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Table: 6, Figs : 8

Effects of *Casuarina equisetifolia* L. leachate on photosynthesis and antioxidant enzymes in seedlings of *Hernandia nymphaeifolia* (C. Presl) Kubitzki

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ABSTRACT

Casuarina equisetifolia L. is major windbreak specie in the south-eastern islands of China, but it facing the great problem of heavy decline due to ageing of forest. To explore the possibility of developing a mixed forest of *C. equisetifolia* and *H. nymphaeifolia*, we in laboratory bioassays, studied the effects of aqueous litter leachate of *C. equisetifolia* (15-20 y old trees) on the germination, photosynthesis parameters and antioxidative activity of *H. nymphaeifolia* seedlings. The leachate of *C. equisetifolia* significantly reduced the net photosynthetic rate, stomatal conductance, transpiration rate, light saturation point, light compensation point, maximal RuBP regeneration rate, apparent quantum yield and carboxylation efficiency on the seedlings of *H. nymphaeifolia*. The higher concentrations (0.125 and 0.25 g/mL) of *C. equisetifolia* leachate, markedly inhibited the activities of superoxide dismutase (SOD) and peroxidase (POD) in the seedlings of *H. nymphaeifolia*. However, the content of malondialdehyde (MDA) and the activity of glutathione (GR) increased with the increasing leachate concentration. The activities of ascorbate peroxidase (APX) and catalase (CAT) were improved at lower leachate concentrations than control but were inhibited at the highest concentration (0.25g/mL). The changes in the antioxidative activity may be an adaptive regulatory mechanism in *H. nymphaeifolia* seedlings in response to the allelochemicals of *C. equisetifolia.*

Key words: Allelopathic effects, antioxidative activity, aqueous extracts, *Casuarina equisetifolia* L., enzymes, germination, *Hernandia nymphaeifolia*, leachates, photosynthesis, seedling growth.

INTRODUCTION

Allelopathy refers to the beneficial or harmful effects of one plant on another plant (26). The plant allelochemicals are released, through leachates, root exudates, volatilization and decomposition of plant residues. These allelochemicals may affect the other plant species or own species seed germination, growth and yield. If it effect is on its own specie, it is called autotoxicity.

Casuarina equisetifolia tree provides fuel wood, land reclamation, dune stabilization, scaffolding for construction, shelter belts, and pulp and paper production (15). Due to its use as windbreak and for soil conservation, it was widely introduced in the 1950s and cultivated as pure stands in the southeast coasts of China as the coastal defence

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forest (14). However, those ageing forest stands are facing problems of autotoxicity and diseases (27). It would be beneficial to plant the forests with the multiple species, especially native species, to improve the soil conservation capability of the fading forest. However, due to the poor growth conditions and the allelopathic effects of *C. equisetifolia*, few native trees can survive with *C. equisetifolia*. The allelochemicals of *C. equisetifolia* reduces the uptake of soil nutrients and plant growth in susceptible plants, leading to forest decline (14,17,27). The *Vatica mangachapoi*, *Thespesia lampas* and *Calophyllum inophyllum* are screened as promising species to mix with *C. equisetifolia* (27). In field research on the coast of Tanmen (QiongHai, Hainan Island, China), we found that *H. nymphaeifolia* was growing with coconut trees and *C. equisetifolia*.

H. nymphaeifolia (Hernandiaceae family) is mangrove specie in the inter-tidal regions. It occurs throughout the tropical coastal areas, along the sea-shore in littoral forest and in coastal swamps, but in China, it was found in limited areas in QiongHai and WenChang (Hainan Island) (31). As an evergreen tree, it has strong wind control abilities and is also an ornamental garden plant due to its white fleshy fruit and shield blade leaves. It is seems to be one of the promising species that can be planted in the shield forest with the *C. equisetifolia* on Hainan Island. However, the current research on *H. nymphaeifolia* is mainly focussed on the alkaloids or terpenes present in its leaves and seeds (28). The effects of allelochemicals from *C. equisetifolia* on the growth of *H. nymphaeifolia* are still unknown, although similar studies are done on *V. mangachapoi* Blanco, *C. inophyllum* and *T. lampas* (13,14). Hence, this study aimed to determine the effects of *C. equisetifolia* leachate on the germination, photosynthesis system activity of antioxidant enzymes of *H. nymphaeifolia* seedlings to explore the possibility of its planting in the declining forests of *C. equisetifolia* to restore the windbreak forest.

