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Effects of substrate types on nitrogen removal efficacy and growth of *Canna indica* L.

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Abstract

Constructed wetlands (CWs), a cost effective technology for wastewater treatment, consist of substrates and wetland plants, which should be selected carefully to gain highest treatment efficiency. However, studies on plant growth and responses to different types of substrates are very few. This study aims to assess the effects of substrate types on growth and root morphology of *Canna indica* L. and nitrogen (N) removal. Twenty-four similar sized approximately 1 month old *C. indica* plants were selected and grown on different substrates (gravel, pumice and biochar). All plants were supplied with a standard growth medium to which 14 mg L^{-1} NH_4^+, 14 mg L^{-1} NO_3^-, 3 mg L^{-1} PO_4^{3-} were added and pH was adjusted to 6.5. The growth solution was renewed every week. The plants were grown under greenhouse conditions for 45 d. Results showed that growth of *C. indica* was not significantly different among treatments but differences on root morphology were found. Plants grown on pumice had the largest root diameters while plants grown on biochar had the longest roots. In the gravel-filled treatment, *C. indica* showed the lowest root diameter and root length but formed more internal air space in its roots. It indicates that types of substrate can affect O_2 supply and root morphological adaptation. Moreover, the porous substrate bed systems were capable of eliminating more NH_4^+ than gravel bed systems, with the NH_4^+ removal rates of 5.6–6.3 mg L^{-1} d^{-1} compared to 4.7 mg L^{-1} d^{-1}, respectively. The results show that porous substrates can act as plant supporting substrates and play important roles in N adsorption. Also, they can improve oxygen supply and stimulate root growth. Thus, application of porous substrates as filter media could help to increase pollutant removal efficacy of CWs.

Keywords: Constructed wetland; Filter media; N adsorption; Root trait; Oxygenation

Introduction

Constructed wetlands (CWs) are well known as an alternative wastewater treatment system which is a cost effective technology that provides highly efficient water treatment. Wetland technology is designed and constructed to treat wastewater using natural methods. In CWs, pollutants are removed from the wastewater

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through various processes such as sedimentation, plant uptake, biodegradation, nutrient cycling and adsorption [1–3]. Substrates are important components of the CWs that can influence treating capacity of the systems. The substrates not only support plant growth, their surfaces also provide habitats for microorganisms. Moreover, some types of substrate are good for ion exchange and adsorption [4–7]. Therefore, there is increased interest in evaluating properties and performance of substrates in CWs.

Natural materials such as sand and gravel have been widely used as filter media in CWs. However, systems using these traditional substrates may face problems such as clogging and low O_2 supply that can lead to low pollutant removal efficiency [4, 8–9]. The selection of alternative substrates has become a challenge to scientists trying to minimize these problems and to increase treatment capacity. Porous substrates are materials that have high porosity and large surface area so they are expected to filter more suspended solid, diffuse more O_2, and support microorganisms development and biofilm formation which enhance organic matter degradation and N transformation in CWs [10]. Some porous substrates have special physicochemical characteristics to adsorb nutrients and pollutants [11]. Biochar is a porous substrate prepared by pyrolyzing organic material at high temperatures (300–700 ºC) in the absence of oxygen [12]. It is mostly used as a soil amendment in agriculture to improve soil fertility and soil water absorption [13]. Currently, biochar technology is gradually being applied to wastewater treatment. It has been revealed that filling biochar produced from bamboo, corn, and Chinese oak into CWs encouraged NO_3^-, NH_4^+, total N, total P, and chemical oxygen demand (COD) removals from wastewater [14–15]. However, efficacy of biochar in removing pollutants from wastewater depends on natural source materials and preparation conditions that result in differences in physical and chemical properties such surface area, pore size and distribution, and surface functional groups [16]. Some kinds of biochar such as biochar derived from oak, wheat straw, and willow released toxic chemical substances that influence plant growth and pollutant removal efficacy in CWs [17–21]. In Northern Thailand, biochar derived from longan (*Dimocarpus longan* Lour.) branches is a new eco-friendly product of local people in Chiang Mai province. Longan biochar is widely used in agriculture [13], meanwhile application of longan biochar as a filter medium to support plants and remove pollutants from wastewater is not well documented. Alternatively, a porous substrate like pumice has been suggested for use as a filter medium in CWs because it provides better root aeration and high surface for pollutants adsorption, particularly phosphorus [4, 22].

