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Arsenic Range Finding Phytotoxicity Test Against *Ludwigia octovalvis* as First Step in Phytoremediation

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ABSTRACT

Studies were conducted to determine the range of arsenic concentration through a phytotoxicity test on the plant species of *Ludwigia octovalvis*. Pots contained 3 kg of sand spiked with As salt of sodium arsenate dibasic heptahydrate. Arsenate [As(V)] concentrations were set as 0 mg kg^{-1} as control 1 without plant, 0 mg kg^{-1} as control 2 with plant, 4, 20, 40, 60 and 80 mg kg^{-1}. The experiment was carried out for 28 days. The parameters such as pH and temperature in spiked sand were also monitored. The As bioavailability in spiked sand was extracted using Na_2-EDTA method and were determined using Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) Optima 7300DV. Based on the range finding test results, *L. octovalvis* can survive up to As(V) concentration of 40 mg kg^{-1}, whereas the plant were wilted and dried at 60 and 80 mg kg^{-1}. The EC_{50} value for single As(V) phytotoxicity to *L. octovalvis* was predicted between 40 and 60 mg As kg^{-1}. Reductions of As(V) bioavailability in spiked sands were calculated as between 13.0±3.3 and 70.4±0.1%. Hence, the next step experiment on a prolonged phytotoxicity test will be based on the value of this As(V) concentration effect.

Key words: Arsenic, range finding phytotoxicity test, *L. octovalvis*, spiked sands

INTRODUCTION

Arsenic (As) is a contaminant public concern since it is highly toxic to humans, plants, animals and also microorganisms. Arsenic concentration typically varies from below 10 mg kg^{-1} in non-contaminated soil (Fitz and Wenzel, 2002), to as high as 30,000 mg kg^{-1} in contaminated soil (Vaughan, 1993). Arsenic contaminated sites with excess of 1000 mg kg^{-1} has been reported at many sites throughout Australia, the United States, Africa and other places of the world (Lou *et al*., 2009). Generally, As(V) is the dominant species in the soil solution under oxidizing conditions, whereas As(III) is the prevailing species under moderately reducing conditions (Quaghebeur and Rengel, 2005).

As is a non essential element for plants and inorganic As is generally highly phytotoxic (Gonzaga *et al*., 2006). The toxicity of As depends on the concentration of soluble As, therefore,

sodium arsenate and arsenic trioxide, formerly used as herbicides, are the most toxic (Kabata-Pendias and Pendias, 2001). In organic form arsenic is more toxic in sands and loams with the reported toxicity thresholds as 40 mg kg^{-1} than in clay soils with 200 mg kg^{-1} (Sheppard, 1992). Phytotoxicity of As is highly dependent on soil properties and while in heavy soil about 90% growth reduction appears at 1000 ppm As addition, but As is equally toxic in light soil (sandy soil) of 100 ppm (Kabata-Pendias and Pendias, 2001). Toxicity of As to plant is due to the damage of chloroplast membrane and cell membrane by peroxidation of membrane lipid (Lou *et al*., 2009).

As(V) uptake by living organisms is via phosphate transportation. As(V) is analogous to phosphate (PO_4^{3-}) in the glycolytic pathway, whereas As(III) binds strongly to sulfhydryl groups of amino acids such as cysteine in proteins inactivating a wide range of enzymes in intermediate metabolism (Wang and Zhao, 2009). Therefore, As(III) is more toxic than As(V). However, exposure to both As(III) and As(V) may result in similar toxicological effects since research studies have shown that most ingested As(V) can be reduced to As(III) by As(V) reductases (Oden *et al*., 1994).

As(V) is a competitive inhibitor of phosphate and acts as an uncoupler of oxidative phosphorylation (Alloway, 1995; Quaghebeur and Rengel, 2005). As(V) can disrupt mitochondrial oxidative phosphorylation and thus, the production of the nucleotide adenosine triphosphate (ATP), which is a main energy source for cells. This process is known as arsenolysis, or the hydrolytic process whose first step is the replacement of As(V) by phosphate (Gonzaga *et al*., 2006). As(V) competes with phosphate for binding to adenosine diphosphate (ADP), the formation of the arsenate-adenosine diphosphate (As(V)-ADP) analogues deprives the cells of energy source, ultimately leading to cell death.

