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Hydrogen peroxide production in *Anubias barteri*, *Echinodorus ozelot* and *Cabomba caroliniana* by induction of 17α-Ethinylestradiol

Suwiya Suttakun*, Surapong Rattanakul* and Parinda Thayanukul*,,***[*]**
*Department of Environmental Engineering, Faculty of Engineering,
King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand
**Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
***Center of Excellences on Hazardous Substance Management (HSM), Bangkok 10330, Thailand
[*]E-mail : P.thayanukul@gmail.com

Abstract

The 17α-ethinylestradiol (EE2), a synthetic estrogen has become public concern on the contamination in the aquatic environment because of its endocrine-disrupting properties which can cause adverse effects on aquatic organisms. EE2 is reported to be effectively removed by advanced wastewater treatment technologies; however, due to its high operating cost other low-cost technologies such as constructed wetland might be more suitable. Since certain plant species were able to produce endogenous hydrogen peroxide (H_2O_2) under stress condition including EE2 and induce hydroxyl radicals (•OH) formation when exposing to external dissolved iron through the Fenton-like reaction. This Phyto-Fenton process might be possible to combine in constructed wetland for EE2 removal. In this study, aquatic commercial plants including Anubias *(Anubias barteri)*, Amazon *(Echinodorus ozelot)*, and Green Cabomba *(Cabomba caroliniana)* were introduced to the solution containing different EE2 concentrations as an environmental stressor to investigate H_2O_2 production. The results revealed the increasing of H_2O_2 production following the increase of EE2 concentration in Amazon, while Anubias and Green Cabomba showed no difference with the control. Based on morphological observation, Amazon branch appeared healthy compared to other plants after exposing to EE2 for 21 days. The results suggested that Amazon was the most suitable aquatic commercial plant for applying to remove EE2 by Phyto-Fenton in constructed wetlands.

Keywords : 17α-ethinylestradiol (EE2); Aquatic commercial plants; Fenton-like reaction;
Hydrogen peroxide (H_2O_2); Phyto-Fenton process; Oxidative stress

Introduction

Nowadays, there is a public concern on the estrogen hormone contamination in the aquatic environment as they can interfere with the endocrine system in aquatic animals. The concerned estrogen hormones include natural estrogens such as estrone (E1), 17β-estradiol (E2), and estriol (E3) and the synthetic estrogen such as 17α-ethinylestradiol (EE2). The estrogen hormones have been detected in surface water at concentrations lower than 0.1-17 ng/L for E1, 0.05-15.5 ng/L for E2, lower than 0.1-3.4 ng/L for E3 and lower than 0.053-30.8 ng/L for EE2 [1]. While the predicted no-effect concentrations (PNEC) were 3 ng/L, 1 ng/L, and 0.1 ng/L for E1, E2, and EE2, respectively [2]. EE2 appeared to be the most recalcitrant compound in environment with no or small degradation rate [3]. Therefore, EE2 was the most concerned chemical among other estrogens. In addition, the occurrence of EE2 in surface water in developing countries was observed at higher levels than in developed countries [4].

Homogeneous fenton reaction is the mechanism involving with hydroxyl radical (•OH) that is produced by the interaction of H_2O_2 and dissolved ferrous salt in a bulk solution (Equation 1). The hydroxyl radical is a powerful oxidizing species (E^0 = 2.80 V vs. NHE) that can eliminate various organic pollutants [5]. Since the ferrous ion is unstable at a pH higher than 4.0, it can easily form unwanted solid iron-oxy hydroxide sludge FeO(OH) [6]. Its application under neutral pH in wastewater treatment plants is not practical. Moreover, the dissolved iron can leach to aqueous environment causing additional post-treatment cost. The heterogeneous Fenton reaction utilizing solid ferric iron materials as a catalyst is feasible over a wide pH range [7]. The reaction of hydrogen peroxide with ferric ion is referred to as a Fenton-like reaction (Equation 2) which produces superoxide radical ($HO_2\bullet$) that has lower oxidation power (E^0 = 1.65 V vs. NHE) than hydroxyl radical. In addition, the ferric iron can be reduced further to ferrous iron (Equation 2-4), which then reacts with H_2O_2 to produce highly reactive •OH via Equation 1 [8].

