



## POTENTIALITY OF USING *A. PINNATA* TO BIOREMEDIATE DIFFERENT HEAVY METALS FROM POLLUTED DRAINING WATER

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**Keywords:** *Azolla pinnata*, phytoremediation, heavy metals, polluted draining water,  $Pb^{+2}$   $Co^{+2}$  and  $Cu^{+2}$ .

### ABSTRACT

*Azolla pinnata* is a small aquatic fern which considered as a multipurpose organism. It is used in bioremediation to remove heavy metals from polluted draining waters. In view of its potentiality, *Azolla pinnata* were tested for their growth on different media (Yoshida, peat moss, and Soil media) to select the best medium for the growth of *Azolla*. Then the best medium was examined for the potentiality of *Azolla* to withstand a different concentration of different heavy metals. Yoshida medium was the most suitable medium, as it recorded the highest significant records in fresh and dry weight, being 25.38 and 1.69 g/ pot respectively, the doubling time was 5.43 days, NPK% being 3.87, 0.85 and 1.95% respectively and nitrogenase activity being 14.32  $\mu\text{mol C}_2\text{H}_4/\text{g dry weight. / hr}$ . The fresh and dry weight of *Azolla* exposed to  $Pb^{+2}$ ,  $Co^{+2}$  or  $Cu^{+2}$ , was found to be increased by increasing the concentration of the metals from 5 to 35 ppm/liter, then they decreased when the fern exposed to 45 ppm/liter for all of them. While, doubling time decreased gradually by increasing the concentration of  $Pb^{+2}$ ,  $Co^{+2}$  or  $Cu^{+2}$  from 5 to 35 ppm/liter and started to increase by increasing the concentration of  $Pb^{+2}$  to 45 ppm/liter in the medium.

### INTRODUCTION

*Azolla* sp. is a small, free-floating fern, less than 1/2 inches diameter (Ferentinos et al 2002). It

is a fern frond consisting of the main stem growing on the surface of the water, with alternate leaves and adventitious roots at regular intervals along the stem (IFAS, 2001). It has a symbiotic relationship with the blue-green algae *Anabaena* (Peters and Mayne, 1974 & Wagner, 1997). The word *Azolla* has a Greek origin. It consists of two words, *azo* and *olly*, which means killed by drought (Moore, 1969 & Ashton and Walmsley, 1976). It has common names of Mosquito Fern, Water velvet, Water fern or simply *Azolla* is a water fern commonly found in tropical and temperate freshwater ecosystem.

Water contaminated by heavy metals is considered one of the most important environmental problems throughout the world because of their toxic effect on the environment and human health (Akpore and Muchie, 2010). A high concentration of lead can be found in industrial wastewater, in domestic detergents and other laundry products and in cigarettes (Celebi and Kendir, 2002). While contamination of aquatic ecosystems with copper is normally due to various anthropogenic sources including copper mine drainage, copper-based pesticides, industrial, domestic wastes and anti-fouling paints (Andrade et al 2004; Ma et al 2003 & Rai, 2009). Cobalt can enter the aquatic environment from a number of sources, both natural and anthropogenic. The natural sources include volcanic emissions, the weathering of rocks by the action of water and decomposition of plant wastes.

The presence of heavy metals pollutants in water bodies possess several risks to the health of humans and ecosystems (Ho et al 2003; Alam et al 2003; Miller et al 2004 & Rai 2008a, 2009).

(Received 14 June, 2017)

(Revised 21 June, 2017)

(Accepted 9 July, 2017)

The term "phytoremediation" is a combination of two words: Greek "phyto" meaning plant and Latin "remediation" meaning to correct or remove an evil (Hazrat et al 2013). While the clear descriptions of the term, phytoremediation is "the use of plants for removal of pollutants from the environment "as it promotes the use of plants for environmental cleanup (Gardea, 2003). Phytoremediation also called green remediation, botano-remediation, agro-remediation and vegetative remediation (Erakhrumen, 2007).

The ability of different plants to remove the toxicity of heavy metals in the environment have been extensively and excellently reviewed by several authors (Orcutt and Nilsen, 2000; Cseh, 2002; Fodor, 2002; Zimmels et al 2004; Pilon, 2005 & Migocka and Klobus, 2007).

The use of aquatic macrophytes, such as *Azolla* that has hyperaccumulating ability is known to be an environmentally friendly option to restore polluted aquatic resources because of it has short doubling time (3-5 days), easy harvest, nitrogen fixation ability and tolerance to and accumulation of a wide range of heavy metals (Sood et al 2012; Rai, 2007; Bennicelli et al 2004; Upadhyay et al 2007; Rai, 2008b & Mashkani and Ghazvini, 2009). Remediation of heavy metals using plants is better in terms of ecology and economy than chemical treatment (Rai, 2011).

The present research highlights the phytoaccumulation potential of *Azolla* as a promising candidate for phytoremediation. The impact of uptake of heavy metals on the growth of *Azolla* has also been discussed for a better understanding and utilization of this symbiotic association for application as a phytoremediation agent in the field.

## MATERIALS AND METHODS

### *Azolla Pinnata* Strain

*Azolla Pinnata* was kindly provided by Agric. Microbial Dept. Soils, Water and Environment Research Institute (S.W.E.R.I), Agric.Res. Center (A.R.C), Giza, Egypt.

### *Azolla* growth media

Modified Yoshida medium (Yoshida et al 1976), peat moss medium, and soil were used. The peat moss medium was prepared by adding (20g) peat moss to (600ml) tap water to each used plastic pot. The composition of peat moss was estimated by El-Shahat (1988).

### Soil medium

The soil was collected from Kaliobia Governorate to study the ability of *Azolla* to grow on it. Soil analyses were estimated according to El-Shahat (1988).

### Propagation of *Azolla Pinnata*

The experiment was carried out in the greenhouse of Soils, Water, and Environmental Res. Ins. (SWERI), Agric. Res. Center (ARC), Giza, Egypt.

