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Phytotoxicity of Copper and Lead to *Oryza sativa* L. with Regard to Different Exposure Durations and Endpoints

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Abstract: *Heavy metal pollution can become a major threat to biota due to its greater potential to bioaccumulate through the food chain. The heavy metal phytotoxicity remains unnoticed in plants because plants are generally less sensitive to heavy metal toxicity. Even though the toxicity is detected, it is difficult to ascertain which toxicant is responsible for toxicity because toxicity tests are usually based on single end point or single exposure duration. The study presented here focuses on toxic response of different organs of O. sativa at different time points to delineate the type of toxicant. The results demonstrated that this approach can be very useful in differentiating between the type of toxicants.*

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I. Introduction

Most heavy metals in the environment originate due to anthropogenic activities [1]. Some heavy metals are also used as reference toxicants – the compounds used to assess the general health and sensitivity of the test species and to compare results obtained from different laboratories [2]. Plants are generally less sensitive to heavy metal toxicity, compared with animals. This poses the risk of heavy metal exposure to consumers through the food chain [3]. Toxicity tests are generally directed towards the detection of toxicity rather than the identification of toxicants [4]. Though this seems to be a limitation, the response pattern produced by the same toxicant to different organisms or different organs of the same organism can be made useful in the identification of toxicants. The ratio between the endpoint estimates (EC, IC, or LC) of different organs (or response) may produce more or less constant values as the toxic response is organ specific. Similarly, the ratio between endpoint estimates of the same organ at different time points may be unique to each organ and the toxicant tested. The problem with the point estimates like IC_{25} is that the confidence intervals produced for them are sometimes unrealistic. To tackle such issues, simultaneous confidence intervals for relative effects between several treatments and control can be computed using recent software packages [5]. These confidence intervals are also useful when interpretations are based on NOEC (no observed effect concentration), for which confidence intervals cannot be computed.

It should also be noted that, instead of looking at point estimates only, the full dose-response curve should be interpreted to reach the conclusion about the toxicant. This is because two toxicants can have the same LC_{50}, but different slopes, and hence the conclusions based on point estimates only may lead to confounding results [6]. In the present study, an effort has been made to assess the toxicity of two heavy metals with regard to different time points and different organs as endpoints. The Cu chosen for this study is one of the standard reference toxicants used in toxicity tests by different agencies.

II. Material and Methods

Two heavy metals ($CuSO_4$, and $Pb(NO_3)_2$) were used in the toxicity test. The $CuSO_4$ is also a standard reference toxicant recommended by agencies like EPA. All the reagents used were analytical grade. *Oryza sativa* (var. Jyothi) seeds were obtained from Regional Agricultural Research Station, Pattamby, Palakkad, Kerala, India. The concentration range used was 0, 6.25, 12.5, 25, 50, 100, and 200 mg/L for $PbN(O_3)_2$, whereas it was 0, 1, 2, 4, 8, 16, and 32 mg/L for $CuSo_4$. Solutions were prepared using distilled water.

Ten seeds were placed in Petri dish filled (without any supporting material) with 10 ml of test solution. Triplicate of Petri dishes with seeds were then kept at 28±2 °C under cool white light (300 µmol/m^2/s, 14:10 light:dark) for 96-h. The photographs of germinating seeds were taken at 72 and 96-hrs using a digital camera. Root, shoot length, seedling length, and seed germination were measured. All measurements were performed using an image analysis software called Fiji [7].

Statistical analysis

Endpoint estimates (LC_{25} for seed germination and IC_{25} for other variables) were computed using an R-software package called drc [8]. Bounds of uncertainty intervals were computed using another package called mratios [9]. The IC_{25} values were compared as per method described in the drc package.

