

Ability of *Ipomoea aquatica* Forssk. to Remediate Phenol in Water and Effects of Phenol on the Plant's Growth

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ABSTRACT

Despite wide applications in industries, phenol pollution leads to many health effects, and one of the technologies used to clean up phenol pollution is phytoremediation. The aim of this research was to assess the remediation ability of *Ipomoea aquatica* Forssk., which is easy to handle and has a fast growth rate. Plantlet was grown in water spiked with 0.05, 0.10, 0.20, 0.30 and 0.40 g/L phenol, followed by daily observation of the plantlets morphology and tracking of phenol concentration in the water and plantlet extracts via 4-aminoantipyrine (4-AAP) assay. Plantlet's roots in 0.10 g/L phenol (57.42 ± 1.41 mm) were significantly longer ($p < 0.05$) than those of the control plantlets (43.57 ± 3.87 mm) in contrast to other phenol concentrations which had stunted roots growth. *I. aquatica* Forssk. was able to survive with 0.30 g/L phenol despite exhibiting yellowing of leaves and increased sensitivity to scarring on the stems. The plantlets were able to completely remove the phenol from the water spiked with phenol at 0.05 g/L after 12 days of growth. However, the highest average rate of phenol removal was 0.021 g/L/day from water spiked with 0.30 g/L phenol. Phenol analysis on the plantlets' extracts revealed that *I. aquatica* Forssk. had degraded the absorbed phenol. This observation is of significant interest as it highlights the potential of *I. aquatica* Forssk. for use as a phytoremediator to clean up phenol contaminated water.

Keywords: 4-aminoantipyrine assay, *Ipomoea aquatica* Forssk., phenol, phytoremediator

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INTRODUCTION

Phenol has wide applications in several industries such as the manufacturing of dyes, plastics, fertilisers, pesticides, polymeric resins and in oil refineries (McCall et al.,

2009). As phenol has high water affinity and low volatility (Prpich & Daugulis, 2005), exposure to untreated phenol released from these industries into the aquatic environment has been reported to be extremely harmful (Huang et al., 2014). Besides being a contributing factor to non-potable and non-palatable drinking water (Sihem, Lehocine, & Miniai, 2012), excessive exposure could also cause health effects on skin, digestive system, brain, kidney, heart, and may also result in genetic damages (Al-Muhtaseb et al., 2011). McCall et al. (2009) reported that oral ingestion might cause vomiting, mouth sores, bloody diarrhoea, nausea, dark urine, and even death in certain cases.

There are currently a few technologies that are being applied to clean up phenol pollution sites. These include (a) extracted enzyme (Deva, Arun, Arthanareeswaran, & Sivashanmugam, 2014), (b) microbial biodegradation (Basha, Rajendran, & Thangavelu, 2010), (c) soil removal (Xia, Ma, Liu, & Fan, 2012) and (d) chemical oxidation (Sihem et al., 2012). These technologies, however, require high operating costs and consist of other limitations, which as a result, reduce their effectiveness. Microbial biodegradation can be easily inhibited and has low microbial survival rate under extended phenol exposure. Both soil removal and chemical oxidation are destructive to the sites.

Phytoremediation is a technology that utilises plants to remove or transform toxic chemicals present in soils, sediments, ground water, surface water, and even the atmosphere to clean up or reduce the level of pollution (Susarla, Medina, & Mccutcheon, 2002). There are many advantages of phytoremediation over other technologies, as reported in various studies. The main advantages of this technology include being environmental friendly due to the usage of plants as a main component (Ahemad, 2015), low cost (Rungwa, Arpa, Sakulas, Harakuwe, & Timi, 2013), ability to remove a wide range of pollutants simultaneously (Batty & Dolan, 2011), as well as being an *in situ* bioprocess, where neither soil nor water is removed from the sites. Thus, the original structure of treated sites remains undisturbed (Ho et al., 2012). Both plants and residuals from phytoremediation are valuable components, which can be used later for biofuel production or carbon sequestration upon completion of the process (Batty & Dolan, 2011).

