容:

烷基酚聚氧乙烯醚 (APEOs) 屬介面活性劑，廣泛使用於各種工業與生活用品中，當排放入環境中會降解成具內分泌干擾特性之毒性代謝物，例如辛基酚、壬基酚，而對特辛基酚為已知普遍並穩定存在環境中之汙染物。為了探討對特辛基酚對水生植物的影響，本研究選用沉水植物金魚藻進行不同濃度 (0、0.5、1、1.5、 2與 3 mg/L) 對特辛基酚為期五天的試驗處理。對特辛基酚之毒性造成金魚藻成長之抑制、葉綠素含量下降並使活性氧族 (超氧陰離子、過氧化氫) 含量上升，顯示對特辛基酚毒性造成金魚藻之氧化逆境，然而 MDA 含量卻沒有顯著變化。抗氧化酵素活性方面，超氧化物歧化酶 (SOD)、愈創木酚過氧化物酶 (POD)、過氧化氫酶 (CAT)、穀胱甘肽還原酶 (GR) 和抗壞血酸過氧化物酶 (APX) 的活性均受對特辛基酚處理下而顯著上升；抗氧化物質方面，抗壞血酸 (AsA) 和穀胱甘肽 (GSH) 的含量也顯著增加，尤其是 GSH 含量尤為顯著。為了確認穀胱甘肽在金魚藻抵抗對特辛基酚所誘導之氧化逆境下所扮演之角色，試驗使用 GSH 生合成抑制劑 (BSO) 進行驗證。結果發現，使用 BSO 預處理後，金魚藻體內總 GSH 含量顯著降低，並伴隨著總 AsA 含量的降低，甚至 GR 和 APX 活性亦顯著下降，因而導致金魚藻在經過 BSO 預處理後暴露於對特辛基酚表現出更嚴重之氧化傷害。因此，金魚藻透過快速調節本身抗氧化系統，特別是穀胱甘肽的生合成，以抵抗對特辛基酚所誘導之氧化逆境。

關鍵字：對特辛基酚、金魚藻、氧化逆境、活性氧族、穀胱甘肽

ABSTRACT

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Alkylphenol ethoxylates (APEOs) are surfactants which have been widely used in a variety of commercial products and can be degraded in the aquatic environment becoming more toxic metabolites. 4-*tert*-octylphenol (OP) is one of the primary breakdown products of APEOs with endocrine disrupting properties that has been known as persistent and ubiquitos pollutant. In order to investigate the effect of OP toxicity to aquatic plant, the submersed macrophyte *Ceratophyllum demersum* was chosen to treat with various concentrations of OP (0, 0.5, 1, 1.5, 2 and 3 mg L^{-1}) for 5 days. The toxic effect and oxidative stress caused by OP resulted in an inhibition of growth rate, reduction of total chlorophyll content (chlorophyll *a* and *b*) and an increase in the levels of reactive oxygen species (ROS), $O_2^{\bullet-}$ and H_2O_2. However, there was no significant change in the content of malondialdehyde (MDA). The antioxidative enzyme activities showed a significant increase in superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), gluthathione reductase (GR) and ascorbate peroxidase (APX). The contents of non-enzymatic antioxidants, ascorbate (AsA) and glutathione (GSH), were also significantly increased under OP exposure. To confirm the role of GSH in *C. demersum* under OP exposure, BSO, a specific and potent inhibitor of GSH biosynthesis, was used. After BSO pretreatment, the total GSH content was significantly reduced. The decreasing of total GSH indicated that the synthesis of GSH has been blocked, it was followed by the decreasing of total AsA content and also GR and APX enzyme activity. Interestingly, *C. demersum* showed much more severe phenotype under OP exposure with BSO pretreatment. In conclusion, *C. demersum* might actively regulate the antioxidant machinery, especially GSH biosynthesis, to against OP-induced oxidative stress.

Keywords: 4-*tert*-octylphenol, *Ceratophyllum demersum*, oxidative stress, ROS, GSH

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1. INTRODUCTION

1.1 Background

Alkylphenol ethoxylates (APEOs) belong to the group of non-ionic surfactants, consisting of a branched chain alkylphenol which has been reacted with ethylene oxide, producing an ethoxylate chain (Renner, 1997). Commercial formulations are usually a complex mixture of homologues, oligomers and isomers. The main alkylphenols used are nonylphenol (NP) and octylphenol, with nonylphenol ethoxylate (NPnEO) taking approximately 80% of the world market, and octylphenol ethoxylate (OpnEO) taking the remaining 20% (White et al., 1994). These products have a large economic relevance and have been used in a wide range of domestic and industrial applications for more than 40 years, such as precursors to the detergents, emulsifiers, as additives for fuels and lubricants, polymers, and as components in phenolic resins as well as manufactured of agricultural chemical products (Renner, 1997; Staples et al., 1999; Oketola and Fagbemigun, 2013).

Recent year, there have been many concerns raised regarding the environmental safety of alkylphenol ethoxylate (APEOs) surfactant because it can be degraded in the aquatic environment becoming more recalcitrant and toxic metabolites. One of the biodegradation products from alkylphenol ethoxylate is 4-*tert*-octylphenol (OP), which can be degraded through photochemically and biologically processes (Ball et al., 1989; Ahel et al., 1994). Since first introduced in 1940s, alkylphenol compounds including OP has been detected in sediments, water, atmosphere and organisms (Van Ry et al., 2000; Chen et al., 2006; Zhang et al., 2008; Dong et al., 2014).

4-*tert*-octylphenol (OP) as prevalent environmental pollutant has been shown to possess intrinsic estrogenic activity, because it competes for binding to the estrogen receptor in higher organisms (Blake and Boockfor, 1997). In addition, it also can suppress growth, decrease photosynthetic pigments and destroy algal ultrastructure (Zhou et al., 2013). Moreover, OP has been reported that it can induce oxidative stress in *Arabidopsis thaliana* by modulating antioxidant enzymes like superoxide dismustase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Chen et al., 2013).

Oxidative stress defined as imbalance conditions in any cell compartment between production of reactive oxygen species (ROS) and antioxidant defense, leading to lipid peroxidation, resulting in damage to cell membranes, protein oxidation, enzyme inhibition, and strand breakage in nucleic acids that are caused by both biotic and abiotic factors (Allen, 1995).

In order to overcome oxidative stress situations, plants have developed elaborate mechanisms, composed by enzymatic and non-enzymatic antioxidant to detoxify these reactive oxygen species (ROS) (Asada, 1992; Li et al., 2008). Major ROS scavenging enzymes in plants include superoxide dismustase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6), ascorbate peroxidase (APX, E.C. 1.11.1.11) and glutathione reductase (GR, E.C. 1.8.1.7). SODs act as the first line of defense against ROS, dismutating superoxide radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) (Apel and Hirt, 2004), while CAT and several classes of peroxidases like APX, scavenge the H_2O_2 into H_2O and O_2 (Aravind and Prasad, 2003). Beside that, APX and GR are two key enzymes in ascorbate glutathione cycle, where APX detoxifies H_2O_2 by consuming ascorbate (AsA) and GR involves in the regeneration of glutathione (GSH) (Apel and Hirt, 2004). The non-enzymatic mechanisms include the major cellular redox buffers AsA and GSH, as well as phenolic compounds, tocopherol, flavonoids, alkaloids, and carotenoids that play a key role in delaying and/or preventing oxidative reactions catalyzed by free radicals (Apel and Hirt, 2004; Singh et al., 2010; Wang et al., 2011).

Although the availability and toxicity of 4-*tert*-octylphenol (OP) has attracted more attention recently, however, to our knowledge only one paper related to oxidative stress and antioxidant responses of plants under OP exposure has been published (Chen et al., 2013). The research related to the physiological effect of OP on aquatic plant is absent. Considering to the occurance of OP in aquatic environment, more attention need to be paid to the effect of OP on aquatic plant.

Aquatic plants have been known as bioindicator that can reflect the environmental conditions. *Ceratophyllum demersum* commonly known as hornwort or coontail is submerged, free-floating aquatic plant with cosmopolitan distribution. It is also well known for its ability to cope with various abiotic stresses (Rama Devi and Prasad, 1998; Sun et al., 2008; Duman et al., 2014). Thus, *C. demersum* is a good candidate for assessing the toxicity of 4-*tert*-octylphenol (OP). The results might provide some valuable information for applying to phytoremediation.

1.2 Objective

The work behind this thesis has been conducted by specific objectives, listed below:

1. To investigate the effect of 4-*tert*-octylphenol (OP) exposure on the alterations of ROS and oxidative stress in *Ceratophyllum demersum*
2. To investigate the effect of OP exposure on the antioxidant responses in *C. demersum.*

2. LITERATURE REVIEW

2.1 Alkylphenols

Alkylphenols, such as nonylphenol and octylphenol are the primary breakdown products from alkylphenol ethoxylates (APEOs). APEOs have been widely used groups of surfactants and manufactured for several commercial purposes (Renner, 1997). APEOs have been introduced in the middle of last century and applied in household products and in agricultural and industrial sector. In the market place, nonylphenol ethoxylates (NPEOs) account takes 80% of APEOs, while octylphenol ethoxylates (OPEs) takes 20% remaining. These products have a large economic relevance. All other alkylphenols are less useful, because the alkyl chain is either too long or too short for a surfactant function. The length of the ethoxylate chain varies between 4 to 20 ethoxy units, depending on the application. APEOs with 8-12 ethoxylates groups are commonly used. Mostly APEOs were used as the raw materials basis for cleaning and washing agent with the surfactant properties, such as foaming behaviour, wetting, dispersing, emulsifying and solubility increase. Most APEOs enter the aquatic environment after disposal in wastewater (White et al., 1994).

Degradation of alkylphenol ethoxylates (APEOs) in wastewater treatment plants or in the environment generates more persistent shorter-chain APEOs and alkylphenols (APs) that have been known can mimic natural hormones and that the levels present in the environment may be sufficient to disrupt endocrine function in wildlife (Blake and Boockfor, 1997; Routledge and Sumpter, 1997; Thiele et al., 1997; Ying et al., 2002).

Alkylphenol ethoxylates (APEOs) are unstable in aquatic environments with a half-life of several days and can be easily degraded to alkylphenols (AP) through photochemical and biochemical process (Yoshimura, 1986). AP such as nonylphenol (NP) and 4-*tert*-octylphenol (OP) are, however, much more stable in the environment with a half-life of approximately 2 months in water and lasting years in sediment (Ying et al., 2002).

2.2 4-*tert*-octylphenol (OP)

4-*tert*-octylphenol (OP) is an important industrial chemical that might pose for fresh and marine waters and sediments, waste water treatment plants (WWTP), soil, air and predatory wildlife. OP (CAS no. 140-66-9) is a high production-volume substance (Brooke et al., 2005). In Taiwan, during 2010-2013, the average of yearly production of nonylphenol (NP) and OP is 36,000 and 11,000 tons, respectively, according to data statisic Bureau of Foreign Trade of Taiwan (TWBOFT). Furthermore, Dong et al (2014) reported that both OP and NP were detected in the sediment of Kaohsiung Harbor, Taiwan and probably pose a potential ecological risk to aquatic life.

The 4-*tert*-octylphenol (OP) is a solid substance (melting point 79-82ºC, boiling point 280-283ºC). It has a vapour pressure of 0.21 Pa at 20ºC, a water solubility of 19 mg L^{-1} at 22ºC and a log octanol–water partition coefficient (log K_{ow}) of 4.12. The log K_{ow} implies a moderate bioaccumulation potential in aquatic biota and the substance mainly partitions to soil and sediment when it is released to the environment (Brooke et al., 2005). OP or 4-(1,1,3,3-tetramethylbutyl) phenol is the primary manufactured isomer with a general formula $C_6H_4(OH)C_8H_{17}$ (Fig. 1), made by alkylating phenol with diisobutylene. Mixtures of OP ethoxylates are often used as detergents, such as Triton X-100 (Blake and Boockfor, 1997).

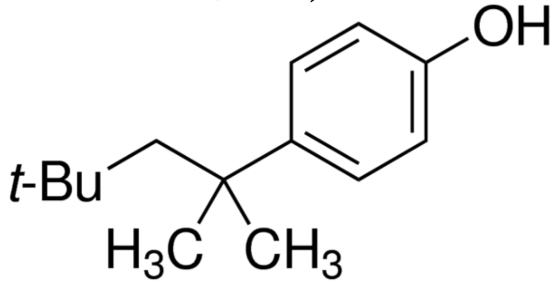


Fig. 1. Molecular structure of 4-*tert*-octylphenol (OP) (Sigma-aldrich).

2.3 Production and Uses of 4-*tert*-octylphenol (OP)

There are two main routes used in the production of 4-*tert*-octylphenol (OP), both of which involve the reaction of phenol and tert-octene (di-isobutene) in the presence of:

a) an ion-exchange resin or boron trifluoride complex in a batch reactor; or

b) a fixed bed ion-exchange resin in a continuous process.

The tert-octene is produced by dimerisation of isobutene which ensures that the octene is branched rather than linear. The purity of isobutene also means no other homologues are expected. Reaction with phenol leads predominantly to substitution by tert-octene in the 4- (para-) position (see Fig. 1). In the first process, the neutralised and/or deactivated catalyst is disposed of via authorised waste facilities in accordance with existing regulations; in the second process, it is discharged directly into an incineration plant (Brooke et al., 2005).

Overall, 4-*tert*-octylphenol (OP) has two main direct uses:

- the production of phenol-formaldehyde resins (or phenolic resins) (and their subsequent derivatives); and
- the production of octylphenol ethoxylates (OPEs) (and their subsequent derivatives).

OP has been widely used in domestic and industrial applications, such as rubber industry; paints, printing inks and coatings industry; chemical industry in emulsion polymerisation and emulsion polymer manufacture; use of ethoxylated resins in the oil industry; textile and leather industry as finishing agents, plant protection and animal health products industry. Other potential uses of OP, include lubricant additives, adhesives, cleaning products, metal cleaning applications, fragrances, pharmaceuticals, the foundry industry, paper coatings, fuel oil stabilisers and injection moulding (Renner, 1997; Blake and Boockfor, 1997; Soares et al., 2008).

2.4 Environmental Fate and Distribution

Based on a log K_{ow} of 4.12, the organic carbon–water partition coefficient (K_{oc}) for 4-*tert*-octylphenol (OP) is estimated as 2740 l/kg (Brooke et al., 2005). However, it is an indication for a high tendency to adsorb at organic material. Johnson et al. (1998) studied the sorption of OP to different river sediments using laboratory batch techniques. The study predicted that suspended sediments might also play a key role in the fate of OP in industrialised areas. In the rural areas a higher proportion OP might be predicted to remain free in solution.

4-*tert*-octylphenol (OP) is a weak acid, because of this pH might have an effect on its adsorptive behaviour. The pKa is thought to be around 10. Hence, in the environment, the substance will be present in the un-dissociated and more hydrophobic form (Brooke et al., 2005). OP is of low volatility and low water solubility, and will sorb strongly to organic matter in soils, sediments and sludges. Degradation processes within these media (biotic and abiotic) are predicted to be relatively slow. If released directly to the atmosphere, degradation occurs rapidly through hydroxyl radical attack. The potential for bioaccumulation in aquatic organisms is expected to be low to moderate (Brooke et al., 2005; Renner, 1997).

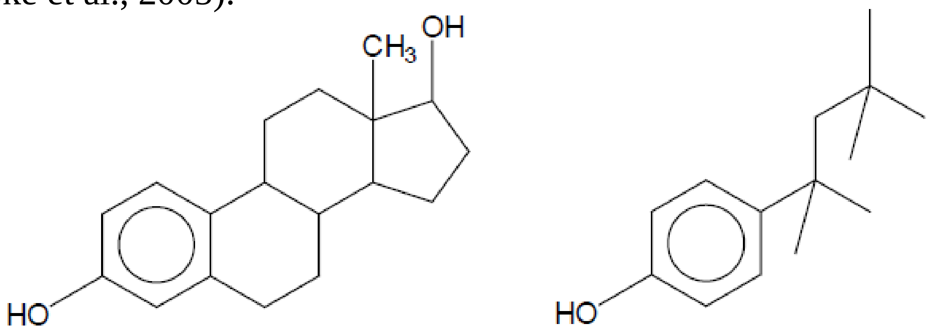
4-*tert*-octylphenol (OP) is not produced naturally. It presence in the environment is solely a consequence of anthropogenic activity. OP enter the environment primarily via industrial and municipal wastewater treatment plant effluents (liquid and sludge), but also due to direct discharge such as through pesticide application (Ying et al., 2002). Many researchers have shown that OP widely exist in various mediums of water environment, such as water, sediments and biological bodies (Chen et al., 2006; Höhne and Püttmann, 2008;

Zhang et al., 2008; Oketola and Fagbemigun, 2013; Dong et al., 2014) especially the sediments, which play the role of storing OP.

2.5 Effect of 4-*tert*-octylphenol (OP) to Organisms

4-*tert*-octylphenol (OP) is known as endocrine disruptors, which possess the ability to mimic natural estrogens and disrupt the endocrine systems of higher organisms by interacting with the estrogen receptor (Blake and Boockfor, 1997). Gray et al. (1999) concluded that exposure to OP during early development through to maturity negatively affected the reproductive performance of male medaka as a result of reductions in courtship intensity and fertilisation rates.

4-*tert*-octylphenol (OP) may exert its effects on organisms by more than one mode of action. Endocrine-mediated responses, on the other hand, are most likely to be mediated by a specific mechanism, and the majority of the data for this substance point towards interference and/or competition with the binding of natural estrogens (such as 17β-estradiol) to receptor sites and mimicry of their effects (i.e., an estrogen agonist). There are some structural similarities between OP and certain hormones (see Fig. 2), and OP has been demonstrated to bind to the estrogen receptor in almost exactly the same way as estradiol (Brooke et al., 2005).



Estradiol

4-*tert* octylphenol (OP)

Fig. 2. Structures of hormone estradiol and 4-*tert*-octylphenol (OP)

In addition, 4-*tert* octylphenol (OP) exposure also can suppress growth, decrease photosynthetic pigments and destroy algal ultrastructure (Zhou et al., 2013). Moreover, OP concentrations higher than 0.062 mg L^{-1}, was shown to decrease by 50% growth of *Microcystis aeruginosa, Pseudokirchneriella subcapitata* (formerly named *Selenastrum capricornutum*) and *Scenedesmus subspicatus* (Baptista et al., 2009); European Commission, 2005). In terestrial plant, *Arabidopsis thaliana* and *Gypsophila elegans*, OP has been reported that can reduce the mean length of roots start at concentration 0.1 and 4.25 mg L^{-1}, respectively (Sinkkonen, et al., 2011; Chen et al., 2013). Moreover, OP has been reported that it can induce oxidative stress in *Arabidopsis thaliana* by modulating antioxidant enzymes like APX, CAT and SOD (Chen et al., 2013).

2.6 Oxidative Stress and Antioxidant System in Plant

2.6.1 Oxidative Stress in Plant

Plants are frequently exposed to a plethora of unfavorable or even adverse environmental conditions, termed abiotic stresses (Fig. 3) such as salinity (Hasanuzzaman et al., 2011a; 2011b; Hossain et al., 2011), drought (Selote and Khanna-Chopra, 2010; Hasanuzzaman and Fujita, 2011), heat (Chakraborty and Pradhan, 2011; Rani et al., 2013), cold (Yang et al., 2011; Zhao et al.,2009), flooding (Li et al., 2011), heavy metal toxicity (Hossain et al., 2010; Wang et al., 2012); UV-radiation (Kumari et al., 2010; Li et al., 2010; Ravindran et al., 2010) and ozone (Yan et al., 2010a; Yan et al., 2010b).

Abiotic stress leads to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Zezulka et al., 2013). Abiotic stresses modify plant metabolism leading to harmful effects on growth, development and productivity. If the stress becomes very high and/or continues for an extended period it may lead to an intolerable metabolic load on cells, reducing growth, and in severe cases, result in plant death (Hasanuzzaman et al., 2012).

Fig. 3. Different types of abiotic stress in plants (Hasanuzzaman et al., 2012).

There are several forms of reactive oxygen species (ROS) including free radicals such as superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and non-radical (molecular) forms: hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Bartosz, 1997). In plants, ROS are unavoidable by-products of aerobic metabolism being produced in various cellular compartments (see Fig. 4) like chloroplasts, mitochondria, and peroxisomes (Gupta and Igamberdiev, 2015). Production and removal of ROS must be strictly controlled. However, the equilibrium between production and scavenging of ROS may be perturbed by a number of adverse abiotic stress factors such as high light, drought, low temperature, high temperature, and mechanical stress (Apel and Hirt, 2004).

