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# Project Summary

# Interlaboratory Root Elongation Testing of Toxic Substances on Selected Plant Species

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**Four contract laboratories and three EPA laboratories participated in the interlaboratory testing of 10 toxic substances on a representative plant species from five families. Seeds were germinated on filter paper saturated in a solution of the toxic substance and incubated for 115 hours. The root lengths were measured to evaluate the toxic effects of the chemical concentrations on the various species. The objective of the testing was to estimate the concentration of chemical which reduced root length to 50% of the control length. This research attempts to determine the precision of this bioassay used to evaluate environmental effects under the Toxic Substances Control Act (TSCA). Although the method proved to give a uniform plant-growth environment, the species variability in relationship to the chemical concentrations that inhibit root growth makes it difficult to use this assay on more than one species at a time.**

***This Project Summary was developed by EPA's Environmental Research Laboratory, Corvallis, OR, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).***

## Introduction

A rapid, simple, and precise bioassay is needed to measure the adverse effects of toxic substances on terrestrial vegetation in the environment. Root elongation tests were conducted on five plant species including lettuce (*Lactuca sativa* L. var. Buttercrunch), radish (*Raphanus sativus* L. var. Cherry Belle), wheat (*Triticum aestivum* L. var. Stephens), cucumber (*Cucumis sativus* L. var. Hybrid Spartan Valor), and red clover (*Trifolium pratense* L. var. Kenland). These species are important economically as agricultural crops. They are also important ecologically in terms of family size, distribution, and abundance. Herbicide bioassays, heavy metal screening tests, salinity and mineral stress tests, and allelopathic studies show these plants to be sensitive to many toxic compounds. Each species was selected to represent a different crop directly or indirectly consumed in the human food chain. These plants germinate quickly and easily, root growth is rapid and relatively uniform, and the cultivars are readily available.

The chemicals used included silver nitrate, sodium fluoride, cadmium chloride, methane arsonic acid (MAA), endosulfan, 2,4-D acid, polyethylene glycol 20,000 linear (Carbowax®), pentachloronitrobenzene (PCNB), cineole, and monuron. These substances represent various types of toxic substances such as inorganic metals, inorganic nonmetals, organometallic compounds, herbicides, fungicides, insecticides, osmoticum, and allelopathic substances.

Test substances were prepared to obtain nominal concentrations and were adjusted to pH 6.5. A controlled environment chamber was used to maintain a uniform testing temperature at 25 ± 1°C. A standardized technique was used to germinate the seeds on filter paper substrate in glass tanks for 115 hours. The root of each plant was measured on a flat surface and germination and seedling morphology data was recorded.

The first test included a wide range of concentrations to determine whether the

test chemical was phytotoxic and to find the appropriate sensitivity range for each species. Subsequent tests included chemical concentrations selected to cover the sensitivity range previously estimated. The chemical concentrations selected were generally equally spaced on a log scale and were used only after they satisfied certain qualifying criteria. For example, one of the following criteria had to be satisfied before a chemical concentration was identified as reducing the root length of a given species by 50% of the control:

1. At least one test must include three concentrations in which mean root length is between 12-85% of the controls with at least one greater than 50% of control and at least one smaller than 50% of the control.
2. At least one test must include one concentration in which the mean root length is between 60-80% of the control and a second concentration in which the mean root length is between 15-40% of the control.

If the mean root length was based on the germination of fewer than 10 seeds per plate for controls on test concentrations, those data were not used under either criteria above.

The EC50's were calculated by selecting data only from the linear portion of the dose-response curve. A linear regression was used to fit the data set selected. Using the regression equation, the chemical concentration required to reduce the root length to 50% of the control was calculated.

## Results

The control root lengths for each species at each laboratory are shown in Table 1. The mean root length ($\bar{x}$) represents 15 roots of each species measured in each of (N) number of control tests. The variation (s) in mean root length within laboratories is a result of seedling variability, the variation in environmental controls, and the experimental error of the testing laboratory. The coefficient of variation ($\bar{V}$) was computed to show the relative precision for control root lengths.

An analysis of variance (Table 2) shows a significant difference in control means among laboratories. There is no significant difference in control means among chemicals tested (Zero dose, chemical). Statistically, there was a significant interaction between laboratory and zero dose (chemical) and between laboratory and species. However, only the laboratory and species interaction was considered large enough to be important.