MATERIALS AND METHODS

Five kg of *C. equisetifolia* litter from middle-aged forests (15-20 y) was collected from the coast of Guilinyang, Haikou, China (110°29’E, 20°1’N), in October 2015. It was washed with distilled water, air dried in shade and then soaked in 10 L distilled water for 24 h. The leachate was filtered through 4 sheets of sterile cotton gauze. It was further diluted with distilled water to get 0.0625 g/mL, 0.125 g/mL and 0.25 g/mL concentrations. The distilled water was used as control. The leachate extracts (The condensed/solidified leachate, extracted with Organic solvents). for the GC-MS analysis were prepared by suspending 1 kg litter in distilled water (1:3 ratio) for 48 h at room temperature in sterile environment and the supernatants were collected using a microfiltration membrane with 0.45 μm micropores and condensed to 1 mL at 70°C by rotary evaporation. Then the samples were freeze-dried and extracted with methanol (27), ethyl ether (24) and n-hexane (29) by GC-MS analysis.

Laboratory bioassay: Seeds of *H. nymphaeifolia* were collected from the coast of Tanmen, Qionghai, Hainan island, China (110°28'E, 19°14'N) in April 2015. These seeds

were soaked in 0.5% $KMnO_4$ for 2 h. Sixty seeds were planted in Petri dishes (11 cm dia) lined with two sheets of filter paper. Then, 15 mL leachate of *C. equisetifolia* (0, 0.0625, 0.125 and 0.25 g/mL) were added to each treatment. Each treatment was replicated thrice in Complete Randomised Design. The humidity of the greenhouse was 80% and the illumination time was 12 h. The seeds were germinated at room temperature (28°C). Germination was recorded daily, but the root and hypocotyl lengths of seedling were measured 15 days after sowing. The following germination parameters were determined:

$$\text{Germination Index (GI)} = \Sigma (Gt/Dt),$$

Where, Gt: Number of seeds that germinated t days after planting, and Dt: Days after germination.

The magnitude of inhibition and stimulation was denoted by the response index (RI) and calculated as under:

$$RI=T/C-1,$$

Where, T: Treatment data, and C: Control data. RI > 0 indicates stimulation and RI < 0 indicates inhibition.

Pot culture: Three seeds were planted per plastic pot (25 cm dia and depth) and 250 mL distilled water or leachate was added as per treatments to keep the culture medium moist every 3-days till the seedlings reached 4- leaf stage. The culture medium was red clay + sandy soil in 1:1 ratio. When the *H. nymphaeifolia* seedlings were at the 4-true leaf stage, 36 pots were chosen for treatment with leachate (0, 0.0625, 0.125 and 0.25 g/mL concentrations) and only one seedling was kept per pot. Six months later, the photosynthetic parameters and activity of protective enzymes were tested.

GC-MS analysis

Extracts of the solidified/condensed leachate (called Leachate Extract) contents were analysed using a Thermo Quest TRACE GC/MS system (Trace 2000, ThermoFinnigan) equipped with a programmable split injector (Port temperature of 250°C throughout the run) and the Xcalibur analysis software. The sample injection volume was 1 µL. The Thermo Quest TRACE mass spectrometer was equipped with an ion source (EI+, 70 eV) and operated in full scan mode (59.60-480.40 atomic mass units). The detailed methods can be found in previous research (27).

Photosynthesis measurements

The leaves of *H. nymphaeifolia* seedlings were analysed with an LI-6400 portable photosynthesis system. The net photosynthesis rate (Pn), stomatal conductance (Cond), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were measured and the photosynthesis-photo and photosynthesis-CO_2 curves were generated. A detailed description of the methods can be found in previous research (13).

Activity of protective enzymes and malondialdehyde (MDA)

The activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (POD) and ascorbate peroxidase (APX) were determined as per the operation manual of the SOD Activity Assay Kit (Jianchen, Nanjing, China), the

Catalase Assay Kit (Jianchen, Nanjing, China), the Glutathione Reductase Assay Kit (Jianchen, Nanjing, China), the Peroxidase Assay Kit (Jianchen, Nanjing, China) and the Ascorbate Peroxidase Assay Kit (Jianchen, Nanjing, China), respectively. The content of MDA was determined as per the manufacturer of the Malondialdehyde Assay Kit (Oxis International, Inc. OXIS).

Statistics analysis

The data were processed by Microsoft Excel 2010. The analysis was done in 3 replicates. Statistical significance was determined by SPSS (16.0) and LSD (the least significant difference method) tests. Differences were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

***H. nymphaeifolia* seed germination and seedling growth**

The higher concentrations of *C. equisetifolia* leachates (0.125 g/mL and 0.25 g/mL) inhibited the seed germination of *H. nymphaeifolia* obviously compared to control. However, the lower concentrations of leachate increased the seed germination than control (Table 1). The root length and Hypocotyl length increased with 0.0625 g/mL concentration and was inhibited in other two concentrations.