Macrophytes are an essential component of CWs; they have specific properties related to wastewater treatment processes [23–24]. Submerged parts of macrophytes help to precipitate suspended sediments and promote biofilm formation. Roots also play important roles in nutrient uptake, and promote degradation and nitrification. Moreover, O_2 released from macrophytes' roots can change the redox potential of the rhizosphere and promote chemical transformation of metals such as iron (Fe^{3+}), manganese (Mn^{2+}) to become insoluble or unabsorbed forms and less toxic to the plants [25]. Because there are several processes depending on the oxygen in the rhizosphere, selection of appropriate macrophytes may help to enhance the performance of CWs. Additionally, substrates can support plants as well as improve dissolved O_2 (DO), making them another component to make CWs successful in treating wastewater. It is expected that porous substrates may alter root morphology and enhance plant performance in CWs. However, effect of substrates used in CWs on plant growth and their ability to take up nutrient is not well studied.

Roots act as bioengineers in O_2 production in CWs. Among aquatic macrophytes, emergent macrophytes have exclusive internal air spaces that enhance O_2 transportation to the buried roots and rhizosphere [26]. This well-developed aerenchyma system makes emergent plants appropriate for all kinds of CWs. *Canna indica* L. is an attractive appearing species used in tropical CWs. This species grows fast, adapts well to both terrestrial and aquatic conditions and has high N uptake [27]. Employment of this species in tropical CWs is currently increasing. Many studies showed that *C. indica* has high N removal efficiency and also provides beautiful landscapes [28–29]. Root adaptation is one plant strategy to cope with changed environmental conditions influenced by different physico-chemical characteristics of each substrate. Nevertheless, the interaction between root and substrate in CWs is rarely studied. A previous study found that adding plastic residues as a filter material did not affect growth and root morphology of *C. indica* [30]. To gain new insight into growth and root traits of this species when grown on other kinds of substrates, this study aims to investigate growth, root morphology of *C. indica* when grown with different filter substrates (gravel, biochar and pumice), as well as to determine N removal efficiency in terms of the whole systems. The results of this study could be informative for selection of substrate and plants to treat wastewater in CWs. For this investigation, we hypothesize that porous substrates could encourage O_2 supply for *C. indica* to increase growth and N uptake and encourage N removal through adsorption onto pumice and biochar particles.

Materials and methods

1) Plant preparation and filter substrates

Rhizomes of *C. indica* were separated from plant stocks growing in the greenhouse of the Department of Biology, Faculty of Science, Chiang Mai University. The rhizomes were cut into 100–150 mm long sections and roots were removed. Then, each rhizome was put into a tray filled with sandy soil. All rhizomes were watered every day for 4 weeks. Then, twenty four new shoots (approximately 25 g fresh weight, 50 cm height) were selected for the experiment. Filter substrates used in this experiment were gravel (Ø = 30–50 mm), pumice (Ø = 20–30 mm) and biochar (Ø = 3–5 mm). The biochar was bought from Warm Heart Foundation, Phrao district, Chiang Mai province, Thailand. This biochar was derived from longan branches and pyrolyzed at 450 ºC for two hours in the absence of oxygen.