Except for Carbowax and cineole, seed germination was not significantly inhibited for those chemical concentrations that inhibited root growth. Although reduced in length, the seedlings were either normal in appearance or showed necrosis, or were weak and spindly. With 2,4-D, the roots were often short, stubby, and thickened.

Figure 1 shows a typical dose-response curve for test data in which root length was plotted against the concentration (expressed as the log 10) of the treatment. Data in the linear portion of the curve where small changes in dose result in large changes in root length best describe the dose-response relationship. The toxicity parameter (EC50) in this assay is defined as the chemical dose (mg/l) required to reduce root length of treated seedlings to 50% of the control.

The EC50 values and the multiplicative standard error were calculated from the toxicity test data from each of the laboratories. Results for endosulfan and pentachloronitrobenzene (PCNB) gave no significant inhibition of root growth for any species or laboratory.

For some chemicals there were large differences in species sensitivity, while in others the EC50 estimates did not differ greatly. For example, EC50's for methane arsonic acid (MAA) range from 464-962 mg/l for wheat and from 9-27 mg/l for red clover. For sodium fluoride, the EC50's for wheat and red clover range from 119-286 mg/l and 217-425 mg/l, respectively. Estimating an EC50 for cineole and Carbowax was difficult because germination and root elongation were inhibited at nearly the same chemical concentrations.

An analysis of variance of the log 10 of the estimated EC50's shows that there is a significant difference among six of the chemicals evaluated for toxicity on the five plant species. The differences due to chemical are considerably larger than those for species. There are no significant differences among the laboratory estimated EC50's for chemicals and species. This implies that all laboratories were equally accurate in estimating EC50's for the chemicals and species. The overall EC50 means were calculated for all laboratories to compare chemicals and species (Table 3). Wheat is consistently the most tolerant species (largest EC50) for the chemicals tested except for sodium fluoride. Lettuce and red clover were generally the most sensitive (smallest EC50) although for sodium fluoride lettuce has the largest EC50.

By comparing the responses of different species to the chemical tested, an indication of the nature of the chemical toxicity can be determined. Sodium fluoride was unique because all the species tested were considerably more tolerant to it than the other five chemicals tested. Also, the response for all species occurred in a similar concentration range. These results indicated a nonselective mode of action for sodium fluoride in inhibiting root growth.

The range of mean EC50's for silver nitrate and cadmium chloride were 3-156 mg/l and 7-92 mg/l, respectively. These results indicate a more selective mode of action in which chemicals inhibit root growth to a greater degree in some species than in others.

For monuron and 2,4-D, the differences in the mean EC50's are small if wheat is excluded. These results indicate a less distinctive selective mode of action.

There is a significant difference in standard error of the EC50 estimate for laboratories. Although the laboratories were able to accurately estimate an EC50,

*Table 1. Control Mean Root Length ($\bar{x}$) in mm, Standard Deviation (s), and Coefficient of Variation ($\bar{V}$) for Laboratories and Species*

| | Red Clover | | | | Lettuce | | | | Wheat | | | | Cucumber | | | | Radish | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Laboratory | N | $\bar{x}$ | s | $\bar{V}$ | N | $\bar{x}$ | s | $\bar{V}$ | N | $\bar{x}$ | s | $\bar{V}$ | N | $\bar{x}$ | s | $\bar{V}$ | N | $\bar{x}$ | s | $\bar{V}$ | $\bar{x}$ |
| 1 | 63 | 25 | 4 | 17 | 64 | 69 | 5 | 7 | 64 | 80 | 8 | 10 | 64 | 103 | 12 | 12 | 64 | 129 | 29 | 23 | 82 |
| 2 | 44 | 23 | 3 | 14 | 35 | 60 | 6 | 10 | 44 | 66 | 12 | 18 | 44 | 111 | 10 | 9 | 44 | 134 | 24 | 18 | 79 |
| 3 | 50 | 27 | 5 | 20 | 49 | 62 | 8 | 12 | 50 | 91 | 12 | 13 | 50 | 118 | 16 | 13 | 50 | 156 | 29 | 19 | 91 |
| 4 | 54 | 25 | 6 | 24 | 54 | 59 | 12 | 20 | 54 | 76 | 17 | 22 | 54 | 112 | 15 | 13 | 54 | 138 | 39 | 28 | 82 |
| 5 | 74 | 22 | 5 | 25 | 76 | 50 | 12 | 23 | 76 | 78 | 11 | 14 | 74 | 87 | 23 | 27 | 75 | 95 | 36 | 37 | 67 |
| 6 | 56 | 32 | 6 | 17 | 54 | 67 | 7 | 10 | 56 | 64 | 10 | 15 | 60 | 113 | 13 | 12 | 58 | 133 | 28 | 21 | 82 |
| 7 | 41 | 28 | 4 | 15 | 41 | 64 | 8 | 13 | 41 | 76 | 7 | 10 | 41 | 95 | 19 | 20 | 41 | 126 | 18 | 14 | 78 |
| $\bar{x}$ | | 26 | | | | 62 | | | | 76 | | | | 106 | | | | 130 | | | |