After optimisation of major parameters such as the phenol concentration, sunlight and treatment time (Wang, et al., 2014a), plants have the potential to remediate phenol efficiently. In the phenol degradation pathway, plants utilise peroxidase (Jha et al., 2013) instead of phenol hydroxylase, as used by other microorganisms (Kwon & Yeom, 2009). According to Jha et al. (2013), plants degrade phenol through the catechol-cleavage pathways, where peroxidase hydroxylates phenol and forms catechol. Catechol will then cleave to form muconic acid and fumaric acid which enter the Krebs cycle, completing the phenol degradation pathway. Above tolerable level, phenol might cause damages such as reduction in root length (Ratsch, 1983), root browning (Jha et al., 2013) and seed germination inhibition (Ibanez, Alderete, Medina, & Agostini, 2012).

Aquatic plants play important roles in biochemical cycles, nutrient mobilisation and sediment dynamics in freshwater ecosystems (Wang, et al., 2014b). They also readily remediate toxic pollutants in freshwater and seawater (Rahman & Hasegawa, 2011). Some aquatic plants can concentrate metals inside the plants to concentrations much higher than the surroundings (Mishra & Tripathi, 2008). The aquatic plant to be studied in this

research is *Ipomoea aquatica* Forssk. of the family Convolvulaceae. It is commonly known as water spinach, “nalanibhaji” and “kalmisag” (Manvar, 2011), or “kangkung” and “ong choy” in Malaysia. It is a semi-aquatic, fast-growing vegetable with hollow stems and arrowhead-shaped leaves (Chen et al., 2010). It has high medicinal values with potential as an anti-tumour agent (Ullah et al., 2013) and the ability to lower the mortality rate in coronary heart disease (Sivaraman & Muralidaran, 2010). This plant has also been used in treating iron-deficiency anaemia (Islam et al., 2013), arsenic poisoning (Alkiyumi et al., 2012) and bronchitis (Manvar, 2011). *I. aquatica* Forssk. is a native plant in sites usually contaminated with excessive nutrients (Chen et al., 2010) and it is often studied as a phytoremediator to remove excessive nitrogen (Jampeetong, Brix, & Kantawanichkul, 2012), chromium (Chen et al., 2010), cadmium (Hseu, Jien, Wang, & Deng, 2013) and manganese (Dassharma, Ravnang, & Nazia, 2014). Thus, the objectives of this study were to study the effects of phenol on *I. aquatica* Forssk. and investigate the ability of the plantlets to remove phenol from phenol-spiked water in order to determine the compatibility of this plant to phytoremediate phenol in water.

MATERIALS AND METHODS

Plant material

The seeds of *I. aquatica* Forssk. were bought from an agricultural seeds shop in Negeri Sembilan, Malaysia (Coordinates 2°43'50.58" N, 101°56'11.76" E). The seeds were kept dry to prevent early germination and spoilage. Germinated plantlets were authenticated by a botanist and deposited at the herbarium of the Biodiversity Unit, Universiti Putra Malaysia, with the specimen voucher number SK2675/15.

Experimental Set-up

Phenol (purchased from Merck, Darmstadt, Germany) concentrations tested in this experiment were 0.05, 0.10, 0.20, 0.30 and 0.40 g/L. This phenol concentrations range was selected based on the reported environmental phenol pollution range of 0.05 to 0.60 g/L (Paisio, Agostini, Gonzalez, & Bertuzzi, 2009), and *I. aquatica* Forssk. could not survive above 0.40 g/L phenol (our unpublished data). Germinated seedlings were grown in containers measuring 30 cm (length), 23 cm (width) and 10 cm (depth). Young plantlets were used as they were more sensitive to pollutants and any morphological changes can be easily noted. Each container held 4 litres of phenol-spiked distilled water, and the water circulation system was supported by a Life Tech AP1000 water pump (Eng Hing Aquatics, Selangor, Malaysia) to circulate the water. Distilled water was used to avoid potential contamination by other microorganisms. No nutrients were added into the system as newly germinated seedlings sustained their growth from nutrients in the cotyledons, while the plantlets were able to photosynthesise. The whole container containing the plantlets was placed on a bench next to a window. All the plantlets were placed equidistant (2.54 cm apart) inside each container to minimise competition and were ensured that roots were always in contact with water, while both leaves and stems were above water level.