One gram of *Azolla Pinnata* was transferred into plastic pots 20cm diameter and 25cm in depth in 5 replicates to estimate the growth of *Azolla Pinnata* during 25 days samples were taken every 5 days. The inoculated pots were incubated at 35 °C±2, with 14 h. light and 10 h. dark for 25 days under greenhouse conditions (EL-Berashi, 2008).

### Parameters measured

**Fresh weight and dry weight** were determined according to El-Shahat, (1997), and they expressed as g / pot.

**Doubling time:** was calculated according to Aziz and Watanabe, (1983), by using the following equation:

$D.T=t/r$  whereas:

t = the duration of *Azolla* growth

$r = \{\log (wt/w0)/0.301\}$

wt = weight of *Azolla* at time t

w0 = weight of *Azolla* at zero time, (weight of inoculum).

### Total nitrogen:

Total nitrogen (%) of *Azolla* fronds was determined in the dried plant materials by the micro kjedahl method according to Black et al (1965).

### Phosphorus percentage:

Phosphorus percentage in *Azolla* fronds was determined in dried samples by Spectrophotometer according to Olsen and Sommers (1982).

### Potassium content

It was determined in plant samples by Flame photometer according to Brown and Lilliland (1946) data exposed as K % of Dry weight.

### Nitrogenase activity

Nitrogenase activity in *Azolla* was assayed by using the acetylene reduction technique as recommended by Hardy et al (1973).

### Cultivation of *A. Pinnata* in Yoshida medium supplemented with different heavy metals

*A. Pinnata* was inoculated by (1g) fresh weight per pot, as standard inoculums in all experiments (El-Berashi, 2008 & Punita and Soma, 2015). The pots were filled with 1000ml of Yoshida medium and supplemented with different concentrations of  $Pb^{+2}$   $Co^{+2}$  or  $Cu^{+2}$ . Three replicates were prepared for each treatment.

Synthetic wastewater samples were prepared by dissolving their corresponding analytical grade salts of  $Pb(NO_3)_2$ ,  $Co(NO_3)_2 \cdot 6H_2O$  or  $CuSO_4 \cdot 5H_2O$  in deionized water at different concentrations 0, 5, 15, 25, 35 and 45 ppm.

The inoculated pots were incubated at  $35 \text{ }^\circ\text{C} \pm 2$ , under 14 h. light (14-17.5 K lux light intensity) and 10 h. dark for 25 days in greenhouse conditions. Samples were taken each five days, using the method adapted by EL-Berashi, (2008).

### Parameters recorded

Fresh weight, dry weight (g/ pot) and doubling time (day) of *Azolla Pinnata*. *Azolla* cultures were estimated every 5 days.

### Statistical analysis

The experiments were carried out as a complete randomized design with three replicates. The statistical analysis was carried out using LSD test to compare means of treatments in experiment (SAS. 2006. Statistical Analysis System, SAS User's Guide: Statistics. SAS Institute Inc. Editors, Cary, NC). Statistical significance was defined as  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Growth performance of *Azolla pinnata* in different media

This experiment was conducted to determine the ability of *A. pinnata* to grow on different media. It was inoculated in three different media namely Yoshida, peat moss and Soil media to select the best growth medium by measuring the growth parameters as fresh and dry weight, doubling time, NPK content and Nitrogenase activity.

The data depicted in (Figs. 1 and 2) show good growth performance of *A. pinnata* in all the tested media. The fresh and dry weight of *A. pinnata* was gradually increased by increasing the incubation periods. The highest fresh and dry weight was recorded within Yoshida medium being 25.38 and 1.69 g/ pot fresh and dry weight, respectively after 25 days from inoculation compared to Peat moss and Soil media, these results are in similar to those obtained by El-Araby et al (1999), who found that *A. pinnata* recorded its maximum growth with increasing the incubation period up to 25 days of growth on the same medium.

Data recorded in (Fig. 3) show that, doubling time of *A. pinnata* increased during incubation period in the first ten days (from 0 to 10 days) in the three tested media then started to decrease during the second 10 days (from 10 to 20 days), then started to increase again after the second 10 days. In accordance with the highest fresh and dry weight of *A. pinnata* at which it was recorded after 25 days in all the tested media, the lowest doubling time was recorded with Soil medium and Peat moss medium, being (5.43 days) for both media.

It should be mentioned the obtained results by Nuzhat et al (2015), stated that *A. pinnata* doubles its biomass in less than two days under laboratory conditions and 5-10 days in normal field conditions.

Nitrogenase activity profile during incubation period (Fig. 4) showed gradual increase during the incubation period. The maximum nitrogenase activity was recorded in Yoshida medium after 25 days, being  $14.32 \mu\text{ mol C}_2\text{H}_4/\text{ g dry weight / h.}$  while both of Soil and Peat moss media recorded their highest Nitrogenase activity after 20 days from inoculation being ( $13.29$  and  $7.63 \mu\text{ mol C}_2\text{H}_4/\text{ g dry weight / h.}$ ) respectively.

These results are in agreement with those of (Abd El-AAI et al 2013), who stated that nitrogenase activity of *Anabaena Azolla* increased gradually by increasing the incubation period. While Pabby et al (2004) reported that prolonged incubation for more than 14 days, in a medium led to the suppression of nitrogenase activity in *Anabaena microphylla*.

The total NPK in *A. pinnata* recorded in (Figs. 5, 6 and 7) show that total NPK% increased gradually during the incubation period in all the three tested media and they reached their maximum record after 25 days in Yoshida medium compared to the other tested media. The highest significant NPK% was recorded within Yoshida medium after 25 days from inoculation being 3.87, 0.85 and 1.95% respectively.

