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Testing the homogenizing effect of low copper sulfate concentrations on the size distribution of *Portulaca oleracea* seedlings *in vitro*

Aki Sinkkonen *, Olli-Pekka Penttinen, Rauni Strömmer

Department of Ecological and Environmental Sciences, University of Helsinki, Niemenkatu 73, 15140 Lahti, Finland

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ABSTRACT

Traditionally, toxicological bioassays rely upon the differences in mean-based statistical tests between the exposed and unexposed plants, and exceptional plant individuals are treated as statistical outliers. Recently, low toxicant concentrations have been observed to affect gene regulation in exposed plant stands and to change the frequency of the largest individuals even if mean plant size remains unchanged. In this paper, we present the results that the latter phenomenon is not restricted to a single toxicant and plant species. Our data analysis consists of two statistical methods that may be of general interest. We utilized the one-tailed Moses extreme reactions test by comparing exposed groups to control plants with and without the trimming of a certain amount of potential outliers from both treatments compared. We also propose that Mann–Whitney *U* or other tests at ordinal scale can be utilized to analyze if the largest plant individuals in exposed and control treatments come from a single 'survivor' population. We conclude that the results supported the hypothesis that very low toxicant concentrations may have ecological effects on fast-growing plant species. Finally, we discuss the limitations of the statistical methods currently in use.

1. Introduction

Today, millions of chemicals are known, and over 100,000 are commonly used, and often discharged to the environment although the fate and effects of most of the chemicals are unknown and often unpredictable. The need to collect adequate information about the distribution, bioavailability, persistence and adverse effects of toxic chemicals at relevant biological organization levels is obvious.

Effects of low toxicant doses have been intensively studied during recent years (Pickrell and Oehme, 2006; Rietjens and Alink, 2006). This has given promise of enhanced control on critical doses on the one hand but raised novel environmental concerns on the other hand. On the negative side, a number of toxicants have been observed to change sexual development of animals at very low concentrations (Hayes et al., 2002; Swan et al., 2005). On the positive side, numerous contaminants at low concentrations produce hormesis i.e. increase the growth of exposed organisms (van der Woude et al., 2005). Such hormetic effects turn to growth inhibition beyond a case-specific toxicant exposure (Sinkkonen, 2006, 2007). These controversial findings have forced toxicologists and environmental authorities to reconsider factors that must be taken into account when determining critical doses and environmental criteria for pollutants. Moreover, many anthropogenic compounds may have ecological implications which have so far remained hidden, and which have to be revealed before environmental quality guidelines for contaminants can be reliably estimated.

There is strong evidence that plants share the soil toxicant pool in high-density stands, which results in low doses per plant and negligible or hormetic toxicant effects in mean values of the variables measured (Sinkkonen, 2001, 2003; Weidenhamer, 2006). However, even if differences in mean values were absent, size differences among individual plants were reduced in high-density stands of annual baby's breath (*Gyposphila elegans*) that grew under very low lead acetate exposure (Sinkkonen et al., 2008). In that study, exposure to very low lead acetate concentrations decreased variation in root growth among the fastest growing seedlings, whereas mean root length remained the same in all treatments. As a result, extreme values were more frequent in the control than in the low lead acetate exposure treatments. A possible reason for this phenomenon is that the potentially fast-growing individuals may have received more toxicant ions quickly due to their high activity, which may have reduced their growth and made all exposed seedlings grow evenly. Another but not mutually exclusive possibility is that low toxicant doses have genotoxic effects that depend on plant growth rate, even if mean plant size remains unchanged (Aina et al., 2006). Low toxicant concentrations may also change gene regulation (Quaggiotti et al., 2007). In a broad chemical context, even a small increment in exposure concentration usually affects measured values although a clear dose–response relationship is missing (Darnerud, 2003; Rietjens and Alink, 2006). Possible effects include behavioral effects, changes in within-population variation and a saw-tooth effect where steep increases and decreases occur step-wise. Although the mechanism behind low-

* Corresponding author. Tel.: +358 9 1912 0344; fax: +358 9 1912 0331.
E-mail address: aki.sinkkonen@helsinki.fi (A. Sinkkonen).

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dose effects is unknown, low toxicant concentrations may have drastic effects on plant populations in environments where rapid growth after germination is crucial.

In this paper, we hypothesized that the frequency of fast-growing plants is reduced under low exposure to copper sulfate *in vitro*. If the hypothesis is proven, this is a second and independent (another plant species, another toxicant) observation about the homogenizing effect of low toxicant concentrations following our published one (Sinkkonen et al., 2008). While our first study focused on a non-essential and non-regulated metal, this second one focuses on an essential and regulated metal. Moreover, we propose an alternative way to use the Moses extreme reactions test in biological studies. We also discuss how the Mann–Whitney *U* or other statistical methods that are based on ordinal scale comparisons can potentially be utilized to analyze differences in the frequencies of the largest individuals between a control and a low dose treatment.

2. Materials and methods

2.1. Toxicant and species selection

Copper sulphate ($CuSO_4$) has been used as an inorganic fertilizer and pesticide. Its long-time usage has lead to overfertilization and accumulation at toxic levels in topsoil layers of perennial agricultural areas, like vineyards and olive fields (Rodríguez Martin et al., 2007). $CuSO_4$ is also widely used to control phytoplankton and mosses, which has caused Cu accumulation in aqueous and riparian habitats (van Hullebusch et al., 2003). Purslane (*Portulaca oleracea* ssp. *sativa*) is among the most widespread plant species in the world. It typically infests agricultural areas in mild and warm climates. Several *Portulaca oleracea* ecotypes are known; all of them germinate rapidly and grow vigorously after germination in environments characterized by warm temperatures and sudden dry periods (Singh, 1973; Zimmerman, 1977).