III. Results

The results demonstrated a dose-dependent response pattern for both the metals. Cu was found to be more toxic than Pb with regard to LC/IC_{25} for root and seedling length, and seed germination (Table 1 and 2). $CuSO_4$ was non-toxic to rice shoot at tested concentration ranges. After 96-hrs of exposure, Cu even produced a stimulatory effect (hormesis) on the shoot at lower ranges of concentration. The Cedergreen-Ritz-Streibig model [10] with $\alpha = 0.25$ was found to be the best fit model for shoot at 96-hr ($p < 0.01$). Although Cedergreen-Ritz-Streibig model and Brain-Cousen's models [11] were found to be non-significant ($p > 0.05$) for root in Cu at 72- and 96-hrs, respectively, they explained the growth curves well (Fig. 1 a and b). However, Cu could not produce any dose-response relationship at earlier stages (72-hrs) of exposure. Though Pb showed a tendency towards stimulatory effect on shoot at 96-hrs, it was found to be non-significant (Fig. 2 a and b). For both the metals, the seed germination was fitted using 2 parameter log-logistic model. The only exception was Pb at 72-hr, for which Weibull model was used. The ratio between IC_{25} of Pb and Cu showed that the root IC_{25} for Pb was several folds (6.46 times) higher than those obtained for Cu at 96-hr (Table 3). The lowest ratio was observed for germination at 96-hr (2.09). Except for seedling length, all other morphometric variables produced different IC values at different time points. The ratio of LC_{25} for germination between 72 and 96-hr in Pb (Table 4) was less than 1 (0.86), indicating that the concentration Pb required to produce inhibition of seed germination was greater as the duration extended (Fig. 2 c). This is in contrast with Cu (1.12) in which the concentration required to inhibit seed germination reduced as the exposure duration extended (Fig. 1 c).

Table 1: IC_{25} (LC_{25} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa a* fter 72-h and 96-h exposure to $PbN(O_3)_2$. BOU = bounds of uncertainty(5).The values are in mg/L.

Duration	Endpoint	LC/IC25		95% CI		BOU (25%)	
				Lower	Upper	Lower	Upper
96-h	Root	29.1		15	43	0	100
	Shoot	96.1		58	135	12.5	> 200
	Seedling	35.3		26	45	12.5	100
	Germination	81.8		65	99	50	200
72-h	Root	35.6		24	47	0	100
	Shoot	192.9		165	221	100	> 200
	Seedling	38.5		25	52	0	100
	Germination	70.1		55	85	25	100

Table 2: IC_{25} (LC_{25} for seed germination) and 95% CI values for different morphological endpoints of *O. sativa* after 72-h and 96-h exposure to $CuSO_4$. BOU = bounds of uncertainty(5).The values are in mg/L.

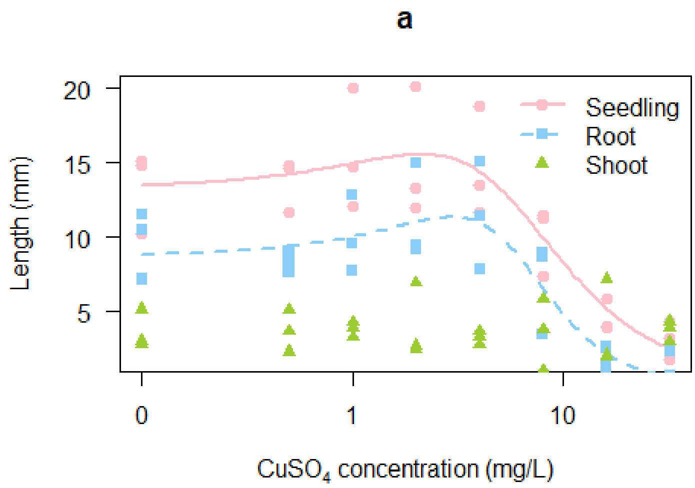
Duration	Endpoint	LC/IC25	95% CI		BOU (25%)	
			Lower	Upper	Lower	Upper
96-h	Root	4.5	2.9	6.1	1	8
	Shoot	>32	19	57	16	> 32
	Seedling	7.25	4.8	9.7	2	16
	Germination	18.4	15	22	8	32
72-h	Root	8.06	5.6	11	0	16
	Shoot	>32	--	--	0	>32
	Seedling	8.01	5	11	0	32
	Germination	20.6	15	26	0	> 32

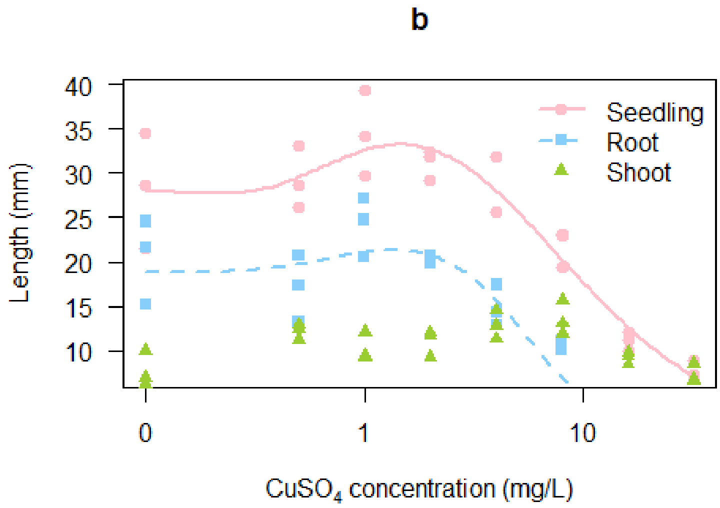
For root elongation in Pb, the BOU value spanned 6 concentrations, whereas for Cu it spanned 4 concentrations. Control was not covered for lower bounds of BOU in the case of root in Cu, whereas it was included for Pb. Contrastingly for shoot length in both the metals, the upper bounds of BOU surpassed the highest concentration tested, indicating the lower sensitivity of shoots towards these metals. For both Pb and Cu, the sensitivity of seedling length was midway between root and shoot length. The seed germination was found to be the least sensitive among the morphometric variables.