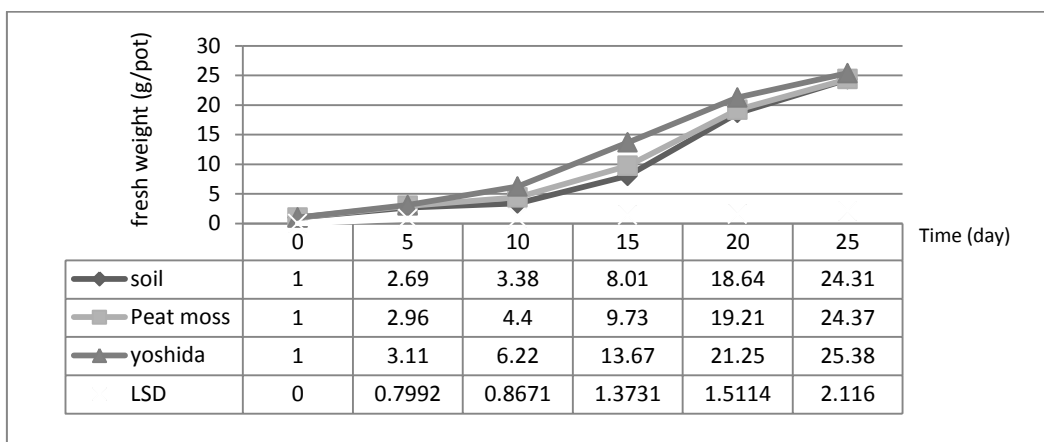


Fig. 1. Fresh weight (g/pot) of *Azolla pinnata* during 25 days of growth on the media used.

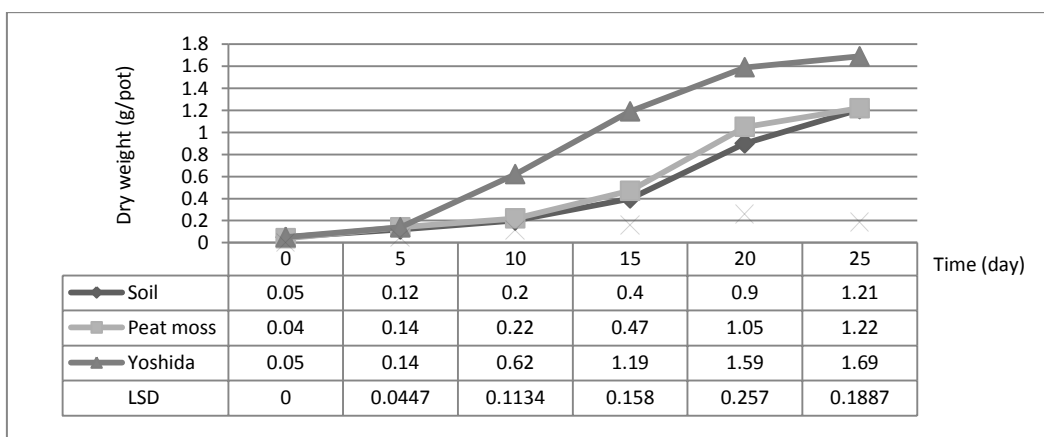


Fig. 2. Dry weight (g/pot) of *Azolla pinnata* during 25 days of growth on the media used.

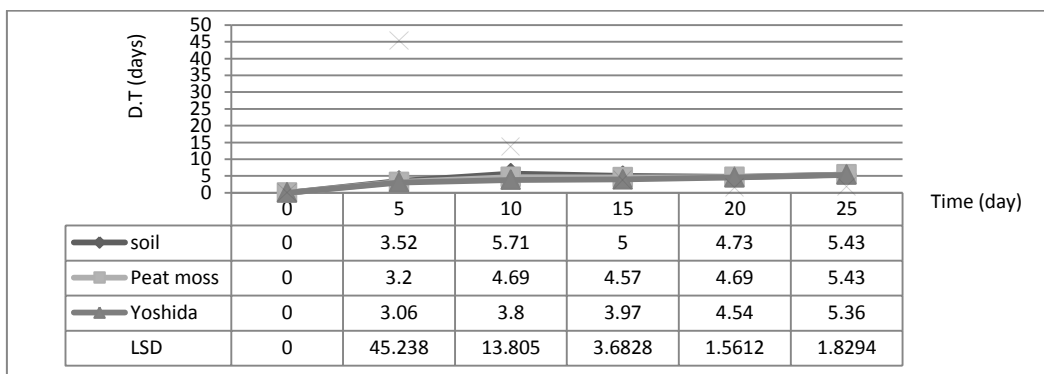


Fig. 3. Doubling time (days) of *Azolla pinnata* during 25 days of growth on the media used.

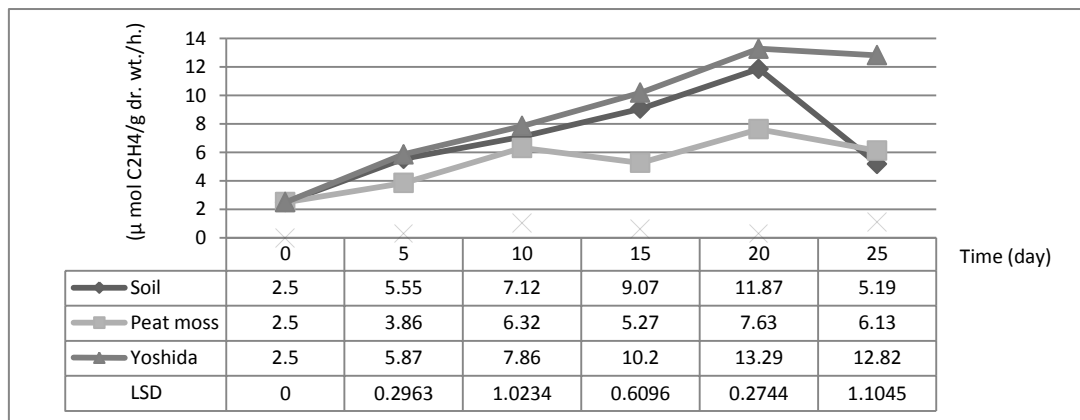


Fig. 4. Nitrogenase activity ( $\mu$  mol C<sub>2</sub>H<sub>4</sub>/g dr. wt./hr.) of *Azolla pinnata* during 25 days of growth on the media used