*Table 2.* *Analysis of Variance Table for Control Mean Root Length by Laboratory, Species, and Zero Dose (Chemical) with the Laboratory x Species x Zero Dose Interaction Designated as Error*

| Source | df | MS | F | P-value |
|---|---|---|---|---|
| Laboratory | 6 | 0.0622 | 41.47 | 2.10 |
| Zero Dose (Chemical) | 7 | 0.0021 | 1.40 | 2.01 |
| Species | 4 | 4.0820 | 2,721.33 | 2.37 |
| Laboratory x Zero Dose | 42 | 0.0035 | 2.33 | 1.38 |
| Laboratory x Species | 24 | 0.0163 | 10.87 | 1.52 |
| Zero Dose x Species | 28 | 0.0017 | 1.13 | 1.48 |
| Laboratory x Zero Dose x Species | 168 | 0.0015 | | |
| Totals | 279 | | | |

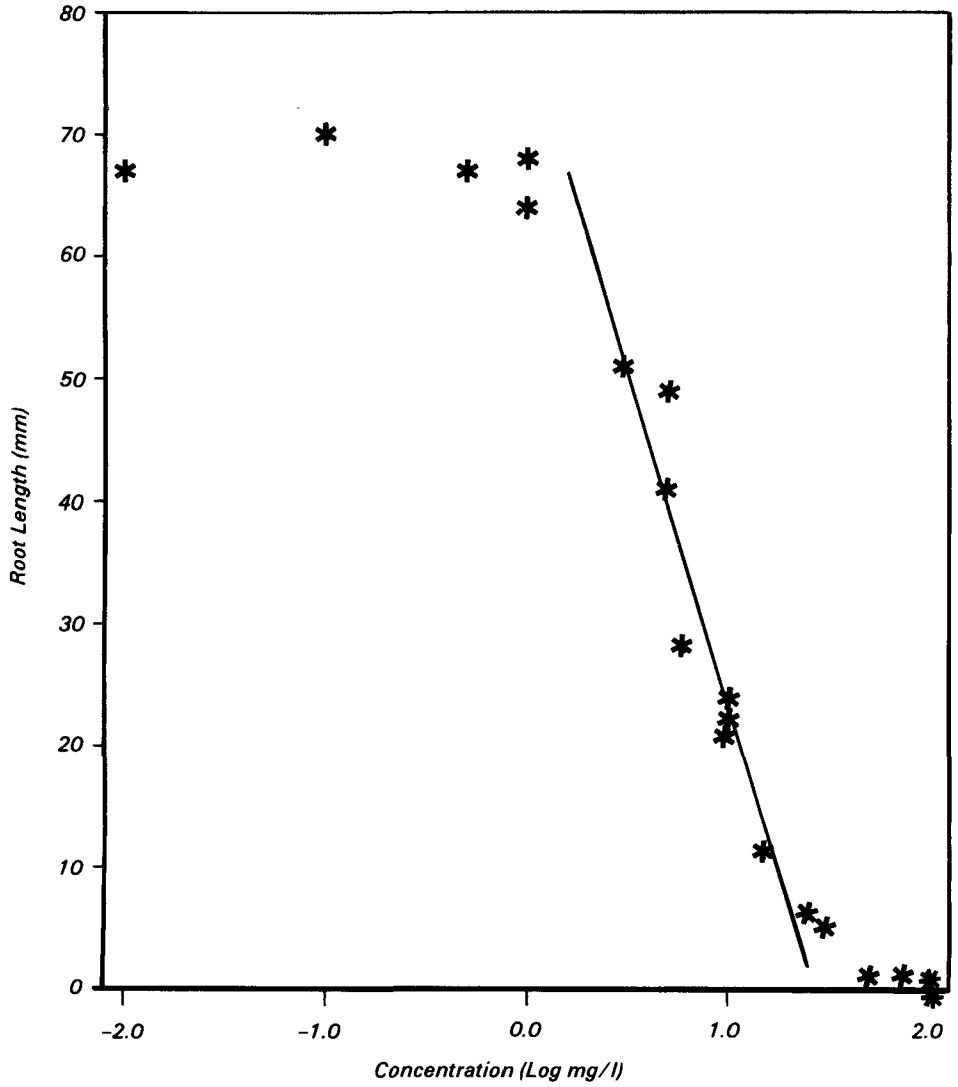


*Figure 1.* *Dose-response curve for lettuce and cadmium chloride using data from laboratory No. 1.*

there was enough variability in technique among laboratories that the precision of the estimates differ significantly. The standard error of the EC50 estimates are also significantly different for species. The interactions between laboratory, chemical, and species do not appear to be an important factor, although the laboratory x chemical and chemical x species interactions are statistically significant.

The precision in which the EC50 is estimated depends on 1) how well the doses were selected in terms of equal spacing on a log scale and the number of doses on the linear part of the response curve, 2) the slope of the regression line, 3) the accuracy of the preparation of the chemical dose, and 4) the variability of the root length response for each species. The critical factor appears to be the allocation of an adequate number of chemical concentrations which give responses in the linear portion of the dose-response curve.

## Conclusions

Reduction in root length is a valid and sensitive plant response to exposure to chemical substances. It is a suitable test for evaluating phytotoxic substances over a wide range of concentrations.

The interlaboratory testing procedures in this bioassay study provided a uniform root growth environment. The results within and among laboratories showed this uniformity. Most variation was due to biological differences among species.

