



Efficacy of ornamental plants for benzene removal from contaminated air and water: Effect of plant associated bacteria



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ABSTRACT

In the present study, eight plant species – *Syngonium podophyllum*, *Sansevieria trifasciata*, *Euphorbia milii*, *Chlorophytum comosum*, *Epipremnum aureum*, *Dracaena sanderiana*, *Hedera helix*, and *Clitoria ternatea* were exposed to benzene under hydroponic conditions to evaluate the benzene uptake rates. Among the tested plants, *C. comosum* showed the highest benzene removal efficiency. However, the results indicated that the removal rates of non-sterilized plants were higher than that of sterilized plants. This variation was due to the different responses of plant associated bacteria. Benzene-resistant bacteria of *C. comosum* were isolated and characterized. Of the 14 isolated bacteria, results showed that 5 isolates are endophytic bacteria, while 9 isolates are epiphytic bacteria. It was revealed that 1 isolate of endophytic bacteria (EN2), and 2 isolates of epiphytic bacteria (EPL1 and EPR2) had the highest benzene removal efficiencies. These species were identified and designated by 16S rDNA as *Enterobacter* EN2, *Cronobacter* EPL1, and *Pseudomonas* EPR2. *Enterobacter* EN2 strain efficiently colonized the inoculated plants. A high rate of IAA production and benzene tolerance was found in the plants inoculated with this strain. These findings further elucidated that plant associated bacteria play essential roles in removing benzene from the contaminated system.

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1. Introduction

Clean air and water, without any pollutant contamination, is essential to humans and other wildlife. Among the most significant environmental pollutants, benzene is a volatile organic compound (VOC) and is exposed to humans primarily through inhalation (Pollution Control Department, 2007). This compound is released to the environment by both natural processes and human activities e.g. forest fires, volcanoes, the release of chemical compounds from industries and manufacturing activities or the use of benzene containing products, including fuels and solvents (WHO, 2000). Furthermore, benzene is also found as a gasoline component and is airborne by vehicular traffic. Cigarette smoke is also a significant source of exposure to benzene (IPCS, 1993). The International Agency for Research on Cancer (IARC) has classified benzene as carcinogenic to humans (Group 1) (IARC, 1987). Although it is highly volatile, however, a minor amount may contaminate surface

water because of its solubility in water. The major sources of benzene in water are atmospheric deposition and leakage/spillage of petrol (WHO, 2003). Therefore, there is a need for developing research methods to clean and circulate both air and water contaminated with this compound.

So far, several techniques have been developed for removing organic pollutants from contaminated air and water e.g. filtration, adsorption, and air stripping (Ball et al., 1984; Kwon and Cho, 2009; Rene et al., 2009). However, these methods require high energy or large capital investment. Phytoremediation is an elegant and low-cost approach for removing organic hydrocarbons from the environment. This method has been proposed as an efficient way to improve the contaminated site (Wolverton et al., 1989; Treesubuntorn and Thiravetyan, 2012). However, there are several factors that impact plant effectiveness including slow growth, small biomass, and long-term commitment. Furthermore, the efficiency of organic compound removal by plants mainly depends on the tolerance of plants to the pollutant. To remedy this situation, an inoculation of plants with plant growth-promoting bacteria (PGPB) or pollutant-degrading bacteria has been proposed as an essential method to enhance phytoremediation activity

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(Glick and Stearns, 2011; Zamioudis and Pieterse, 2012). Therefore, the purpose of this study was to evaluate the abilities of eight plant species (*Syngonium podophyllum*, *Sansevieria trifasciata*, *Euphorbia milii*, *Chlorophytum comosum*, *Epipremnum aureum*, *Dracaena sanderiana*, *Hedera helix*, and *Clitoria ternatea*) on removing benzene from contaminated air and water. The uptake rates of benzene by these plants after exposure to benzene under hydroponic conditions were evaluated and compared. The benzene-resistant bacteria from the highest benzene removal efficiency of non-sterilized plant was isolated and identified by 16S rDNA neighbor-joining phylogenetic tree. The bacteria strain which possesses the ability to tolerate benzene was inoculated into the sterilized plant, *C. comosum* to analyze the ability of bacteria to help plants reduce benzene stress. In addition, the removal efficiency of inoculated sterilized and non-sterilized plant was also studied.

2. Materials and methods

2.1. Screening plants for benzene removal

Eight plant species – *S. podophyllum*, *S. trifasciata*, *E. milii*, *C. comosum*, *E. aureum*, *D. sanderiana*, *H. helix*, and *C. ternatea* – were purchased from plant shops, and then grown in a greenhouse at the Remediation Laboratory of King Mongkut's University of Technology Thonburi (KMUTT), Thailand. Before initiating experiments, plants were thoroughly cleaned with water to remove adhering algae and insect larvae. Then, plants were grown in bottles containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with Gamborg vitamin (Gamborg et al., 1968) for at least 5 days prior to use in remediation experiments. Stock and experimental cultures were maintained at a temperature of 25 °C under fluorescent light with a 16 h photoperiod.