Table 3: Ratios between IC_{25} (LC_{25} for seed germination) of Cu and Pb for different morphometric endpoints of *O. sativa* after 72-h and 96-h exposure to heavy metals. Upper and Lower represents 95% CI values.

Duration	Endpoint compared	Estimate	Std. Error	Lower	Upper
72	Root	4.419	0.950	2.557	6.282
72	Shoot	-	-	-	-
72	Seedling	4.808	1.168	2.519	7.097
72	Germination	3.395	0.597	2.224	4.566
96	Root	6.457	2.271	2.005	10.909
96	Shoot	2.520	0.767	1.017	4.023
96	Seedling	4.869	1.017	2.876	6.863
96	Germination	2.091	0.410	1.287	2.895

– IC_{25} was greater than the tested concentration ranges of $CuSO_4$.





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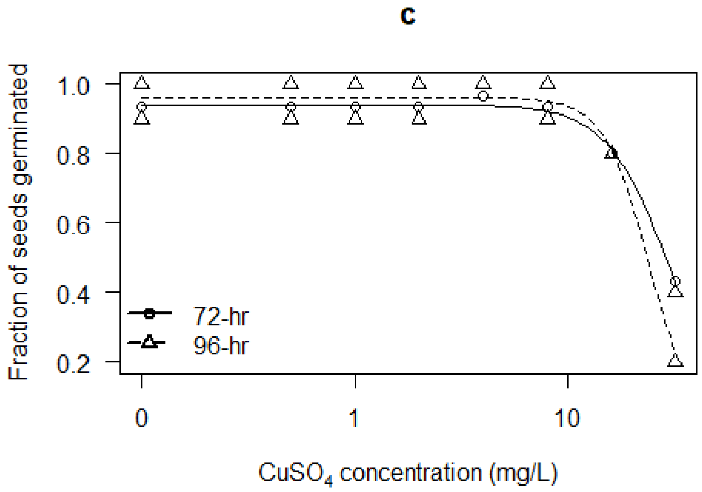
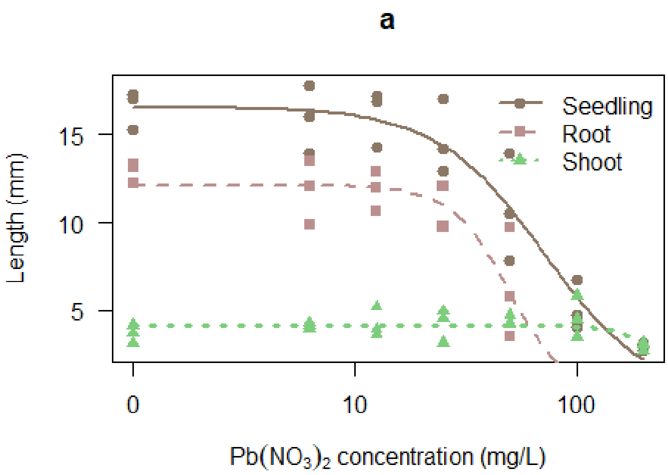
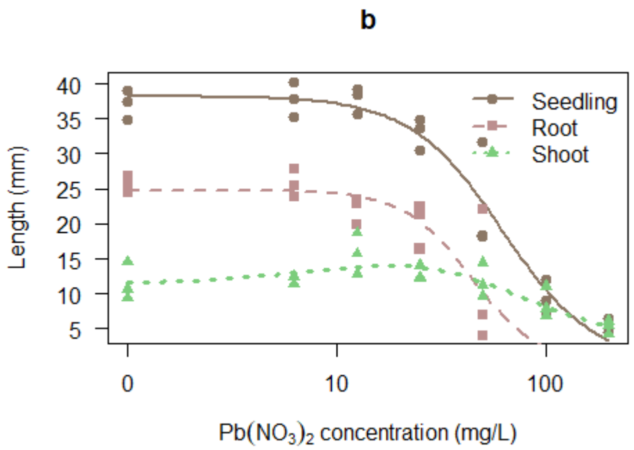


Fig. 1: Dose-response curves for root, shoot, and seedling lengths at 72-h (a), 96-h (b), and seed germination (c) of *O. sativa* after exposure to $CuSO_4$. Points without lines denote no significant dose response relationship.