Range finding phytotoxicity test is a preliminary test that is conducted to establish the definitive test as the main toxicology experiment (USEPA, 1996). The range-finding test help to determine the concentrations of the toxicant that will be administered to the test organisms. The range finding test is usually conducted at five concentrations to determine a concentration-response ratio of mortality over several level of concentration of the chemical tested. The measurement parameter at acute test is mortality number of dead individual or effect on growth inhibition in plants. It indicates as LC_{50} (lethal concentration) or EC_{50} (effect concentration). LC_{50} is defined as the concentration at which 50% lethal of the population of experimental organisms, whereas EC_{50} is defined as the concentration at which 50% effect of the experimental organisms estimated by graphical or computational means (Landis and Yu, 1995; Lam *et al*., 2004). According to Rombke and Moltmann (1996), mortality data usually from acute tests is discrete data, with values which may range between 0 and 100%.

According to Essoka *et al*. (2006), there is contribution of the oil industry with the heavy metal contamination. Screening of plants survived in crude oil-contaminated area as a preliminary study toward toxicity testing in phytoremediation was carried out. *Ludwigia octovalvis* is one of plants that could grow and survive in that contaminated area. *L. octovalvis* is locally known as buyang samalam, lakom ayer and pujang malam. Other names of this plant are false primrose, primrose willow, swamp primrose, water primrose, willow primrose and yellow willow herb. *L. octovalvis* is a cosmopolitan plant with a mainly tropical distribution (Barau, 2010).

The aim of this study of single As(V) range finding phytotoxicity test against *L. octovalvis* was to determine the maximum concentration of As(V) that the *L. octovalvis* have ability to survive. The results for this study will then be used for further research since it is a part of a research project that aims to uptake and accumulate As using phytoremediation method. Before any plant can be used in phytoremediation, it should be tested first to know the tolerance of the plant to As exposure in the range finding phytotoxicity test and the prolonged phytotoxicity test.

MATERIALS AND METHODS

Propagation of the plant species: At the first step, the seeds were obtained from *L. octovalvis* originally from the crude oil contaminated site in Malacca, Malaysia. The propagation of *L. octovalvis* to prepare plant stock was conducted using the seeds of *L. octovalvis* in garden soil at the greenhouse for second generation. The seeds (diameter 0.6-0.75 mm) were planted in plastic crates (37×27×10 cm). After three weeks, individual seedlings were transferred into polybags. The wet weight of the plant at three weeks was within 0.1307±0.0361 g. All of the plants used in the experiment were eight weeks old. The wet weight of the eight weeks old plant was within 8.7867±1.8111 g.

Range finding phytotoxicity test: The experiment was conducted in a greenhouse in UKM, Malaysia in 2009 using As spiked sand. According to OECD 208 (Guidelines for Testing of Chemicals 208 to Terrestrial Plants and Growth Test) (OECD, 1984), the size of sand was sieved (5 mm in diameter) to remove coarse fragments, in the range finding phytotoxicity test sand was used 4.75 mm in diameter. The macro nutrient (N, P, K, S, Mg, Ca, K) and one micro nutrient (Cl) were analyzed through Ion Chromatography (IC) (Metrohm 882 Compact IC plus, Switzerland), while the micro nutrient (Fe, Zn, Mn) and trace elements (Pb, As) were determined using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) Optima 7300DV (Perkin Elmer, USA). Based on analysis, sand contents for the macro nutrient were 29.2 mg kg^{-1} N (nitrate) and 1.2 mg kg^{-1} K, 13.0 mg kg^{-1} SO_4^{2-}, 86.5 mg kg^{-1} Ca, 7.4 mg kg^{-1} Mg, whereas the micro nutrient contents were; 6.4 mg kg^{-1} Cl^-, 5.5 mg kg^{-1} Fe, 0.04 mg kg^{-1} Zn and 1.62 mg kg^{-1} Mn. The trace elements were not detected.

Pots contained sand spiked with As salt of sodium arsenate dibasic heptahydrate ($AsHNa_2O_4.7H_2O$) (FlukaChemika, Switzerland) at six different concentrations of As(V): 0 mg kg^{-1} as control 1 without plant, 0 mg kg^{-1} as control 2 with plant, 4, 20, 40, 60 and 80 mg kg^{-1}. The equation used to convert the As(V) concentration from mg L^{-1} to mg kg^{-1} is as below and the conversion results are in Table 1:

$$\text{As(V) conc. in mg kg}^{-1} = \frac{\text{As(V) concentration (mg L}^{-1}) \times \text{Volume (L)}}{\text{Sand weight (kg)}} \quad (1)$$

Sand (3 kg) was mixed thoroughly in order to obtain homogeneity and was then allowed to equilibrate for seven days in plastic pots under controlled greenhouse conditions (Hartley and Leep, 2008). The range finding phytotoxicity test was conducted for 28 days. During range finding phytotoxicity test, pH and temperature of spiked sand was monitored. All samplings were conducted in three replicates. Watering was conducted at every two day.