Table 1. Effects of leachate of *C. equisetifolia* on germination of *H. nymphaeifolia*

Leachate concentration (g/ml)	RI		
	Germination Index	Root length	Hypocotyl length
0.0625	0.08754a	0.04332a	0.06609a
0.125	-0.02643b	-0.0245b	-0.04402b
0.25	-0.03742c	-0.03362c	-0.04609b

The same letter is not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test
GIU : Germination index

Six months after treatment with different *C. equisetifolia* leachate concentrations, all the treated seedlings survived with different growth patterns (Figure 1). The growth of the *H. nymphaeifolia* seedlings was inhibited and the inhibition increased with the increasing concentrations of leachate. Based on the field investigation, the growth of *H. nymphaeifolia* was also inhibited, when grown near the *C. equisetifolia* (5). Phytochemicals released into the environment inhibited the germination and growth of adjoining plants by altering their metabolism or impacting their soil community mutualists (4).

Figure 1. The seedling growth of *H. nymphaeifolia* treated with different concentrations of leachate of *C. equisetifolia*. Right to left: 0 g/mL, 0.0625 g/mL, 0.125 g/mL and 0.25 g/mL.

GC-MS analysis of *C. equisetifolia* leachate extracts

Extracts from the litter of *C. equisetifolia* middle-aged forests were analysed by GC-MS chromatograms by methanol, ethylether and n-hexane treatments. The representative chemical components with relatively high contents in each extract are listed in Table 2. The GC-MS analysis showed that 36 chemical compounds were found in litter extracts, but none were found in all three treatments. Among them, 21 compounds were extracted by methanol, 5 were extracted by n-hexane and 13 were extracted by ethylether treatment. It can also be found that the 3 same compounds were analysed from the methanol and n-hexane extracts including phenol and 2,4-bis (1,1-dimethylethyl)-, which has heavy inhibitory effects on the seed germination and seedling growth of rice, barnyard grass, lettuce (19) and sesame (25). Only (-)-Tricyclo[6.2.1.0(19,4)]undec-5-ene, 1,5,9,9-tetramethyl-cisocary ophylene-I1 was found in both the methanol and ethylether treatments. In the ethylether extracts, Lup-20(29)-en-3-one and lupenone were found with high relative contents (7.391% and 3.227%, respectively), which can be found in *Erica multiflora* leaf extracts and can stimulate melanogenesis in B16 murine melanoma cells (23).

Photosynthetic response of *H. nymphaeifolia* to photosynthetically active radiation

Light availability can affect enzyme activity and the stomatal aperture of plant leaves (30). In this study, six months after treatment with leachate, the net photosynthetic rates (Pn), photosynthetically active radiation (PAR) - stomatal conductance and PAR - transpiration rate all decreased with the increasing leachate concentrations (Figs 2,3,4). Meanwhile, there were significant differences between the different concentrations and the control, except for the stomatal conductance between the 0.125 g/mL and 0.25 g/mL

Table 2. GC-MS analysis of the litter of *C. equisetifolia* in a middle-aged forest