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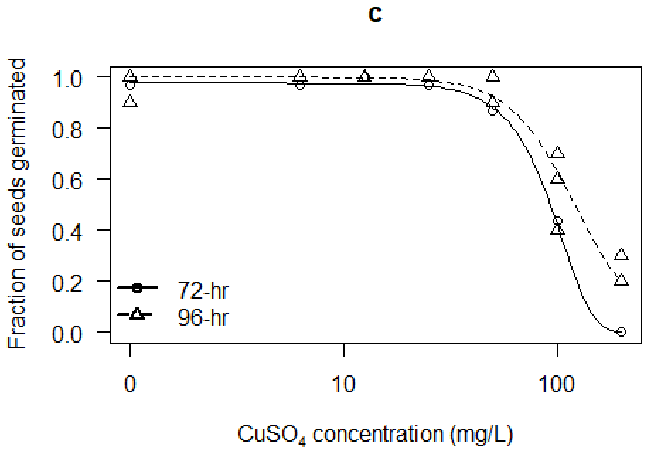

Fig. 2: Dose response curves for root, shoot, and seedling lengths at 72-h (a), 96-h (b), and seed germination (c) of *O. sativa* after exposure to $PbN(O_3)_2$.

Table 4: Ratios between IC_{25} (LC_{25} for seed germination) of different time points (72-hr/96-hr) for each heavy metal and morphometric endpoints of *O. sativa* after 72-h and 96-h exposure to $CuSO_4$. Upper and Lower represents 95% CI values.

Metal	Endpoint	Estimate	Std. Error	Lower	Upper
Pb	Root	1.23	0.34	0.56	1.89
Pb	Shoot	2.01	0.40	1.23	2.80
Pb	Seedling	1.09	0.23	0.64	1.54
Pb	Germination	0.86	0.13	0.60	1.11
Cu	Root	1.79	0.41	0.99	2.60
Cu	Shoot	-	-	-	-
Cu	Seedling	1.10	0.27	0.58	1.63
Cu	Germination	1.12	0.20	0.74	1.51

– IC_{25} was greater than the tested concentration ranges of $CuSO_4$.

IV. Discussion

From the results presented here, it is inferred that both Cd and Pb have their unique toxic behaviour on different organs (endpoints) of rice plant. This uniqueness can be useful in specific identification of such compounds. The varied response of $CuSO_4$ might be of interest as it produced the stimulatory effect on one organ (shoot) and inhibitory effect on another organ (root) at the same time. Such response was not observed previously in a similar study by Wang [12]. One reason for this is that the concentration ranges chosen by him might have skipped the hormetic effect. It has already been reported that the concentration ranges chosen and the spacing between them (especially at lower ranges) affects the determination of hormesis in bioassays [13, 14]. It should also be noted that such stimulatory effect is due to the function of Cu as a micro nutrient. Higher LC_{25} for Pb points to the low sensitivity of *O. sativa* to this metal compared to Cu. To cause toxicity, the Pb was required at higher concentrations with the longer exposure duration in contrast with Cu which was required at lower concentration with the shorter exposure duration. This is an important observation in the study. This behaviour indicates the ability of *O. sativa* to attain resistance with time against the toxicity of Pb. This resistance might be due to a special mechanism in which the Pb in the solution is adsorbed by the oxalate secreted by the rice [15]. Comparing the entire dose-response curve is also found to be advantageous in identifying specific patterns unique to each toxicant. From the ongoing discussion, it is clear that BOU for point estimates draws a boundary line for uncertainties of reaching wrong conclusions. Thus it supplements the confidence intervals produced from point estimates. The confidence intervals of LC_{25} in combination with BOU can be very useful in environmental management aspects.

V. Conclusion

The results presented here clearly indicate that the inclusion of different endpoints and different point estimates may be helpful in specifically identifying the toxicants. The study has also proven that pattern of change in point estimates (IC/EC/LC) at different time points is unique to each compound, a property which can be made use of in the identification of toxicants.

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