Seeds were rinsed to remove dirt on the seed coats, and then soaked in distilled water for 72 hours before transferring to the container. This germination step before placing the seedlings into the container enables selection of healthy seedlings with similar root size to start the experiment. A total of thirty germinated seedlings, with roots measuring approximately 2.00 cm, were placed in the container with phenol-spiked water (designated as Day 0) and grown for 14 days. The experiment was conducted in triplicate for each of the phenol concentration used, which involved a total of ninety seeds distributed equally in three different containers. Growth parameters such as lengths of root, stem, primary leaf and petiole were measured every day for 14 days. Extra phenotypic information such as lengths of secondary leaf, root hair, thickness of root and formation of roots at the nodes was also recorded at the end of the experiment. Phenol remaining in the water and retained in the plantlets were quantified daily and at the end of the experiment, respectively. The pH of the water was not monitored in this experiment since plant peroxidase responsible for phenol degradation was reported to be capable of functioning in a wide pH range from pHs 4 to 9 (Gonzalez et al., 2006).

Phenol assay: 4-aminoantipyrine (AAP) assay

Quantification of phenol remaining in the water was carried out daily throughout the 14 days of growth by removing 1 ml of the water from each container for the 4-aminoantipyrine (4-AAP) assay, following the protocol of Ettinger, Ruchloft, and Lishka (1951). Likewise, the 4-AAP assay was also used to measure absorbed phenol within the plantlets. All the surviving plantlets were pooled and ground in a pestle and mortar, followed by centrifugation at $10,625 \times g$ for 15 minutes. The supernatant was then subjected to the 4-AAP assay. Extract from *I. aquatica* Forssk., grown in distilled water without phenol, was used as a control in the 4-AAP assay. Each sample was assayed in triplicate to obtain the mean concentration of phenol present. All the chemicals used were purchased from Sigma Aldrich, St. Louis, USA.

Statistical Analysis

Measurements of the plantlet's length and phenol assay were carried out daily throughout the 14-day experiments. Roots, stems, leaves, and petioles from all surviving plantlets were measured daily and presented as mean and standard deviation as error bars in the graph. Statistical analysis of the data by one way analysis of variance (ANOVA) was conducted using GraphPad Prism version 5.00 for Windows. Significant differences between means were determined using Tukey's Multiple Comparison Test, where p value less than 0.05 was regarded as significant.

RESULTS AND DISCUSSION

Plantlets growth in distilled water

Ninety germinated plantlets were transferred and grown in containers with phenol-free distilled water for 14 days, and observations on the changes of the roots, stems, leaves, and petioles were recorded daily, as shown in Figure 1(a). Throughout the 14 days of growth, all the plantlets exhibited rapid growth during the early stage, but plateaued as they matured towards the end

of the growth period (Figure 2). The roots were visible and increased significantly in length from Day 1 until Day 10. Meanwhile, measurements of leaves were taken only starting on Day 3 after most of the cotyledons had withered and fallen off, exposing the young green leaves. The leaf length increased until Day 7, with an average increment of 7.66 ± 1.35 mm daily, which remained constant after Day 8.

The stems, however, exhibited a different growth pattern. While both leaf and petiole lengths increased from Day 0 until Day 14, the stem length reached its maximum increment of 9.69 ± 0.62 mm on Day 4, following which, no significant increment was observed. It was also observed that root hairs continued to form and grow throughout the stems.