No	Compound name	Relative content (%)		
		Methanol	Ethylether	n-hexane
1	Phenol, 2,4-bis(1,1-dimethylethyl)-	0.016	-	3.100
2	4 - hydroxy butyric acid lactone	0.033	-	-
3	Hexadecanoic acid, methyl ester	0.038	-	-
4	Sulfurous acid, 2-propyl undecyl ester	0.023	-	-
5	2-Pyrrolidinone, 1-butyl-	0.031	-	-
6	2H-Pyran-2-one, tetrahydro-4-hydroxy-4-methyl-	0.026	-	-
7	2(3H)-Furanone, dihydro-4,5-dimethyl-	0.035	-	-
8	1(3H)-Isobenzofuranone	0.015	-	-
9	7,8-Epoxy-.alpha.-ionone	0.021	-	-
10	Phenol, 3-isopropoxy-5-methyl-	0.0393	-	-
11	(-)-Globulol	0.044	-	-
12	Sulfuric acid, butyl octadecyl ester	2.888	-	-
13	2,4,7,9-Tetramethyl-5-decyn-4,7-diol	7.797	-	3.246
14	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2, 8-dione	3.596	-	-
15	Anthracene, 9,10-dihydro-9,9,10-trimethyl-	25.756	-	12.952
16	Pyridine-3-carboxamide oxime, N-(2-trifluoromethylphenyl)-	0.470	-	-
17	1(3H)-Isobenzofuranone	0.054	-	-
18	1-Nitro-.beta.-d-arabinofuranose, tetraacetate	0.015	-	-
19	2-Amino-8-[3-d-ribofuranosyl]imidazo[1,2-a]-s-triazin-4-one	0.017	-	-
20	2-Piperidinone, N-[4-bromo-n-butyl]-	0.293	-	-
21	Hexanedioic acid, bis(2-ethylhexyl) ester	6.697	-	-
22	(-)-Tricyclo[6.2.1.0(4,11)]undec-5-ene, 1,5,9,9-tetramethyl-cisocary ophylene-I1	-	5.454	-
23	2 - (phenyl acetylene) - pyridine	-	-	1.042
24	7 -, 9 - two tertiary butyl - 1 oxygen mixed screw (4, 5) decyl - 6, 9 - diene - 2, 8 - diketone	-	-	2.232
25	n-Hexadecanoic acid	-	1.962	-
26	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	-	1.473	-
27	1,2-Benzenedicarboxylic acid, diisooctyl ester	-	3.913	-
28	Vitamin E	-	2.072	-
29	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a, 5,6,6a,6b,7,8,8a,9,10,11,12, 12a, 14,14a,14b-octadecahydro-2H-picen-3-one	-	29.91	-
30	Olean-18-ene	-	4.211	-
31	alpha.-Amyrin	-	19.640	-
32	Lup-20(29)-en-3-one	-	7.391	-
33	Lupeol	-	3.227	-
34	5,8-Dimethoxy-2,3,10,10a-tetrahydro-1H,4aH-phenanthrene-4,9-dione	-	1.071	-
35	7-Oxabi cyclo[4.1.0]heptane, 2,2,6- trimethyl-1-(3-methyl-1,3- butadienyl)-5 -methylene-	-	1.483	-
36	Stigmast-4-en-3-one	-	1.914	-

leachate concentrations (Table 3). These reactions may be the stress response to the effects of leachate on the expression of chlorophyll synthesis and photosystem genes (2).

Table 3. Effects of PAR on photosynthesis of *H. nymphaeifolia* treated with leachate of *C. equisetifolia*

Leachate concentration (g/mL)	PAR-Pn	PAR-Stomatal conductance	PAR-Transpiration
0(Control)	2.7533±0.0974a	0.0601±0.0015a	0.8149±0.0189a
0.0625	2.3071±0.1309b	0.0146±0.0006b	0.6224±0.0286b
0.125	1.8089±0.0621c	0.0090±0.0006c	0.4846±0.0364c
0.25	1.3802±0.0727d	0.0083±0.0007cd	0.1940±0.0056d

The same letter is not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test. Pn: net photosynthesis rate; PAR: photosynthetically active radiation.

The Pn of the control reached a maximum when the PAR was approximately 1000 μmol m^{-2} s^{-1}, but under the 0.0625 g/mL treatment, the Pn reached a maximum when the PAR was 800 μmol m^{-2} s^{-1}; the maximum Pn reached in the other two treatments when the PAR was 600 μmol m^{-2} s^{-1}. The Pn of all the treated groups declined after it reached the maximum than control (Figure 2), which shows that photoinhibition of *H. nymphaeifolia* occurred with the leachate treatments when PAR was > 600 μmol $m^{-2}s^{-1}$.

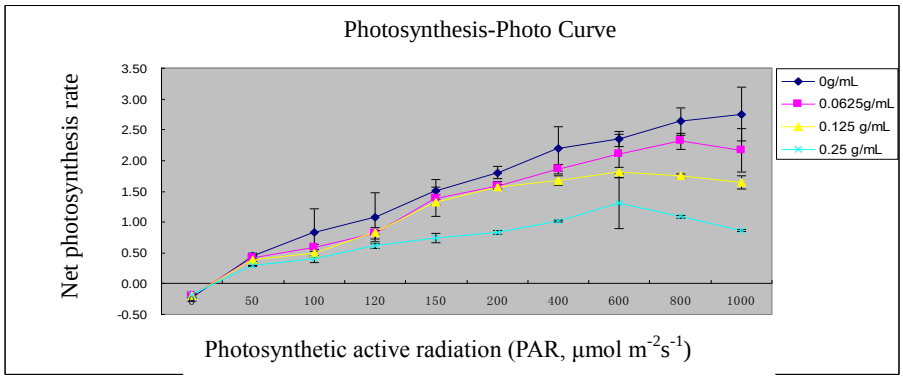

Figure 2. Photosynthesis-photo curve of *H. nymphaeifolia* treated with leachate of *C. equisetifolia*

Figure 3 shows the stomatal conductance response curve of *H. nymphaeifolia* seedlings, which were treated with various leachate concentrations of *C. equisetifolia*. When the PAR increased, the conductance of all groups increased and stabilized at a PAR of 400 μmol $m^{-2}s^{-1}$, except for the group with the 0.0625 g/mL treatment and the control. Meanwhile, there was no difference between the two groups with the 0.125 g/mL and 0.25 g/mL leachate concentrations. The obvious differences between the leachate treatment groups and the control showed that leachate decreased the stomatal conductance of *H. nymphaeifolia* seedlings.