In chloroplasts $O_2^{\bullet-}$ production takes place at PSI and PSII; it is converted by SOD to H_2O_2 (Gupta and Igamberdiev, 2015). In peroxisomes, glycolate oxidase (GO), acyl-CoA oxidase and xanthine oxidase (XO) are major sites of ROS production, SOD is scavenger. On the other hand, the generation of $O_2^{\bullet-}$ involves both the reaction of xanthine oxidase (XO) in the organelle matrix and a small electron transport chain at the peroxisomal membrane level (Hasanuzzaman et al., 2012). The plant mitochondrial electron transport chain is also an important source of ROS production in plant cells and consists of several dehydrogenase complexes that reduce a common pool of ubiquinone (Q). ROS production is likely to occur mainly in complex I (NADH dehydrogenase) and complex III (Møller 2001 ; Blokhina et al. 2003). Although mitochondrial ROS production is much lower compared to chloroplasts, mitochondrial ROS are important regulators of a number of cellular processes, including stress adaptation and PCD (Robson and Vanlerberghe, 2002). In glyoxysomes, acyl-CoA oxidase is the primary enzyme responsible for the generation of H_2O_2. Plasma membrane-bound NADPH oxidases (NADPHox) as well as cell-wall associated peroxidases (POX) are the main sources of $O_2^{\bullet-}$ and H_2O_2 producing apoplastic enzymes activated by various forms of stress (Mittler, 2002; Mhamdi et al., 2010).

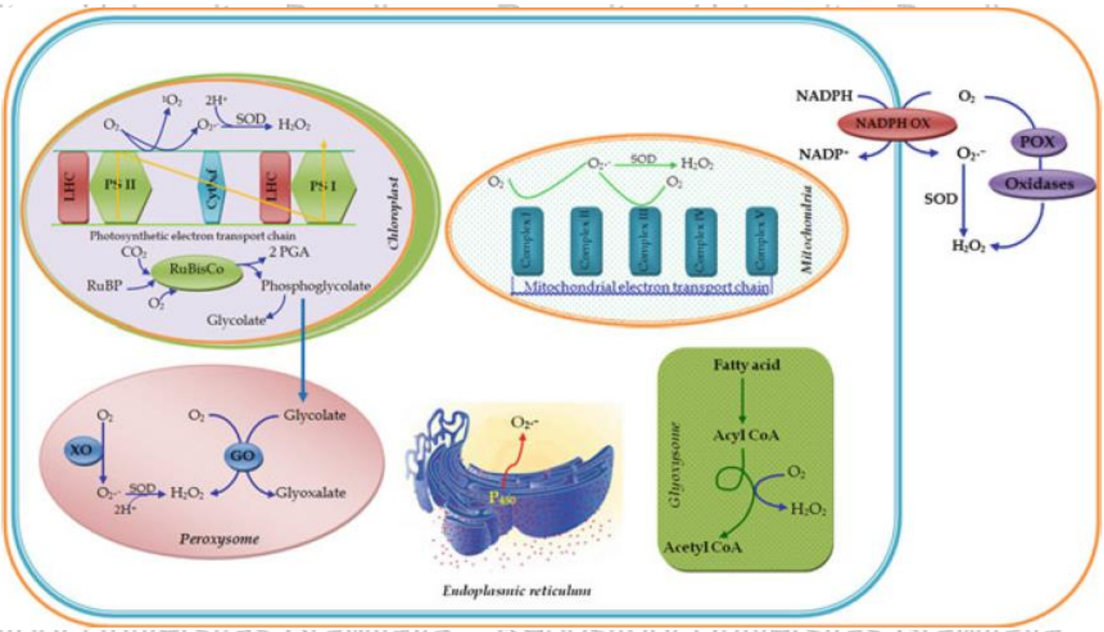


Fig. 4. ROS-generating pathways in various compartments of plant cell (Hasanuzzaman et al., 2012).

2.6.2 Detoxification of ROS by the Antioxidant Defense System

Certain environmental stresses or genetic defects cause the production of ROS to exceed the management capacity. ROS play two divergent roles in plants: at low concentrations, they act as signaling molecules for the activation of defense responses under stresses, whereas at high concentrations, they cause exacerbating damage to cellular components. If prolonged, abiotic stresses, through enhanced production of ROS, can pose a threat to cells by causing the peroxidation of lipids, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of the programmed cell death (PCD) pathway and ultimately cell death (Sies and Cadenas, 1985; Apel and Hirt, 2004; Møller et al., 2007).

An imbalance between the excess production of ROS and the ability of the organisms to counteract or detoxify their harmful effects through neutralization by antioxidants defined as oxidative stress (Bartosz, 1997; Demidchik, 2015). Plants have developed elaborate mechanisms to withstand an oxidative stress. These mechanisms can be conveniently divided into two groups, viz. non-enzymatic and enzymatic antioxidants (Apel and Hirt, 2004; Gupta and Igamberdiev, 2015).

Plants possess an efficient non-enzymatic antioxidants, such as ascorbate and glutathione and enzymatic antioxidants, including superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; glutathione peroxidase, and peroxidases, POD as defense systems which work in concert to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging ROS (see Fig. 5) (Mittler et al., 2004; Gill and Tuteja, 2010).

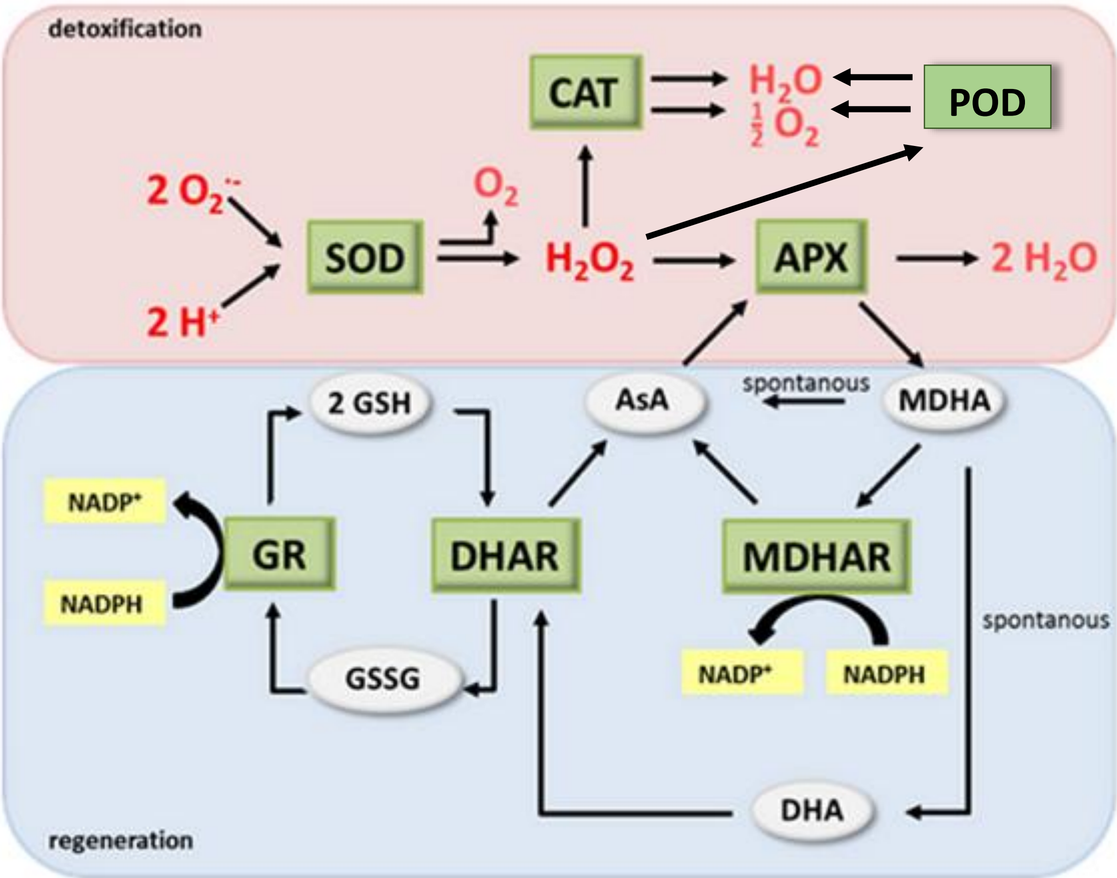


Fig. 5. Mechanisms of ROS detoxification by different antioxidant enzymes (Groß et al., 2013).

2.6.2.1 Non-enzymatic Antioxidants

a. Ascorbate (AsA)

Ascorbate (AsA) is an important antioxidant in plant tissues which is synthesized in the cytosol of higher plants primarily from the conversion of D-glucose to AsA. AsA is present in all subcellular compartments, including the apoplast (cell wall), chloroplasts, cytosol, vacuoles, mitochondria, and peroxisomes (Rautenkranz et al., 1994; Foyer and Lelandais, 1996; Jimenez et al., 1997). It reacts with a range of ROS such as H_2O_2, $O_2^{\cdot-}$ and $^1O_2$, which are the basis of its antioxidant action. AsA, the terminal electron donor in these processes, scavenges free radicals in the hydrophilic environments of plant cells. It also scavenges OH• at diffusion-controlled rates (Yu, 1994). In the

AsA-GSH cycle, two molecules of AsA are utilized by APX to reduce H_2O_2 to water with the concomitant generation of monodehydroascorbate reductase (MDHA). MDHA is a radical with a short life span that can disproportionate into dehydroascorbate (DHA) and AsA. The electron donor is usually NADPH and the reaction is catalyzed by MDHAR or ferredoxin in a water–water cycle in the chloroplasts (Asada, 1992; 1997).

In plant cells, the most important reducing substrate for the removal of H_2O_2 is AsA (Wu et al., 2007). AsA is also thought to maintain the reduced state of the chloroplastic antioxidant, α-tocopherol. AsA in plants may be involved in the synthesis of zeaxanthin, which dissipates excess light energy in the thylakoid membranes, preventing oxidative damage (Conklin et al., 1996). Improvement of ascorbate content in plants will increase plant stress tolerance, while decreasing ascorbate content will result in stress sensitivity of plants (Zhang, 2013).

b. Glutathione (GSH)

Glutathione (GSH) is a multifunctional water-soluble tripeptide containing a sulfhydryl (-SH) group and is a substrate for DHAR in the AsA-GSH pathway. GSH is an abundant metabolite in plants which directly scavenges OH• and $^1O_2$ and may protect enzyme thiol groups and also known to involve in signal transduction in virtually all cellular components such as chloroplasts, mitochondria, endoplasmic reticulum, vacuoles, and cytosol (Noctor and Foyer, 1998; Gill et al., 2013). Additionally, GSH detoxifies herbicides by conjugation, either spontaneously or by the activity of a glutathione-S-transferase, and also regulates gene expression in response to environmental stress and pathogen attack (Noctor et al., 2002). Furthermore, GR catalyzes the NADPH-dependent formation of a disulphide bond in glutathione disulphide (GSSG) and is thus important for maintaining the reduced pool of GSH. Together, GSH and GR perform the scavenging of ROS and its reaction products; thereby provide tolerance to stress-exposed plants (Gill et al., 2013). Other functions of GSH include the formation of phytochelatins (PCs), which have an affinity to heavy metal and are transported as complexes into the vacuole, thus allowing plants to have some level of resistance to heavy metal (Wang et al., 2012). The role of GSH in the antioxidant defense systems provides a strong basis for its use as a stress marker. The change in the ratio of its reduced (GSH) to oxidized (GSSG) form during the degradation of H_2O_2 is important in certain redox signaling pathways (Bloem et al., 2015).

2.6.2.2 Enzymatic Antioxidants

a. Superoxide Dismustase (SOD)

In plant cells, SODs are considered the first line of defense against damage by the superoxide radical. It removes $O_2^{\bullet-}$ by catalyzing its dismutation, one $O_2^{\bullet-}$ being reduced to H_2O_2 and another oxidized to O_2. SODs occur in different isoforms with different metal cofactors, namely copper and zinc (Cu/ZnSOD), manganese (MnSOD), and iron (FeSOD). Cu/ZnSOD is localized in the cytosol and chloroplasts, MnSOD in the matrix of mitochondria and peroxisomes, and FeSOD in the chloroplasts of some higher plants, but they are also generally found in prokaryotes (Scandalios, 1993; Elavarthi and Martin, 2010).

b. Catalase (CAT)

Catalase (CAT) is a tetrameric hemecontaining enzyme that is found in all aerobic organisms and serves to rapidly degrade H_2O_2. CATs are present in peroxisomes, glyoxysomes, and related organelles where H_2O_2-generating enzymes are located (Agrawal et al., 2009). CAT has one of the highest turnover rates of all enzymes: one molecule of CAT can convert around six million molecules of H_2O_2 to H_2O and O_2 per minute. Thus, CAT is important in removing H_2O_2, which is generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration, and purine catabolism (Gill and Tuteja, 2010). It has also been reported that apart from its reaction with H_2O_2, CAT also reacts with some hydroperoxides (Willekens et al., 1995).

c. Ascorbate-Glutathione (AsA-GSH) Cycle Enzymes

The AsA–GSH cycle is the major defense system against ROS in chloroplasts, cytosol, mitochondria, peroxisomes and apoplasts. The AsA–GSH cycle involves four enzymes (APX, MDHAR, DHAR and GR) as well as AsA, GSH and NADPH which work together to detoxify H_2O_2 in a series of cyclic reactions and further regenerate AsA and GSH. In this cycle APX catalyses the reduction of H_2O_2 to H_2O with the simultaneous generation of monodehydroascorbate (MDHA), which is converted to AsA by the action of NADPH-dependent MDHAR or disproportionates nonenzymatically to AsA and dehydroascorbate (DHA) (Mittler, 2002). DHA undergoes irreversible hydrolysis to 2, 3-diketogulonic acid or is recycled to AsA by DHAR, which uses GSH as the reductant (Chen et al., 2003). This results in the generation of GSSG, which is regenerated to GSH by GR.

- **Ascorbate Peroxidase (APX)**

  The scavenging of H_2O_2 by APX is the first step of the AsA-GSH cycle and may play the most essential role in scavenging ROS and protecting cells in higher plants of electrons from AsA to H_2O_2, producing DHA and water (Raven, 2002). The APX family consists of at least five different isoforms including mitochondrial (mAPX), thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Noctor and Foyer, 1998).

- **Glutathione Reductase (GR)**

Glutathione reductase (GR) is a potential enzyme of the AsA-GSH cycle and plays an essential role in the defense system against ROS. Increased GR activity confers stress tolerance and has the ability to alter the redox state of important components of the electron transport chain. This enzyme catalyzes the reduction of GSH, involved in many metabolic regulatory and antioxidative processes in plants where GR catalyses the NADPH-dependent reduction of disulphide bond of GSSG and is thus important for maintaining the GSH pool (Yousuf et al., 2012). Thus, GR also maintains a high ratio of GSH/GSSG in plant cells, also necessary for accelerating the H_2O_2 scavenging pathway, particularly under stress conditions (Gill et al., 2013).

d. Guaiacol Peroxidase (POD)

Guaiacol peroxidases (PODs) are involved in many physiological processes in plants, involving responses to biotic and abiotic stresses and the biosynthesis of lignin. Lignin is a polymer responsible for rendering the plant stronger and more rigid and also making the cell walls hydrophobic. Peroxidases are involved in the polymerization of the precursors of lignin. They are also involved in the scavenging of reactive oxygen species (ROS), which are partially reduced forms of atmospheric oxygen, highly reactive, and capable of causing oxidative damage to the cell. POD can be a source of hydrogen peroxide (H_2O_2) but also are capable of scavenging it (Vicuna, 2005). POD can decompose H_2O_2 become water and oxygen. It is predominantly located in the cytosol, cell wall, vacuolar and extracellular spaces (Mishra et al., 2006).

2.7 *Ceratophyllum demersum*

Ceratophyllum demersum L. (hornwort or coontail) grows fast in shallow, muddy, quiescent water bodies at low light intensities (Aravind and Prasad, 2005). It is a submerged, rootless, free floating, perennial and is cosmopolitan in distribution (Fig. 6). This submerged macrophyte has a high capacity for vegetative propagation and biomass production even under the modest nutritional conditions. It is useful as an oxygenator for use in the Closed Equilibrated Biological Aquatic System (CEBAS) (Chorom et al., 2012). *C. demersum* can be biofilter for heavy metals, such as Cd (Aravind and Prasad, 2005), Pb (Mishra et al., 2006) and Ni (Chorom et al., 2012).

Some studies have been reported that *C. demersum* is tolerant to oxidative stress due to different abiotic stress, such as heavy metals (Rama Devi and Prasad, 1998; Mishra et al., 2006; Chorom et al., 2012), organic contaminants (Menone and Pflugmacher, 2005; Yin et al., 2008) and brominated flame retardant (Sun et al., 2008) through activation of antioxidant system.

Fig. 6. *Ceratophyllum demersum* (Wikipedia).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Plants

Ceratophyllum demersum were collected from Pingtung Agricultural Biotechnology Park (PABP). Before 4-*tert*-octylphenol (OP) treatments, plants (3 cm tip portion) were acclimatized in aquaria for 1 week under laboratory conditions (55 µmol m^{-2} s^{-1} light with 12 h photoperiod at 25 ± 2°C) in 10% Hoagland's solution (Appendix I) (Hoagland and Arnon, 1950). The solutions were refreshed everyday during acclimation periods and the pH value was maintained at 6.5.

3.1.2. 4-*tert*-octylphenol (OP) Preparation

4-*tert*-octylphenol (OP) was purchased from Sigma-Aldrich, USA. The stock solution was prepared in DMSO at a concentration of 20,000 mg L^{-1} and stored at 4°C in the dark.

3.2. Experimental Design

The experimental design was described by the flow chart as shown in Fig.7 and the detail procedures were as follows: plants were exposed to different concentration of OP (0, 0.5, 1, 1.5, 2 and 3 mg L^{-1}) after acclimation for 1 week. The stock solution of each compound was spiked into 300 ml 10% hoagland solution in 500 ml glass beaker according to the concentrations. The plants maintained under above mentioned laboratory conditions and keep in the growth chamber (FIRSTEK, GC-101). The experiments were conducted in triplicate and the density of each replicate was 1 g/300 ml. The toxicity of DMSO to *C. demersum* was checked and no observed effect concentration of

DMSO was determined to be 0.025% (v/v) to the growth rate and lipid peroxidation from the preliminary experiment.

All glass beakers received a 100% solution exchange for every 24 h in the 5 days exposure. The pH of all solutions were maintained at 6.5 at all treatments and fresh weight of the plants were measured everyday. The relative growth rate (RGR) for 5 days cultivation was calculated using the following equation (Watanabe et al., 2000):

$$\text{Relative Growth Rate (RGR)} = (\ln W_t - \ln W_0)/(t_t - t_0)$$

where W_t is the fresh weight at time t_t, W_0 is the initial fresh weight at the beginning of the treatment. After the experimental treatments, leaves were harvested, rinsed with distilled water, blotted and stored at -80°C for the further analysis.

Further experiment were conducted using BSO, a specific and potent inhibitor of ɣ-ECS, the first enzyme that play role in biosynthesis of GSH to confirm the involvement of GSH in *C. demersum* defense mechanism under OP exposure. The leaves of *C. demersum* pretreated with 0.5 mM BSO, for 8 h (Chao et al., 2011). After 8h, the solution were renewed and the leaves were treated with and without 3 mg L^{-1} OP for 5 days. For chemical preparation and the detailed analysis procedure, please refer to Appendix II-IV.

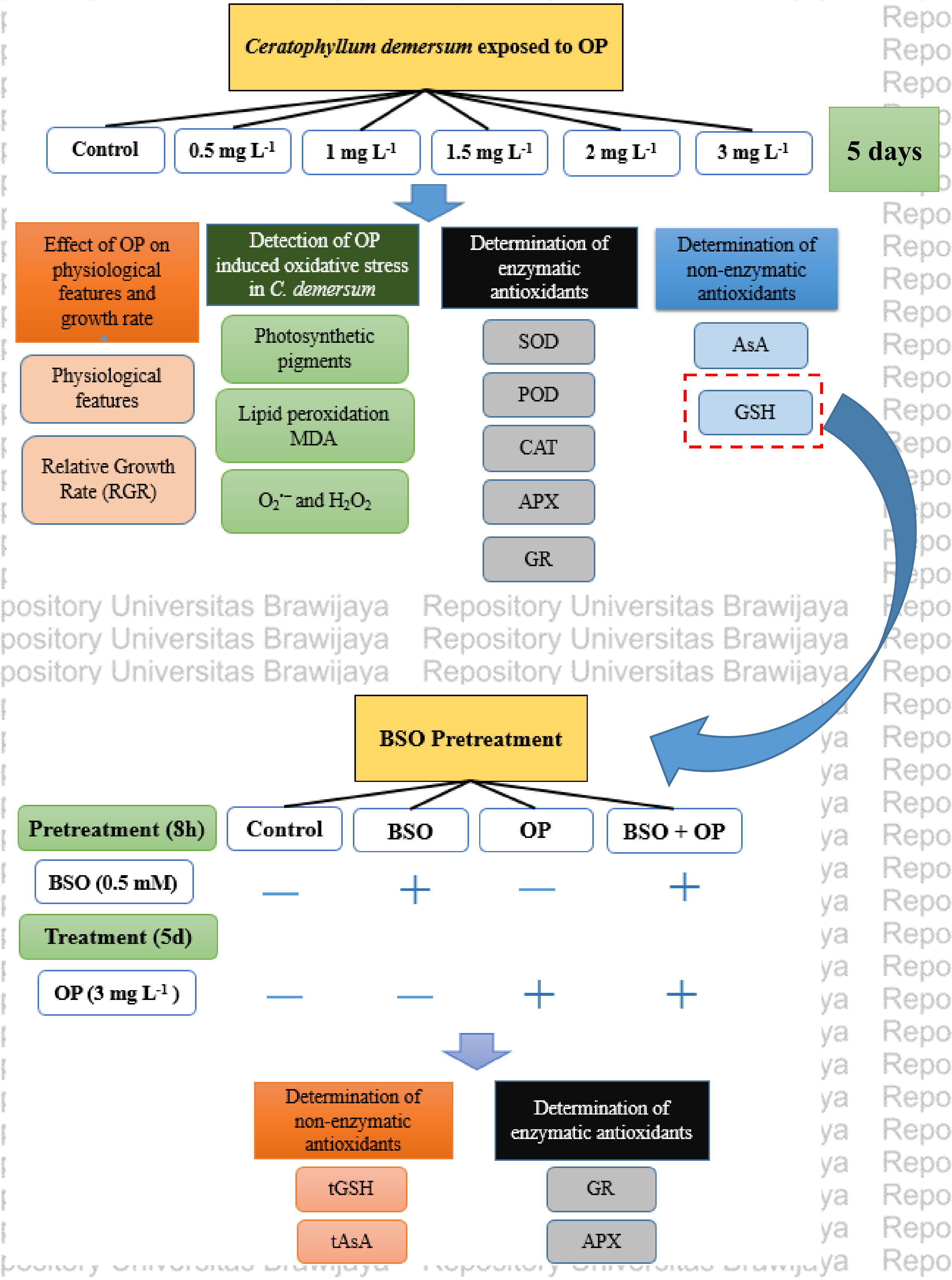


Fig. 7. The flow chart of experimental design.