2) Experimental set-up

Each filter substrate, including gravel, pumice, and biochar, was filled into perforated black plastic bags (15×30 cm) up to 150 mm from the bottom (four bags for each substrate, 12 bags in total). Two selected *C. indica* were planted in each bag (total 24 plant samples). Then, each individual bag was placed in a 5 liter plastic bucket. After that, standard growth medium solution modified from Smart and Barko [31] with 14 mg L^{-1} NH_4^+, 14 mg L^{-1} NO_3^-, 3 mg L^{-1} PO_4^{3-} and 80 mg L^{-1} Fe-EDDHA (consisted of 10.6 mg L^{-1} Fe) was added onto the bucket. The volume of growth medium in the gravel and biochar-filled treatments was 3.8 liters (78% porosity), while the volume in the pumice-filled treatment was 4 liters (80% porosity). The pH of nutrient solution was measured using a multi-parameter analyzer (Cyber-Scan PC 300, Eutech Instruments Pte Ltd., Singapore) and was adjusted to 6.5 by HCl with and NaOH. In order to prevent porous filter substrates (biochar, pumice) from floating up, the medium surface was topped up with gravel. The nutrient solution was renewed every week and all plants were grown under greenhouse conditions for 45 d. The air temperature in the greenhouse was 35± 2ºC :25±1 ºC day:night.

3) Growth study

After harvest, all plants were cleaned and the substrate was removed from roots gently. Total height of the plants was recorded. Shoot elongation rates (SER; mm d^{-1}) were calculated as the increase of height divided by time (days) of the experiment. Afterwards, the plants were separated into roots, rhizomes and leaves. All fractions were dried at 60 ºC using a hot-air oven. Some parts of all fractions were dried using a freeze-dryer. The freeze-dried plant materials were then used for chlorophyll and nitrogen analysis. Total dry mass was measured. The relative growth rate (RGR, g g^{-1} d^{-1}) based on initial and final plant dry mass (W_1 and W_2) and initial and final day (t_1 and t_2) was calculated following the Eq. 1 [32];

$$RGR = (\ln W_2 - \ln W_1)/(t_2 - t_1) \qquad \text{(Eq. 1)}$$

4) Root study

Three days before harvest, mature roots (four replicates per treatment) were selected. Root diameters at the middle part were measured and root cross sections were prepared by free-hand sectioning technique.

The radial oxygen loss (ROL) of roots was observed following Armstrong and Armstrong [33]. An agar solution was prepared and dissolved methylene blue (0.012 g L^{-1}) was added. Then, dissolved O_2 in the agar solution was completely reduced by adding sodium dithionite (0.12 g L^{-1} $Na_2S_2O_4$). Plants (four replicates from each treatment) were selected and placed in glass vials containing a 2 L O_2-reduced agar solution. After a blue area all over the roots developed, photos were taken. Root porosity was determined according to Sojka [34]. Fresh roots were cut into pieces 250–300 mm long. Four replicates of the selected roots from each treatment (100 – 300 mg) were gently blotted with tissue paper and weighed (Wr). The pycnometer was filled with distilled water and weighed (Ww). Then, the roots were placed into the pycnometer and weighed (Ww+r). After weighing, the roots were taken out from the pycnometer and crushed in the mortar in order to remove air from the roots. All pieces of crushed roots were placed back into the pycnometer and weighed again after filling the pycnometer with distilled water (Wh). The root porosity was calculated using the formula (Eq. 2) [34].

5) Plant tissue analysis

Freeze-dried leaf samples were cut into small pieces and 8 mg samples were weighed. They were soaked with 8 mL of 95% ethanol and placed in a dark room for 24 h. Then, the absorbance of the extracts was measured at 470, 648.6 and 664.2 nm using a UV-VIS spectrophotometer (Lambda 25 version 2.85.04, USA). The concentrations of chlorophyll a (Chl *a*), chlorophyll b (Chl *b*), and carotenoids were calculated following Lichtenthaler [35]. For N concentrations in plant tissue, 50 mg samples of freeze-dried root and leaf samples were weighted and analyzed for N content using the Kjeldahl method [36].

$$\% \text{ root porosity} = 100 * [Wh-(Ww+r)]/[(Ww+Wr)-(Ww+r)] \qquad \text{(Eq. 2)}$$

6) N removal

Forty-five days after the start of the experiment, the depletion of ammonium (NH_4^+) and nitrate (NO_3^-) was measured. The standard growth medium was renewed. The growth solution was sampled (10 mL) at the beginning and then daily to determine NH_4^+ and NO_3^- concentrations until both NH_4^+ and NO_3^- were depleted. NH_4^+ was measured using the salicylate method [37], while NO_3^- concentration was measured by the UV method [38]. Then, NH_4^+ and NO_3^- removal rates (mg L^{-1} d^{-1}) were calculated from the depletion curves with linear regression analyses.