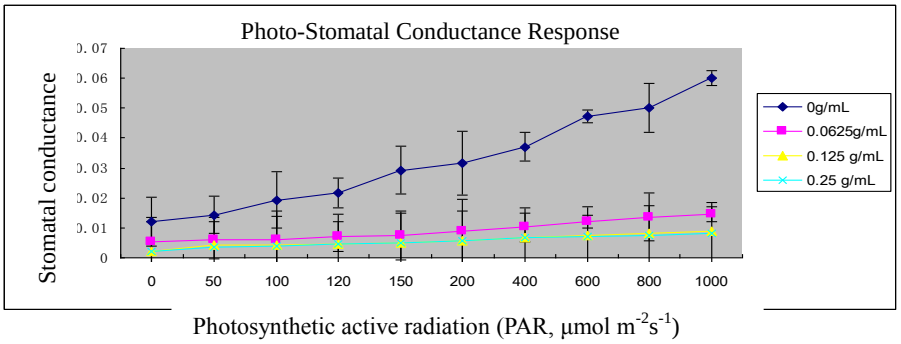

Figure 3. PAR-stomatal conductance response of *H. nymphaeifolia* treated with leachate of *C. equisetifolia*

Photoresponse parameters calculated from the Farquhar model (Table 4) indicated that the light saturation point (LSP), light compensation point (LCP), maximal RuBP regeneration rate (Pnmax) and apparent quantum yield (AQY) of *H. nymphaeifolia* were reduced significantly with increases in the leachate concentration. The leachate can obviously effect photosynthesis, which has been observed in other plants (12,13,15,21). The LSP in plants is important under high light conditions as the dark reaction follows the light reaction in photosynthesis and then limits the instantaneous photosynthetic rate (3,10,30). The higher LSP and lower LCP mean an increasing ability to use light (33), but in our study, both the LSP and LCP were inhibited by allelopathic effects. This finding may be caused by the increasing number of injured cells under the higher leachate concentrations. Pn, together with AQY, express the rate of electron transport and the activity of photophosphorylation. The simultaneous decrease in Pn and AQY is a prominent characteristic of photoinhibition when photosynthetic organs in seedlings are damaged (14,21).

Table 4. Photoresponse parameters calculated from the Farquhar model

Leachates conc (g/mL)	LSP ($\mu mol.m^{-2}.s^{-1}$)	LCP ($\mu mol.m^{-2}.s^{-1}$)	Pnmax ($\mu mol.m^{-2}.s^{-1}$)	AQY ($\mu mol.m^{-2}.s^{-1}$)
0 (Control)	887.3333±9.0738a	24.6010±1.0150a	2.5237±0.0744a	0.0167±0.0013a
0.0625	809.3333±6.6583b	20.8797±0.2991b	2.1040±0.0561b	0.0111±0.0012b
0.125	762.0000±2.6458c	18.2513±0.4769c	1.8527±0.0586c	0.0096±0.0002b
0.25	651.6667±11.0604d	14.2783±1.0413d	1.2079±0.0714d	0.0063±0.0003c

The same letter is not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test. LSP: light saturation point; LCP: light compensation point; Pnmax: maximum RuBP regeneration rate; and AQY: apparent quantum yield.

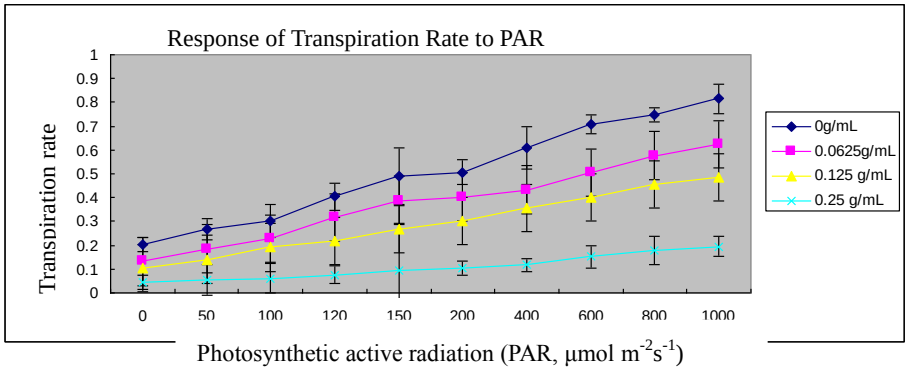

Figure 4. PAR-transpiration curve of *H. nymphaeifolia* treated with leachate of *C. equisetifolia*