2.2. Benzene treatment to plants, sampling and analysis

Plants of each species (3–4 leaves, 5–7 cm in height) were placed in sterilized clear glass serum bottles, 120 mL nominal volume, containing 20 mL of liquid MS medium. Then, the bottles were capped with septum stoppers, and 170 µg of benzene from a stock solution in water was injected into each bottle. The mass of benzene in the bottles was calculated based on volume, solubility in water and temperature, assuming equilibrium using a dimensionless Henry's law constant at 25 °C of 0.228 (US EPA, 1996). The experiments were carried out in triplicate, at a temperature of 25 °C under fluorescent light with a 16 h photoperiod. In addition, MS medium without plant was used as a negative control.

To analyse the rate of removal of benzene, 50 µL of air in each bottle was sampled and manually injected into a gas chromatograph with flame ionization detector (GC–FID) (GC–430, Bruker) using a VF–1ms column (15 m × 0.25 mm, 0.25 µm I.D.). The experimental conditions of injection, oven and detector temperature for GC–FID were 200 °C, 50 °C and 200 °C, respectively. Sample injections were performed in triplicate. Initial headspace samples were taken 1 h after the introduction of benzene into the bottles to allow for air-liquid equilibrium. Headspace samples were analysed every 24 h for 96 h.

2.3. Isolation of benzene resistant bacteria from the highest benzene removal efficiency plant species

The highest benzene removal efficiency plant from the previous study was grown under 170 µg of benzene for 96 h, and then plant associated bacteria including epiphytic and endophytic bacteria from the plant were isolated. Epiphytic microorganisms were isolated by shaking the leaf or root for 1 h with 100 mL of sterile

distilled water. Then, 1 mL from the leaf or root stem wash was plated on nutrient broth medium (NB). For endophytic microbial isolations, microorganisms were isolated from the surface sterilized plants by submerging in 10% (v/v) sodium hypochlorite for 20 min. Afterward sample materials were rinsed 2–3 times with sterile distilled water, and dried in laminar flow. Then 1 g of sterilized sample was ground in a sterile mortar containing 10 mL of sterilized distilled water. Then, 1 mL of tissue extracts was added into the NB medium and incubated at 32 °C for 24 h. After 24 h of incubation, all morphologically different bacteria colonies were selected and subcultured 3 times to ensure purities and stability. Furthermore, in order to confirm that the surface sterile process was successful, 100 µL of the last washing water was spread onto an NA medium plate and then incubated at 32 °C for 24 h.

2.4. Benzene removal efficiency by isolated microorganisms

The ability of the isolated bacteria to grow in the presence of benzene in a semisolid phase was studied by culturing them on a minimal agar medium containing 1000 mg L⁻¹ of benzene (0.25 g NH₄Cl, 0.266 g MgSO₄·7H₂O, 3 g KH₂PO₄, and 11.32 g Na₂HPO₄·7H₂O in 1 L of water). Then all culturing bacteria were incubated at 32 °C for 7 days.

Furthermore, the ability of the isolated bacteria to grow in the presence of benzene gas in the system was studied by culturing bacteria in a 120 mL serum bottle. Then benzene gas was injected in the system to produce a benzene concentration of 100 ppm. Sample injections were performed in triplicate. Then, 50 µL of headspace samples were taken from each bottle and analysed every 24 h for 48 h.

2.5. Quantification indole-3-acetic acid (IAA) production by isolated bacteria

To determine the amount of IAA produced by each isolate, a colorimetric technique was performed using Salkowski's reagent (Gordon and Weber, 1951). The isolates bacteria were grown in the NB and incubated at 32 °C for 24 h on a rotary shaker. After 24 h, 1 mL aliquot of the culture were transferred into tubes containing 5 mL of Dworkin and Foster (DF) salts minimal medium (Dworkin and Foster, 1958) supplement with L-tryptophan at a concentration of 500 mg L⁻¹ and were incubated in darkness at 32 °C for 5 days. The bacterial culture suspensions were centrifuged at 10,000 g for 20 min. Then, the supernatant was transfer to a new tube, and equal volume of Salkowski's reagent was added in the supernatant. After 30 min, a pink color was developed, which indicated IAA production. The absorbance of pink color was read at 530 nm using a spectrophotometer. IAA concentrations were determined using a calibration curve of pure IAA as a standard following the linear regression analysis of authentic IAA (Sigma–Aldrich).

2.6. Identification of plant growth promoting benzene degradation bacteria

Isolated genomic DNA of bacteria were isolated and identified. Samples were submitted to a PCR that amplified a 1457 bp fragment of the 16S rDNA gene using universal bacterial primer primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTACGACTT-3') (Pitulle et al., 1999). pGEM-T easy vector cloning kit was used to amplify fragments. Specific descriptions were shown in the manufacturer's instructions. The ClustalW program was used to create a phylogenetic tree.

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