Using a single method to evaluate five species simultaneously was cumbersome. It is difficult to satisfy all criteria for all the species at the same time. The precision of the EC50 estimates varied significantly indicating the need for more data points to adequately define the dose-response relationships for all five species. A minimum of four observations in the linear portion of the dose-response curve should be used. These points should also be equally spaced on a log scale with the interval between doses approximately 1.5 to 2.0.

Although this test is difficult to use on five species at once, it might be used more efficiently for one species at a time. The results would give some indication of the general response of most species but would not reflect the response of all species.

**Table 3.** *EC50 (mg/l) Geometric Means for All Laboratories for Chemical and Species†*

| Species | $AgNO_3$ | $CdCl_2$ | Monuron | MAA | NaF | 2,4-D | PCNB | Carbowax | Cineole | Endosulfan |
|---|---|---|---|---|---|---|---|---|---|---|
| Cucumber | 19 (4) | 18 (2) | 83 (3) | 87 (2) | 165 (5) | 0.03 (2) | * | 74,000(5) | 1695(2) | * |
| Lettuce | 3 (5) | 7 (5) | 79 (4) | 18 (4) | 489 (1) | 0.03 (3) | * | 147,000[c](3) | 717[a](5) | * |
| Radish | 65 (3) | 17 (3) | 91 (2) | 25 (3) | 345 (2) | 0.04 (4) | * | 99,000[c](4) | 1257[b](3) | * |
| Red Clover | 96 (2) | 16 (4) | 60 (5) | 18 (5) | 303 (3) | 0.04 (5) | * | 157,000[a](2) | 2158(1) | * |
| Wheat | 156 (1) | 92 (1) | 176 (1) | 655 (1) | 236 (4) | 2.54 (1) | * | 207,000(1) | 1084(4) | * |

*(†) Species ranks within chemical where 1 is most tolerant and 5 is most sensitive are in parentheses.*
** No significant inhibition of root growth.*
*([a]) Geometric mean based on estimates from 4 laboratories.*
*([b]) Geometric mean based on estimates from 5 laboratories.*
*([c]) Geometric mean based on estimates from 6 laboratories.*

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*The complete report, entitled "Interlaboratory Root Elongation Testing of Toxic Substances on Selected Plant Species," (Order No. PB 83-226 126; Cost: $8.50, subject to change) will be available only from:*
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