Photosynthetic response of *H. nymphaeifolia* to CO_2 concentration

The net photosynthetic rate is influenced by stomatal factors (number, diameter and opening of stomata) and non-stomatal factors (intracellular enzyme activities and photosynthesis control) (11). To understand the effectors of Pn, it is necessary to simultaneously examine the stomatal conductance and intercellular CO_2 concentration (Ci). In this study, Pn decreased with the increase in the leachate concentration, but Pn increased with the increase of the CO_2 concentration (Figure 5). In Table 5, it can be seen that all the treated groups were significantly different from the control. The same tendency can also be found in studies of transpiration rate and stomatal conductance (Figs. 6 and 7). However, there were no differences between the 0.125 g/mL and the 0.25 g/mL treatment groups ($P < 0.05$) for the *H. nymphaeifolia* stomatal conductance in response to CO_2 (Table 5). The change in stomatal conductance is a regulated physiological process and is advantageous to the survival of the plant under stress. The partial closure of stomata under allelopathic effects can help the plant to avoid excess water loss and avoid damage to photosynthetic organelles (2,14).

Table 5. Effects of CO_2 on photosynthesis of *H. nymphaeifolia* treated with leachate of *C. equisetifolia*

Leachates conc (g/mL)	CO_2-Pn	CO_2-stomatal conductance	CO_2-transpiration
0 (Control)	3.0452±0.1060a	0.0580±0.0047a	0.4508±0.0360a
0.0625	1.4996±0.0175b	0.0250±0.0267b	0.3128±0.0150b
0.125	0.8854±0.0342c	0.0111±0.0008c	0.1425±0.0038c
0.25	0.6640±0.0218d	0.0082±0.0001c	0.1027±0.0028d

The same letter is not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test.

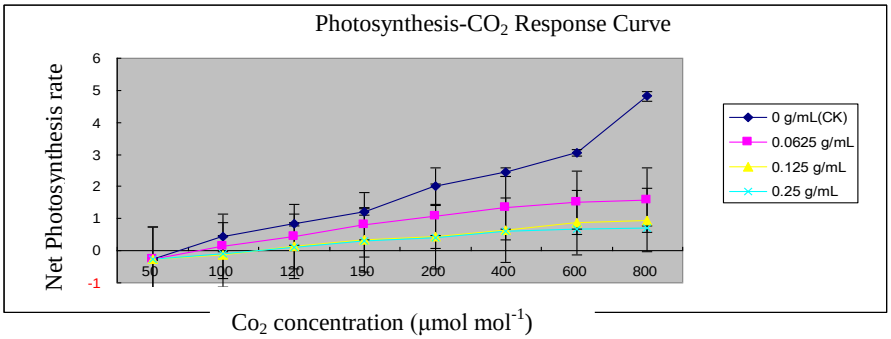

Figure 5. Photosynthetic rate of *H. nymphaeifolia* in response to CO_2 after treatment with various concentrations of leachate of *C. equisetifolia*

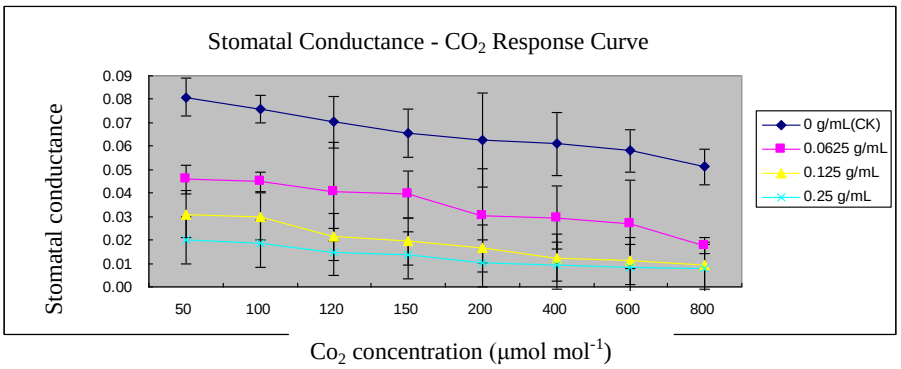

Figure 6. Stomatal conductance of *H. nymphaeifolia* in response to CO_2 after treatment with various concentrations of leachate of *C. equisetifolia*

