Bioassay with Humics: A Statistical Approach to Data Collection

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Keywords: bioassay, experimental design, statistics

1. INTRODUCTION

Humic substances (HS) have been the subject of numerous scientific studies due both to their mitigating effects on contaminants toxicity to biota, and to their anti-stress effects under abiotic stress conditions (unfavourable temperature, pH, salinity, et al.) (1 and citations in it). In spite of numerous studies on the biological effects of HS, the mechanism of their action remains unclear. The main reason seems to be the stochastic nature of HS. They are characterized as polydisperse materials having elemental compositions that are non-stoichiometric, and structures, which are irregular and heterogeneous (*2*). The above features hamper a use of common biological approaches to study biological activity of HS. Of importance is also that in contrast to the substances with a well-defined mode of action, HS effects drastically depend on the environmental conditions (3, 4) what elucidates another peculiar feature of HS – instability and poor reproducibility of the biological effects. Finally, effect of HS does not usually exceed 20 % what makes difficulties for data interpretation as bioassay techniques are often characterized with relatively high experimental error due both to measurements errors and heterogeneity of biological responses. To avoid the above mentioned problems and to obtain reliable and statistically significant results, the statistical approach to the data collection should be applied. In spite of data treatment *post factum*, a pilot study can be conducted to choice sample size for prescribed accuracy of the bioassay.

This study was aimed to estimate metrological performance of the bioassay on the example of one of most extensively used bioassay technique with seedlings.

2. MATERIALS AND METHODS

Humic material: Leonardite humic acid (HA) was a commercially available preparation Powhumus (Humintech, Germany) desalted using dialysis before the experiments. Elemental analysis of HA was determined with a Carlo Erba Strumentazione analyzer and showed that HA contained (on ash-free and moisture-free bases) 45.9 % C, 3.4 % H, and 1.6 % N. Ash content was 1.1 %.

Bioassay: Wheat plants (*Triticum aestivum* L. var. Inna) were used as a biotarget, and distilled water was used as a blank. When required, HA preparation was added to distilled water at the concentrations of 5, 15, 30, 50 or 100 mg/l. Wheat seeds were germinated in the test solutions in the dark at 25°C for 72 hours. Shoot and root length of seedlings was used as a response. The pilot study consisted of 60 replicate seeds allocated in 6 Petri dishes for each variant of the experiment. The experiment was repeated 14 times.

Statistical data treatment: the normality of distribution of data was assessed by the Kolmogorov-Smirnov test; two-sizes tests were applied. The homogeneity of the obtained data was estimated using analysis of variance (5). Calculation of sample size n (i.e. required replications) was conducted as follows (*6*):

$$n = \frac{s^2 t_P^2 (1 + 1\,2m)}{I_P^2}$$

where s – a standard deviation, t_P – Student's statistics at probability P and number of observations m in the pilot experiment, and I_P was a relative prescribed accuracy, i.e. preset maximum difference between "true" and estimated average, %. For this study, I_P = 5 % and P = 90, 95 and 99 % were chosen.

3. RESULTS AND DISCUSSION

Kolmogorov-Smirnov test revealed that the distributions of both roots and shoots length values can be approximated by the normal distribution. This allowed us to apply other procedure of parametric statistics for data treatment. Analysis of variance did not show significant differences between roots and shoots length in different Petri dishes used in the experiment, the obtained data was therefore consolidated in one sample for each variant. The number of replications n was calculated for each examined variant in each of 14 experiments. The averaged value of n varied from 44±13 to 230±67 depending

on variant, probability level, and response used. Calculated values for I_P= 5 % at P = 99, 95, and 90 % are summarized in Table 1.

Table 1: Required number of replications in seedlings bioassay at relative prescribed accuracy I_P = 5% and different HA concentrations and probability P.

	HA concentration, mg/l					
Response	**0 (blank)**	**5**	**15**	**30**	**50**	**100**
			P = 99 %			
Shoots	104±31	118±48	116±51	123±60	102±20	150±31
Roots	143±16	208±80	203±94	162±25	170±35	230±67
			P = 95 %			
Shoots	62±18	66±27	65±29	69±34	57±11	84±17
Roots	85±10	117±45	113±53	90±14	95±20	122±38
			P = 90 %			
Shoots	44±13	47±19	46±20	49±24	40±8	60±12
Roots	61±7	83±32	81±37	64±10	68±14	86±27

± denotes confidence level

The data presented in Table 1 showed that experiments with usage of roots length as a response requires more replications than those with a use of shoots length. This finding is probably resulted from higher variability of the latter compared with former. Indeed, average relative standard deviation for the shoots length was 17 %, while that for roots reached 22 %. So, the bioassay with seedling implied roots length as response needs to be performed with larger replications.

On the other hand, comparing to the blank variant, all the variants with HA needed more replications to reach the same accuracy. In spite of statistical insignificance of the observed differences due to high confidence levels, it is possible to say for the trend above mentioned. The trend established signified that a pilot study aimed at experimental design of bioassay with humics should include both blank and HS variants.

4. CONCLUSIONS

To perform seedlings bioassay with humics at prescribed relative accuracy 5% it can be recommended to make about 80 replications if using shoots length as a response is implied; if roots length are to be used as a response, the number of replications should be increased to about 120.

ACKNOWLEDGEMENTS

This work was supported by the Research Center for Environment and Health (GSF, Neuherberg, Germany) FE 75184, BA 31/139166/02/U and ISTC (KR-964).

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