3.3. Biochemical analysis

3.3.1. Determination of photosynthetic pigments

A 0.1 g leaves sample was extracted in 4 ml extraction buffer (sodium phosphate buffer 50 mM, pH = 6.8) under 4°C. The homogenate was taken 40 µl and added 960 µl ethanol (100%), and was mixed together. The mixture was put in the dark chamber at 4°C for 30 minutes and centrifuged at 1000 g for 15 minutes under 4°C. The absorbance of the supernatant was measured at 649 and 665 nm and calculated the chlorophyll content using these formula according to Wintermans and De Mots (1965) :

Chlorophyll *a* $= (13.7 \times A_{665}) - (5.76 \times A_{649})$ [µg Chl $(40\ \mu l)^{-1}$]

Chlorophyll *b* $= (25.8 \times A_{649}) - (7.6 \times A_{665})$ [µg Chl $(40\ \mu l)^{-1}$]

Total Chlorophyll $= (6.1 \times A_{665}) + (20.04 \times A_{649})$ [µg Chl $(40\ \mu l)^{-1}$]

Chlorophyll *a* content (mg g^{-1} FW)

=Chlorophyll *a* × 50 (dilution) ÷ 1000 ÷ FW (g)

Chlorophyll *b* content (mg g^{-1} FW)

=Chlorophyll *b* × 50 (dilution) ÷ 1000 ÷ FW (g)

Total Chlorophyll content (mg g^{-1} FW)

=Total Chlorophyll × 50 (dilution) ÷ 1000 ÷ FW (g)

3.3.2. Determination of Lipid Peroxidation (MDA contents)

Lipid peroxidation was expressed as MDA content. MDA content was determined according to the method decribed by Heath and Packer (1968). Briefly, a 0.1 g leaves sample was extracted in 1 ml 5% trichloroactic acid (TCA) under 4°C. The homogenate was centrifuged at 12,000 g for 10 min under 4°C. A sample of 0.5 ml of the supernatant was mixed with 2 ml of 20% TCA containing 0.5% thiobarbituric acid (TBBA). The mixture was incubated at 95°C for 30 min and the reaction was terminated after transferred to ice box. The 2 ml of the above reaction solution was taken and centrifuged at 12,000 g for 10 min under 4°C. The amount of MDA content was calculated from the difference in absorbance at 532 nm and 600 nm using an extinction coefficient of 155 mM^{-1} cm^{-1}.

3.3.3. Determination of Hydrogen Peroxide (H_2O_2) Activity

The level of H_2O_2 was determined according to Jana and Choudhuri (1981). Briefly, a 0.1 g leaves sample was extracted in 2 ml 50 mM sodium phosphate buffer pH 6.8 containing 1 mM hydroxylamine under 4°C. The homogenate was centrifuged at 12,000 g for 10 min under 4°C. A sample of 0.5 ml of the supernatant was mixed with 0.5 ml $TiCl_4$ [Titanium Chloride (0.1%, v/v) diluted in 20% (v/v) H_2SO_4] and centrifuged at 12,000 g for 10 min under 25°C. The absorbance of supernatant was measured at 410 nm. The content of H_2O_2 was calculated using an extinction coefficient of 0.28 μmol^{-1} cm^{-1}

3.3.4. Determination of Superoxide Radical ($O_2^{\bullet-}$) Activity

The level of $O_2^{\bullet-}$ was determined according to Panda (2007) and Elstner and Heupel (1976). Briefly, a 0.1 g leaves sample was extracted in 1 ml 65 mM sodium phosphate buffer pH 7.8 under 4°C. The homogenate was centrifuged at 12,000 g for 20 min under 4°C. A sample of 0.5 ml of the supernatant was mixed with 0.45 ml 65 mM sodium phosphate buffer pH 7.8 and 0.05 ml 10 mM hydroxylamine. The mixture was put under 25°C for 20 minutes. After 20 minutes, 0.5 ml of the mixture was taken and mixed with 0.5 ml 17 mM sulfanilic acid and 0.5 ml 7 mM α-naphtylamine, the mixture was put under 25°C for 20 minutes. After 20 minutes, 0.7 ml of the mixture was taken and mixed with 0.7 ml ether and centrifuged at 1,500 g for 5 min under 25°C. The absorbance of supernatant was measured at 530 nm. The content of $O_2^{\bullet-}$ was calculated according to the standard curve generated using different concentration of sodium nitrite (0, 1, 2, 5, 10 and 20 µM).

3.4. Effect of 4-*tert*-octylphenol (OP) on Enzymatic Antioxidant

3.4.1. Spectrophotometric measurement

A 0.1 g sample was extracted in 1 ml 50 mM sodium phosphate buffer (pH 7) containing 2 mM Na_2EDTA and 1 mM PMSF, with the addition of 0.5 mM ascorbate for the APX assay. The homogenate was centrifuged at 12,000 g for 10 minutes under 4°C at least 3 times and the supernatant used for the enzyme assays. Protein contents were determined following the methods described by Bradford (Bradford, 1976), using bovine serum albumin as standard and measured the absorbance at 595 nm using ELISA reader (Spectramax 190, Kim Forest Enterprise). The average protein content that was used for spectrophotometric measurements was 0.2 µg μl^{-1}.

3.4.1.1 Ascorbate Peroxidase (APX; EC 1.11.1.11) Assay

APX activity was determined as described by Nakano and Asada (1981). Briefly, 1 ml reaction solution contained 0.1 ml supernatant, 50 mM sodium phosphate buffer (pH 7.0), 1.5 mM Na_2EDTA, 0.5 mM Ascorbate, 0.25 mM H_2O_2. The decrease in absorbance at 290 nm was measured and activity was calculated using the extinction coefficient $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. One unit of APX activity was defined as the amount required to decompose 1 nmol ascorbic acid min^{-1} mg $protein^{-1}$.

3.4.1.2 Catalase (CAT; EC 1.11.1.6) Assay

CAT activity was determined as described by Kato and Shimizu (1987). Briefly, 1 ml reaction solution contained 0.1 ml supernatant, 25 mM sodium phosphate buffer (pH 7.0) and 20 mM H_2O_2. The decrease in absorbance at 240 nm was measured that accompanied the consumption of H_2O_2 and activity was calculated using the extinction coefficient $\varepsilon = 40\ mM^{-1}\ cm^{-1}$. One unit of CAT activity was defined as the amount required to decompose 1 nmol H_2O_2 min^{-1} mg $protein^{-1}$.

3.4.1.3 Glutathione Reductase (GR; EC 1.8.1.7) Assay

GR activity was determined as described by Sgherri et al. (1994). Briefly, 1 ml reaction solution contained 0.1 ml supernatant, 0.2 M sodium phosphate buffer (pH 7.5), 0.2 mM Na_2EDTA, 1.5 mM $MgCl_2$, 0.25 mM GSSG and 25 µM β-NADPH. GR activity was quantified by following the reduction of NADPH reflected as a change of the absorbance at 340 nm and calculated using the extinction coefficient $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$. One unit of GR activity was defined as the amount of enzyme required to decompose 1 µmol β-NADPH min^{-1} mg $protein^{-1}$.

3.4.1.4 Superoxide Dismustase (SOD; EC 1.15.1.1) Assay

SOD activity was determined as described by Beauchamp and Fridovich (1971). Briefly, 475 µl reaction solution contained 20 µl supernatant, 0.025 M sodium phosphate buffer (pH 7.8), 0.16 mM Na_2EDTA, 20.52 mM methionine, 99.5 µM NBT and 2.34 µM riboflavin. The reaction mixtures were illuminated under the light in incubator for 15 minutes. SOD activity was quantified by monitoring the inhibition of nitro blue tetrazolium (NBT) photochemical reduction at 340 nm. One unit of SOD activity was defined as 50% inhibition of the reduction of NBT mg $protein^{-1}$ h^{-1}.

3.4.1.5. Peroxidase (POD; EC 1.11.1.7) Assay

POD activity were carried out spectrophotometrically as described by Kato and Shimizu (1987). Briefly, 1.29 ml reaction solution contained 0.1 ml supernatant, 7.7 mM sodium phosphate buffer (pH 6.8), 0.77 mM guaiacol and 11.54 mM H_2O_2. POD enzyme will converted H_2O_2 to H_2O and O_2, then oxygen reacts with guaiacol to produce a brown color. The increase absorbance as a result of formation oxidized product (tetraguaiacol) was measured at λ= 470 nm and calculated using the extinction coefficient ε = 26.6 mM^{-1} cm^{-1}. One unit of POD activity was defined as 1 µmole tetraguaiacol formation min^{-1} mg $protein^{-1}$.

3.4.2. Zymography Assay

A 0.3 g sample was extracted in 0.5 ml 50 mM sodium phosphate buffer (pH 7.0) containing 2 mM Na_2EDTA and 1 mM PMSF, with the addition of 5 mM ascorbate for the APX zymography assay. The homogenate was centrifuged at 12,000 g for 10 minutes under 4°C at least 3 times and the supernatant was used for the zymography assays. Protein contents were determined following the methods described by Bradford (Bradford, 1976), using bovine serum albumin as standard and measured the absorbance at 595 nm using ELISA reader (Spectramax 190, Kim Forest Enterprise). The average protein content that used for zymography assay was 0.4 µg μl^{-1} The supernatant was mixed with 10X protein dye (Appendix III) and stored in -80°C for further analysis.

3.4.2.1 Ascorbate Peroxidase (APX; EC 1.11.1.11) Assay

The zymography assay of APX was based on the principle that nitroblue tetrazolium will react with ascorbate and TEMED generated formazan purple blue color. While APX will scavange ascorbate, so if there is no ascorbate, there is no purple-blue color on the gel. APX zymography assay was conducted using 10% native PAGE resolving gel which was prepared according to the composition of 1.5 mm gel as shown in the Table 1.

Table 1. Composition of one 1.5 mm gel

	Resolving gel (ml)			**Stacking gel (ml)**
	12%	**10%**	**8%**	**4%**
Solution A (40%)	2.40	2.00	1.60	0.40
Solution B	2.00	2.00	2.00	-
Solution C	-	-	-	1.00
TEMED	0.0072	0.0072	0.0072	0.0040
H_2O	1.98	2.38	2.78	1.78
50% glycerol	1.6	1.6	1.6	0.80
10% APS	0.02	0.02	0.02	0.02
Total Volume		8 ml		4 ml

Solution A: 40% polyacrylamide mix

Solution B: 1.5 M Tris-HCl pH 8.8

Solution C: 0.5 M Tris-HCl pH 6.8

The gel was run under 4°C using 1X TG Buffer (Appendix III) containing 2 mM ascorbate as running buffer. The gel was prerun at 80 V for 30 minutes to ensure the gel full of ascorbate. After prerun, the running buffer in the inner tank was changed freshly. Then, 5 µg protein were loaded in each well. The gel was run at 80 V until the sample into resolving gel for 99 minutes. After that, the voltage was changed into 120 V and run for 160 minutes.

After electrophoresis, activity of APX was stained using the method described by Mittler and Zilinskas (1993). Briefly, the gel was equilibrated with 15 ml 50 mM $NaPO_4$ pH 7.0 containing 2 mM ascorbate for 10 min (3 times). After that, the gel was incubated in 50 mM $NaPO_4$ pH 7.0 containing 4 mM ascorbate and 2 mM H_2O_2 for 25 minutes. The gel was washed with 50 mM $NaPO_4$ pH 7.8 containing 28 mM TEMED and 2.45 mM NBT for 3-5 minutes in the dark (stop while the bands are disguisable). After that, 10% acetic acid was added to stop the reaction and photographed using scanner (EPSON Perfection V370). Stored the gel in 10% acetic acid at 4°C up for several months.

3.4.2.2 Peroxidase (POD; EC 1.11.1.7) Assay

The zymography assay of POD was based on the principle that POD enzyme will converted H_2O_2 to H_2O and O_2, then oxygen reacts with guaiacol to produce a brown color. POD zymography assay was conducted using 10% native PAGE resolving gel which was prepared according to the composition of 1.5 mm gel as shown in the Table 1.

The gel was run under 4°C using 1X TG Buffer as running buffer. The gel was prerun at 80 V for 30 minutes to eliminate APS. After prerun, the running buffer in the inner tank was changed freshly. Then, 5 µg protein were loaded in each well. The gel was run at 80 V until the sample into resolving gel for 60 minutes. After that, the voltage was changed into 120 V and run the gel for 5 – 6 hours.

After polyacrylamide gel electrophoresis, activity of POD was stained using the method described by Köksal and Gülçin (2008). The gel was washed with distillated water to remove the buffer. Then, the gel was incubated in 4.5 mM guaiacol and 22.5 mM H_2O_2 in 100 mM phosphate buffer (pH 7.0) at 25°C and gently shake (stop while the bands are disguisable). The staining solution is then poured off; the gel was briefly rinsed. After that, 10% acetic acid was added to stop the reaction and photographed using scanner (EPSON Perfection V370). Stored the gel in 10% acetic acid at 4°C up for several months. The POD bands are brown on the transparent gel and stable for at least several hours. The gel was stored in 10% acetic acid at 4°C up for several months

3.4.2.3 Glutathione Reductase (GR; EC 1.8.1.7) Assay

GR zymography assay was conducted using 10% native PAGE resolving gel which was prepared according to the composition of 1.5 mm gel as shown in the Table 1.

The gel was run under 4°C using 1X TG Buffer as running buffer. The gel was prerun at 80 V for 30 minutes to eliminate APS. After prerun, the running buffer in the inner tank was changed freshly. Then, 5 µg protein were loaded in each well. The gel was run at 80 V until the sample into resolving gel for 75 minutes. After that, the voltage was changed into 120 V and run the gel for 115 minutes.

After electrophoresis, activity of GR was stained using the method described by Foyer et al. (1991). Briefly, the gel was immersed with the 10 ml staining solution contained 250 mM Tris-HCl pH 7.5, 3 mM Na_2EDTA, 0.4 mM NADPH, 0.68 mM 2,6 dichlorophenolindophenol (DCIP), 0.48 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 3.4 mM GSSG, gently shake in the dark at least 1 h until the bands were disguisable. Duplicate gel was stained in the absence of GSSG as control. After staining, the gel was briefly rinsed and immersed in 10% acetic acid until the background become transparent. Take a picture using scanner (EPSON Perfection V370). The gel was stored in 10% acetic acid at 4°C up for several months.

3.4.2.4 Superoxide Dismustase (SOD; EC 1.15.1.1) Assay

The zymography assay of SOD was conducted using 10% native PAGE resolving gel which was prepared according to the composition of 1.5 mm gel as shown in the Table 1.

The gel was run under 4°C using 1X TG Buffer as running buffer. The gel was prerun at 80 V for 30 minutes to eliminate APS. After prerun, the running buffer in the inner tank was changed freshly. Then, 5 µg protein were loaded in each well. The gel was run at 80 V until the sample into resolving gel for 99 minutes. After that, the voltage was changed into 120 V and the gel was run until the dye is at the end of gel for 60 minutes.

After electrophoresis, activity of total SOD was stained using the method described by Beauchamp and Fridovich (1971). Briefly, the gel was immersed in 0.1% NBT for 15 minutes in the dark and gently shake. The gel was rinsed with distillated water three times. The gel was added with 20 ml 0.1 M sodium phosphate buffer pH 7.0 containing 66.7 µl TEMED and 74.7 µl 7.5 mM riboflavin, shake for 15 minutes. The gel was briefly rinsed with distillated water three times and added the small amount of 0.1 M sodium phosphate buffer pH 7.0, gently shake under light for 10 minutes until the bands were disguisable. After that, 10% acetic acid was added to stop the reaction and photographed using scanner (EPSON Perfection V370). Stored the gel in 10% acetic acid at 4°C up for several months.

To identified the SOD isoenzymes, H_2O_2 and KCN were added. For detected the MnSOD, the H_2O_2 were used to inhibit CuZnSOD and FeSOD. Briefly, the gel was immersed in 20 ml 0.1 M sodium phosphate buffer pH 7.0 containing 16.5 µl 9.7 M H_2O_2 for 30 minutes under 4°C. The gel was briefly rinsed with distillated water three times. Then, the gel was stained followed the total SOD staining procedure and photographed using scanner (EPSON Perfection V370).

MnSOD and FeSOD were detected by adding KCN to inhibit CuZnSOD. Briefly, the gel was immersed in 0.1% NBT for 15 minutes in the dark and gently shake. The gel was rinsed with distillated water three times. The gel was added with 20 ml 0.1 M sodium phosphate buffer pH 7.0 containing 66.7 µl TEMED, 74.7 µl 7.5 mM riboflavin and 80 µl 2 M KCN, shake for 15 minutes. The gel was briefly rinsed with distillated water three times and added the small amount of 0.1 M sodium phosphate buffer pH 7.0, gently shake under light for 10 minutes until the bands were disguisable. After that, 10% acetic acid was added to stop the reaction and photographed using scanner (EPSON Perfection V370). Stored the gel in 10% acetic acid at 4°C up for several months. Finally, the three gels were compared to determine the SOD isoenzymes.

3.4.2.5 Protein Staining

Protein staining was conducted using 10% native PAGE resolving gel which was prepared according to the composition of 1.5 mm gel as shown in the Table 1.

The gel was run under 4°C using 1X TG Buffer as running buffer. The gel was prerun at 80 V for 30 minutes to eliminate APS. After prerun, the running buffer in the inner tank was changed freshly. Then, 5 µg protein were loaded in each well. The gel was run at 80 V until the sample into resolving gel for 99 minutes. After that, the voltage was changed into 120 V and the gel was run until the dye is at the end of gel for 60 minutes.

After electrophoresis, the gel was stained using Coomassie Brilliant Blue R 250 (Appendix III) and gently shake for 30 minutes. For destain procedure, the gel was immersed in destain buffer I (50% methanol + 10% glacial acetic acid) and then replace with destain buffer II (5% methanol + 7% glacial acetic acid). Take a picture using scanner (EPSON Perfection V370) when the background turned to transparent. Stored the gel in ddH_2O + destain buffer II at room temperature up for several months.

3.5. Effect of 4-*tert*-octylphenol (OP) on Non-enzymatic Antioxidant

3.5.1 Determination of Ascorbate (AsA)

Ascorbate (AsA) and total ascorbate (AsA+dehydroascorbate (DHA)) were determined according to the method described by Hodges et al. (1996). A 0.1 g leaves samples was extracted in 1 ml of 5% (v/v) TCA. The homogenate was centrifuged at 12,000 g for 10 minutes under 4°C. For determination of total ascorbate, 0.1 ml of supernatant was added to 0.5 ml of 120 mM sodium phosphate buffer (pH7.4) containing 0.2 ml of 15 mM Na_2EDTA and 0.2 ml of 10 mM dithiothreitol (DTT). After reaction at 25°C for 10 min, 0.1 ml of 40 mM N-ethylmaleimide, 0.4 ml of 10% TCA (v/v), 0.4 ml of 8 M H_3PO_4, 0.4 ml of 0.26 M α,α'-dipyridyl in 70% ethanol (w/v) and 0.2 ml of 0.19 M $FeCl_3$ were added and mixed well in sequence. AsA was assayed in a similiar procedure except that 0.1 ml ddH_2O was used to replace 0.1 ml of DTT and 40 mM N-ethylmaleimide. The mixtures were incubated at water bath under 40°C for 1 h. The absorbance of the mixture were assayed at 525 nm. The content of ascorbate was calculated according to the standard curve generated with different concentrations of L-ascorbate (0-40 nmole). The difference between total ascorbate and AsA was considered to represent the content of DHA.