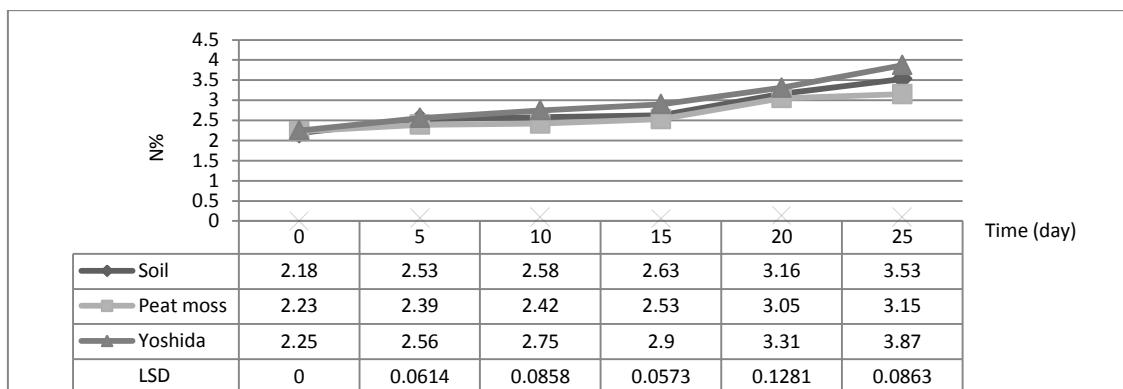


Fig. 5. Nitrogen content (%) of *Azolla pinnata* during 25 days of growth on the media used.

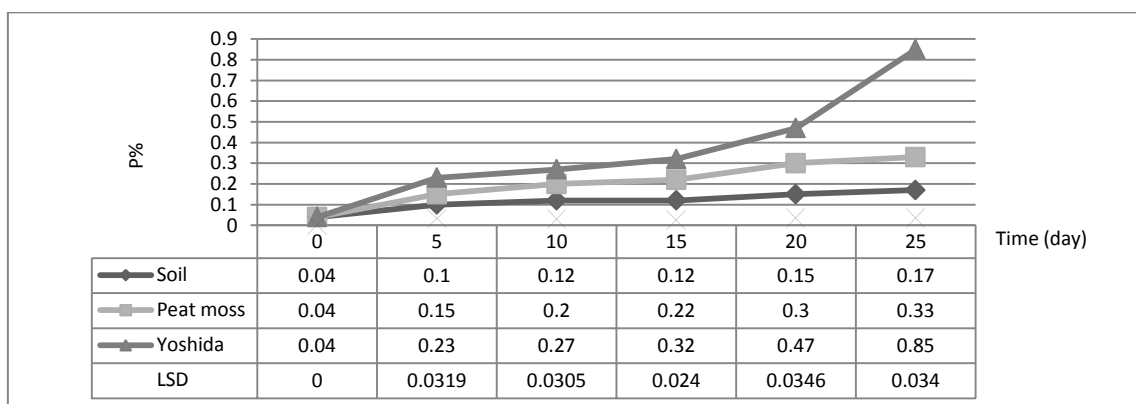


Fig. 6. Phosphorus content (%) of *Azolla pinnata* during 25 days of growth on the media used.

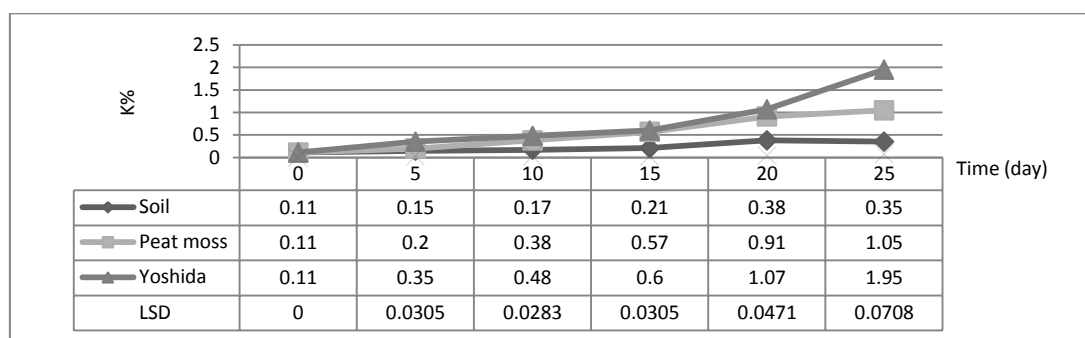


Fig. 7. Potassium content (%) of *Azolla pinnata* during 25 days of growth on the media used

Similar results are in agreement with those recorded by **El-Shaymaa, (2005)**, who showed that in Yoshida medium, *A. pinnata* gave its highest value of total nitrogen content compared to soil and Van Hove media after 30<sup>th</sup> days of incubation. Also, **Nour El-Din, (1997)** found that total nitrogen percentage of *A. pinnata* reached to 5.10% after 25 days of incubation.

The results of this experiment validate that Yoshida medium is considered the best medium for the best performance of *Azolla* this statement was similar with those reported by **El-Shaymaa, (2005) & Shoe and Anita, (2011)**, who reported that Yoshida medium is considered the most suitable medium as it had the essential nutrients needed for *Azolla* growth.

#### Growth performance of *Azolla pinnata* during incubation period as affected by the addition of $Pb^{+2}$ on Yoshida medium.

Data recorded in (**Figs 8 and 9**) revealed that fresh and dry weight of *Azolla* increased in all treatments by increasing incubation periods until the 20<sup>th</sup> day, then started to decrease. Also, the fresh and dry weight of *Azolla* exposed to  $Pb^{+2}$  in the form of  $Pb(NO_3)_2$  recorded an increase by increasing the concentration of  $Pb^{+2}$  from 5 to 35 ppm/liter then they decrease values when it exposed to 45 ppm/liter of  $Pb^{+2}$ . The highest significant fresh and dry weight were recorded within *Azolla* inoculated in Yoshida medium treated with  $Pb^{+2}$  35 ppm/liter after 20 days from inoculation, being 9.5 and 0.57 g/pot respectively.