2.2. Plant material and growth conditions

Seeds of purslane (Portulaca *oleracea* ssp. *sativa*) were bought from a commercial horticultural company (Oy Schetelig Ab, Vantaa, Finland). Sixty seeds per dish were germinated in tightly closed 45 mm diameter polyethylene Petri dishes in 1 ml of aqueous solution that was made of deionized Milli-Q grade water and 0.4 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $MgSO_4 \cdot 7H_2O$, 0.01 mM KCl, and 0.15 mM $NaHCO_3$ (total Ca + Mg: 0.5 mM, ratio Ca:Mg = 4:1) for 1 day. On the second day, seedling number was harmonized to 45–47 per dish by removing ungerminated seeds and an additional ten random seedlings per dish. The procedure harmonized the mean dose per seedling (Sinkkonen, 2001, 2007). Thereafter, treatments as six randomized replicates were established by adding 2 ml of 0 mg l^{-1}, 1 mg l^{-1}, or 5 mg l^{-1} copper sulfate ($CuSO_4$) in the aqueous solution. The dishes were closed airtight and the seedlings were grown at 28 °C and 16 h light:8 h darkness diurnal rhythm in a growth chamber. After three more days, the lengths of the roots were measured.

2.3. Statistical analyses

The EC50 for purslane roots was estimated according to the logistic model by Haanstra et al. (1985):

$$Y = Y_{max} / \left(1 + (C_{\mathrm{tox}} / \mathrm{EC50})^b\right)$$

where Y is plant response at a certain toxicant concentration, $Y_{\max}$ is the maximum response in the untreated controls, C_{tox} is the toxicant concentration, EC50 is the concentration at which response is 50% of that in the controls, and b is the slope of the dose–response curve. Other statistical analyses were executed with SPSS for Windows 15.0.1. Univariate analysis of variance was performed with Tukey honestly significant difference tests. Since the assumption of normality was not met, the probability of rejecting a true null hypothesis was higher than indicated by the analysis. Therefore, two-treatment differences between medians were analyzed non-parametrically with Mann–Whitney *U* tests. If Mann–Whitney *U* tests indicated no difference, Moses test of extreme reactions was executed without trimming of Group 1. Because the Moses extreme reactions test tests for one-tailed differences (Moses, 1952), $CuSO_4$-exposed treatments were set as Group 1 in the test. Because this test also is affected by extreme values that may occur due to experimental or systematic errors, the three longest seedling roots were excluded as potential outliers at the right tails of the two distributions compared.

The rationale for the use of the Moses extreme reactions test in dose–response studies is to discover if large individuals are sensitive to low toxicant levels in the substratum. The reason is that rapid root growth is crucial for the survival of the seedlings of several plant species because drought and declining water table cause selective mortality (Mahoney and Rood, 1991, Horton and Clark, 2001). However, the Moses test *per se* cannot separate if a significant difference is due to large or small seedlings. To solve this problem, we hypothesized that all seedlings that survive beyond the seedling stage come from a single population of survivors at the right tail of size distribution diagram, and that there are no size differences between plants growing at various toxicant levels within the survivor population. To test this, we selected the seven longest plants in every treatment, and compared the $CuSO_4$-free control treatment with the $CuSO_4$-exposed treatments in Mann–Whitney *U* tests. Thereafter, we enlarged the number of seedlings included in the analysis by six. The selection of seven and 13 plants was based on 99% and 97% percentiles adjusted for ties, respectively. Thereafter, seedling number was increased by six until an insignificant test result occurred. Since Mann–Whitney *U* tests require that the distributions compared are similar, we additionally performed Kolmogorov–Smirnov *Z* tests which do not share this limitation.

3. Results

The median effective concentration (EC50 ± SE; 3.5 ± 0.69 mg/l) for growth reduction determined by logistic model had a low coefficient of determination ($r^2 = 0.08$, $p < 0.01$) due to the selected exposure regime. Mean root lengths in 0-mg, 1-mg and 5-mg treatments were 18.3, 17.8 and 15.2 mm, respectively. The roots in 5-mg treatment were significantly shorter than the roots in 1-mg and 0-mg treatments (Fig. 1: $F = 35.9$; df = 802,2; $p < .001$; Tukey HSD: $p < .05$). Also Mann–Whitney

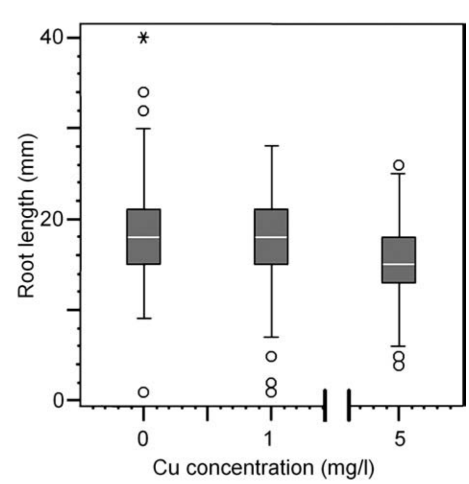

Fig. 1. Root lengths of *Portulaca oleracea* at three concentrations of $CuSO_4$. Bars represent median (white line), 25 and 75% percentile (box ends), 95% percentiles (line segments), statistical outliers (circles) and extreme values (asterisks).