3.5.2 Determination of Glutathione (GSH)

The glutathione pool was assayed as described by Anderson (1985) with little modification. A 0.1 g leaves samples was extracted in 1 ml of 5% (v/v) TCA. The homogenate was centrifuged at 12,000 g for 10 minutes under 4°C. Then, 0.4 M phosphate buffer pH 8.0 was added into TCA extracts for neutralization with ratio 1 : 1. Then, the supernatant was divided into 2 tubes for measuring total GSH (tGSH) and GSSG. For GSSG quantification, 0.1 ml supernatant was added with 2 µl 1M 2-vinylpiridine and incubated in 25°C for 1 h to eliminate GSH. After 1 h, then the extract keep in 4°C and used for determination of GSSG. For tGSH assay, 1 ml of reaction mixture containing 0.2 mM β-NADPH, 100 mM phosphate buffer (pH 7.5), 5mM Na_2EDTA, 0.6 mM 5,5' dithiobis (2-nitrobenzoic acid) prepared in 0.2 M $NaPO_4$ pH 7.5 and 0.1 ml of supernatant were mixed. The reaction was started by adding 0.1 ml GR (1U/ml) then monitored by measuring the change in absorbance at 412 nm for 2 min. GSSG was assayed in a similiar procedure with tGSH. A standard curve was prepared based on solutions with different concentrations of 1 mM GSSG (0-20 nmole). The difference between tGSH and GSSG content was considered to represent the content of GSH.

3.6 Statistical Analysis

Data from experiments were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis at $P < 0.05$ to identify significant differences among treatments using the software SPSS 20.0 package. Data are presented as mean ± standard deviation (mean ± SD).

4. RESULT

4.1. Effect of 4-*tert*-octylphenol (OP) on the Growth Rate and Physiological Features

After 5 days treatments under 4-*tert*-octylphenol (OP) exposure, the physiological features in *Ceratophyllum demersum* were observed. Physiological features, such as chlorosis and yellowing leaves were shown obviously at concentration 3 mg L^{-1}. In the higher concentration, some leaves were also fall down (Fig. 8A). The growth rate based on the fresh weight of plant fragments were gradually decreased with the increasing of OP concentrations. Relative growth rates were decreased significantly by about 26, 66, 69, 70 and 92% in the *C. demersum* treated with 0.5, 1, 1.5, 2 and 3 mg L^{-1} for 5 days, respectively, compared with the control (Fig. 8B).

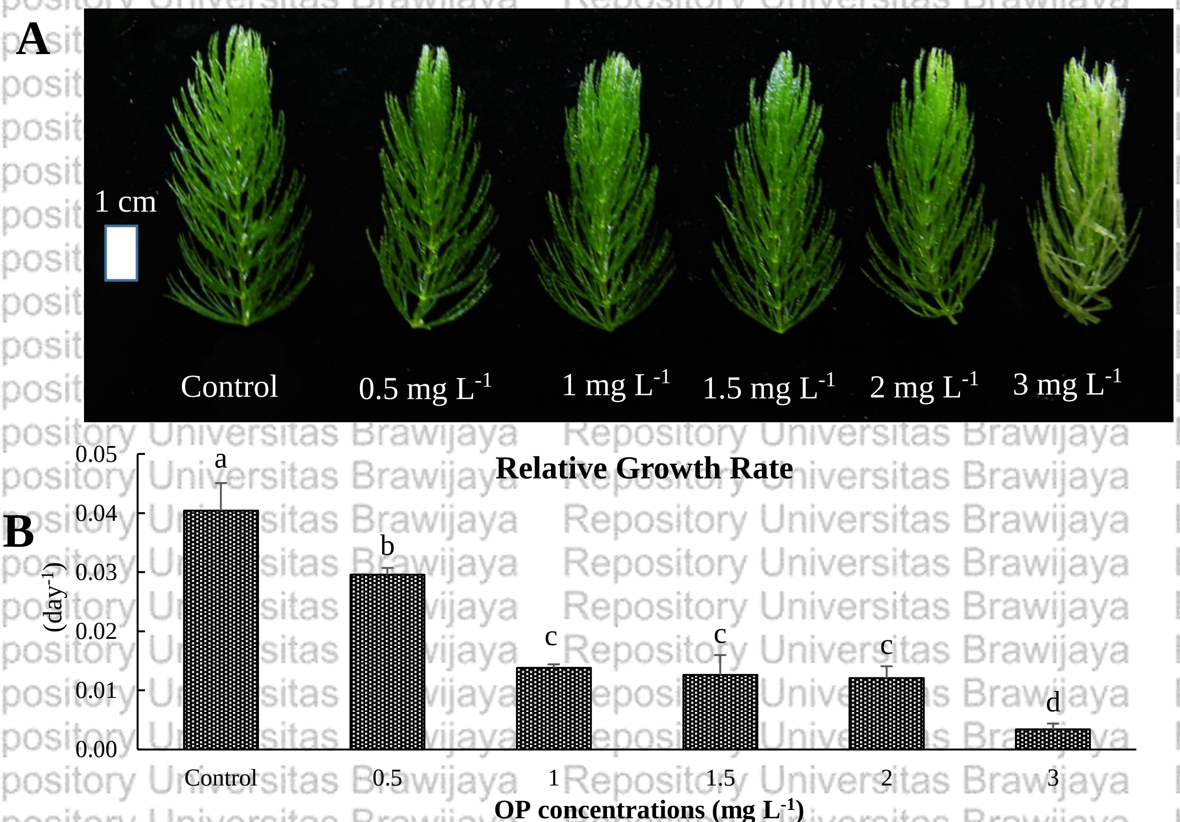


Fig. 8. Physiological features in *C. demersum* leaves after exposed to 0, 0.5, 1, 1.5, 2 and 3 mg L^{-1} OP for 5 days (A); Effects of OP on growth rates of *C. demersum* (B). Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

Photosynthetic pigments, such as total chlorophyll (chlorophyll *a* and *b*) were also decreased under OP treatments. For total chlorophyll, the significant decreased were found at 1.5, 2 and 3 mg L^{-1} by about 20, 23 and 43%, compared with the control, respectively, and that of chlorophyll *a* by about 20, 21 and 42%, respectively. Chlorophyll *b* did not significantly alter in plants treated with OP, while a significant decrease was detected at concentration 3 mg L^{-1} (Fig. 9).

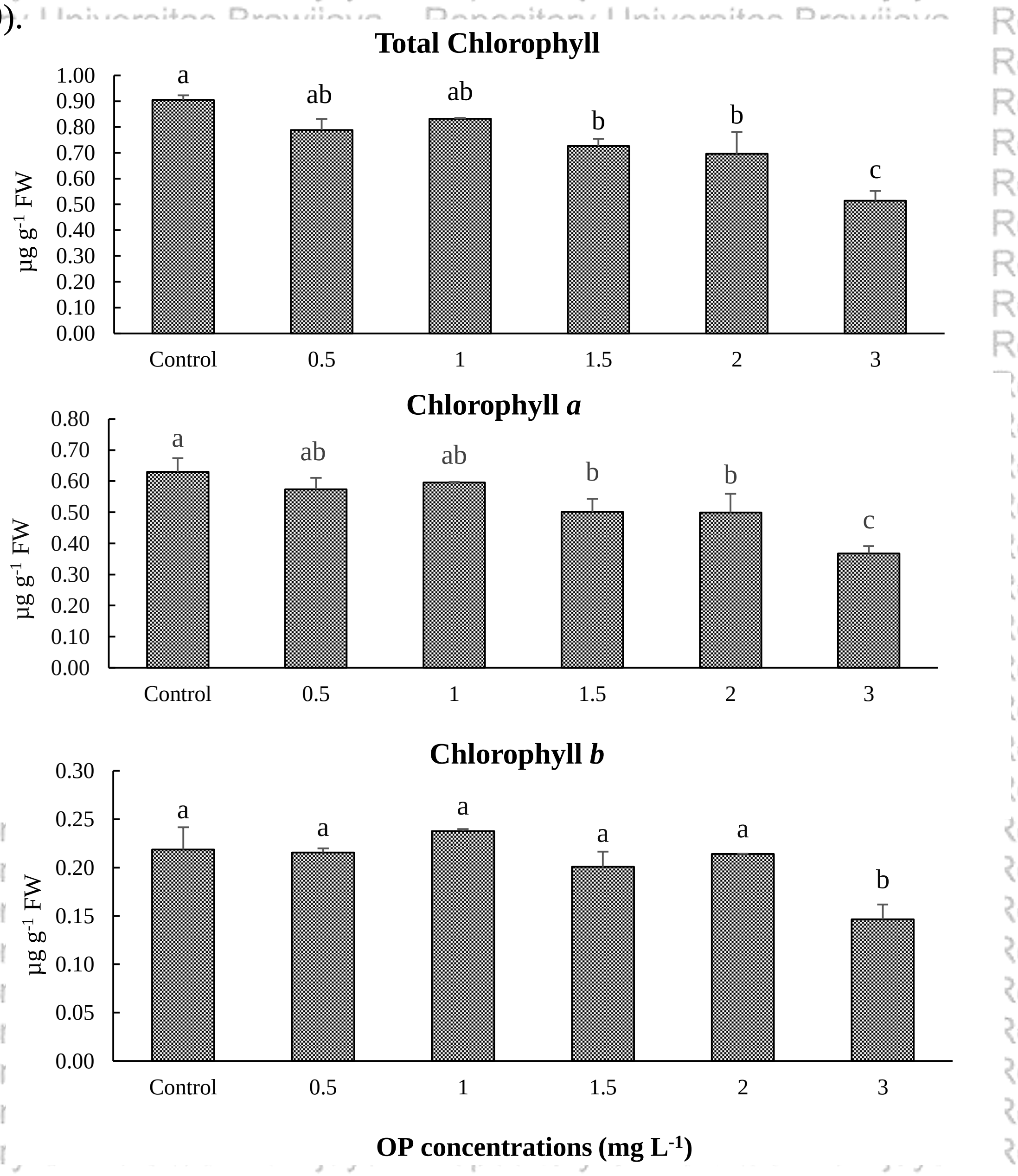


Fig. 9. Effects of exposure to OP on the total chlorophyll (chlorophyll *a* and *b*) content of *C. demersum*. Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

4.2. Effects of 4-*tert*-octylphenol (OP) on the Levels of ROS and Lipid Peroxidation

Treatment with 3 mg L^{-1} OP increased the content of $O_2^{\bullet-}$ significantly by about 12% (Fig. 10A), when compared with control plants; and that of H_2O_2 were increased in 2 mg L^{-1} and 3 mg L^{-1} OP treatments by about 15 and 29% (Fig. 10B), respectively. The content of MDA in plants treated with OP did not increase as expected, the MDA contents were not altered under OP treatments (Fig. 10C).

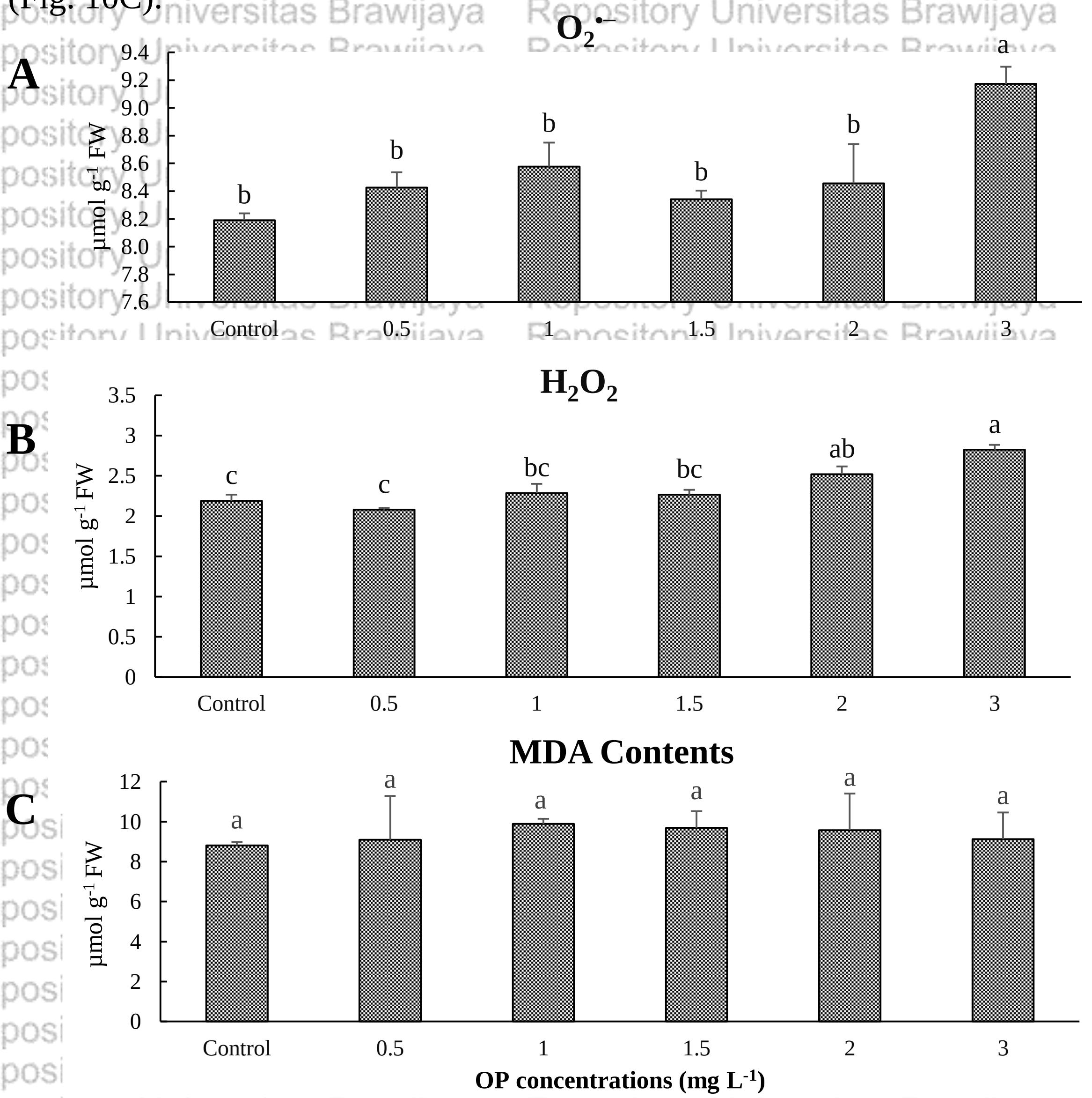


Fig. 10. Effects of OP on the levels of ROS, $O_2^{\bullet-}$ (A), H_2O_2 (B) and MDA contents (C) in *C. demersum.* Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

4.3. Effects of 4-*tert*-octylphenol (OP) on the Enzymatic Antioxidants

A significant increase of SOD activity was observed in *C. demersum* leaves treated with 3 mg L^{-1} OP after 5 days exposure, while no significant alterations were detected at the lower concentration (0.5, 1, 1.5 and 2 mg L^{-1}) (Fig. 11B). At least three SOD isoenzymes, CuZnSOD, FeSOD 1 and FeSOD 2, were detectable in both, the control and OP-treated leaves (Fig. 11A) and their activities were highest in plants treated with 3 mg L^{-1} OP.

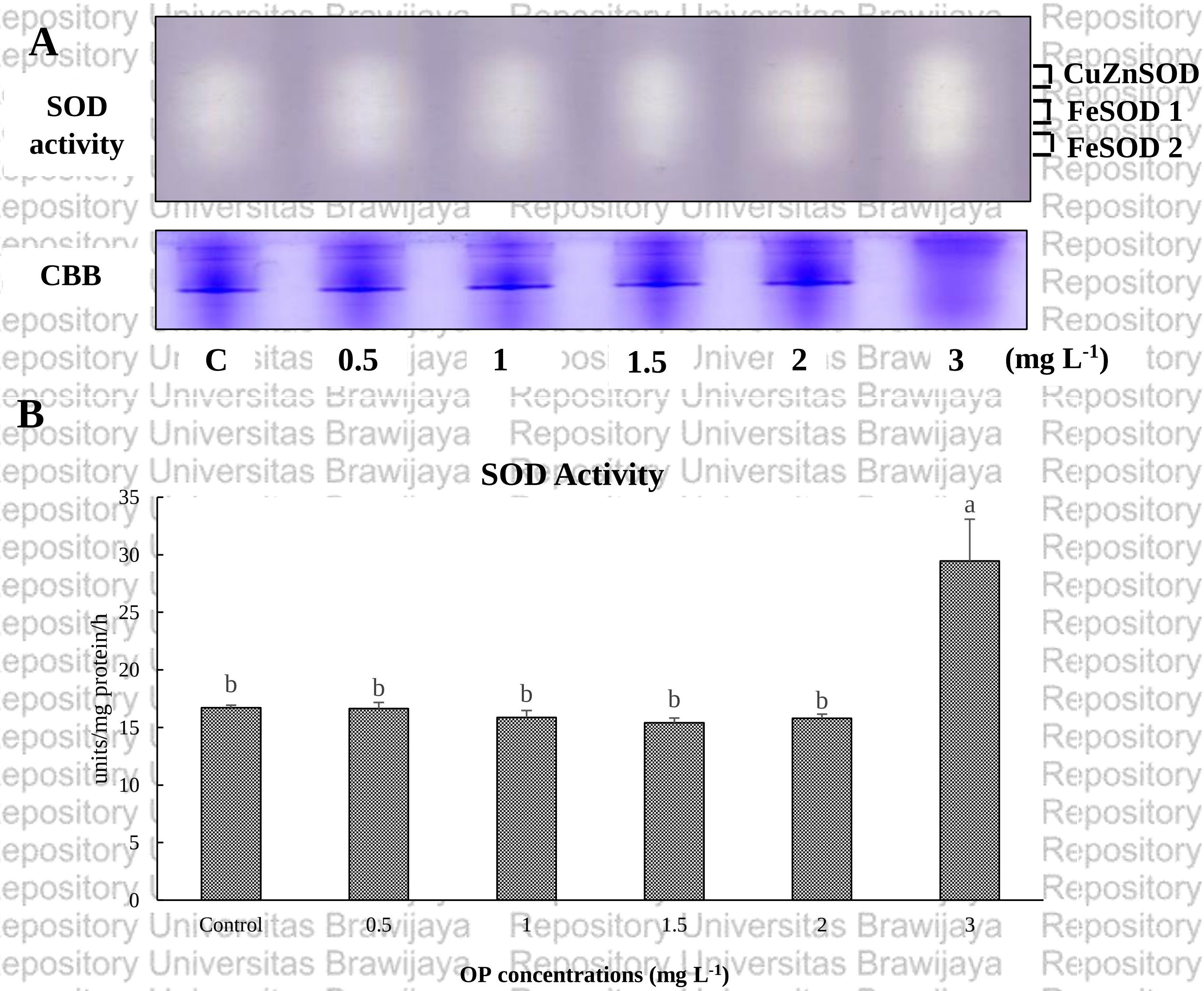


Fig. 11. Effects of OP on SOD activity in *C. demersum*. Zymography assay of SOD activity in native PAGE (A); Spectrophotometric measurement of SOD activity (B). CBB are indicated Coomassie Brilliant Blue staining. Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

The enzymatic activity of POD (Fig. 12B) were observed spectrophotometrically showed increasing activity by about 31% in leaves of *C. demersum* after exposure to 3 mg L^{-1} for 5 days. Furthermore, POD isoenzymes were visualized using guaiacol and H_2O_2 in *C. demersum* (Fig. 12A). Activity of POD isoenzymes increased after treatments with 3 mg L^{-1} for 5 d, however no significant different was detected in lower concentration (0, 0.5, 1, 1.5 and 3 mg L^{-1}).