Data recorded in (**Fig. 10**) show that inoculation of *Azolla* in Yoshida medium contain  $Pb^{+2}$  in the form of  $Pb(NO_3)_2$  recorded decrease gradually in the doubling time by increasing the concentration of  $Pb^{+2}$  from 5 up to 35 ppm/liter then doubling

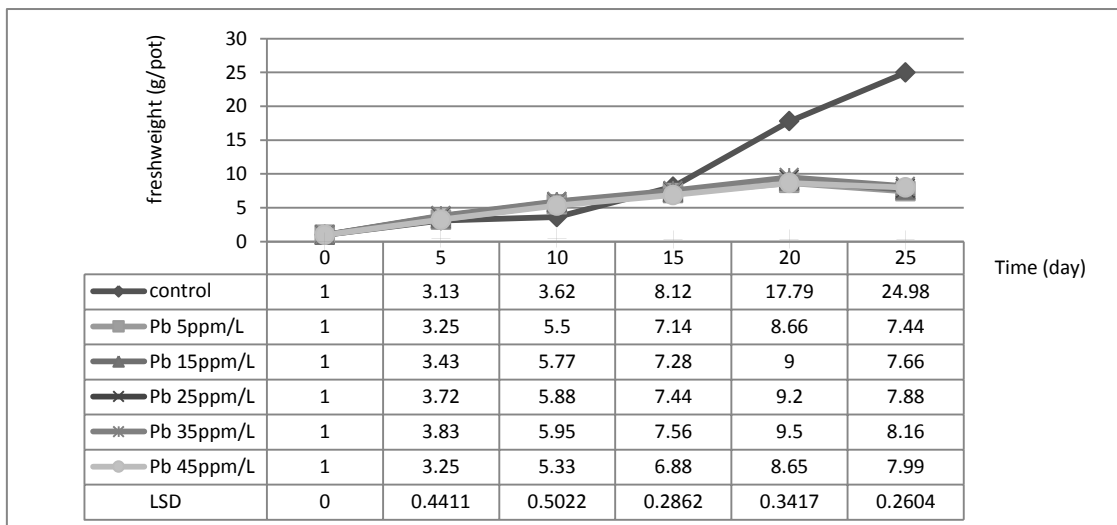
time started to increase by increasing the concentration of  $Pb^{+2}$  to 45 ppm/liter in the medium, as well, the doubling time of *Azolla* increased by increasing incubation period. The lowest doubling time was recorded within *Azolla* inoculated in Yoshida medium that contains 35 ppm/liter, being 6.17days.

These results are in agreement with those reported by **Monica et al (2014)**, who stated that *Azolla pinnata* doesn't express any noticeable toxicity symptoms in low concentrations from  $Pb(NO_3)_2$ . Also, **Thayaparan et al (2013)**, reported that the relative growth of *A. pinnata* exposed to lead was significantly reduced when metal concentration was increased.

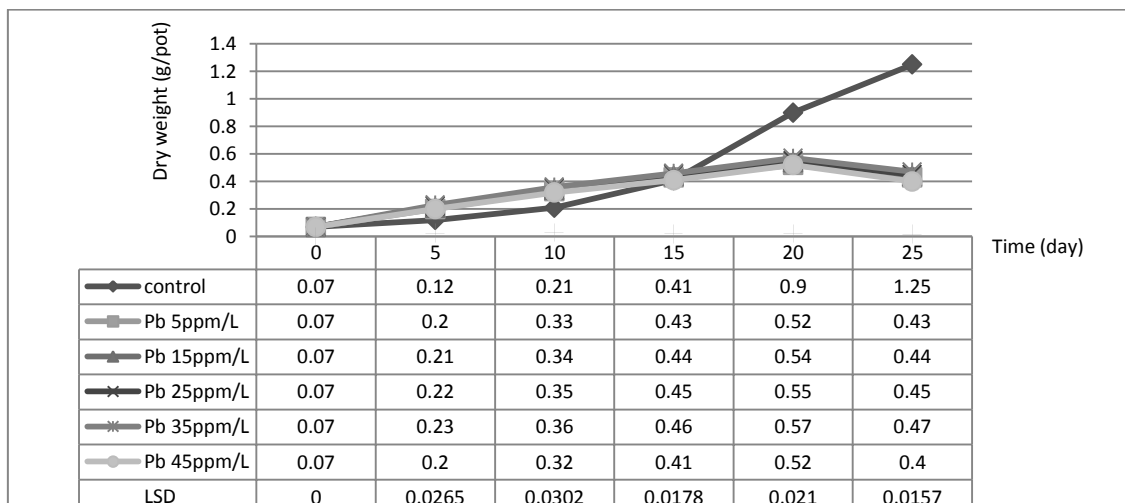
In this respect, **Arora et al (2006)**, reported that *A. pinnata* have the ability to absorb  $Pb$ , and other heavy metals and showed tolerance when present in low concentrations.

#### Growth performance of *Azolla pinnata* during incubation period as affected by the addition of $Co^{+2}$ on Yoshida medium.