$$Fe^{2+} + H_2O_2 + H^+ \longrightarrow Fe^{3+} + HO^- + \bullet OH \quad (1)$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + HO_2\bullet + H^+ \quad (2)$$

$$Fe^{3+} + HO_2\bullet \longrightarrow Fe^{2+} + O_2 + H^+ \quad (3)$$

$$Fe^{3+} + O_2^-\bullet \longrightarrow Fe^{2+} + O_2 \quad (4)$$

Plants can generate reactive oxygen species (ROS) responded to biotic or abiotic stresses. It is regulated by autonomous factors such as age, reproductive development, and phytohormone levels and by environmental signals including photoperiod, stresses, drought, ozone, nutrient deficiency, wounding, and shading [9]. Under normal condition, the production of ROS in cells is maintained at low level by antioxidant enzymes. This balance can be disrupted by a depletion of antioxidants or an accumulation of ROS, leading to an increasing of oxidative stress, and consequently to damage cellular macromolecules and membranes [10]. H_2O_2 is one of the major ROS in plant tissues. It is produced in chloroplasts and mitochondria via electron transport systems during normal metabolic processes [11]. H_2O_2 plays an important role in plants under stress conditions as a signaling molecule that mediates between different physiological processes in response to environmental stress [12].

Recent studies indicated that H_2O_2 is produced at a concentration range between 0.4-1.5 μmol/gFW in typha (*Typha spp.*), vetiver (*Vetiveria zizanioides*), reed (*Phragmites australis*) and bird of paradise (*Strelitzia reginae*) under E2 stress conditions [13]. Also, H_2O_2 produced in response to EE2 stress at concentration of 0.6-1.5 μmol/gFW in large-flowered waterweed (*Egeria densa*), green cabomba (*Cabomba caroliniana*), amazon frogbit (*Limnobium laevigatum*) and greater duckweed (*Spirodela polyrhiza*) [7]. As plants can produce endogenous H_2O_2, it is possible to react with iron catalysts and eliminate recalcitrant pollutants. This technology is defined as Phyto-Fenton process [7] and possible to incorporate into a constructed wetland with fenton like reaction at neutral pH.

Based on these background, this study investigated how the EE2 can affect to H_2O_2 production in different EE2 concentrations in three aquatic commercial plants including; Anubias, Amazon and Green Cabomba. The important criteria for aquatic plant selection were easily growing and economic value as they can be benefit to livestock farmer when apply to wastewater treatment. The plant with highest H_2O_2 production level might be possible to apply to the advanced oxidation Phyto-Fenton wetland for EE2 removal.

Methodology

Experimental setup and aquatic plant acclimatization

The study was conducted in glass containers with a dimension of 21 cm × 21 cm × 21 cm (Figure 1). There were three aquatic plants used in this study including Anubias (*Anubias barteri*), Amazon (*Echinodorus ozelot*), and Green Cabomba (*Cabomba caroliniana*). They were acclimatized in Hoagland's nutrient solution (59.04 mg/L $Ca(NO_3)_2{\cdot}4H_2O$, 25.28 mg/L KNO_3, 24.65 mg/L $MgSO_4{\cdot}7H_2O$, 6.80 mg/L KH_2PO_4, 0.18 mg/L $MnCl_2{\cdot}4H_2O$, 0.01 mg/L $ZnCl_2$, 0.005 mg/L $CuCl_2{\cdot}2H_2O$, 0.005 mg/L $Na_2MoO_4{\cdot}2H_2O$, and 0.286 mg/L H_3BO_3) [7] before starting the experiment. Each type of plant was cultured in an individual container with tree plants per tank. Initial fresh weights of Anubias, Amazon and Green Cabomba were 9 ± 0.7 g, 7 ± 0.9 g and 12 ± 0.5 g, respectively. Three concentrations of EE2 (Sigma-Aldrich, China) including 1 ng/L, 1 μg/L, and 1 mg/L were applied to triplicate sets. There was also a control set which was free of EE2.