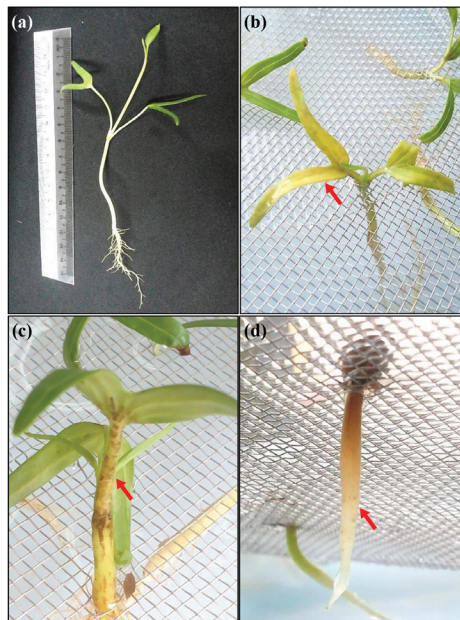


Figure 1. Daily observation of the *I. aquatica* Forssk. including (a) Measurement of structure sizes, and physical effects of phenol on the plantlets (arrows) such as (b) Yellowish leaves, (c) Scars on stems, and (d) Soggy roots.

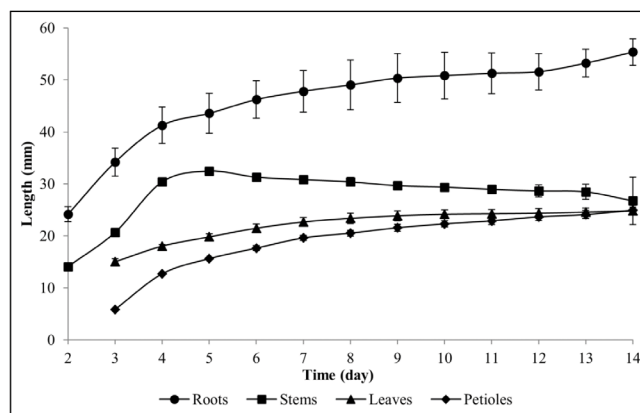


Figure 2. Growth of Control plantlets in distilled water. Values plotted are means \pm standard deviation (SD).

Plantlets subjected to phenol-spiked water

Figure 3 shows that all the plantlet parts exhibited similar growth profiles as that of the control. This observation indicated that at different concentrations, phenol affected the growth rate but not the growth profiles of treated plantlets.

From the data obtained in this study, it was evident that *I. aquatica* Forssk. was able to grow in all the phenol concentrations, except for 0.40 g/L. As phenol concentration increased, the plantlets' growth slowed and the plantlets were mostly stunted. Interestingly, the plantlets grown in 0.05 g/L phenol were always shorter than those in 0.10 g/L phenol, including their root length. This condition could be due to a basal growth induction triggered by phenol concentration of 0.10 g/L. For instance, as displayed in Figure 3(a), the roots of plantlets grown in 0.10 g/L phenol were significantly longer ($p < 0.05$) than all other concentrations, including the Control plantlets. The highest effect was achieved on Day 5 as the plantlets grown in 0.10 g/L have root lengths (57.42 ± 1.41 mm) which were 13.85 ± 1.11 mm longer ($p < 0.05$) than the control plantlets (43.57 ± 3.87 mm). Upon termination of growth (Day 14), plantlets in 0.10 g/L (61.02 ± 1.50 mm) were 5.67 ± 3.11 mm longer than the Control plantlets (55.36 ± 2.55 mm). Growth induction by phenol could occur as plant peroxidase degrades the phenol through the catechol-cleavage pathway (Jha et al., 2013) to generate fumaric acid for subsequent production of useful metabolites such as pyruvic acid, acetic acid and acetyl-CoA in the Krebs cycle.

In this study, plantlets exposed to phenol up to 0.20 g/L exhibited green and fully expanded leaves. However, plantlets in 0.30 g/L phenol were withered and the leaves were yellowish, as shown in Figure 1(b), postulating a compromised on the photosynthesis apparatus of the plantlets. This result is similar to the recent study by Wang et al. (2014a), where the leaves of *Polygonum orientale* turned yellow and brown upon treatment with phenol above 0.10 g/L. The effects that phenol had on the photosynthetic mechanism of plants were discussed in the recent studies of Hang and Zhao (2010), and Li, Zhang, Xiew, Li, and Zhang (2015). Hang and Zhao (2010) reported that phenol stress inactivated the Photosystem II, which is the photosynthetic reaction centre, and caused a restriction on excited electrons transfer resulting in a reduction of the actual photochemical reactions. This could probably explain the yellowing of leaves observed in this study. Li et al. (2015) reported that phenol concentration above 0.20 g/L inhibited the photosynthetic activities of *Salix babylonica* besides reducing their growth and repair capabilities. Nonetheless, further studies need to be done to investigate the actual effects of phenol on *I. aquatica* Forssk. photosynthetic system during phenol treatment.