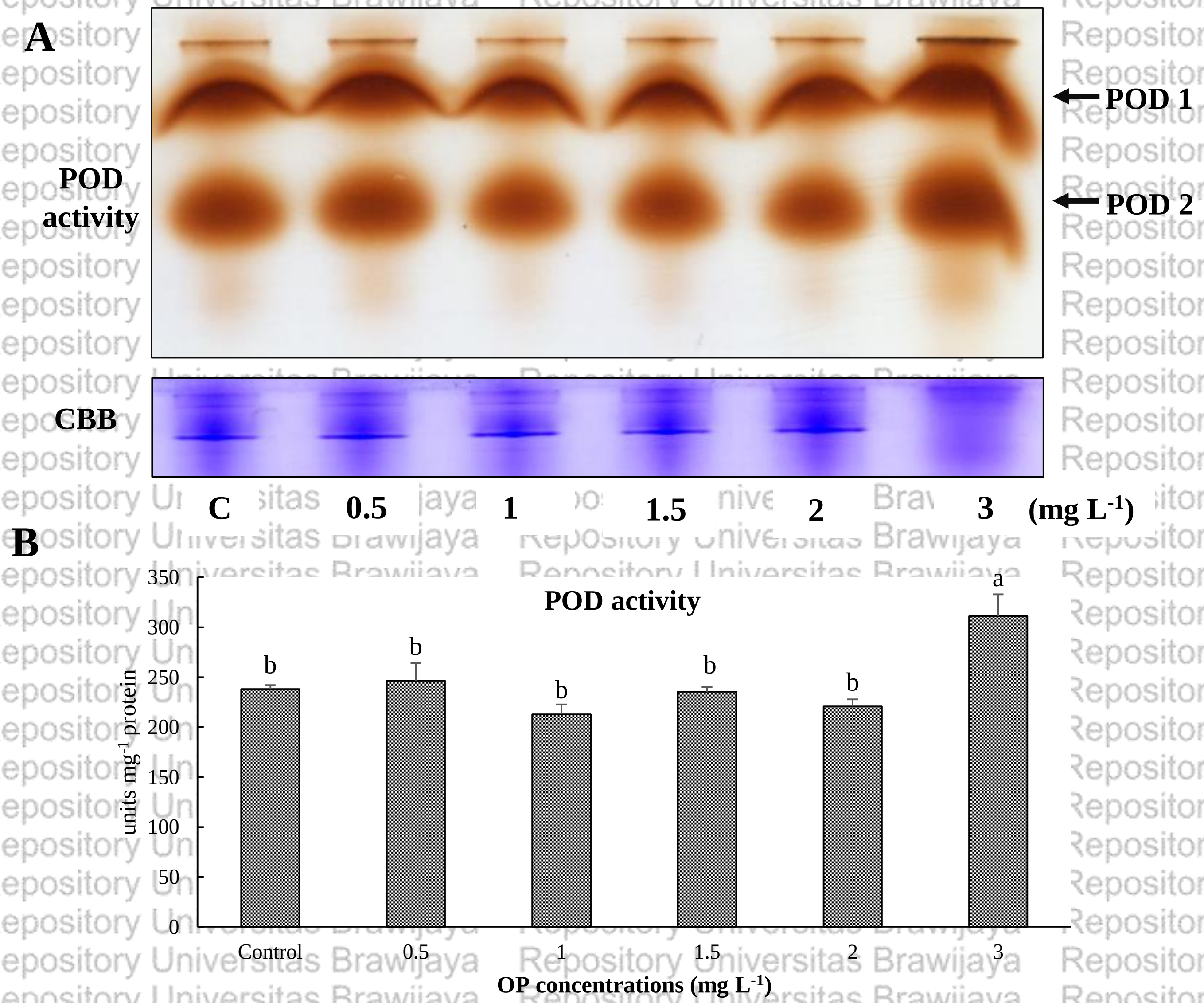


Fig. 12. Effects of OP on POD activity in *C. demersum*. Zymography assay of POD activity in native PAGE (A); Spectrophotometric measurement of POD activity (B). CBB are indicated Coomassie Brilliant Blue staining. Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

Exposure of plants to 0.5 and 1 mg L^{-1} did not show any alterations in APX activity compared to the control, while in 1.5, 2 and 3 mg L^{-1}, APX activity increased gradually by about 33, 51 and 62%, respectively (Fig. 13B). Zymography assay were conducted to detect APX activity using ascorbate as electron donor, APX isoenzyme also showed an increasing in OP treatments 1.5, 2 and 3 mg L^{-1} (Fig. 13A).

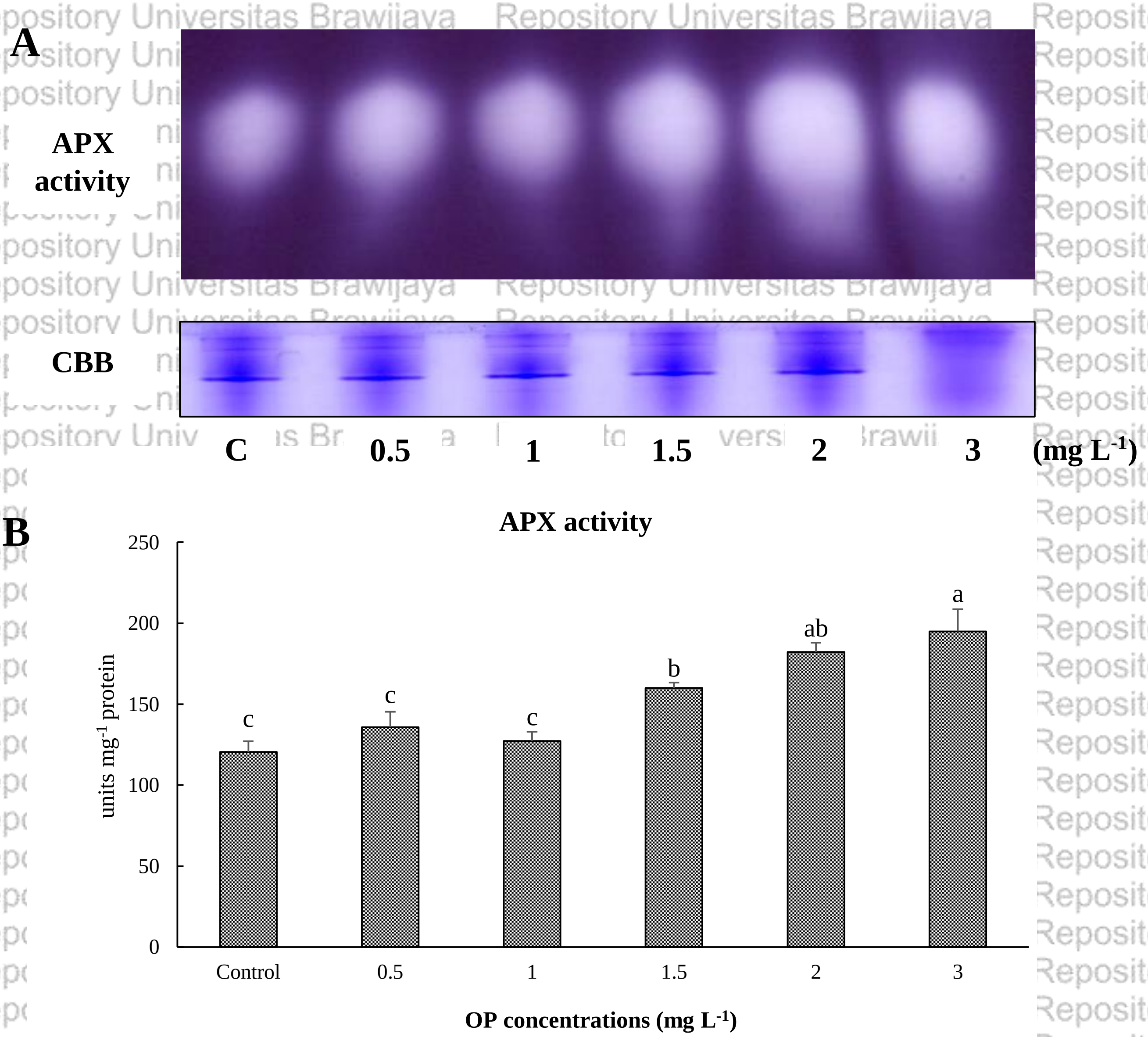


Fig. 13. Effects of OP on APX activity in *C. demersum*. Zymography assay of APX activity in native PAGE (A); Spectrophotometric measurement of APX activity (B). CBB are indicated Coomassie Brilliant Blue staining. Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

A significant increase in the activity of GR was observed in plants treated with 0.5, 1, 1.5 and 2 mg L^{-1} by about 9, 27, 27 and 18% compared with the control, respectively (Fig. 14B). However, in the highest concentration OP treatment 3 mg L^{-1} the GR activity decreased 27%, compared with the control. GR activity assay using native PAGE also shows the same pattern with spectrophotometric measurement. Two GR isoenzymes were detected in gel and increased after treatments with 0.5-2 mg L^{-1} OP and decreased at OP concentration 3 mg L^{-1} (Fig. 14A)

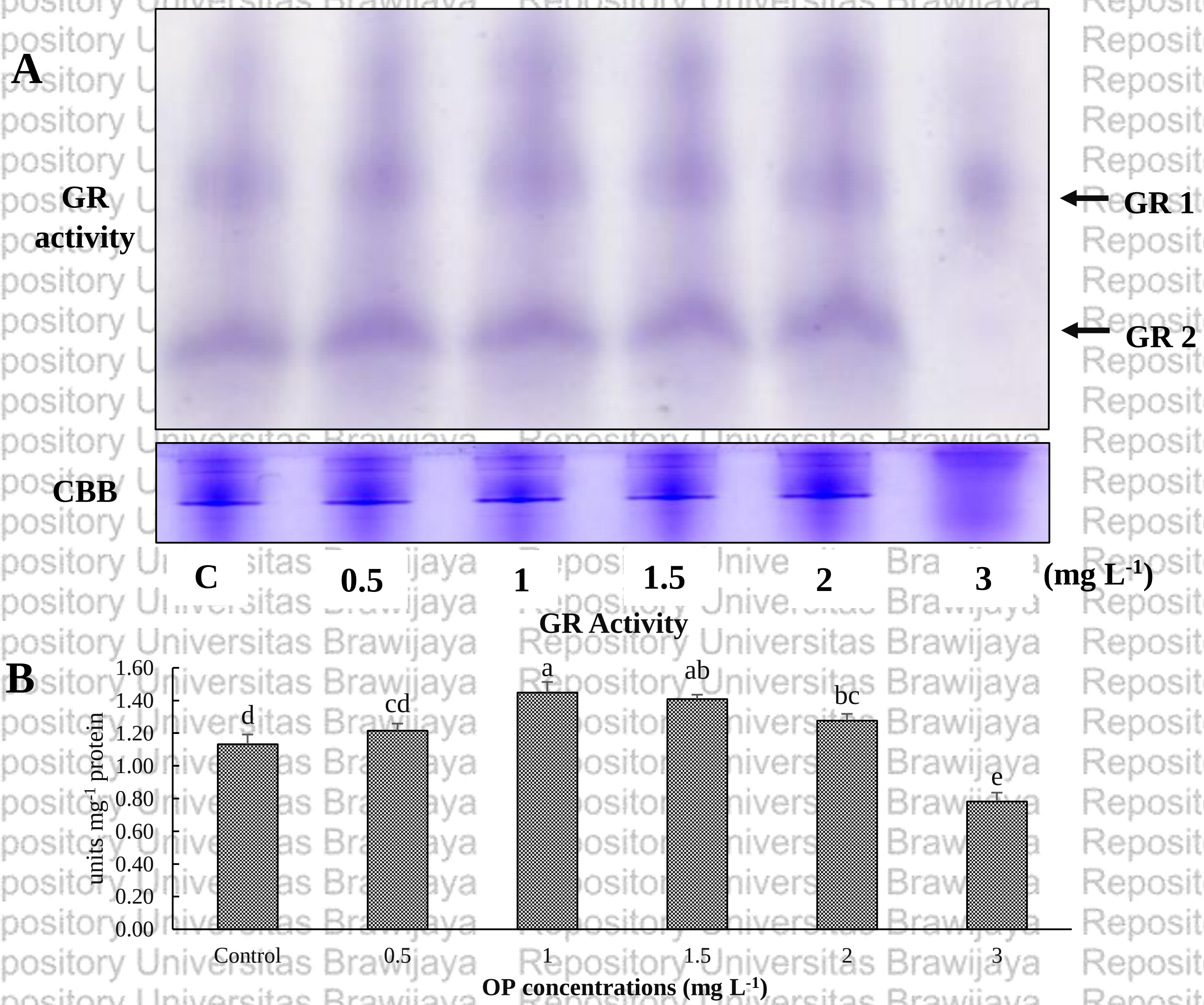


Fig. 14. Effects of OP on GR activity in *C. demersum*. Zymography assay of GR activity in native PAGE (A); Spectrophotometric measurement of GR activity (B). CBB are indicated Coomassie Brilliant Blue staining. Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

Plants treated with various concentrations OP in the growth medium caused significant alterations of the activity of CAT (Fig. 15) in leaves. The activity of CAT was increased significantly at 0.5, 1, 1.5, 2 and 3 mg L^{-1}. The maximum activities of CAT were observed in leaves at 1, 2 and 3 mg L^{-1} OP, which were ~112, ~100 and ~78% higher than the activities in controls, respectively.

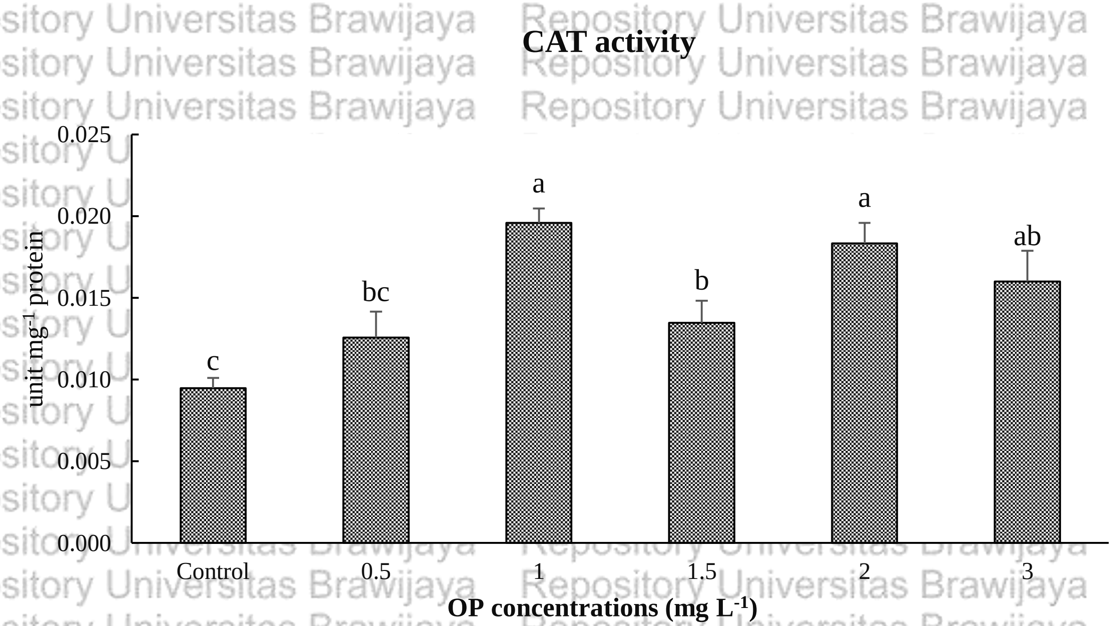


Fig. 15. Effects of OP on CAT activity in *C. demersum*. Data are expressed as a mean ± SD. Different letters indicate significant differences ($P < 0.05$).

4.4. Effects of 4-*tert*-octylphenol (OP) on the Non-Enzymatic Antioxidants

4.4.1 Ascorbate (AsA)

Fig. 16A shows that the contents of total ascorbate increased significantly between concentration 1 mg L^{-1} and 3 mg L^{-1} of OP, compared with the control, the increasing percentage in 1, 1.5, 2 and 3 mg L^{-1} by about 42, 43, 38 and 43%, respectively. A significant enhancement in AsA content was also observed in leaves treated with concentration 1 until 3 mg L^{-1} of OP compared with the control, while the AsA contents at 2 and 3 mg L^{-1} were decreased slightly compared with 1 and 1.5 mg L^{-1} OP but still higher than the control (Fig. 16B), while contents of DHA increased significantly between 0.5 and 3 mg L^{-1} OP (Fig. 16C). The ratio of ASC/DHA were observed and it decreased in the concentration 2 and 3 mg L^{-1} OP compared with the control, by about 24 and 14%, respectively (Fig. 16D).

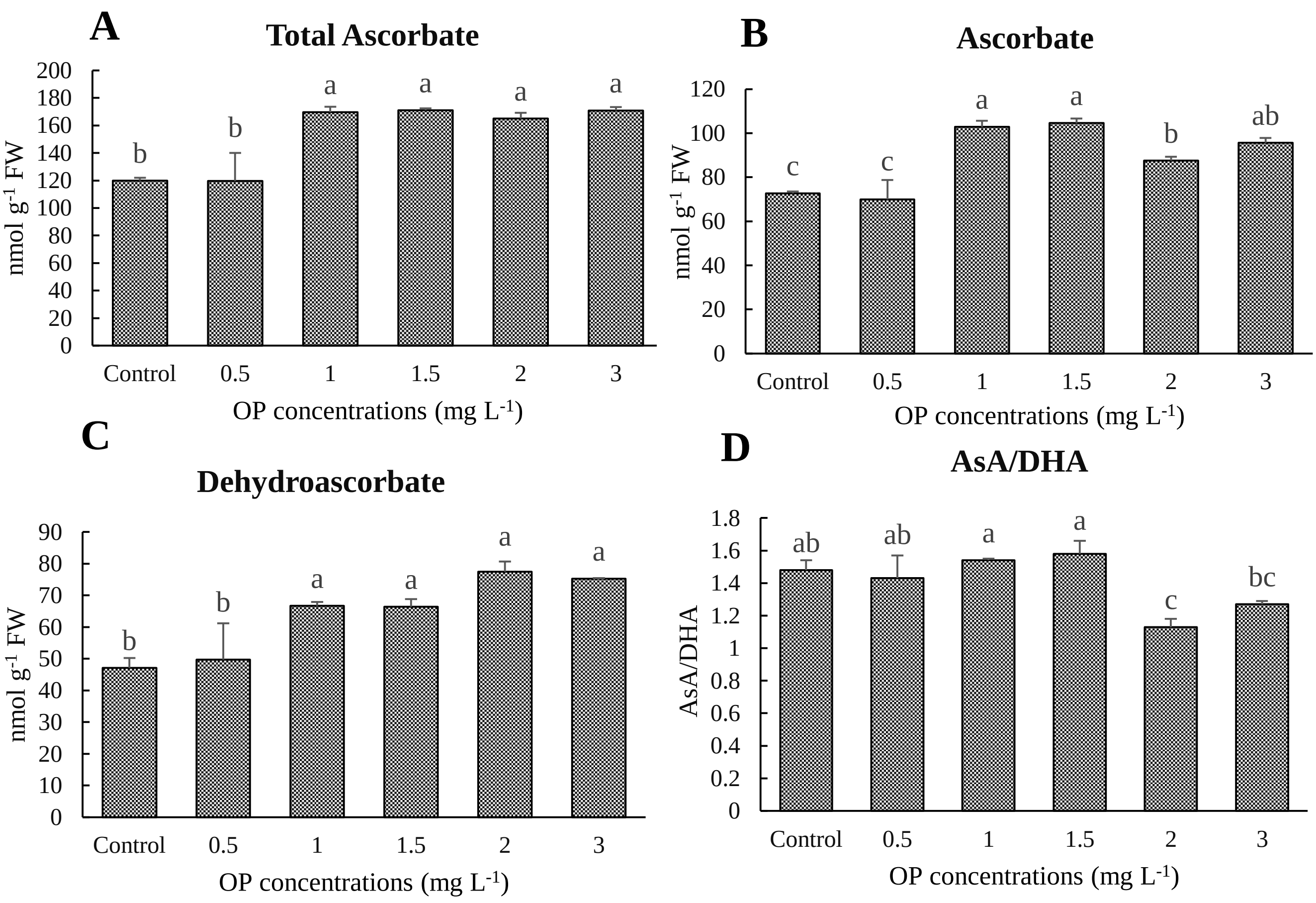


Fig. 16. Effects of OP on total ascorbate (A), AsA (B), DHA (C) and AsA/DHA (D) in *C. demersum*. Data are expressed as a mean ± SD. Different indicate significant differences ($P < 0.05$).