7) Data analysis

Statistics were analyzed using the software Past326b [39]. All data were tested by one-way analysis of variance (ANOVA). Differences between treatments were identified by the Tukey HSD post hoc procedure at the 5% significance level.

Results

1) Plant growth

Growth of *C. indica* was not affected by the different substrates. The relative growth rates and shoot elongation rates in the three treatments were 0.038–0.039 g g^{-1} d^{-1} and 11.6–12.9 mm d^{-1}, respectively. The filter substrates did also not influence on plant biomass and shoot: root ratio (Table 1).

2) Root morphology

Types of substrate affected root diameter, root length, and aerenchyma formation of *C. indica*. The plants grown on gravel showed significantly lower root diameter than the plants grown on pumice and also had significantly shorter roots than the plants grown on biochar. However, root internal air space and porosity of *C. indica* grown on gravel were significantly higher than of the plants grown on pumice and biochar (Table 1; Figure 1).

Table 1 Growth rates, dry mass, chlorophylls and carotenoids and root morphology of *Canna indica* (mean ± SD) grown on different filter substrates. Different letters indicate significant differences between treatments (P<0.05).

	Substrate		
	Gravel	**Pumice**	**Biochar**
Relative growth rate (g g^{-1} d^{-1})	0.039 ± 0.005^a	0.038 ± 0.006^a	0.039 ± 0.006^a
Shoot elongation rate (mm d^{-1})	12.9 ± 1.9^a	12.0 ± 2.7^a	11.6 ± 2.5^a
Leaf dry mass (g)	8.8 ± 1.7^a	10.5 ± 3.5^a	10.6 ± 3.8^a
Rhizome dry mass (g)	1.0 ± 1.1^a	1.0 ± 0.4^a	1.0 ± 0.3^a
Root dry mass (g)	1.0 ± 0.3^a	1.1 ± 0.4^a	1.1 ± 0.3^a
Total dry mass (g)	10.7 ± 2.8^a	12.6 ± 3.9^a	12.5 ± 4.3^a
Shoot:root ratio	9.2 ± 1.6^a	9.6 ± 3.0^a	10.0 ± 2.9^a
Chl *a* (mg g^{-1} DW)	8.0 ± 0.2^a	7.9 ± 0.1^a	7.9 ± 0.1^a
Chl *b* (mg g^{-1} DW)	2.5 ± 0.4^{ab}	3.0 ± 0.3^b	2.4 ± 0.5^a
Total carotenoids (mg g^{-1} DW)	1.3 ± 0.09^a	1.6 ± 0.2^b	1.4 ± 0.3^{ab}
N in root tissue (mg g^{-1} DW)	17.9 ± 1.9^b	16.6 ± 1.3^{ab}	14.7 ± 1.1^a
N in leaf tissue (mg g^{-1} DW)	21.1 ± 1.9^a	28.6 ± 1.8^b	26.1 ± 1.6^b
Root diameter (µm)	$1{,}382.8 \pm 67.3^a$	$1{,}662.8 \pm 122.7^b$	$1{,}489.9 \pm 214.1^{ab}$
Root length (cm)	20.1 ± 3.1^a	22.5 ± 5.9^{ab}	24.9 ± 3.3^b
Aerenchyma area (%)	9.9 ± 2.3^b	2.7 ± 2.2^a	2.8 ± 1.1^a
Root porosity (%)	43.6 ± 3.9^b	22.5 ± 11.0^a	21.0 ± 8.5^a

Table 2 N-removal rate of the systems filled with different substrates (mean ± SD). Different letters superscripts indicate significant differences between treatments (P<0.05)

	Substrate		
	gravel	**pumice**	**biochar**
NH_4^+ removal rate (mg L^{-1} d^{-1})	4.7 ± 0.8^{a}	6.3 ± 0.3^{b}	5.6 ± 0.8^{ab}
NO_3^- removal rate (mg L^{-1} d^{-1})	3.7 ± 0.1^{ab}	3.6 ± 0.1^{a}	3.8 ± 0.0^{b}