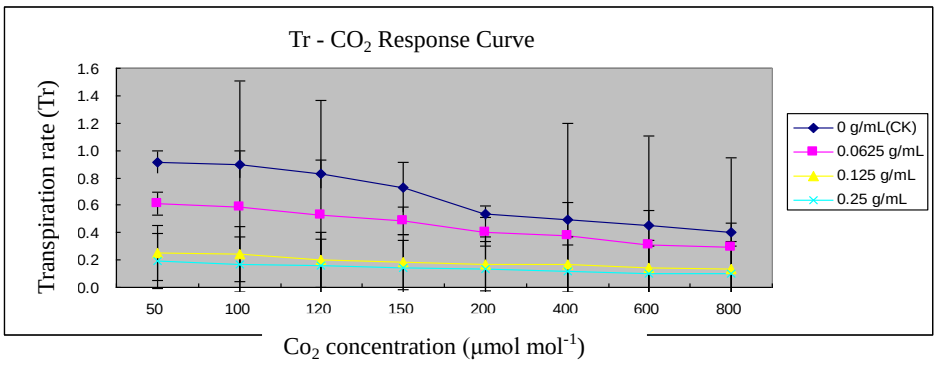

Figure 7. Transpiration rate of *H. nymphaeifolia* in response to CO_2 after treatment with leachate of *C. equisetifolia*

The same letter is not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test. CSP: CO_2 saturation point; CCP: CO_2 compensation point; Pnmax: maximum RuBP regeneration rate; and CE: carboxylation efficiency CO_2 is the most important substrate in photosynthesis. Within a certain concentration range, enhanced CO_2 concentrations can promote an instantaneous photosynthetic rate change. In this study, with the Farquhar model, CO_2 response parameters were calculated in Table 6; the CO_2 compensation point (CCP) was elevated with the increased leachate concentrations, but net photosynthesis rate (Pn) and carboxylation efficiency (CE) decreased. The CO_2 saturation point (CSP) increased at the low leachate concentration of 0.0625 g/mL and decreased with the higher leachate concentrations. The decline in CE meant a reduction in ribulose 1,5-bisphosphate carboxylase (RuBPCase) activity and therefore a reduction in the use of CO_2, and the use of CO_2 must affect the CSP and CCP during the growth of the plant (13).

Table 6. CO_2 response parameters calculated from the Farquhar model

Leachates conc (g/mL)	CSP ($\mu mol.m^{-2}.s^{-1}$)	CCP ($\mu mol.m^{-2}.s^{-1}$)	Pnmax ($\mu mol.m^{-2}.s^{-1}$)	CE ($\mu mol.m^{-2}.s^{-1}$)
0 (Control)	1183.6433±3.7277b	86.3333±0.4382d	8.2360±0.0888a	0.0094±0.0002a
0.0625	1265.5000±7.7488a	94.7133±0.4375c	5.7191±0.0333b	0.0063±0.0003b
0.125	1133.5167±25.3571c	157.5833±2.5525b	5.1612±0.0185c	0.0041±0.0001c
0.25	1079.0200±4.2822d	197.5900±2.6251a	3.9865±0.0596d	0.0036±0.0001d

ANTIOXIDANT ENZYMES

Cell membrane peroxidation

MDA is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for damage to cell membranes (9,20). An increase in the amount of MDA occurred in *H. nymphaeifolia* in all leachate concentrations used, which significantly increased with the increase in the *C. equisetifolia* leachate concentrations (Fig. 8). When the leachate concentration was 0.25 g/mL, the MDA content of the treatment was approximately 80% higher than the control. The results showed that allelochemicals caused the accumulation of MDA. Then, the metabolites became unbalanced because of the increase of active oxygen and free radicals. Furthermore, the peroxidation of cell membranes damaged the structure and function of the cell membranes (13,20).

Protective enzymes

Under environmental stress, increased active oxygen activates the chemical defence signals to start-up antioxidant enzyme systems, including many reactions of other signals (18). Enzymes such as SOD, POD, APX, GR and CAT were tested in the treatment groups of *H. nymphaeifolia*, and they are important enzymes for clearing free radicals and

maintaining the plant membrane system (6,9,20). The activities of SOD and POD in the 0.0625 g/mL concentration treatment were higher than those of the control, but when the concentration of the leachate increased more, their activities both decreased. The activity of APX was also higher than the control when the treated leachate concentration was no more than 0.125 g/mL. However, when the concentration reached 0.25 g/mL, the activity of APX decreased 36%, and the same result was observed with CAT (22%). For lower leachate concentrations, the activities of APX and CAT increased with the increase in the leachate concentration. For the GR of the treated groups, the activities were all higher than the control and increased with the increase in the leachate concentration (Fig. 8). All activities of the enzymes tested in *H. nymphaeifolia* seedlings, except for GR, were inhibited by the heavy stress in the 0.25 g/mL concentration of *C. equisetifolia* leachate. The inhibition can also be found in the seedlings of *V. mangchapoi* (14) by treatment with leachate of *H. nymphaeifolia*.