4.4.2 Glutathione (GSH)

Plants treated with various concentration of OP (0.5, 1, 1.5, 2 and 3 mg L^{-1}) cause significant alteration in total glutathione (tGSH) contents. The increasing of tGSH reached 73% at 0.5 mg L^{-1} and more than 100% between concentration 1 and 3 mg L^{-1} (112-132%) (Fig. 17A). The same pattern also were observed at the GSH contents, the increasing of GSH contents reached more than 100% in each concentration of OP treatments 0.5, 1, 1.5, 2 and 3 mg L^{-1} by about 107, 154, 177, 174 and 168%, respectively, compared with the control (Fig. 17B). Meanwhile, the GSSG contents decreased by 86, 85, 85, 71 and 50%, respectively, in plants treated with 0.5, 1, 1.5, 2 and 3 mg L^{-1} (Fig. 17C). The ratio of GSH/GSSH were increased significantly under OP treatments compared with the control (Fig. 17D)

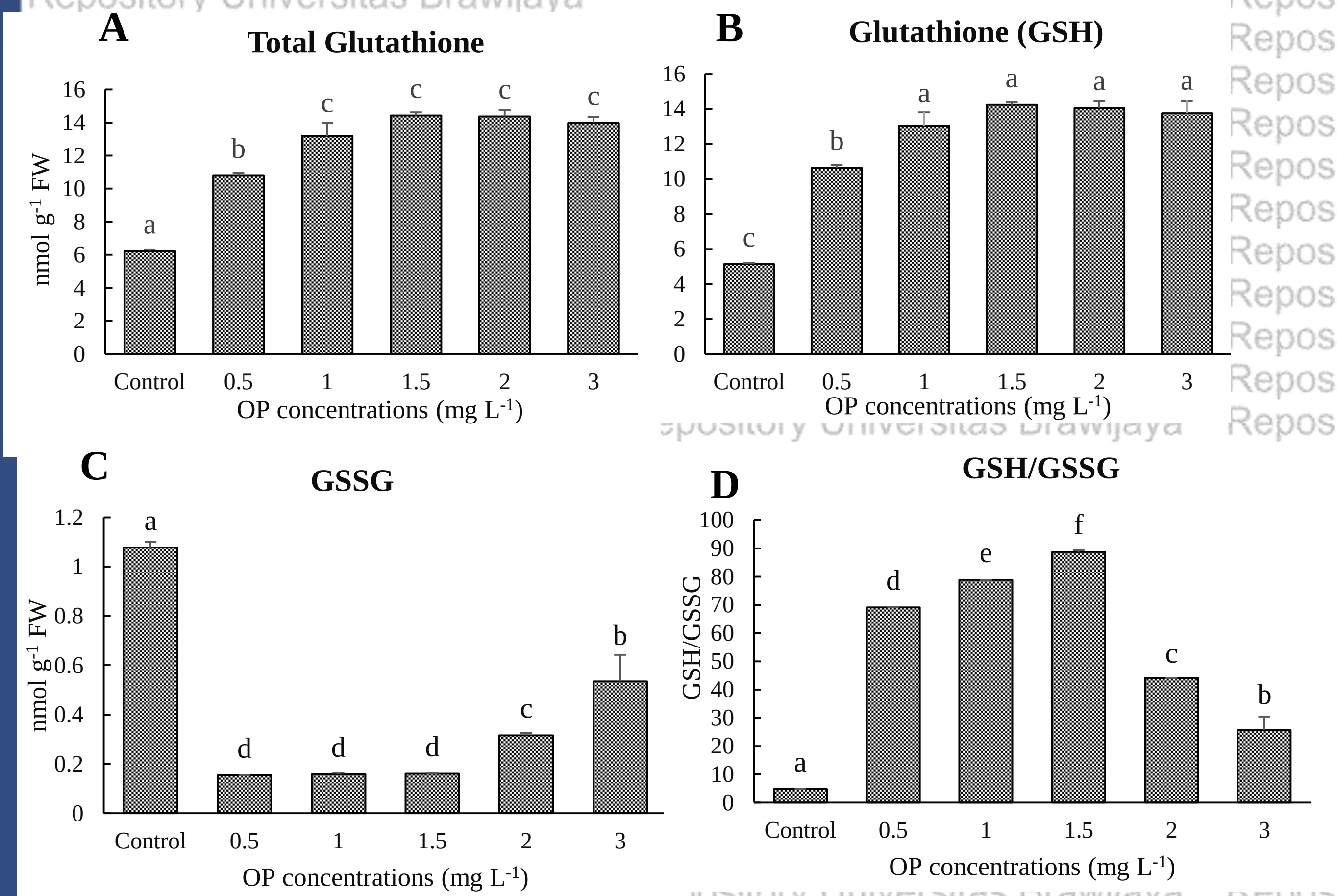


Fig. 17. Effects of OP on total glutathione (A), GSH (B), GSSG (C) and GSH/GSSG (D) in *C. demersum*. Data are expressed as a mean ± SD. Different letters indicate significant differences (P < 0.05).

4.5 Pretreatment of *C. demersum* leaves with BSO

The data from previous treatment shown that the tGSH and GSH increased in the leaves treated with various concentrations of OP (Fig. 17A and 17B). It also shown that tAsA and AsA increased under OP treatments. We hypothesized that GSH may play an important role in *C. demersum* to cope with OP induced oxidative stress.

To test the hypothesis, the leaves of *C. demersum* pretreated with 0.5 mM BSO, a specific and potent inhibitor of ɣ-ECS, the first enzyme that play role in biosynthesis of GSH for 8 h (Chao et al., 2011). After 8h, renew the solution, then the leaves were treated without and with 3 mg L^{-1} OP for 5 days. After 5 days treatments under OP exposure, the physiological features in *C. demersum* were observed. Physiological features, such as chlorosis and yellowing leaves were shown obviously at OP treatment and BSO pretreatment + OP, compared to the control and BSO pretreatment. Some leaves also fell down at OP treatment and the worst condition was found at BSO pretreatment + OP which most of the leaves fell down (Fig. 18).

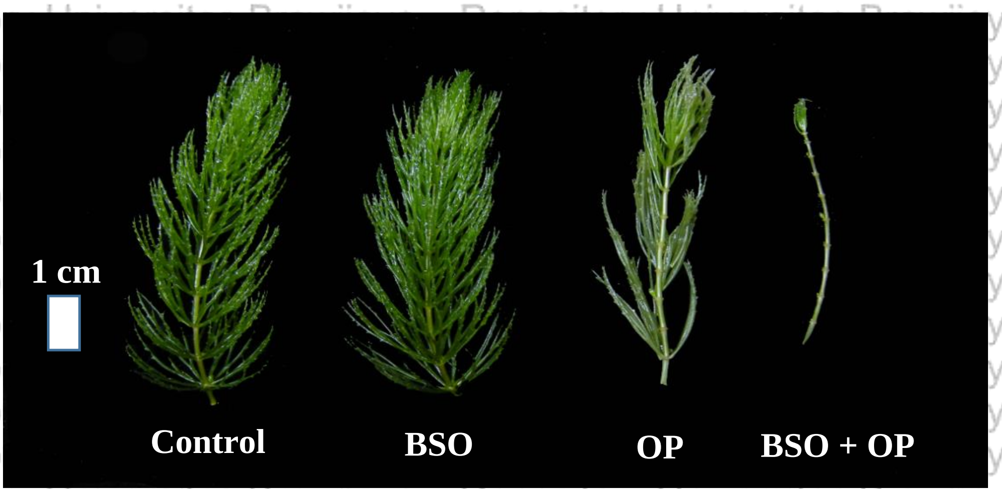


Fig. 18. Physiological features in *C. demersum* leaves after 8h BSO pretreatment and continued with 3 mg L^{-1} OP exposure for 5 days.

It was observed that the content of total glutathione (tGSH) (Fig. 19A), total ascorbate (tAsA) (Fig. 19B), and the activities of GR (Fig. 19C) and APX (Fig. 19D) were decreased in leaves of *C. demersum* pretreated with BSO.

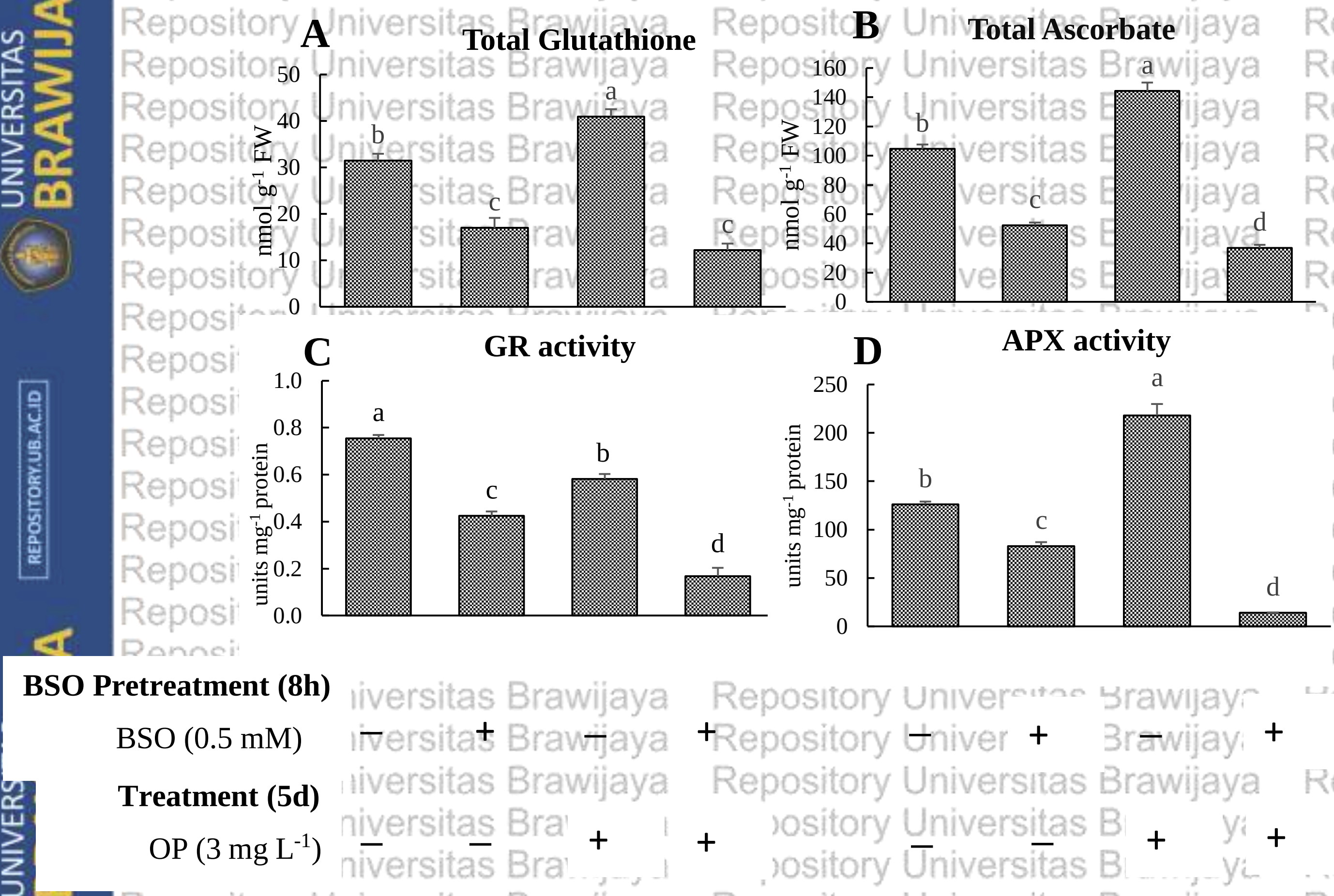


Fig. 19. Effect of BSO and BSO + OP on the contents of tGSH (A), tAsA(B), GR (C) and APX activity (D) in the leaves of *C. demersum* pretreated with or without BSO for 8h, then transferred to hoagland solution with or without 3 mg L^{-1} OP for 5 days. Data are expressed as a mean ± SD. Different letters in each graph indicate significant differences ($P < 0.05$).

5. DISCUSSION

The toxicity effect of 4-*tert*-octylphenol (OP) in aquatic plant has not been widely studied, meanwhile OP has been reported by Chen et al. (2013) that it can affect in the physiological and morphological features of *Arabidopsis thaliana* during growth and induced oxidative stress. In addition, at higher OP levels (0.25 and 1 mg L^{-1}) *Microcystis aeruginosa* growth was impaired, indicating toxic effects (Baptista et al., 2009). OP also can suppress growth, decrease photosynthetic pigments and destroy algal ultrastructure in freshwater green microalgae *Scenedesmus obliquus* (Zhou et al., 2013). In present study, OP exposure in *Ceratophyllum demersum* caused negative effect on plant growth. Growth of plant, as determined by an increase in fresh wight, was significantly reduced in concentration dependent manner by OP treatment beginning with 0.5 mg L^{-1} OP. As the growth is reduced with increasing OP concentrations it can be hypothesized that one of the toxicity effects is the growth inhibition. OP was previously found to reduce the mean length of roots and inhibit the growth in *A. thaliana* and *Gypsophila elegans* start at concentration 0.1 and 4.25 mg L^{-1}, respectively (Sinkkonen et al., 2011; Chen et al., 2013).

Photosynthesis is the most fundamental and intricate physiological process in all green plants. Reduction of photosynthetic pigment content is a physiological marker of abiotic stress in plant (Ashraf and Harris, 2013). The physiological features, such as chlorosis or yellowing leaves have been shown in *C. demersum* under OP exposure. Accordingly, total chlorophyll contents (*a* and *b*) also decreased significantly. This loss in pigment contents could be due to the damage of photosynthetic apparatus. In green algae and cyanobacteria, OP had adverse effects on photosystem II energy fluxes (Perron and Juneau, 2011). Photosystem II (PSII) electron transport is one of the most sensitive indicators of damage in the photosynthetic apparatus (Krause and Weis, 1991).

Under stress condition, excess H_2O_2 and $O_2^{\bullet-}$ can induces oxidative stress, causing the lipid peroxidation, oxidation of proteins, damage to nucleic acids, enzyme inhibition and ultimately cell death (Mittler, 2002; Apel and Hirt, 2004). Our result confirmed that OP induced the generation of $O_2^{\bullet-}$ and H_2O_2 in *C. demersum* significantly. In plant cells, ROS (H_2O_2 and $O_2^{\bullet-}$) can play dual role, both an important signaling molecule and a toxic byproduct of cell metabolism, its cellular levels are under tight control, and their maintenance has hallmarks of homeostatic regulation (Apel and Hirt, 2004). Studies with exogenously applied H_2O_2 confirm the role of H_2O_2 as a cell death trigger and show that high concentrations can cause necrosis instead of PCD (Yao et al., 2001).

MDA is a reactive aldehyde known as the end product of oxidative degradation of lipid and have been used as the marker of lipid peroxidation (Ayala et al., 2014). MDA contents in plant cells usually have positive correlation with the level of ROS (Cheng, 2011; Wang et al., 2012). However, in this present study, the increasing of ROS level did not accompany with the increasing of malondialdehyde contents (MDA). Similiar phenomena were also found in *C. demersum* under Cd^{2+} exposure, the MDA contents did not change at concentration 0.01 – 0.5 mM Cd^{2+} while at 1 mM the MDA contents were decline (Dhir et al., 2004). The reduced contents of MDA were also reported in *Hydrilla verticilata* and *Vallisneria natans* under ammonium stress even the high level of ROS were detected (Wang et al., 2008; 2010). Gupta et al. (1996) suggested that the decreased of MDA under Cu^{2+} in aquatic plant may be due to the decreasing of polyunsaturated fatty acids content, while Nimptsch and Pflugmacher (2007) considered that the increase of antioxidant defense mechanisms in *Myriophyllum mattogrossense* under ammonia stress can avoid and obstruct the peroxidation of lipid.

Plant have developed the antioxidant defense mechanism to maintain the equlibrium status between production and scavenging of ROS, consist of enzymatic and non-enzymatic antioxidants (Bartosz, 1997; Gill and Tuteja, 2010). ROS can act as the signal molecule to the defense mechanisms system, resulting in the enhancement or suppression of the antioxidant enzyme activity (Wang et al., 2009). SOD is metalloenzyme that has been known as the first line defense mechanism againts the ROS, convert $O_2^{\bullet-}$ become H_2O_2 (Apel and Hirt, 2004). SODs locate in cytosols, chloroplasts, mitochondria, apoplast and peroxisomes (Hasanuzzaman et al., 2012). In this present study, SOD activities were increased under highest concentration of OP exposure. Similiar result was also reported that SOD activity increased under OP treatments in *A. thaliana*, mainly CuZnSOD (Chen et al., 2013). In addition, the increasing of SOD activity was also reported in *C. demersum* under various stress, such as brominated flame retardant (Sun et al., 2008), heavy metal (Rama Devi and Prasad, 1998; Mishra et al., 2006) and PAH (Yin et al., 2008). The induction of SOD can occur during high production of $O_2^{\bullet-}$, therefore, an increase of SOD activity indicates an increase at $O_2^{\bullet-}$ production (Oruç and Üner, 2000). According to Fig. 10A and Fig. 11B, the result is similiar to the paper that the SOD activity showed elevated activity in response to the increase of $O_2^{\bullet-}$ levels. There are at least three major forms of SOD (Fe-SOD, Cu/Zn-SOD or Mn-SOD) in plant kingdom (Mishra et al., 2006). In this study, the increases of three SOD isoenzymes activities, CuZnSOD, FeSOD 1 and FeSOD 2 were identified by zymogram staining (Fig. 11A), suggesting that three SOD isoenzymes were activated in *C. demersum* under OP exposure.

Further detoxification for SOD product, H_2O_2 was conducted by other enzymatic and non-enzymatic antioxidants in all compartments in plant. CAT and POD have responsibility to detoxify H_2O_2 become water and oxygen. CAT is distributed mainly in peroxisomes and mitochondria (Willekens et al., 1995), while POD can be found mostly in cytosol, cell wall, vacuole and extracellular spaces (Mishra et al., 2006). In this present study, the enzyme activities of CAT and POD were increased under OP treatments concomitant with the increase of

H_2O_2 level. OP exposure has been reported that can induced CAT activity in *A. thaliana* (Chen et al., 2013). The activities of CAT and POD in *C. vulgaris* and *S. capricornutum* were also induced under NP treatment (Gao and Tam, 2011). *C. demersum* also have been reported to employ the CAT and POD to cope with various stress, like salt stress, heavy metals and organobromine compounds (Mishra et al., 2006; Sun et al., 2008; Cheng, 2011). Peroxidases (PODs) have been reported as the primal enzymes on endocrine disrupting chemicals (EDCs) degradation. Oxidation of most phenolic EDCs (include OP) catalyzed mainly by peroxidases or biological Fenton reaction through the utilization of H_2O_2 (Reis and Sakakibara, 2012). Histochemical localization using guaiacol and H_2O_2 shown the oxidation sites of EDCs by peroxidases in *C. demersum* cells and have been proposed to degradation mechanism of EDCs in aquatic plant. Further, in *C. demersum*, which has a high POD activity also showed higher removal efficiencies of most EDCs in the enzymatic *in vitro* treatments (Reis et al., 2014). In this present study, the H_2O_2 and POD activity were increased significantly, these findings might indicate the role of H_2O_2 and POD in removal mechanisms of OP in *C. demersum.*

APXs are heme-containing enzymes involved in scavenging H_2O_2 in water-water and AsA-GSH cycles (Asada, 1992). APX isoenzymes are distributed in at least five distinct cellular compartments: mitochondrial (mAPX), thylakoid (tAPX) and glyoxisome membrane forms (gmAPX), as well as chloroplast stromal soluble form (sAPX), cytosolic form (cAPX) (Noctor and Foyer, 1998). In this present study, the enzyme activity of APX was significantly increased under OP treatments. Increased APX was also detected in leaves of *A. thaliana* under OP and NP treatment (Chen et al., 2013; Chen and Yen, 2013). APX activity is enhanced in *C. demersum* in response to during different abiotic stress conditions (Rama Devi and Prasad, 1998; Mishra et al., 2006). Together with APX, GR plays an essential role in the defense system against ROS through AsA-GSH cycles. Increased GR activity confers stress tolerance and has the ability to alter the redox state of important components of the electron transport chain (Gill et al., 2013). Thus, an increased activity of GR in the present observation might have contributed to maintain the homeostasis in the plant cells under OP exposure.

As mentioned above, APX and GR are well known as two key enzymes in AsA-GSH cycles. AsA is utilized by APX to detoxify H_2O_2 become water, resulting in generation of MDHA. MDHA has a short life span, that can disproportionate into DHA and AsA (Hasanuzzaman et al., 2012). In this present study, both enzymes activities were enhanced under OP treatments. The increase of APX activity was accompanied by AsA and DHA enhancement, resulting in the decrease of AsA/DHA ratio in the higher concentration of OP treatment. In the normal conditions, the plants will maintain the redox status homeostatis through keeping the ratio of AsA/DHA remains high. While the changes in AsA/DHA ratio are considered to be a redox status indicator (Brossa et al., 2013). In plant cells, AsA has been known as the major metabolite that can act as antioxidant by scavenging ROS directly or in association with other antioxidant to protect from oxidative stress damage (Smirnoff, 1996).

GSH play an important role as a substrate for DHAR in the AsA-GSH pathway, it also can directly scavenges $OH^{\bullet}$ and $^{1}O_2$ and may protect enzyme thiol groups and also known to involve in signal transduction (Foyer and Shigeoka, 2011). In this present study, total glutathione contents were increased significantly under OP treatments, concomitant with the increase of reduced glutathione (GSH) and decrease of oxidized glutathione (GSSG), resulting in the elevation of GSH/GSSG ratio. The decreased of GSSG and the high ratio of GSH/GSSG may be ascribed to the increase of GR activity. GR converts oxidized glutathione (GSSG) to reduced glutathione (GSH) thus helps in maintaining high ratio of GSH/GSSG under various abiotic stresses (Trivedi et al., 2013). GSSG consists of two GSH linked by a disulphide bridge which can be converted back to GSH by GR (Halliwell, 2006). Thereby, GR helps in maintaining GSH pool and reducing environment in the cell, which is crucial for the active functioning of proteins. However, we also found that the tGSH of *C. demersum* still high even the GR activity has already declined under 3 mg L^{-1} OP treatment. We suggested the GSH biosynthesis might play an important role in *C. demersum* under OP exposure. Generation and maintenance of reduced GSH, can occur either by *de novo* synthesis and via recycling by GR (Pessarakli, 2014). Its *de novo* synthesis occurs two well characterized steps: the first involving the formation of ɣ-glutamylcysteine from glutamate and cysteine is catalyzed by enzyme ɣ-glutamylcysteine synthase; second step the ɣ-glutamylcysteine is converted to glutathione by glutathione synthase catalyzing the reaction (Noctor and Foyer, 1998). The induction of cysteine and glutathione synthesis during salt stress in the wildtype plants of *Brassica napus* as reported by Ruiz and Blumwald (2002) suggests a possible protective mechanism by GSH.