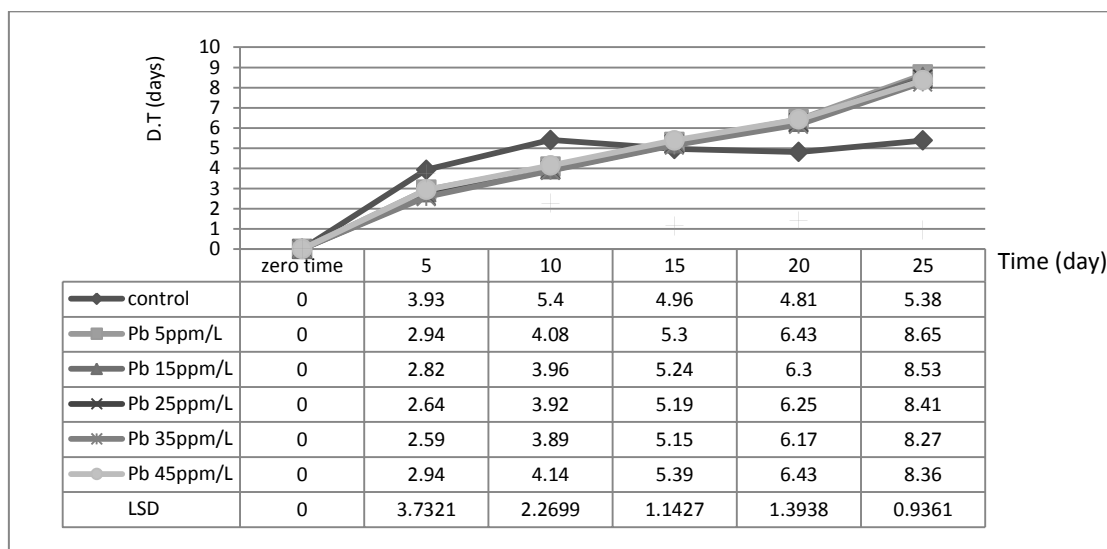
Data recorded in (**Fig 11 and 12**) show an increase in the fresh and dry weight of *Azolla* by increasing the incubation period until the 20<sup>th</sup> day then started to decrease. The fresh and dry weight of *Azolla* exposed to  $Co^{+2}$  in the form of  $Co(NO_3)_2 \cdot 6H_2O$  increased gradually by increasing the concentration of  $Co^{+2}$  from 5 to 35 ppm, then it recorded lower value when it exposed to 45 ppm. The highest significant dry weight was recorded with *Azolla* growth on Yoshida medium treated with 45 ppm, being (0.72 g/pot). While the highest significant fresh weight was recorded within *Azolla pinnata* inoculated in Yoshida treated with 15 ppm  $Co^{+2}$  after 20 days from inoculation, being (9.8 g/pot).



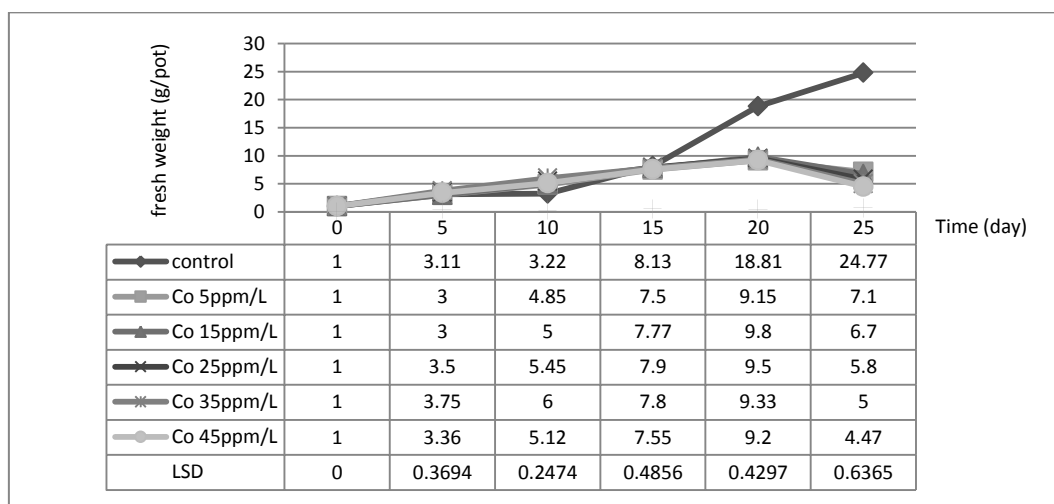
**Fig. 8.** Effect of different concentrations of Pb<sup>+2</sup> on fresh weight (g/pot) of *Azolla pinnata* grown on Yoshida medium



**Fig. 9.** Effect of different concentrations of Pb<sup>+2</sup> on dry weight(g/pot) of *Azolla pinnata* grown on Yoshida medium

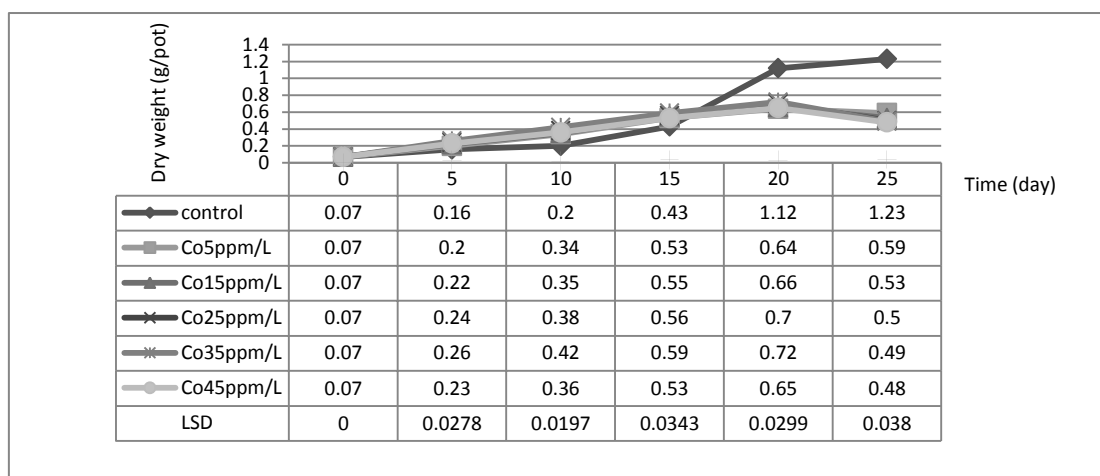


**Fig. 10.** Effect of different concentrations of  $Pb^{+2}$  on doubling time(days)of *Azolla pinnata* grown on Yoshida medium