Operation and Sampling

The artificial sunlight was provided with the 12 h of light followed by 12 h of dark cycle. The irradiation intensity ranged 2000-4000 lux in different locations. The 16 W LED daylight tubes (Ecofit LED tubes T8, PHILIPS) were used. The temperature in the growth chamber was about 26-30°C. The experiments were run in batch mode for 21 days and plants samples were collected at day 1, 3, 5, 7, 9, 11, 13, 17, 19 and 21.

H_2O_2 Analysis

The method of endogenous H_2O_2 analysis was followed Velikova *et al*. [14] with some modification. Leaf tissues (100-500 mg) were homogenized in liquid nitrogen and then introduced to 5 mL of 0.1% (w/v) Trichloroacetic acid (Merck, Germany). The homogenate was centrifuged at 12,000×g for 15 min and 0.5 mL of supernatant was subsequently transfered to a new tube containing 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M Potassium iodide. The absorbance of the supernatant was measured at 390 nm by Spectorphotometer (Thermo Scientific GENESYS20, USA). The content of H_2O_2 (30%, Merck, Ausalia) in samples were determined relative to a standard curve.

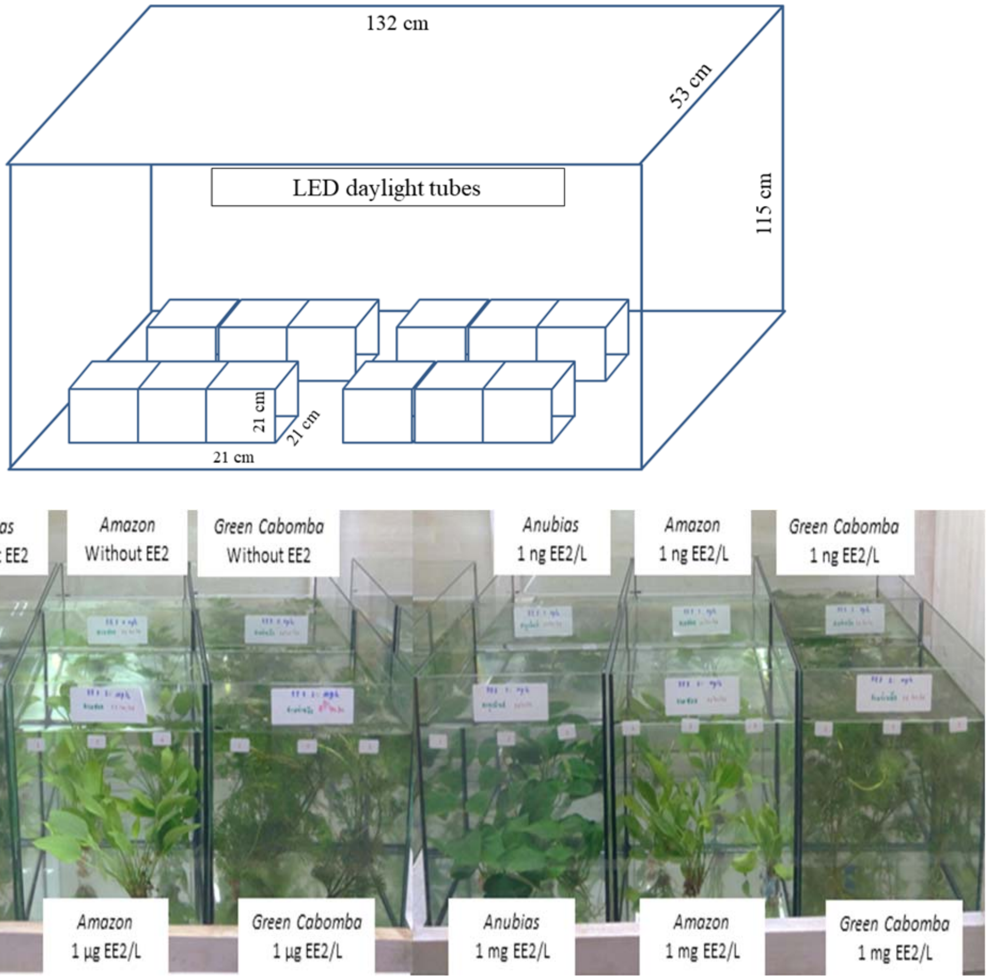

Figure 1 Experimental setup

Results and Discussions

Plant Morphology

In order to apply the aquatic commercial plants for EE2 removal with H_2O_2 induction, the resistance of the plants to the study conditions is important. We observed the physical appearance or morphology of the selected plants at 1 mg/L EE2 concentration during 21 days period. Figure 2 shows the morphology of three aquatic plants at day zero and the end of experiment at day 21. Judging from the appearance, Amazon was the most healthy compared to Anubias and Green Cabomba. Some leaf or stalk of Anubias and Green Cabomba were spoilage and roots were not regenerated as observed in Amazon. Therefore, amazon was more suitable to apply to the EE2 treatment among all selected plant species.

(a) *Anubias bateri* at day 0

(b) *Anubias bateri* at day 21th

(c) *Echinodorus ozelot* at day 0

(d) *Echinodorus ozelot* at day 21th

(e) *Cabomba caroliniana* at day 0

(f) *Cabomba caroliniana* at day 21th

Figure 2 The appearance of three aquatic plants at the start (day 0) and end (day 21th) of the operation

Concentration of endogenous H_2O_2

Figure 3 demonstrates the production of endogenous H_2O_2 under different EE2 concentrations. For the baseline condition without EE2 addition (Figure 3a), the H_2O_2 concentrations in Anubias were quite high during the beginning period and then reduced; however, the concentration in Amazon and Green Colomba returned to the high level at the end period.

The H_2O_2 concentrations after exposed to EE2 were shown in Figure 3b – d. The H_2O_2 concentrations in all plant species followed the same trend with the EE2 free condition. While increasing the EE2 concentrations, Anubias and Green Cabomba did not respond to the changes. Except for Amazon, the H_2O_2 concentration increased at higher EE2 concentrations. This indicated that only Amazon responded to the EE2 stress.