A graph describing the response of cumulative of effect to a range of As(V) concentration is called the concentration-response curve (Landis and Yu, 1995). The cumulative effect (wilting,

Table 1: Conversion of As(V) concentration in mg L^{-1} to mg kg^{-1}

As(V) (mg L^{-1})	As(V) (mg kg^{-1})
15.5	4
77.5	20
155	40
232.5	60
310	80

dried and death) to the plant as response versus As(V) concentration is drawn and the As(V) concentration that results in 50% of the measure effect can be determined from the graph.

Determination of As(V) bioavailability in sand: The As(V) bioavailability was determined through extracting using 0.05 mol L^{-1} ethylene diamine tetraacetic acid disodium (Na_2-EDTA) solution (Merck, Germany) (Quevauviller, 1998). Approximately 5 g of sand from each As(V) concentration was extracted for the As analysis. All samples were conducted in three replicates. The sand samples were placed into plastic tubes and after adding Na_2-EDTA, the samples were agitated using a BIOSAN multi RS-60 (Latvia) at 30 rpm for 1 h at room temperature. Each sample was then centrifuged at approximately 3000 rpm for 10 min using an Eppendorf centrifuge type 5810 R (USA). The samples were filtered through filter paper with a porosity of 0.2-1.1 μm (Whatman, UK) using a vacuum filter, GAST model DOA-P504-BN (USA). The supernatant was collected in polyethylene bottles and stored at 4°C for further As analysis using an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) Optima 7300DV Perkin Elmer (USA).

RESULTS AND DISCUSSION

Table 2 summarizes the observation of *L. octovalvis* growth in the range finding phytotoxicity using As(V) and Fig. 1 depicts the image of *L. octovalvis* during the range finding phytotoxicity test. Based on plant observation during the range finding phytotoxicity, *L. octovalvis* can survive up to 40 mg kg^{-1} As(V). Plants were wilting after 3 days of exposure in 40, 60 and 80 mg kg^{-1} As(V), but plants can still survive and became healthy in 40 mg kg^{-1} after 7 days of As(V) exposure. Meanwhile *L. octovalvis* were wilted and dried at 60 and 80 mg kg^{-1} As(V) after 3 days of exposure until the end of exposure (Day-28). Several leaves in control fell after 21 days could be due to decreasing nutrient since no additional nutrient was added. The dropping leaves in As(V) concentrations of 4 and 20 mg kg^{-1} also significantly occurred after 21 days due to the additional

Table 2: Plant observation during As(V) range finding phytotoxicity test

As(V) conc. (mg kg^{-1})	Time of exposure (day) 0	1	3	7	14	21	28
0 (control)	Plants were healthy	Plants were healthy	Plants were healthy	Plants were healthy	Plants were healthy, flower grew	Plants were healthy, flower grew, several leaves fell	Plants were healthy flower grew, several leaves fell
4	Plants were healthy	Plants were healthy	Plants were healthy	Plants were healthy	Plants were healthy, flower grew	Plants were healthy, flower grew, several leaves fell	Plants were healthy, flower grew, several leaves fell
20	Plants were healthy	Plants were healthy	Plants were healthy	Plants were healthy	Plants were healthy, flower grew	Plants were healthy, flower grew, several leaves fell	Plants were healthy, flower grew, several leaves fell
40	Plants were healthy	Plants were healthy	Plants became wilt	Plants could survive, plants became healthy again, several leaves fell	Plants were healthy, several leaves fell	Plants were healthy, several leaves fell	Plants were healthy, several leaves fell
60	Plants were healthy	Plants were healthy	Plants became wilt	Plants became wilt, leaves and stems were brown	Plants became wilt, leaves and stems were brown	Plants became wilt, leaves and stems were brown	Plants became wilt, leaves and stems were brown
80	Plants were healthy	Plants were healthy	Plants became wilt	Plants became wilt,	Plants became wilt, leaves and stems were brown	Plants became wilt, leaves and stems were brown	Plants became wilt, leaves and stems were brown