**Fig. 11.** Effect of different concentrations of  $Co^{+2}$  on fresh weight(g/pot) of *Azolla pinnata* grown on Yoshida medium



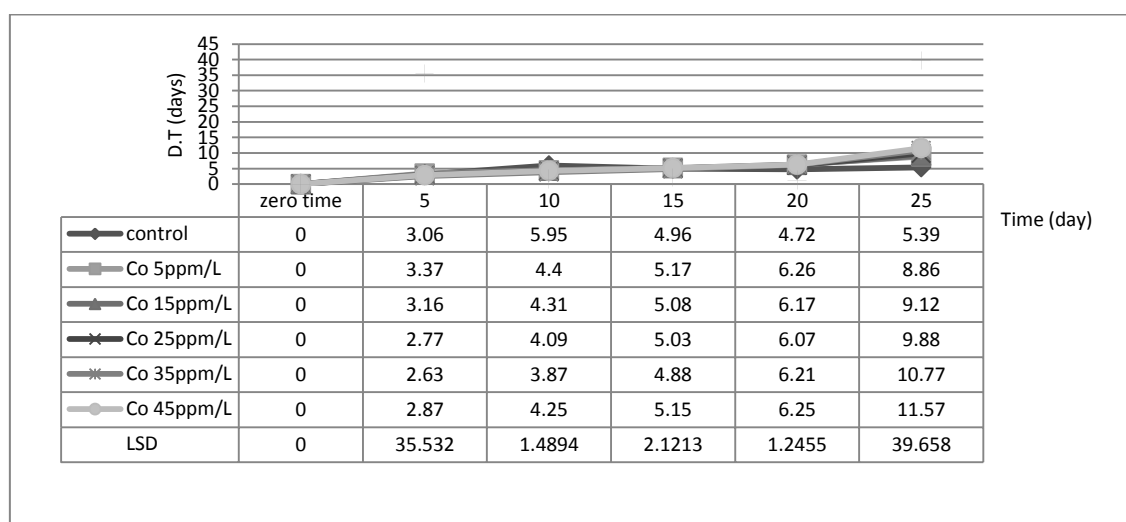


**Fig. 12.** Effect of different concentrations of  $\text{Co}^{+2}$  on dry weight (g/pot) of *Azolla pinnata* grown on Yoshida medium

Data recorded in (Fig. 13) show that inoculation of *Azolla* in Yoshida medium containing  $\text{Co}^{+2}$  in the form of  $\text{CO}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  gradually decreased doubling time such decrease was conceded with increasing the concentration of  $\text{Co}^{+2}$  in the medium from 5 to 35 ppm and starting to increase by increasing the concentration of  $\text{Co}^{+2}$  to 45 ppm in the medium. Also, the doubling time of *Azolla* increased by increasing the incubation period. After 20 days from inoculation, *Azolla* exerted the high

est significant fresh and dry weight, the lowest doubling time was observed within *Azolla* inoculated in Yoshida medium that contains 25 ppm from  $\text{Co}^{+2}$ , being (6.07 days).

El-Berashi (2008), indicated that by increasing the concentration of the heavy metals in the growth medium and incubation period, the doubling time would increase which is an indicator for decreasing the growth rate of *A. pinnata*.



**Fig. 13.** Effect of different concentrations of  $\text{Co}^{+2}$  on doubling time(days)of *Azolla pinnata* grown on Yoshida medium

### Growth performance of *Azolla pinnata* during incubation period as affected by the addition of $\text{Cu}^{+2}$ on Yoshida medium

Data recorded in (Figs. 14 and 15) show that inoculation of *Azolla* in Yoshida medium contain  $\text{Cu}^{+2}$  in form of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  exerted slight increase in the fresh and dry weight of *Azolla* in the first 20 days from incubation period. Increasing the concentration from 5 to 45 ppm the both parameters started to decrease. While from the 20<sup>th</sup> to the 25<sup>th</sup> day, the fresh and dry weight of *Azolla* decreased by increasing the concentration of the  $\text{Cu}^{+2}$ . The highest significant increase in fresh weight was recorded within *Azolla* in Yoshida contain 15 ppm  $\text{Cu}^{+2}$  after 20 days, being 5.36 and dry weight in 5 ppm  $\text{Cu}^{+2}$  after 20, being 0.43 g/pot.

Data presented in (Fig 16) revealed that inoculation of *Azolla* in Yoshida medium contain  $\text{Cu}^{+2}$  in the form of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  recorded lower values of doubling time by increasing the concentration of  $\text{Cu}^{+2}$  in the medium from 5 to 35 ppm and starting

to increase by increasing the concentration of  $\text{Cu}^{+2}$  to 45 ppm in the medium. Likewise, the doubling time of *Azolla* increased by increasing the incubation period in all  $\text{Cu}^{+2}$  concentrations. After 20 days from the inoculation at which *Azolla* revealed the highest significant fresh and dry weight, the lowest doubling time was recorded within *Azolla* inoculated in Yoshida medium that contains 5ppm from  $\text{Cu}^{+2}$ , being (8.26 days).

Arora et al (2006), reported that exposing *Azolla* to heavy metals suppressed the vegetative growth and sporulation in different species and the suppression percentage depends on the species and the concentration of heavy metals.

Rai (2008a), reported that copper solution at low concentration is essential to develop the growth of *A. pinnata* while in higher concentration the color of *Azolla* changed into yellow which cause suppression in *A. pinnata*'s growth when it exposed to copper. While Wagner, (1997) reported that *Azolla* tolerates different concentration of copper.