Phenol was also observed to have significantly reduced stem length ($p < 0.05$) by all except 0.10 g/L phenol in comparison with the Control, as shown in Figure 3(b). Scars were frequently observed on the stems of plantlets exposed to 0.20 g/L and above phenol despite precautions taken during the handling of plantlets throughout the experiment [Figure 1(c)]. Increased phenol concentrations also led to the formation of shorter petioles [Figure 3(d)].

Plantlets exposed to phenol above 0.30 g/L were fatal, whereby 27.8% of them in 0.30 g/L phenol died on Day 14 and 87.8% in 0.40 g/L phenol died on Day 6 (Table 1). The highest death rate in 0.40 g/L phenol was achieved on Day 4. The physical effects observed prior to death in 0.40 g/L phenol were the roots became soggy, transparent and brittle to touch as depicted

in Figure 1(d). Root was the first plant's part to establish direct contact and responded to the phenol (Zhou, Tang, & Wu, 2013; Park et al., 2016), explaining the apparent damages observed prior to death of the whole plantlet.

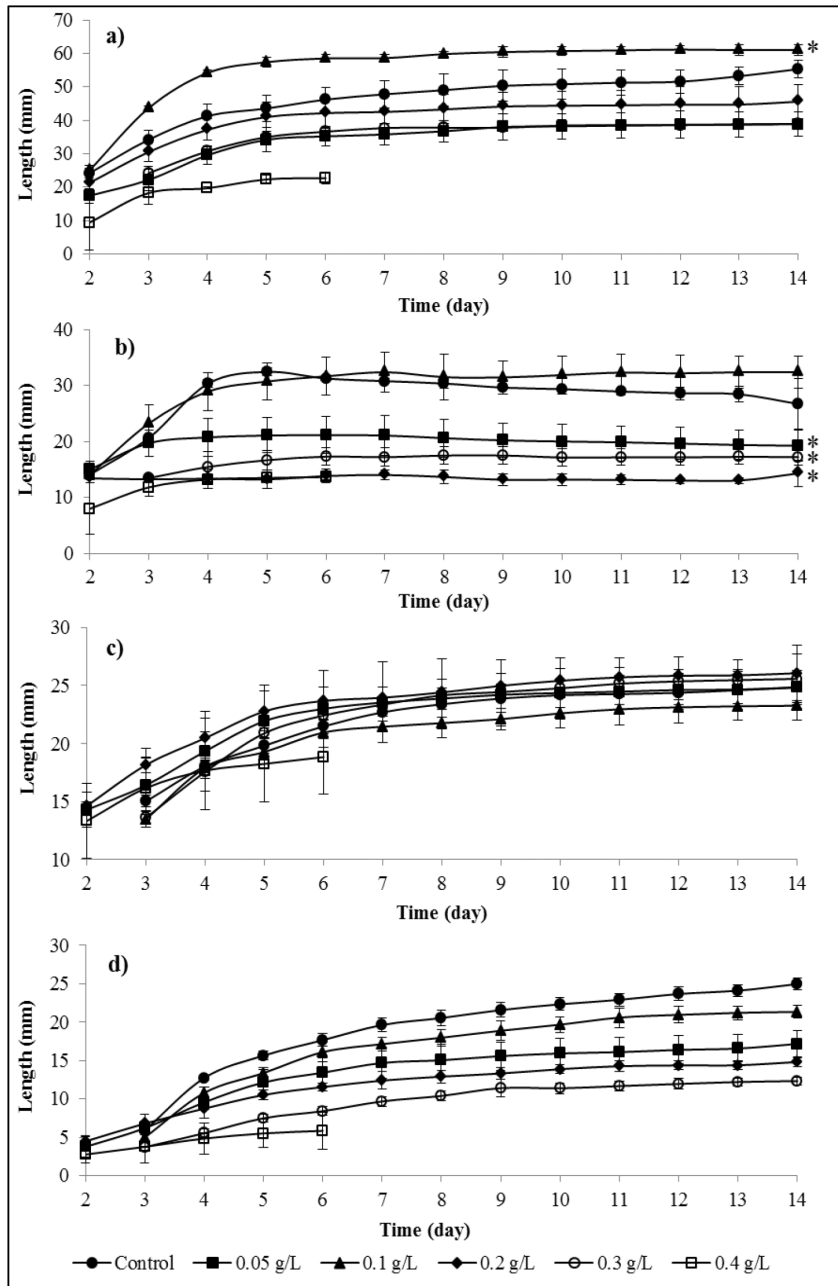


Figure 3. Growth of *I. aquatica* Forssk. in different phenol concentrations: (a) Roots, (b) Stems, (c) Leaves, and (d) Petioles. Values plotted are means \pm standard deviation (SD). *Growth was of significant difference ($p < 0.05$).

Table 1

Total plantlets survived and phenol assay on plantlet extracts