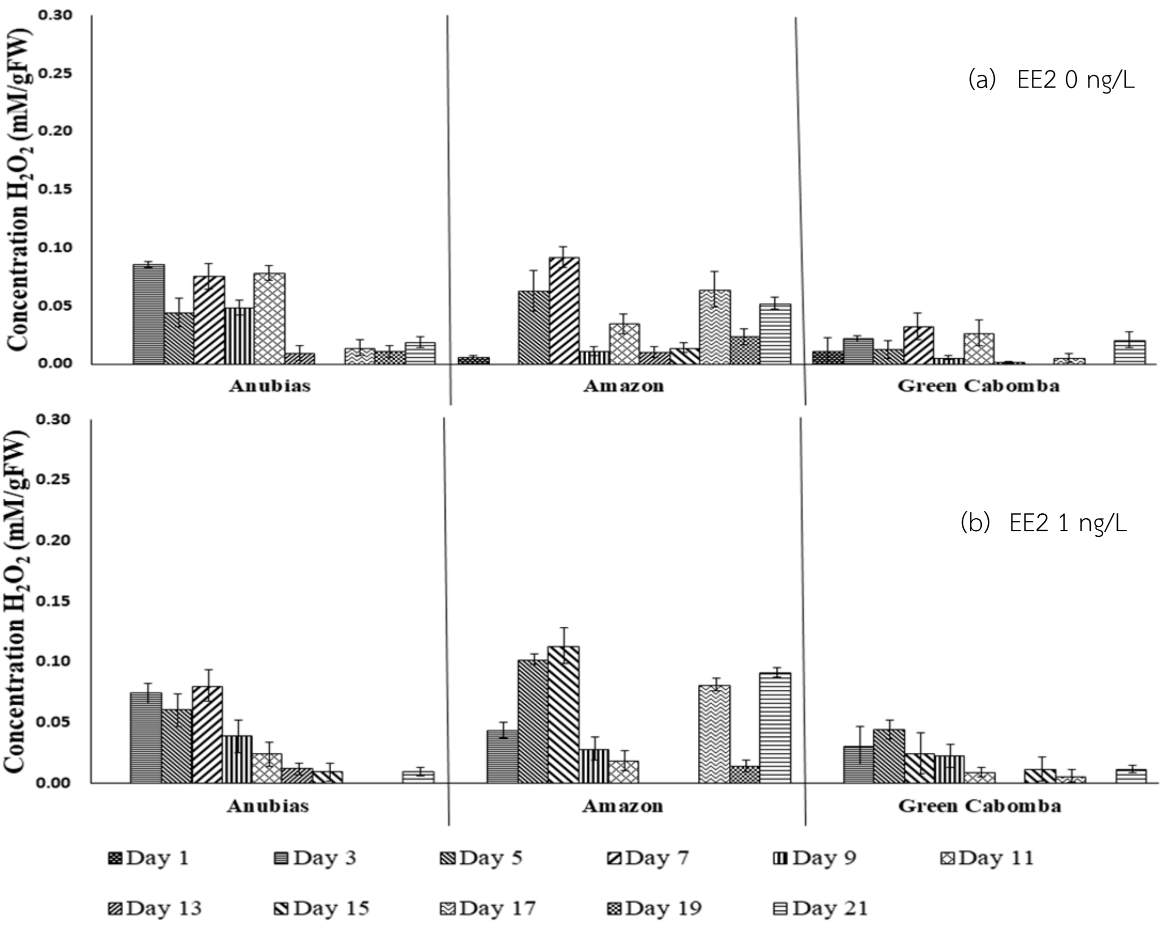

Figure 3 H_2O_2 concentration in tested aquatic plants induced by different EE2 concentrations. Error bars indicate standard error