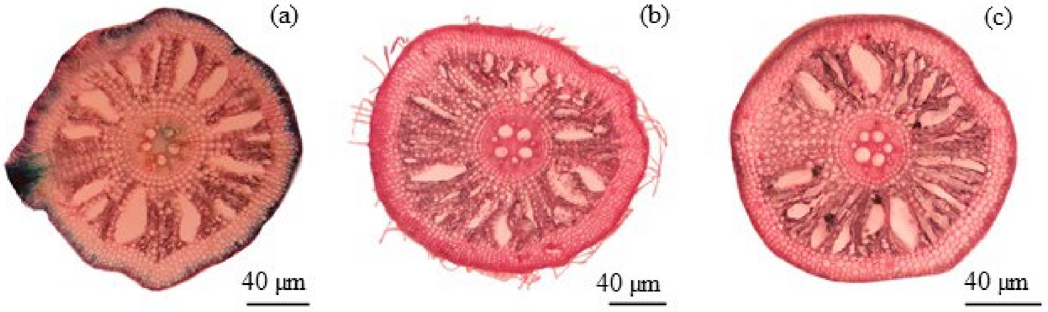

Figure 1 Root cross sections of *Canna indica* grown on (a) gravel, (b) pumice and (c) biochar, respectively.

3) Plant tissue analysis

The concentrations of Chl *a* were not significantly different among treatments. In contrast, Chl *b* and carotenoids in leaves of *C. indica* grown on pumice and biochar were higher than Chl *b* and carotenoids in leaves of *C. indica* grown on gravel. Like N accumulation, especially in leaf, *C. indica* planted on pumice and biochar showed significantly higher N concentrations than leaves of *C. indica* planted on gravel. Meanwhile, a slightly different N accumulation (14.7–17.9 mg g^{-1}) was found in the roots (Table 1).

4) N-depletion

From this study we found NH_4^+ was removed within 3 d but there was a significant difference of NH_4^+ removal rates among treatments. The NH_4^+ removal rate in the pumice-filled treatment was 6.3 mg L^{-1} d^{-1} which was considerably higher than the gravel-filled treatment (4.7 mg L^{-1} d^{-1}). NO_3^-, removal rates were lower than NH_4^+ removal rates. The NO_3^- removal rates were in the range of 3.6–3.8 mg L^{-1} d^{-1} (Table 2).

Discussion

Generally, growth and biomass production of *C. indica* were not affected by different types of substrate. Similarly, Zamora-Castro et al. [40] found that growing plants in different substrate materials (porous river rocks and tepezyl) did not affect growth of *C. indica*, *Pontederia sagittata* C. Presl, and *Spathiphyllum wallisii* Regel, but they found significant growth differences among these three species. In our study, however, it seems that the plants grew better and had larger roots with higher fine root production when grown on porous substrates. Both pumice and biochar are extremely porous, which increases aeration in the rhizosphere and enhances water and nutrient uptake by the root system [41–44]. Small holes distributed around the substrates allow diffusion of air for root respiration. Also, their high surface area aids lateral roots to grow and penetrate throughout the substrates. Results from a meta-analysis by Xiang et al [43] showed that most plants increased root biomass, root length, root diameter, root volume and surface area under biochar addition. It was assumed that biochar may adsorb nutrient ions particularly inorganic N leading to greater nutrient deficiency, so changes in root morphology including increasing root length and root surface area could be an important root trait that plants have developed in order to prevent or alleviate nutrient deficiency. Therefore, biochar application has been introduced for use in constructed wetlands due to its adsorption property and also because it can stimulate root growth and increase nutrient demand for plant growth [43]. In the present study, we found the plants grown on the porous substrates had dense roots and long lateral roots compared with the roots of the plants grown on gravel. Moreover, roots of *C. indica* under the biochar treatment tended to be longer than roots of the plants under the pumice treatment. Regarding substrate properties, pumice is an inert inorganic substrate that does not alter nutrients availability in the root medium. For plants grown on pumice it is not necessary to invest in additional root biomass, so there was no change in root:shoot ratio [42–43].