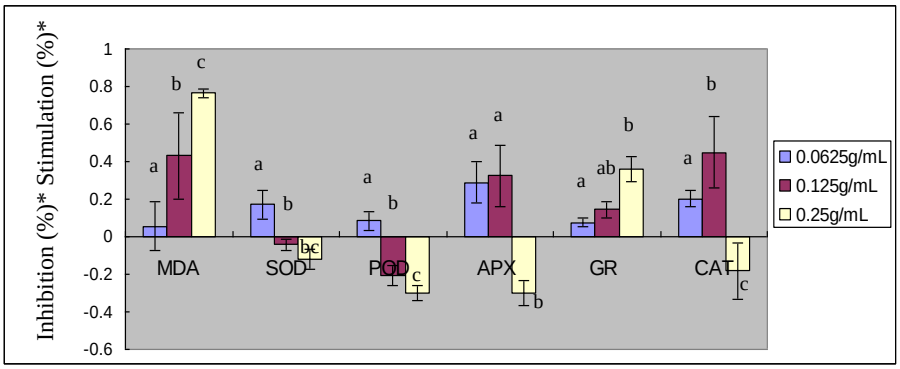

Figure 8. Effects of leachate of *C. equisetifolia* on the activity of antioxidant enzymes of *H. nymphaeifolia*. *: % Inhibition/Stimulation over control. MDA: malondialdehyde; SOD: superoxide dismutase; POD: peroxidase; APX: ascorbate peroxidase; GR: glutathione reductase; and CAT: catalase. The same letter is not significantly different at the 0.05 level as determined by the Student-Newman-Keuls test.

Plant oxidative stress (OS) can be caused by environmental stressors such as salt, drought and allelochemicals effects (8,20). OS is generated as a result of insufficient activity of the endogenous antioxidant defence system against reactive oxygen species (ROS). On the one hand, excessive ROS could exert oxidative damage to lipids, proteins, and DNA (7), which yield relatively stable oxidized biomolecule products such as MDA. On the other hand, the levels of antioxidants (vitamins E, C, A, and B6 and folate) and antioxidant enzyme activity (SOD, CAT, POD, APX, GR and GPx) significantly decreased, although the expression levels of some of them also increased (32). The higher MDA contents of all treatment groups implied that the allelochemical-induced ROS generation and cell membrane peroxidation damaged the structure and function of the cell membrane

system, which has also been reported for allelopathy of *Lantana canmara*, *Ipomoea cordifolia* and *V. mangachapoi* (14,20,22). For the heavy allelopathic effects, the SOD, POD and CAT were damaged and reduced the clearing of H_2O_2 in the cells of *H. nymphaeifolia* seedlings. However, in *V. mangachapoi*, the activities of the above enzymes increased under allelopathic stress (14). The reduced enzyme activity can be caused by the increase in damaged cells from heavy allelopathic stress. In the damaged cells, GR reduces GSSG into GSH and is related to plant photosynthesis (1). In this study, with increasing allelopathic stress, the activity of GR increased. GR can clear the ROS and reduce the damage to cells caused by allelochemical stress in cooperation with other oxidant enzymes (7).

CONCLUSIONS

The leachate extracts from *C. equisetifolia* were inhibitory to *H. nymphaeifolia* seed germination and seedling growth at higher concentrations and were stimulatory at low concentrations. The leachate from *C. equisetifolia* significantly reduced the net photosynthetic rate, stomatal conductance, transpiration rate, light saturation point, light compensation point, maximal RuBP regeneration rate, apparent quantum yield and carboxylation efficiency. For the antioxidant enzymes, the activities of superoxide SOD and peroxidase POD were inhibited markedly by higher concentration leachate treatments. The content of MDA and the activity of GR increased with the increases in the leachate concentration. The activities of APX and CAT were improved compared with the control under the lower leachate concentrations but were inhibited by the highest concentration treatment. These changes may be an adaptive regulation of *H. nymphaeifolia* seedlings in response to the allelochemicals of *C. equisetifolia.*

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