Control at day-1, plants healthy

Control at day-3, plants healthy and grew

Control at day-28, plants healthy and grew, leaves dropping

4 mg kgG^{1} at day-1, plants healthy

4 mg kgG^{1} at day-3, plants healthy and grew

4 mg kgG^{1} at day-28, plants healthy and grew, leaves dropping

20 mg kgG^{1} at day-1, plants healthy

20 mg kgG^{1} at day-3, plants healthy and grew

40 mg kgG^{1} at day-28, plants healthy and grew, leaves dropping

40 mg kgG^{1} at day-1, plants healthy

60 mg kgG^{1} at day-3, plants healthy and grew

40 mg kgG^{1} at day-28, plants healthy and grew, leaves dropping

Fig. 1(a-r): Continue

Fig. 1(a-r): Images of *L. octovalvis* during range finding phytotoxicity test

effect of As(V) toxicity. Meanwhile the dropping leaves in As(V) concentrations of 40 mg kg^{-1} started at 7 days after exposure. According to Kabata-Pendias and Pendias (2001) and Quaghebeur and Rengel (2005), the symptoms of As toxicity to plants are variously described as leaf wilting, violet coloration (increased anthocyanin), root discoloration, inhibition of root growth, cell plasmolysis and plant death. Wilting, especially of the leaves, was observed as an initial symptom of phytotoxicity resulting from As(V) exposure. The roots of *L. octovalvis* were collected at the end of experiment. Roots grew well in control, 4, 20 and 40 mg kg^{-1}, but roots did not grow and dried in 60 and 80 mg kg^{-1} As(V). The dried roots were in black color, whereas the roots in control crates were still white in color.

Figure 2 depicts EC_{50} value for single As(V) toxicity to *L. octovalvis* which was predicted between 40 and 60 mg kg^{-1} As(V). According to Kabata-Pendias and Pendias (2001), 100 ppm As (65 mg kg^{-1}) is equally toxic to plants in sandy soil or light soil. Based on the observations, the higher the concentration of As(V), the greater the effect percentage and the faster wilting condition occurred. So, the next test (prolonged phytotoxicity test) of As(V) against *L. octovalvis* will be conducted on As(V) concentration range of 5 to 65 mg kg^{-1} As(V).

Figure 3 shows that As bioavailability in sand spiked with various As(V) concentrations significantly decreased during the range finding phytotoxicity test. Reductions of As(V) bioavailability in spiked sands were calculated as between 13.0±3.3 and 70.4±0.1%. The decreasing As(V) bioavailability in the spiked sand suggests the As(V) was taken up and accumulated by *L. octovalvis*.

The pH during the range finding phytotoxicity test was 5.45±0.01 to 7.00±0.02, this value were within still the range of pH (5-7.5) as mentioned in OECD (1984) (Fig. 4). Temperature ranging from 25.5±0.1-27.8±0.1°C showed the room temperature at tropical climate (Fig. 5).

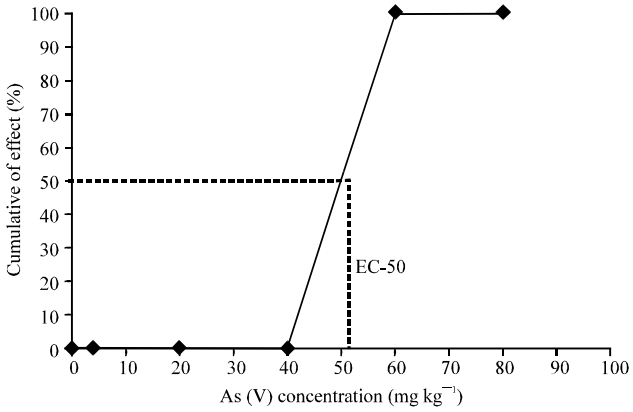

Fig. 2: As(V) toxicity to *Ludwigia octovalvis* concentration-response curve

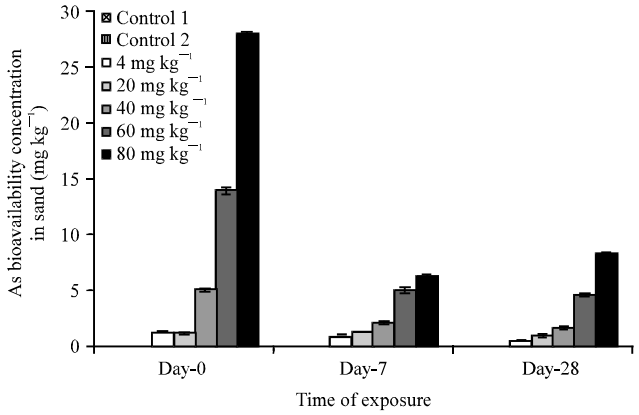

Fig. 3: As bioavailability concentration in spiked sand, control 1 is without plant and control 2 with plant, both of them are without contaminant