To confirm the involvement of GSH in *C. demersum* defense mechanisms under OP exposure, *C. demersum* leaves were pretreated with BSO, a specific and potent inhibitor of ɣ-ECS, the first enzyme that play role in biosynthesis of GSH for 8 h (Chao et al., 2011) then transferred to hoagland solution with or without OP and cultivated for 5 days. After 5 days, under BSO pretreatment, the tGSH were decreased. The decreasing of tGSH was followed by the decreasing of tAsA contents and also GR and APX enzyme activity. *C. demersum* also showed much more severe phenotype damage under OP exposure with BSO pretreatment. Exogenous application of BSO also has been reported that can reduce GSH and AsA content, also decreased the activities of GR and APX in Cd tolerance of rice seedlings (Chao et al., 2011). Based on the data obtained in this study, it could be concluded that GSH biosyntheis plays an important role in *C. demersum* to cope with the OP-induced oxidative stress.

In the present study, we found that OP was able to cause oxidative stress in aquatic plant *C. demersum*, and to induce production of a large number of free radicals. OP also caused growth inhibition and reduced photosynthesis pigments, chlorophyll *a* and *b*. Aditionally, the changes of antioxidant defense systems were observed. Among these parameters, both enzymatic and non-enzymatic antioxidants were enhanced under OP treatments. SOD, APX, GR, CAT and POD were increased, while GSH and AsA levels were also elevated. BSO pretreatment were conducted to confirm the role of GSH biosynthesis in *C. demersum* under OP exposure. The decreasing of tGSH after BSO pretreatment indicated that the synthesis of GSH has been blocked by BSO, it was followed by the decreasing of tAsA content and also GR and APX enzyme activity. Interestingly, *C. demersum* with BSO pretreatment showed the worst conditions under OP exposure. These results suggest that antioxidative system were actively regulated by plants *C. demersum* especially GSH biosynthesis to against OP-induced oxidative stress, but it could not prevent the increase levels of ROS or damage of the photosynthetic system in plants exposed to higher concentrations of OP. In the highest concentration (3 mg L^{-1}), *C. demersum* showed the worst physiological features because the increase production of ROS ($O_2^{\bullet -}$ and H_2O_2) might exceed the management capacity of antioxidant defense system and caused exacerbating damage to cellular components, while in the lower concentration, *C. demersum* can cope with the OP-induced oxidative stress by modulating the antioxidant defense system to scavenging the ROS and maintain the equilibrium status.

6. CONCLUSIONS

These studies show that the toxicity of 4 tert-octylphenol (OP) induces oxidative stress and alters the antioxidant machinery in *Ceratophyllum demersum* plants. The main findings and conclusions of this study are summarized as follows:

1) OP was able to cause oxidative stress in aquatic plant *C. demersum*, and to induce production of a large number of free radicals.
2) OP also caused growth inhibition and reduced photosynthesis pigments, chlorophyll *a* and *b*.
3) Both enzymatic and non-enzymatic antioxidants were enhanced under OP treatments. SOD, APX, GR, CAT and POD were increased, while GSH and ASC levels were also elevated.
4) Antioxidative system were actively regulated by *C. demersum* in response to the OP stress, mainly GSH biosynthesis. BSO pretreatment confirmed the important role of GSH biosynthesis in *C. demersum* to against OP-induced oxidative stress.

Overall, this study has been shown that at concentration 0.5 mg L^{-1} of OP, *C. demersum* can cope with the stress and activate the defense mechanism to againt the OP-induced oxidative stress. Considering the ability of *C. demersum* to cope with the stress under OP exposure, we propose *C. demersum* as the phytoremediator in aquatic environment to eliminate the pollutant such as, OP, but until now we lack an important data about absorption. Further work is necessary to evaluate the accumulation of OP in *C. demersum*.

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Appendix I. Hoagland's Solution Recipe

The composition of Hoagland's solution was shown in the Table 2, and the detail procedures were as follows:

Table 2. Hoagland's solution recipe

Stock conc.	**Components**	**Formula**	**Mol. Wt**	**cc in a litre of nutrient solution**
1 M	Ammonium acid phosphate	$NH_4H_2PO_4$	115.02	1
1 M	Potassium nitrate	KNO_3	101.1	6
1 M	Calcium nitrate	$Ca(NO_3)_2$	236.15	4
1 M	Magnesium Sulfate	$MgSO_4$	246.47	2
5 g L^{-1}	Iron chelate	Fe-EDTA		1
Micronutrient stock solution				1
Micronutrient stock composition				**Gram dissolved in 1 litre H_2O**
	Boric Acid	H_3BO_3	61.84	2.86
	Manganese Chloride	$MnCl_2.4H_2O$	197.91	1.81
	Zinc Sulfate	$ZnSO_4.7H_2O$	287.54	0.22
	Copper Sulfate	$CuSO_4.5H_2O$	249.69	0.08
	Molybdic Acid	$H_2MoO_4.H_2O$		0.02

1. Use RO water to prepare the stock solution as shown in Table 2
2. Autoclave all solutions using a cycle with 15 min at 121°C.
3. Store Fe-EDTA and $MgSO_4$ at 4°C and all others at room temperature.

Appendix II. Biochemical Analysis Protocol

Prepare Sodium Phosphate Buffer

1. Prepare separate stock solutions of (a) disodium hydrogen phosphate (Na_2HPO_4, FW 141.96) and (b) sodium dihydrogen phosphate (NaH_2PO_4, FW 137.99), both at 1 M concentration in 500 ml ddH_2O.
2. Buffer solutions (at 1 M) are then prepared at the required pH by mixing together the volume of each stock solution shown in the Table 3.

Table 3. Preparation of sodium phosphate buffer solutions for use at 25°C

Required pH at 25°C	Volume of stock Na_2HPO_4 (ml)	Volume of stock NaH_2PO_4 (ml)
6.0	6.2	43.8
6.2	9.3	40.7
6.4	13.3	36.7
6.6	18.8	31.2
6.8	24.5	25.5
7.0	30.5	19.5
7.2	36.0	14.0
7.4	40.5	9.5
7.6	43.5	6.5
7.8	45.8	4.2
8.0	47.4	2.6

Determination of superoxide radical ($O_2^{\bullet -}$) activity

Materials:

1. 0.1 g leaves
2. 65 mM pH 7.8 phosphate buffer
3. 10 mM hydroxylamine
4. 17 mM sulfanilic acid
5. 7 mM α-naphthylamine (dark)
6. Ether

Method:

1. Take the sample (about 0.1 g), put into the mortar, take 1 mL sodium phosphate buffer (65mM, pH 7.8), grind the mixture in the cold area
2. Centrifuge 12,000 g for 20 minutes under 4°C
3. Take supernatant 0.5 mL, add 0.45 mL sodium phosphate buffer (65 mM, pH 7.8) and 0.05 mL hydroxlamine, put in the room temperature for 20 minutes
4. After 20 minutes, take liquid 0.5 mL, add 0.5 mL sulfanilic acid (17 mM) and 0.5 mL α-naphthylamine (7 mM), put in the room temperature for 20 minutes
5. After 20 minutes, take 0.7 ml liquid and mix with 0.7 mL ether, centrifuge at 1,500 g for 5 minutes under 25°C
6. Take the bottom liquid and measure in the spectrophotometer at 530 nm [use 0, 1, 2, 5, 10, and 20 µM sodium nitrite for standard curve line].

Chemicals:

➢ **17 mM sulfanilic acid**

0.825 g sulfanilic acid in 187.5 mL H_2O + 62.5 mL glacial acetic acid, exactly to 250 mL

➢ **7 mM α-naphthylamine: (M)**

0.25 g α-naphthylamine in 50 mL boiling H_2O + 62.5 mL glacial acetic acid, exactly to 250 mL.

Determination of photosynthetic pigments

1. The leaves sample is ground in extraction buffer (sodium phosphate buffer 50 mM, pH = 6,8)

   PS : plant material (mg) : extraction buffer (ml) = 1 g : 4 ml
2. Take 40 µl from the mixture and put into 1,5 ml eppendorf tube + add 960 µl ethanol (100%), and mix them together.
3. Put the mixture in the dark chamber at 4°C and wait until 30 minutes.
4. Centrifuge at 1,000 g for 15 minutes under 4°C.
5. Measure the absorbance in the spectrophotometer at 649 ad 665 nm and calculated the chlorophyll content using these formula:

   Chlorophyll *a* = $(13.7 \times A_{665}) - (5.76 \times A_{649})$ [µg Chl $(40\ \mu l)^{-1}$]

   Chlorophyll *b* = $(25.8 \times A_{649}) - (7.6 \times A_{665})$ [µg Chl $(40\ \mu l)^{-1}$]

   Total Chlorophyll = $(6.1 \times A_{665}) + (20.04 \times A_{649})$ [µg Chl $(40\ \mu l)^{-1}$]

   Chlorophyll *a* content (mg g^{-1} FW)

   - Chlorophyll *a* × 50 (dilution) ÷ 1000 ÷ FW (g)

   Chlorophyll *b* content (mg g^{-1} FW)

   - Chlorophyll *b* × 50 (dilution) ÷ 1000 ÷ FW (g)

   Total Chlorophyll content (mg g^{-1} FW)

   - Total Chlorophyll × 50 (dilution) ÷ 1000 ÷ FW (g)

Determination of Lipid Peroxidation (MDA contents)

Extraction buffer:

5% TCA (trichloroacetic acid)

Procedure :

1. The leaves sample is extracted in 5% TCA
   PS : plant materials (g) : 5% TCA (ml) = 0.1 : 1
2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant (keep supernatant under 4°C)
4. Add the following chemicals in the **covered test tube** and mix well (Table 4)

Table 4. Chemicals composition for MDA measurement

		Add volume (ml) **total : 2.5 ml**	Final conc.
	Supernatant	0.5	
0.5%	Thiobarbituric acid (**TBBA**)	2	

PS : "blank" = 0.5 ml supernatant is replaced by 0.5 ml **5% TCA**

5. Incubate the mixture at water bath under **95°C** for **30 min**
6. Terminate the reaction after transferred to ice box
7. Take 2 ml of the above reaction solution and centrifuge it at 12,000 g for 10 min under 4°C
8. Collect the supernatant and incubate it for 20 min under room temperature
9. Determine the absorbency at **532** nm and **600** nm
10. **E = 155 mM^{-1} cm^{-1}**
11. MDA contents (μmol/g FW) = $[(A_{532}-A_{600})*5)]/(155*FW)*1000$

Chemicals :

a. 5% TCA : 5 g TCA / 100 ml dist. H_2O

b. 0.5 % TBBA : 0.5 g TBBA/ 100 ml 20% TCA

Determination of Hydrogen Peroxide (H_2O_2) Activity

1. A 0.1 g leaves sample is extracted in 2 ml 50 mM sodium phosphate buffer pH 6.8 containing 1 mM hydroxylamine under 4°C.
2. The homogenate is centrifuged at 12,000 g for 10 min under 4°C.
3. A sample of 0.5 ml of the supernatant is mixed with 0.5 ml $TiCl_4$ [Titanium Chloride (0.1%, v/v) diluted in 20% (v/v) H_2SO_4]
4. Centrifuged at 12,000 g for 10 min under 25°C. The absorbance of supernatant is measured at 410 nm. The content of H_2O_2 was calculated using an extinction coefficient of 0.28 $\mu mol^{-1}cm^{-1}$.

H_2O_2 contents = $A_{410} \div 0.28$ (K, $\mu mol^{-1}cm^{-1}$) × 1.5 (dilution) ÷ FW (g)

Chemicals :

a. 20% H_2SO_4: 200 ml H_2SO_4/ 1000 ml dist. H_2O

b. $TiCl_4$: 1 g TiC_3/ 1000 ml 20% TCA

Appendix III. Enzymatic Antioxidant Assay Protocol

1. Spectrophotometric measurement

Ascorbate Peroxidase (APX; EC 1.11.1.11) Assay

Extraction buffer:

1 ml 50 mM $NaPO_4$ (pH 7) + 2 mM Na_2EDTA + 1 mM PMSF + 0.5 mM ascorbate

Procedure :

1. The algae sample is and extracted in extraction buffer

   PS : plant material (g) : extraction buffer (ml) = 0.1 : 1
2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant and assay APX activity within 2 h (keep supernatant under 4°C)
4. Add the following chemicals in the **cuvette** and mix well (Table 5)

Table 5. Chemicals composition for APX Activity Assay

		Add volume (ml) **total : 1 ml**	Final conc.
	Supernatant	0.1	
0.1 M	phosphate buffer pH = 7	0.5	50 mM
5 mM	ascorbate	0.1	0.5 mM
0.5 mM	Na_2EDTA	0.2	1.5 mM
10 mM	H_2O_2	0.1 (**add at last and then measure**)	0.25 mM

   PS : "blank" = 0.1 ml supernatant is replaced by 0.1 ml **extraction buffer**
5. Determine the decrease of absorbance at 290 nm within 1 min at room temperature as compared with the blank
6. **$E = 2.8\ mM^{-1}\ cm^{-1}$**

An unit of APX is defined as decrease in 1 nmol ascorbate/min

Specific activity = unit of APX activity/mg protein

Chemicals :

a. **<u>0.1 M phosphate buffer (50 ml, pH = 7)</u>**

5 ml 1 M $NaPO_4$ pH 7 / 50 ml H_2O

b. **<u>5 mM ascorbate (1 ml, FW 176.13, fresh prepared)</u>**

0.05 ml 0.1 M ascorbate (stock) / 1 ml dist. H_2O

c. **<u>0.5 mM Na_2EDTA (15 ml, FW 372.24)</u>**

0.0028 g / 15 ml dist. H_2O

d. **<u>10 mM H_2O_2 (1 ml, 9.7912 M, fresh prepared)</u>**

1 µl 9.7912 H_2O_2 / 1 ml dist. H_2O

Catalase (CAT; EC 1.11.1.6) Assay

Extraction buffer:

1 ml 50 mM $NaPO_4$ (pH 7) + 2 mM Na_2EDTA + 1 mM PMSF

Procedure :

1. The algae sample is and extracted in extraction buffer

   PS : plant material (g) : extraction buffer (ml) = 0.1 : 1

2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant and assay CAT activity within 2 h (keep supernatant under 4°C)
4. Add the following chemicals in the **cuvette** and mix well (Table 6).

Table 6. Chemicals composition for CAT Activity Assay

		Add volume (ml) **total : 1 ml**	Final conc.
	Supernatant	0.1	
31.25 mM	phosphate buffer pH = 7	0.8	25 mM
200 mM	H_2O_2	0.1 (**add at last and then measure**)	20 mM

   PS : "blank" = 0.1 ml supernatant is replaced by 0.1 ml **extraction buffer**

5. Determine the decrease of absorbance at 240 nm within 2 min at room temperature as compared with the blank
6. **E = 4.0 mM^{-1} cm^{-1}**

   An unit of CAT is defined as decrease in 1 nmol H_2O_2 /min.

   Specific activity = unit of CAT activity/mg protein

Chemicals :

a. **<u>31.25 mM phosphate buffer (50 ml, pH = 7)</u>**

   1.5625 ml 1 M $NaPO_4$ pH 7 / 50 ml dist. H_2O

b. **<u>200 mM H_2O_2 (1 ml, 9.7912 M, fresh prepared)</u>**

   200 µl 9.7912 H_2O_2 / 1 ml dist. H_2O

Glutathione Reductase (GR; EC 1.8.1.7 or EC 1.6.4.2) Assay

Extraction buffer:

1 ml 50 mM $NaPO_4$ (pH 7) + 2 mM Na_2EDTA + 1 mM PMSF

Procedure :

1. The algae sample is and extracted in extraction buffer

   PS : plant material (g) : extraction buffer (ml) = 0.1 : 1

2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant and assay GR activity within 2 h (keep supernatant under 4°C)
4. Add the following chemicals in the **cuvette** and mix well (Table 7)

Table 7. Chemicals composition for GR Activity Assay

		Add volume (ml) **total : 1 ml**	Final conc.
	Supernatant	0.1	
0.4 M	phosphate buffer pH = 7.5	0.5	0.2 M
2 mM	Na_2EDTA	0.1	0.2 mM
15 mM	$MgCl_2$	0.1	1.5 mM
2.5 mM	GSSG	0.1	0.25 mM
0.25 mM	β-NADPH	0.1 (**add at last and then measure**)	25 µM

   PS : "blank" = 0.1 ml supernatant is replaced by 0.1 ml **extraction buffer**

5. Determine the decrease of absorbance at 340 nm within 2 min at room temperature as compared with the blank
6. **E = 6.2 mM^{-1} cm^{-1}**

   An unit of GR is defined as decrease in 1 nmol β-NADPH /min

   Specific activity = unit of GR activity/mg protein

Chemicals :

a. **<u>0.4 M phosphate buffer (50 ml, pH = 7.5)</u>**

   20 ml 1 M $NaPO_4$ pH 7.5 / 50 ml dist. H_2O

b. **<u>2 mM Na_2EDTA (50 ml, FW 372.24)</u>**

   0.0372 g / 50 ml dist. H_2O

c. **<u>10 mM $MgCl_2$ (50 ml, FW 203,3)</u>**

   0.1525 g / 50 ml dist. H_2O

d. **<u>25 mM GSSG stock (5 ml, FW 656.6, stored in -20 ^{0}C)</u>**

   0.082 g / 5 ml dist. H_2O

   PS : each eppendorf has 0.15 ml 25 mM GSSG

e. **<u>2.5 mM GSSG</u>**

   Add **1.35** ml dist. H_2O in each eppendorf

f. **<u>0.25 mM β-NADPH (30 ml, FW 833.4, stored in -20 ^{0}C)</u>**

   6.25 mg / 30 ml dist. H_2O

   PS : each eppendorf has 1.5 ml 0.25 mM β-NADPH

Peroxidase (POD; EC 1.11.1.7) Assay

Extraction buffer:

1 ml 50 mM $NaPO_4$ (pH 7) + 2 mM Na_2EDTA + 1 mM PMSF

Procedure :

1. The algae sample is and extracted in extraction buffer

   PS : plant material (g) : extraction buffer (ml) = 0.1 : 1

2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant and assay POD activity within 2 h (keep supernatant under 4°C)
4. Add the following chemicals in the **cuvette** and mix well (Table 8)

Table 8. Chemicals composition for POD Activity Assay

		Add volume (ml) **total : 1.3 ml**	Final conc.
	Supernatant	0.1	
0.1 M	phosphate buffer pH = 6.8	1	7.7 mM
10 mM	guaiacol	0.1	0.77 mM
150 mM	H_2O_2	0.1 (**add at last and then measure**)	11.54 mM

PS : "blank" = 0.1 ml supernatant is replaced by 0.1 ml **extraction buffer**

5. Determine the increase of absorbance at 470 nm within 5 min at room temperature as compared with the blank

   PS : guaiacol $\longrightarrow$ tetraguaiacol

6. **E = 26.6 mM^{-1} cm^{-1}**

An unit of POD is defined as 1 μmol tetraguaiacol formation/min

Specific activity = unit of POD activity/mg protein

Chemicals :

a. **<u>0.1 M phosphate buffer (50 ml, pH = 6.8)</u>**

   5 ml 1 M $NaPO_4$ pH 6.8 / 50 ml dist. H_2O

b. **<u>10 mM guaiacol (50 ml, 8.9576 M)</u>**

   0.558 ml 8.9576 M / 50 ml dist. H_2O

c. **<u>150 mM H_2O_2 (1 ml, 9.7912 M, fresh prepared)</u>**

   15 µl 9.7912 M / 1 ml dist. H_2O

Superoxide dismustase (SOD; EC 1.15.1.1) Assay

Extration buffer:

1 ml 50 mM $NaPO_4$ (pH 7) + 2 mM Na_2EDTA + 1 mM PMSF

Procedure :

1. The algae sample is and extracted in extraction buffer

   PS : plant material (g) : extraction buffer (ml) = 0.1 : 1

2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant and assay SOD activity within 2 h (keep supernatant under 4°C)
4. Add the following chemicals in the **test tube** and mix well (Table 9)

Table 9. Chemicals composition for SOD Activity Assay

		Add volume (ml) **total : 0.475 ml**	Final conc.
	Supernatant	0.02	
0.15 M	phosphate buffer pH = 7.8	0.25	79 mM
130 mM	methionine	0.075	20.52 mM
1 mM	Na_2EDTA	0.075	0.16 mM
0.63 mM	NBT	0.075	99.5 μM
7.5 μM	riboflavin	0.15 (**add at last and then measure**)	2.34 μM

PS : "blank" = 0.02 ml supernatant is replaced by 0.02 ml **extraction buffer**

5. Reaction is carried out in test tubes at room temperature under illumination in incubator and run for 10 min
6. Determine the absorbance at 560 nm
7. An unit of SOD activity is defined as a 50% inhibition of the nitro blue tetrazolium (NBT).