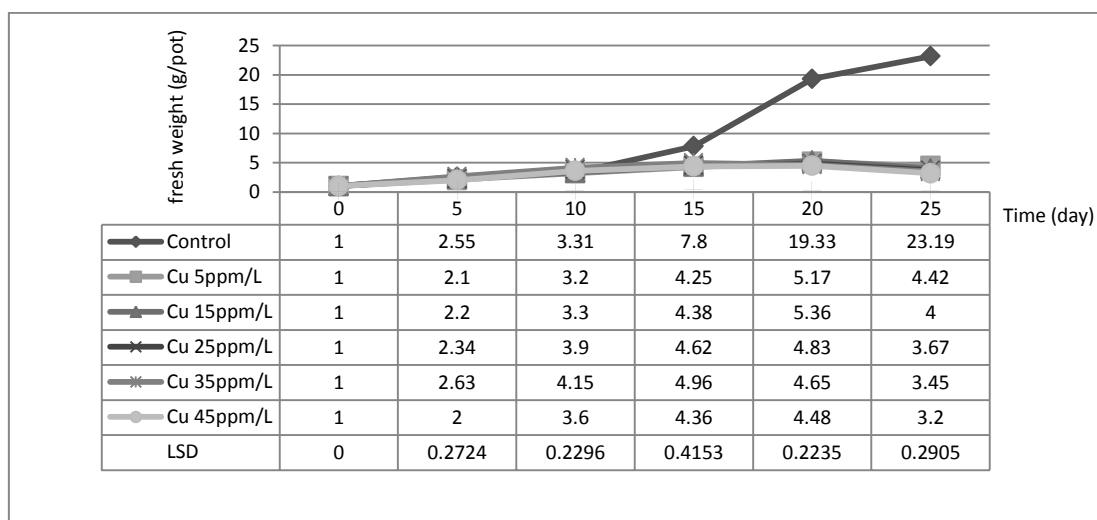


Fig. 14. Effect of different concentrations of  $\text{Cu}^{+2}$  on fresh weight (g/pot) of *Azolla pinnata* grown on Yoshida medium

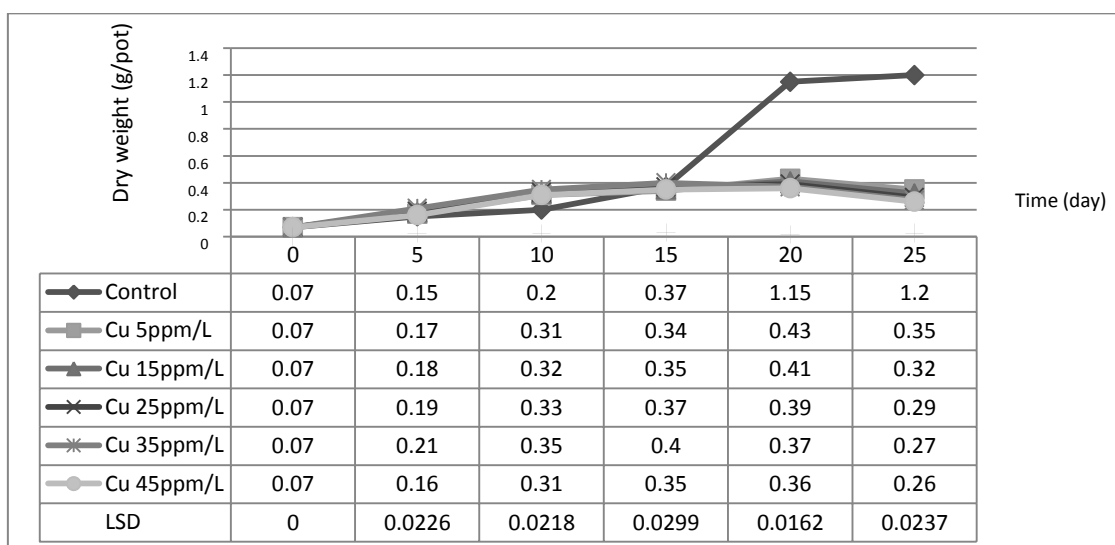


Fig. 15. Effect of different concentrations of  $\text{Cu}^{+2}$  on dry weight (g/pot) of *Azolla pinnata* grown on Yoshida medium

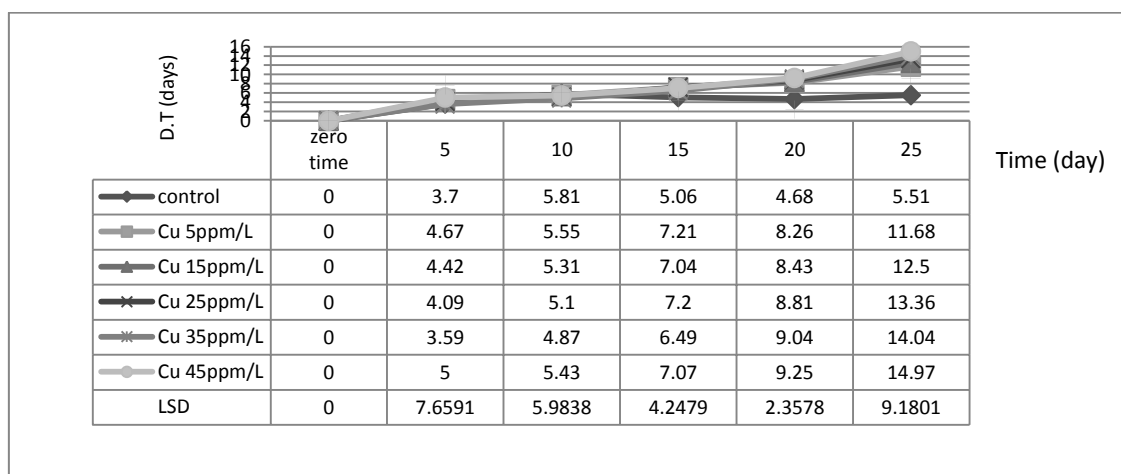


Fig. 16. Effect of different concentrations of  $\text{Cu}^{+2}$  on doubling time (days) of *Azolla pinnata* grown on Yoshida medium

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