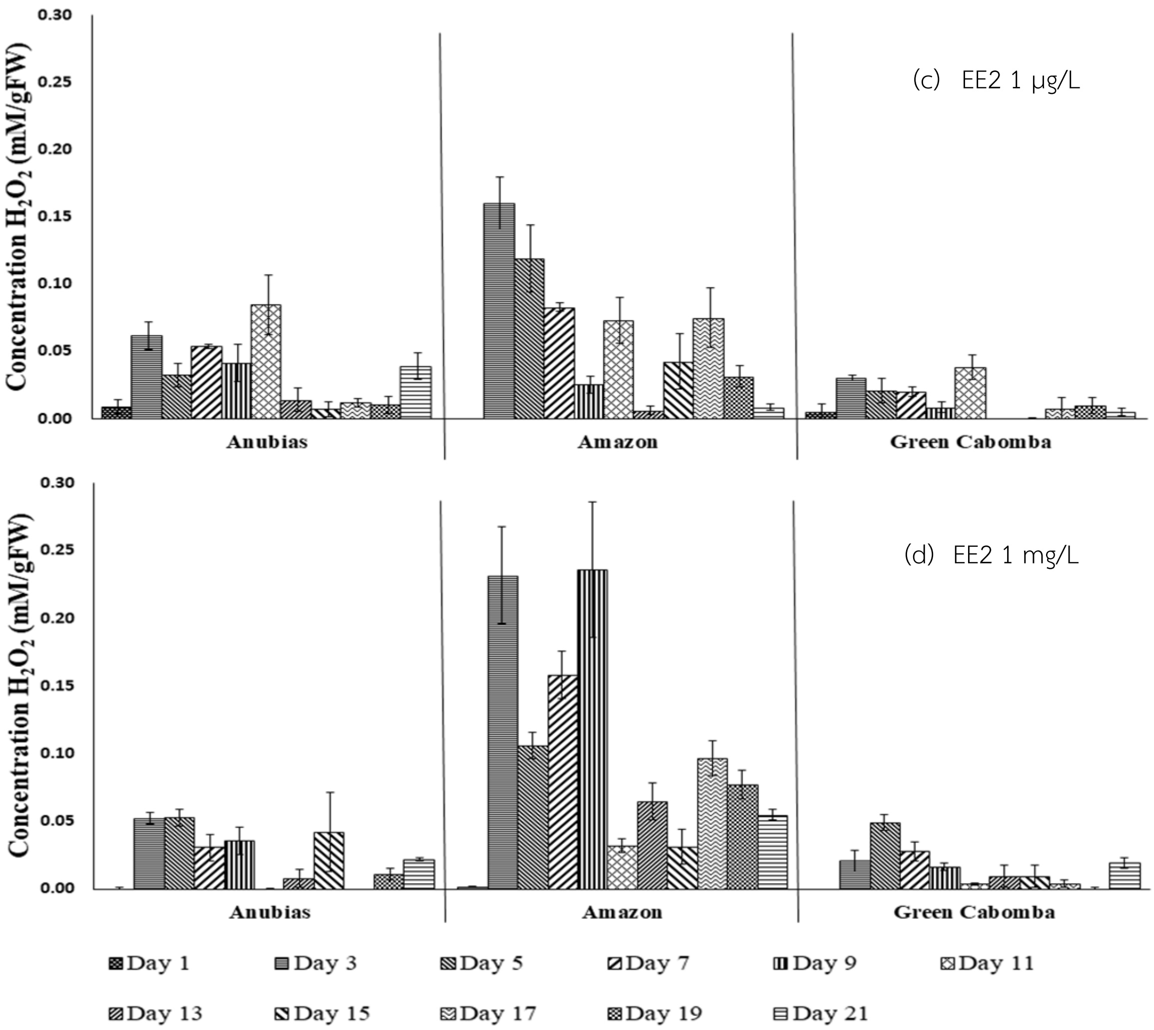

Figure 3 H_2O_2 concentration in tested aquatic plants induced by different EE2 concentrations. Error bars indicate standard error (cont')

Table 1 showed the averaged H_2O_2 concentrations in Anubias, Amazon, and Green Cabomba during operation were 0.035, 0.034, and 0.013 mM/gFW for without EE2, 0.028, 0.045, and 0.014 mM/gFW for 1 ng/L, 0.033, 0.057, and 0.013 mM/gFW for 1 μg/L, and 0.023, 0.099, and 0.015 mM/gFW for 1 mg EE2/L, respectively. Elevated levels of H_2O_2 at higher EE2 concentration were observed only in Amazon. The change of H_2O_2 with the EE2 addition did not observe in Anubias and Green Cabomba. These results suggested Amazon was the most suitable plant to apply to phyto-fenton advanced oxidation wetland among all selected plant species based on H_2O_2 production.

Table 1 H_2O_2 concentrations in three aquatic plants during operation pediod (21 day)

EE2 concentration	Aquatic plant	Concentration H_2O_2 (mM/gFW)		
		Average	Minimum	Maximum
Control	Anubias	0.035	<0.001	0.086
	Amazon	0.034	<0.001	0.092
	Green Cabomba	0.013	<0.001	0.032
1 ng/L	Anubias	0.028	<0.001	0.080
	Amazon	0.045	<0.001	0.113
	Green Cabomba	0.014	<0.001	0.044
1 µg/L	Anubias	0.033	0.007	0.085
	Amazon	0.057	<0.001	0.160
	Green Cabomba	0.013	<0.001	0.038
1 mg/L	Anubias	0.023	<0.001	0.053
	Amazon	0.099	0.002	0.236
	Green Cabomba	0.015	<0.001	0.049

Conclusion

Based on the experimental results obtained in this study, the following conclusions are made:

- The highest H_2O_2 concentration was found in Amazon (0.034-0.099 mM/gFW), followed by Anubias (0.023-0.035 mM/gFW) and Green Cabomba (0.013-0.015 mM/gFW).
- The H_2O_2 concentrations in Amazon increased with the higher EE2 concentration which was likely due to the environmental stress accumulation. The induction trend was not observed in the other selected plants.
- Amazon was more resistance to EE2 exposure than Anubias and Green Cabomba during 21 days.
- Among tested aquatic commercial plants, Amazon was the most suitable plant to apply for testing EE2 removal by Phyto-Fenton process.

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