Specific activity = unit of SOD activity/mg protein/h

(Blank-sample)/(blank/2)*6/mg protein

Chemicals :

a. **0.15 M phosphate buffer (50 ml, pH = 7.8)**
   7.5 ml 1 M $NaPO_4$ pH 7.8 / 50 ml dist. H_2O
b. **130 mM methionine (50 ml, FW 149.2, stored in -4°C)**
   0.9698 g / 50 ml dist. H_2O
c. **1 mM Na_2EDTA (50 ml, FW 372.24)**
   18.612 mg / 50 ml dist. H_2O
d. **0.63 mM NBT stock (30 ml, FW 817.6, stored in -4°C)**
   25.75 g / 30 ml dist. H_2O
e. **7.5 mM riboflavin stock (10 ml, FW 376.4, stored in -20 ^{0}C)**
   28.23 mg / 10 ml dist. H_2O
f. **7.5 µM riboflavin**
   10 µl 7.5 mM riboflavin / 10 ml dist. H_2O

2. Zymography Assay

Zymography assay of GR activity

Principle:

$$\text{NADPH} + \text{GSSG} + H^+ + \text{DCIP} \xrightarrow{\text{GR}} 2\ \text{GSH} + \text{NADP}^+ + \text{DCIP}$$

(reduced form)

$$\text{MTT} \xrightarrow{\text{Reduced DCIP}} \text{Formazan (purple-blue color)}$$

Extraction buffer for GR

50 mM $NaPO_4$ pH 7.0 containing 2 mM Na_2EDTA, 1 mM PMSF

Table 10. 10 mL Extraction Buffer for Enzyme (GR, SOD, POD) and Protein Assay

	Volume	Final conc.
1 M $NaPO_4$ pH 7.0	0.5 mL	50 mM
0.5 M Na_2EDTA	40 µl	2 mM
0.1 PMSF	10-100 µl	0.1-1 mM
H_2O	9.36 ml	
Total	10 ml	

Sample Extraction

1. Take 0.3 g leaf tissue
2. Extract with 0.5 ml extraction buffer;
3. Centrifuge in 12,000 g at 4°C for 10 min;
4. Collect the supernatant and repeat centrifugation at least 3 times;
5. Determine the protein content;
6. Add 10X protein dye and mix well;
7. Store in -80°C for NATIVE PAGE.

Native PAGE (Run at 4°C) 10% Resolving Gel (Table 1)

1. Using 1X TG buffer as running buffer;
2. Prerun at 80V for 30 min to eliminate APS;
3. Change freshly running buffer in the inner tank;
4. Loading sample (5 μg/well);
5. Run at 80V until the sample into resolving gel for 75 min;
6. Change voltage to 120V, run for 115 min;
7. The gel is ready for staining.

Staining procedure (Method I, All steps are performed at RT.)

Immersed gel in Staining buffer (0.25 M Tris-HCl (pH 7.5) containing 3 mM Na_2EDTA, 0.4 mM NADPH, 0.68 mM 2,6 dichlorophenolindophenol (DCIP), 0.48 mM 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and 3.4 mM GSSG) (Table 5) in the dark for 1 hr. (Duplicate control gels were stained in the absence of GSSG.). Use 10% acetic acid to stop the reaction. Store gel in 10% acetic acid at 4°C up for several month.

Table 11. Composition of Staining Buffer for GR Activity

Staining buffer	Volume (ml)		Final conc.
0.5 M Tris-HCl pH 7.5	5	7.5	250 mM
0.5 M Na_2EDTA	0.06	0.09	3 mM
40 mM NADPH	0.1	0.15	0.4 mM
34 mM DCIP	0.2	0.3	0.68 mM
16 mM MTT	0.3	0.45	0.48 mM
85 mM GSSG	0.4	0.6	3.4 mM
H_2O	3.94	5.91	
Total	10 ml	15ml	

Chemicals

1. 0.5 M Tris-HCl pH 7.5 (MW 121.14, 24.288g/400 ml)
2. 40 mM NADPH (MW 833.35, 0.034g/1ml, 100 µl per tube) (stored in -20 ^{0}C)
3. 85 mM GSSG (MW612.63, 0.1044g/2ml, 1 ml per tube)
4. 34 mM DCIP (MW290.08, 0.0986g/10ml, 1 ml per tube)
5. 16 mM MTT (MW414.32, 0.06g/9ml, 1 ml per tube)
6. 10X protein dye (dissolved in water): 0.01% Bromophenol Blue + 50% glycerol
7. 10X TG buffer (pH 8.3): (30.28g Tris + 144.13g Glycine)/1000 ml H_2O
8. 500 ml 1X TG: 50 ml 10X TG diluted to 500 ml with Qwater.

Zymography assay of APX activity

Principle:

$$NBT \xrightarrow{ASC + TEMED} \text{formazan (purple-blue)}$$

$$H_2O + ASC \xrightarrow{APX} H_2O + DHA$$

So, if there's no ASC, there's no purple-blue color on the gel

Extraction buffer for APX

50 mM $NaPO_4$ pH 7.0 containing 2 mM Na_2EDTA, 1 mM PMSF, 5 mM ASC

Table 12. 10 mL Extraction Buffer for APX

	Volume	Final conc.
1 M $NaPO_4$ pH 7.0	0.5 mL	50 mM
0.5 M Na_2EDTA	40 µl	2 mM
1 M ASC	50 µl	5 mM
0.1 PMSF	10-100 µl	0.1-1 mM
H_2O	9.36 ml	
Total	10 ml	

Sample Extraction

1. Take 0.3 g leaf tissue
2. Extract with 0.5 ml extraction buffer;
3. Centrifuge in 12,000 g at 4°C for 10 min;
4. Collect the supernatant and repeat centrifugation at least 3 times;
5. Determine the protein content;
6. Add 10X protein dye and mix well;
7. Store in -80°C for NATIVE PAGE.

Native PAGE (Run at 4°C) 10% Resolving Gel (Table 1)

1. Using 1X TG buffer as running buffer containing 2 mM ASC
2. Prerun at 80V for 30 min to eliminate APS;
3. Change freshly running buffer in the inner tank;
4. Loading sample (5 μg/well);
5. Run at 80V until the sample into resolving gel for 99 min;
6. Change voltage to 120V, run for 160 min;
7. The gel is ready for staining.

p.s. prepare 500 ml running buffer per run

Staining procedure (All steps are performed at RT and gentle shaking)

1. Equilibrate with 50 mM $NaPO_4$ pH 7.0 containing 2 mM ASC for 10 min (3 times)
2. Incubate with 50 mM NaPO4 pH 7.0 containing 4 mM ASC and 2 mM H_2O_2 for 20 min.
3. Wash with 50 mM $NaPO_4$ pH 7.0 for 1 min.
4. Incubate with 50 mM $NaPO_4$ pH 7.8 containing 28 mM TEMED and 2.45 mM NBT for 3-5 min in the dark (stop while the bands are disguisable). Use 10% acetic acid to stop the reaction.
   (10 ml → 42 μl TEMED, 0.02 g NBT)
5. Store the gel in 10% acetic acid at 4°C up for several month.

Chemicals

1. Ascorbate: 1 M stock stored in -20°C (disending into 0.5 ml/valve)
2. 500 ml 1X TG: 50 ml 10X TG diluted to 500 ml with Qwater, and add 1 ml 1 M ASC

Zymography assay of SOD activity

Extraction buffer for SOD

50 mM $NaPO_4$ pH 7.0 containing 2 mM Na_2EDTA, 1 mM PMSF as shown in Table 10.

Sample Extraction

1. Take 0.3 g leaf tissue
2. Extract with 0.5 ml extraction buffer;
3. Centrifuge in 12,000 g at 4°C for 10 min;
4. Collect the supernatant and repeat centrifugation at least 3 times;
5. Determine the protein content;
6. Add 10X protein dye and mix well;
7. Store in -80°C for NATIVE PAGE.

Native PAGE (Run at 4°C) 10% Resolving Gel (Table 1)

1. Using 1X TG buffer as running buffer
2. Prerun at 80V for 30 min to eliminate APS;
3. Change freshly running buffer in the inner tank;
4. Loading sample (5 µg/well);
5. Run at 80V until the sample into resolving gel for 99 min;
6. Change voltage to 120V, run for 60 min;
7. The gel is ready for staining.

p.s. prepare 500 ml running buffer per run

Total SOD Staining procedure (All steps are performed at RT.)

1. Immersed the gel in 0.1 % NBT (0.01g / 10 ml) and shake for 15 min in the dark, rinsed with ddH_2O three times.

2. Add 20 ml 0.1 M potassium phosphate buffer pH 7.0 containing 66.7 µl TEMED + 74.7 µl 7.5 mM riboflavin, shake for 15 min, and rinsed with ddH_2O three times.
3. Add small amount of 0.1 M potassium phosphate buffer pH 7.0, gently shake for 10-20 min until the bands are disguisable.
4. Use 10% acetic acid to stop the reaction.
5. Take a photo.
6. Store the gel in 10% acetic acid at 4°C up for several month.

MnSOD (Use H_2O_2, to inhibit CuZnSOD and FeSOD)

1. Immersed the gel in 20 ml potassium phosphate buffer 16.5 µl H_2O_2 (9.7 M , final conc. = 8 mM) 30 min in 4°C.
2. Rinsed with Qwater three times
3. Followed the total SOD staining procedure

MnSOD and FeSOD (Use KCN to inhibit CuZnSOD))

1. Immersed the gel in 0.1 % NBT (0.01g / 10 ml) and shake for 15 min in the dark, rinsed with ddH_2O three times.
2. Add 20 ml 0.1 M potassium phosphate buffer pH 7.0 containing 66.7 µl TEMED + 74.7 µl 7.5 mM riboflavin + 80 µl KCN (2 M, final conc. = 8 mM, shake for 15 min, and rinsed with ddH_2O three times.
3. Add small amount of 0.1 M potassium phosphate buffer pH 7.0, gently shake for 10-20 min until the bands are disguisable.
4. Use 10% acetic acid to stop the reaction.
5. Take a photo.
6. Store the gel in 10% acetic acid at 4°C up for several month.

Zymography assay of POD activity

<u>Extraction buffer for POD</u>

50 mM $NaPO_4$ pH 7.0 containing 2 mM Na_2EDTA, 1 mM PMSF as shown in Table 10.

<u>Sample Extraction</u>

1. Take 0.3 g leaf tissue
2. Extract with 0.5 ml extraction buffer;
3. Centrifuge in 12,000 g at 4°C for 10 min;
4. Collect the supernatant and repeat centrifugation at least 3 times;
5. Determine the protein content;
6. Add 10X protein dye and mix well;
7. Store in -80°C for NATIVE PAGE.

<u>Native PAGE (Run at 4°C) 10% Resolving Gel (Table 1)</u>

1. Using 1X TG buffer as running buffer
2. Prerun at 80V for 30 min to eliminate APS;
3. Change freshly running buffer in the inner tank;
4. Loading sample (5 µg/well);
5. Run at 80V until the sample into resolving gel for 60 min;
6. Change voltage to 120V, run for 5-6 h;
7. The gel is ready for staining.

p.s. prepare 500 ml running buffer per run

<u>Total POD Staining procedure (All steps are performed at RT.)</u>

1. The gel was washed with distillated water to remove the buffer.
2. Immersed the gel in 4.5 mM guaiacol (4.5 ml 10 mM guaiacol) and 22.5 mM H_2O_2 (23.19 µl 9.7 M H_2O_2) in 100 mM phosphate buffer

(pH 7.0) at 25°C and gently shake (stop while the bands are disguisable).

3. Use 10% acetic acid to stop the reaction.

4. Take a photo

5. Store the gel in 10% acetic acid at 4°C up for several month.

Protein Staining

Extraction buffer for protein staining

50 mM $NaPO_4$ pH 7.0 containing 2 mM Na_2EDTA, 1 mM PMSF as shown in Table 10.

Sample Extraction

1. Take 0.3 g leaf tissue
2. Extract with 0.5 ml extraction buffer;
3. Centrifuge in 12,000 g at 4°C for 10 min;
4. Collect the supernatant and repeat centrifugation at least 3 times;
5. Determine the protein content;
6. Add 10X protein dye and mix well;
7. Store in -80°C for NATIVE PAGE.

Native PAGE (Run at 4°C) 10% Resolving Gel (Table 1)

1. Using 1X TG buffer as running buffer
2. Prerun at 80V for 30 min to eliminate APS;
3. Change freshly running buffer in the inner tank;
4. Loading sample (5 µg/well);
5. Run at 80V until the sample into resolving gel for 60 min;
6. Change voltage to 120V, run for 99 min;

7. The gel is ready for staining.

p.s. prepare 500 ml running buffer per run

Protein Staining procedure (All steps are performed at RT.)

1. The gel was washed with distillated water to remove the buffer.
2. Immersed the gel in Coomasie Brilliant Blue R 250, gently shake for 30 min.
3. After 30 min, for destain procedure, the gel is immersed in destain buffer I (50% methanol + 10% glacial acetic acid) until the gel background become transparent light blue.
4. Replace the destain solution I with destain buffer II (5% methanol + 7% glacial acetic acid). Gently shake until the gel background become transparent and the bands are disguisable.
5. Take a picture using scanner (EPSON Perfection V370) when the background turned to transparent. Add water and a small amount of destain buffer II to store the gel at room temperature.

Chemical:

Coomassie Brilliant Blue R 250 Solution (250 ml)

Coomassie Brilliant Blue R 250	0.625 g
Acetic acid	25 ml
50% methanol	225 ml)

Appendix 4. Non-enzymatic Antioxidant Assay Protocol

Ascorbate (ASA) Assay

Extraction buffer:

5% TCA (trichloroacetic acid)

Procedure :

1. The fresh algae sample is extracted in extraction buffer
   PS : plant materials (g) : extraction buffer (ml) = 0.1 : 1
2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant and assay ascorbate (keep supernatant under 4°C)
4. Add the following chemicals in the **covered test tube** and mix well (Table 13)

Table 13. Chemicals composition for Ascorbate Assay

		Add volume (ml)	Final conc.
	Supernatant	**a.** 0.2 **b.** 0.2	**a.** Total ascorbate **b.** ASA
120 mM	phosphate buffer pH = 7.4	0.5	60 mM
15 mM	Na_2EDTA	0.2	3 mM
10 mM	DTT (dithiothreitol)	0.1 **a.** DTT **b.** Replaced by H_2O	1 mM
		25°C **10 min**	
40 mM	N-ethylmaleimide	0.1 **a.** N-ethylmaleimide **b.** Replaced by H_2O	
10%	TCA	0.4	
8 M	H_3PO_4	0.4	
0.26 M	α,α'-dipyridyl	0.4	
7.5 µM	$FeCl_3$	0.2	
		Incubate at water bath under **40**°C for **1 h**	

PS : Total Ascorbate = ASA + DHA (dehydroascorbate)

5. Determine the absorbance at 525 nm
6. A standard curve is prepared based on solutions with different concentrations of L-ascorbate (MERCK) (0-40 nmole). The difference between total ascorbate and AsA is considered to represent the content of DHA.

Chemicals :

a. **<u>0.12 M phosphate buffer (50 ml, pH = 7.4)</u>**

   6 ml 1 M $NaPO_4$ pH 7.4 / 50 ml dist. H_2O

b. **<u>15 mM Na_2EDTA (50 ml, FW 372.24)</u>**

   0.279 g / 50 ml dist. H_2O

c. **<u>40 mM N-ethylmaleimide (15 ml, FW 125.13, stored in -4°C)</u>**

   0.075 g / 15 ml dist. H_2O

   PS: each eppendorf has 1 ml

d. **<u>100 mM DTT stock (10 ml, FW 154.2, stored in -20°C)</u>**

   0.1542 g / 10 ml dist. H_2O

   PS: each eppendorf has 0.1 ml 100 mM DTT

e. **<u>10 mM DTT</u>**

   Add **0.9** ml dist H_2O in each eppendorf

f. **<u>8 M H_3PO_4 (50 ml, 14.8316 M, stored in -4°C)</u>**

   26.97 ml 85% H_3PO_4 / 50 ml dist. H_2O

g. **<u>0.26 M dipyridyl (100 ml, FW 156.19, stored in -20 ^{0}C)</u>**

   4.06 g / 100 ml 70% EtOH

   PS: each eppendorf has 1 ml

h. **<u>0.19 M $FeCl_3$ (50 ml, FW 270.3)</u>**

   2.568 g / 50 ml dist H_2O

Glutathione (GSH) Assay

Extraction buffer:

5% TCA (trichloroacetic acid)

Procedure :

1. The fresh algae sample is extracted in extraction buffer
   PS : plant materials (g) : extraction buffer (ml) = 0.1 : 1
2. Centrifuge at 12,000 g for 10 min under 4°C
3. Collect the supernatant
4. Add 300 µl 0.4 M $NaPO_4$ pH 8.0 in 300 µl TCA extracts for neutralization (keep supernatant under 4°C)
5. Add the following chemicals in the **cuvette** and mix well (Table 14)

Table 14. Chemicals composition for Glutathione Assay

		Add volume (ml)	Final conc.
	Supernatant	0.2	
250 mM	phosphate buffer pH = 7.5	0.4	100 mM
50 mM	Na_2EDTA	0.1	5 mM
2 mM	β-NADPH	0.1	0.2 mM
6 mM	DTNB (prepared in 0.2 M $NaPO_4$ pH 7.5)	0.1	0.6 mM
0.5 U/ml	GR	0.1	START

PS : Total GSH = GSH + GSSG

6. Determine the absorbance at 412 nm within 2 min
7. A standard curve is prepared based on solutions with different concentrations of 1 mM GSSG (SIGMA) (0-20 nmole)

GSSG

2 µl supernatant / 0.1 ml extract ⟶ vortex and incubate in room temperature for 1 h to eliminate GSH ⟶ then the extract was for the determination of GSSG

Chemicals :

a. **0.25 M phosphate buffer (50 ml, pH = 7.4)**

   12.5 ml 1 M $NaPO_4$ pH 7.4 / 50 ml dist. H_2O

b. **50 mM Na_2EDTA (50 ml, FW 372.24)**

   0.9306 g / 50 ml dist. H_2O

c. **40 mM β-NADPH (2,9 ml, FW 834,4 stored in -20°C)**

   0.1 g / 2.9 ml dist. H_2O

   PS: each eppendorf has 0.1 ml 40 mM β-NADPH

d. **6 mM DTNB (50 ml, FW 396.36, stored in -4°C)**

   0.119 g / 50 ml 0.2 M $NaPO_4$ pH 7.5

e. **1 M 2-vinylpyridine (1 ml, 8.995 M, stored in -20°C)**

   111.18 µl 2-vinylpyridine / 1 ml EtOH

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Research Experience

Year	Title	Description
2013	Vertical Distribution of Phytoplankton Communities in Gondang Reservoir, Lamongan, East Java	Field Work Practice
2013	Study on Heavy Metal Content Hg, Pb and Cd in Oysters (*Crassostrea* sp.) from Gresik Coastal Waters, East Java	Minor Thesis

Organizations Experience

Years	Organizations	Position
2010-2011	Communication and Study Forum in Faculty of Fisheries and Marine Science	Member of Research and Technology Department
2010-2011	Research and Technology University of Brawijaya	Member of Research
2011-2012	Research and Technology University of Brawijaya	Member of Human Resources Management

2012-2013	HUMANERA (Student Association , Department of Water Resources Management)	General Secretary

Working Experiences		
Years	**Institution**	**Description**
2012	Chemistry Lab Assistant	Staff of Value Recapitulation and Laboratory
2012	Ichtyology Lab Assistant	General Secretary, Field Coordinator, Speakers of material course
2011-2013	Aquatic Animal Physiology Lab Assistant	General Secretary, Field Coordinator, Speakers of material course
2012-2013	Fishery Resources Lab Assistant	Field Coordinator, Speakers of material course

Seminar, Training Course and Workshop		
Year	**Event**	**Location**
2010	Education and Training Discipline and Nationality Insights on Regiment Military Region V/ Brawijaya	Regiment Military Region V / Brawijaya, Malang
2011	Training and Workshop on Scientific Writing	University of Brawijaya
2011	National Seminar on Technology "Technology Innovation for Nation Building"	University of Brawijaya
2011	Aquatic National Seminar and Workshop "Contribution of Coastal and Marine Conservation Area in Reducing the Impact of Global Warming and Support the National Food Security"	University of Brawijaya

Reward and Achievement	
Years	**Description**
2009	Participant in Java-Bali English Olympiad
2010	Second Winner of Student Creativity Programme in Faculty of Fisheries and Marine Science, University Of Brawijaya
2011-2013	Recipients of PPA (Academic) Scholarship (Academic and Non-Academic Achievement) University Of Brawijaya
2014	Recipients of Fast Track Scholarship Studies S2 (Masters), Concentration of Fisheries and Marine Biotechnology, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, University of Brawijaya
2015	Finalist of NPUST Singing Competition