Phenol Concentration (g/L)	Number of Plantlets Survived	Time of Assay on Absorbed Phenol	Phenol in Plantlet Extract (g/L)
0*	90	Day 14	0.02 ± 0.00
0.05	76	-	-
0.1	58	i) Day 9 ii) Day 11	i) 0.10 ± 0.01 ii) 0.08 ± 0.01
0.2	60	-	-
0.3	65	Day 14	0.07 ± 0.01
0.4	11	-	-

*Control plantlets (no phenol exposure) were grown in distilled water only.

Phenol Degradation

I. aquatica Forssk. was capable of gradually removing phenol starting from the first day of growth (Figure 4), with over 80.0% of the phenol removed from the water in all the phenol concentrations below 0.40 g/L. Total removal of phenol (100%) from water spiked with 0.05, 0.10 and 0.30 g/L phenol took 14 days. However, only 0.17 ± 0.01 g/L (84.6%) of phenol was removed from the 0.20 g/L phenol-spiked water, while 0.14 ± 0.01 g/L (34.2%) was removed from the 0.40 g/L phenol-spiked water (the experiment was terminated on Day 6 for 0.4 g/L phenol as almost all the plantlets had died). Compared to *I. aquatica* Forssk., *Vetiveria zizanoides* L. Nash plants removed 0.05 and 0.10 g/L phenol in 4 days (Singh, Melo, Eapen & D'Souza, 2008), *Vicia sativa* removed 0.10 g/L phenol in 7 days, while alfalfa removed 0.10 g/L phenol in 30 days (Flocco et al., 2002).

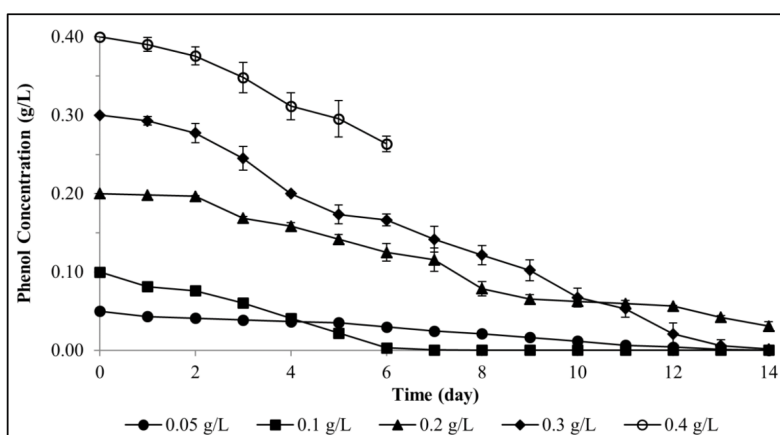


Figure 4. Daily analysis of phenol remaining in the water. Aliquot (1.00 mL) of water from each container was subjected to 4-AAP assay to determine daily concentration of phenol remaining in the water. Data plotted are means ± standard deviation (SD).