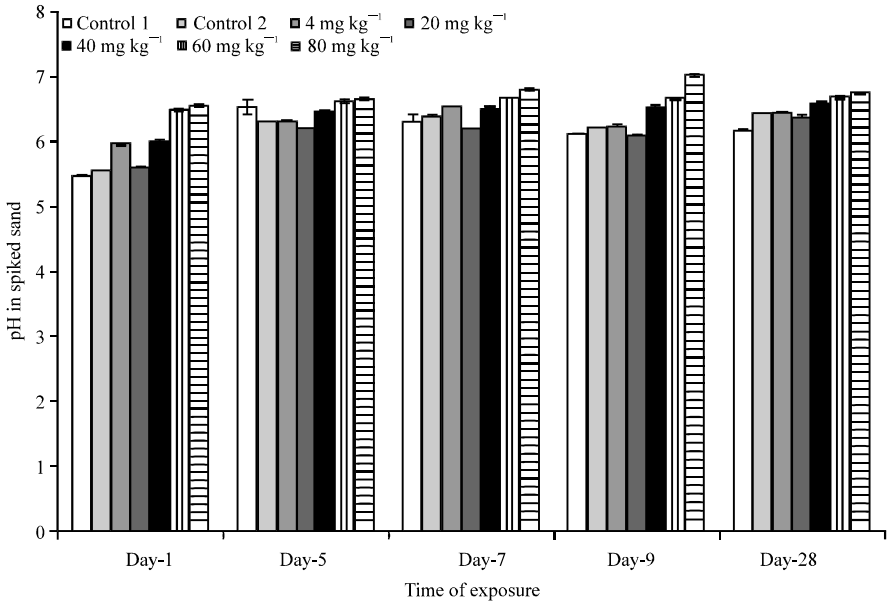

Fig. 4: Monitoring of pH in spiked sand during As(V) range finding phytotoxicity test

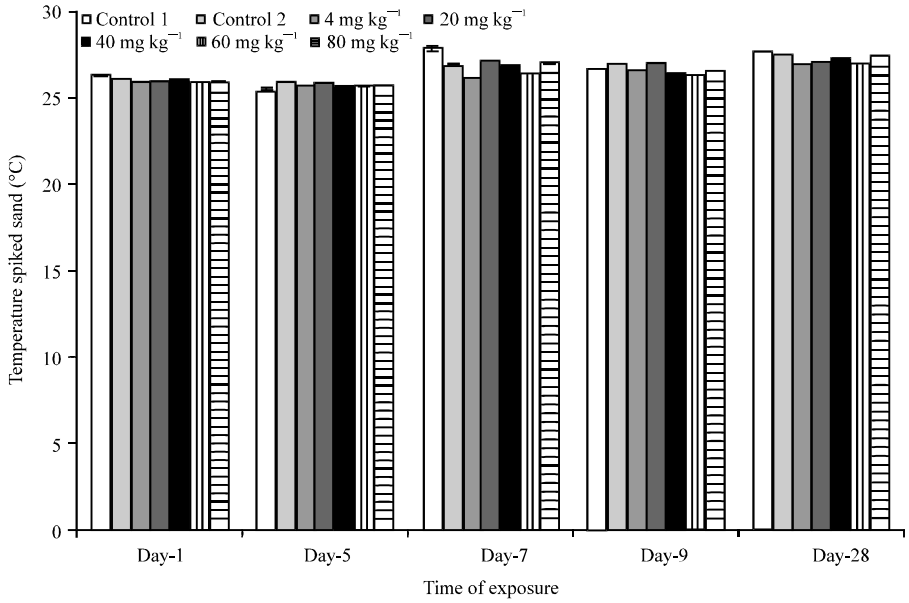

Fig. 5: Monitoring of temperature in spiked sand during As(V) range finding phytotoxicity test

CONCLUSIONS

The results showed that as the As(V) concentration increased in spiked sand, the wilting leaves and dried plant of *L. octovalvis* were also increased. This study demonstrated that *L. octovalvis* has the ability to survive up to the As(V) concentration of 40 mg kg^{-1} in the range finding phytotoxicity test. However, the plant did not survive at As(V) concentration of 60 and 80 mg kg^{-1}. The effect concentration with 50% effect for single As(V) phytotoxicity to *L. octovalvis* was predicted between 40 and 60 mg kg^{-1}. Reductions of As(V) bioavailability in spiked sands reached 13.0±3.3 to 70.4±0.1%. Hence, the future prolonged phytotoxicity test will be conducted on As(V) concentration of 5 to 65 mg kg^{-1}.

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