Because the high porosity of pumice and biochar facilitates aeration in the rhizosphere, it may affect aerenchyma development in the roots. It is well known that aerenchyma provides internal pathway for gas transportation from the shoots to the below ground tissue. Under submerged or water saturated soil, the ability to form aerenchyma can alleviate O_2 stress in plants [45–46]. A previous study found that wheat, maize, and rice did not develop aerenchyma in their roots when grown on dry and well-drained soil, while plants grown on wet soil had well produced aerenchyma [47]. However, the degree of internal air space formed in the root cortex seems to depend on the O_2 supply. In the present study, roots of the plants grown on pumice and biochar have less aerenchyma development compared with the plants grown on gravel. The slight response of the roots may indicate that dissolved oxygen in the rhizosphere did not decrease much in the pumice and biochar systems. Similar results found in the study of Fu et al. [48] revealed that porous substrates such as ceramsite and activated carbon can retard the reduction of DO and enhance reoxygenation in CWs. Therefore, porous filter substrates provide not only a major function as supporting substrates, but can also improve oxygen supply and stimulate root growth.

In CWs, removal of NH_4^+ is complex because it can be removed by various processes such as adsorption onto substrate, plant uptake, and transformation to nitrate by nitrification [49]. In this study, the concentrations of NH_4^+ in systems with plants growing on porous substrates dramatically decreased within 3 d with high removal rates (6.3 mg L^{-1} d^{-1} and 5.6 mg L^{-1} d^{-1} for pumice and biochar, respectively). Meanwhile, the NH_4^+ removal rate in the gravel-filled systems was approximately 4.7 mg L^{-1} d^{-1}. However, the removal rate for NO_3^- in all treatments was 3.6–3.8 mg L^{-1} d^{-1}. It is obvious that porous substrates affected NH_4^+ removal more than NO_3^-. Because high surface areas and porosity can promote growth of microorganisms and aeration in the rooting media, decreasing of NH_4^+ may be caused by nitrification processes whereas some of the NH_4^+ was taken up by plants. In this study, adequate O_2 in rhizospheres of the systems planted with porous filter substrates (pumice and biochar) encouraged root growth and enhanced N uptake as well. As a result, photosynthetic pigments and N concentrations in the plant tissue increased. Konnerup and Brix [27] found that high NH_4^+ uptake rate of *C. indica* resulting in high N accumulation in the plant tissue, and high concentrations of chlorophylls and carotenoids in their leaves, but the RGR was insignificantly different. However, a study by Liu et al. [5] found that NH_4^+ removal depended on types of substrates with differences in physicochemical characteristics such as specific surface area, micropore distribution, cation exchange capacity (CEC) and adsorption capacity. Biochar has a negative charge and high CEC, so its properties are beneficial for NH_4^+ adsorption [50]. Many previous studies showed high efficiency of biochar on NH_4^+ adsorption [51–53]. Also, pumice containing -OH groups is suggested for use as an adsorbent [54]. High efficiency of pumice on NH_4^+ adsorption was reported as well [55]. As stated, biochar and pumice could enhance N removal directly by N adsorption onto the filters and indirectly by increasing root growth leading to higher N uptake. Therefore, the use of these two kinds of substrate in CWs have been increasing, and they showed high N removal efficiency which is influenced by microbial development (encouraging N transformation), N adsorption, and plant uptake [15, 56–57]. However, removal efficiency of porous substrates may decrease after being used for a while [5, 58]. In order to maintain adsorption capacity of these filter materials, refreshing materials by washing with water or acid has been suggested [59–60].

Conclusions

Pumice and biochar showed high efficiency of N removal, especially NH_4^+ (6.3 mg L^{-1} d^{-1} and 5.6 mg L^{-1} d^{-1}, respectively). They encouraged root diameter increase, root elongation, and N uptake of *C. indic*a, and reduced aerenchyma formation (only 2% aerenchyma area compared with 9% in *C. indica* root grown on gravel). These two substrates and *C. indica* are good candidates to create CWs systems. However, further studies should evaluate the effects of these two porous substrates on plant growth and wastewater treatment efficiency in full-scale CWs.

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