Phenol removal activity was calculated as quantity of phenol removed from the water daily throughout the experimental growth period. The highest rate was attained in 0.30 g/L phenol, averaging around 0.021 g/L per day (Figure 5). Although *I. aquatica* Forssk. achieved the highest phenol degrading activity in 0.30 g/L, the growth of the plantlets was compromised (signs of inhibition), as discussed in the previous section (plantlets subjected to phenol-spiked water). *I. aquatica* Forssk. was the least affected by 0.10 g/L phenol treatment, exhibiting a phenol removal rate of 0.013 g/L per day, which was significantly lower compared to 0.30 g/L phenol treatment ($p < 0.05$). Nonetheless, phenol removal activity at 0.20 g/L was 0.012 g/L per day, while much a lower activity was observed in 0.05 g/L phenol (0.004 g/L per day).

Further analysis of the crude extracts (Table 1) indicated that *I. aquatica* Forssk. metabolised or transformed the absorbed phenol instead of accumulating it in the plantlet tissues. Theoretically, a complete removal of 0.30 g/L phenol from the water and its subsequent accumulation in the plantlets without degradation would total up to be 0.32 g/L. However, our analysis on the plantlets extract detected only 0.07 ± 0.01 g/L phenol as compared with 0.02 g/L phenol in the control (plantlets grown in distilled water only). This observation implies that the absorbed phenol has either been biodegraded or biotransformed within the plantlets. Similar results were obtained when plantlets exposed to 0.10 g/L phenol were extracted and assayed on both Day 9 and Day 11, indicating a decrease in phenol concentration over two days of growth. Biodegradation of phenol by *I. aquatica* Forssk. was supported by Cataldo et al. (1987), which reported that plants had the tendency to metabolise phenol readily instead of accumulating it due to their high respiratory decomposition rate. Meanwhile, necrotic lesions and leaves fall were observed if there was accumulation of pollutants in plants (Hawrylak-Nowak, Kalinowska, & Szymanska, 2012). Such leaf defects were absent in this study.

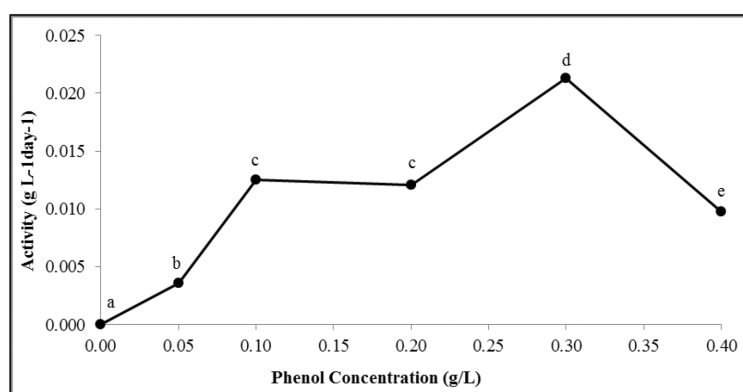


Figure 5. Rate of phenol removal by *I. aquatica* Forssk. Daily removal of phenol from the water by plants was assayed. Data plotted represent means of triplicates. Rates with different letters indicate significant differences ($p < 0.05$). The standard deviation bars are too small to be visible.

CONCLUSION

The maximum tolerable phenol concentration for *I. aquatica* Forssk. was 0.30 g/L, with a survival rate of 72.2% despite various physical and physiological damages exhibited by the plantlets. The positive effect of 0.10 g/L phenol on plantlets root lengths was observed and peaked at Day 5, with a value of 57.42 ± 1.41 mm, which was significantly longer ($p < 0.05$) than the control plantlets by 13.85 ± 1.11 mm. The maximum phenol removal rate by the plantlets was achieved with 0.30 g/L phenol at 0.021 g/L/day. The findings also showed that *I. aquatica* Forssk. was capable of removing phenol from water and possibly biodegrading it with zero post-treatment, and it is a natural low cost, easy to handle and fast growing phenol phytoremediator.

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