



Standard Practice for Conducting Early Seedling Growth Tests¹

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1. Scope

1.1 This practice covers a standard procedure for obtaining useful, reproducible laboratory data on the toxicity to representative seedlings of chemical substances, industrial and domestic effluents, industrial and domestic sludges, and site soils. The procedures lead to the determination of a concentration or percent that either inhibits or enhances the growth of terrestrial plants. The results of this practice can be used to predict the likelihood of adverse plant effects if the test substance is released onto the land.

1.2 The first days of seedling growth are often the most sensitive stages of plant development. Adverse effects due to chemical substance exposure are most likely to take place during these phases. Because of the rapid growth phase, damage to the plant roots will be most readily discernible.

1.3 A major consideration in the development of this practice was a choice of the test organisms. The choice of test species was based on species' sensitivity to many chemicals, and if species were widely used by agronomists, plant physiologists, horticulturists, agricultural workers, and other plant scientists. Additional criteria included that the seeds have a reasonable germination response (germinating within several days), should be compatible with the testing environment, should be of economic or ecological importance, or model systems, and represent the two major groups of plants (monocots and dicots).

1.4 Modifications of these procedures might be justified by special needs or circumstances. Although using appropriate procedures is more important than following prescribed procedures, results of tests conducted using unusual procedures are not likely to be comparable to results of many other tests. Comparison of results obtained using modified and unmodified versions of these procedures might provide useful information on new concepts and procedures for conducting early seedling studies.

1.5 This practice is arranged as follows:

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1.6 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.* Specific precautionary statements are given in Section 5.

2. Terminology

2.1 Definitions:

2.1.1 *chlorosis*—the condition of green plants in which chlorophyll concentration is depressed and the leaves are pale green or yellow in color.

2.1.2 *control*—those plants exposed to the control soil and water. Seedlings exposed to the control soil are compared to those seedlings exposed to the treated soil to determine if there are any physical differences.

2.1.3 *decade-level concentrations*—concentrations that are separated by a factor of 10, for example, 1 mg/kg, 10 mg/kg, 100 mg/kg, etc.

2.1.4 *defoliated*—the condition in which a plant does not have a normal complement of leaves due to some internal or external cause.

2.1.5 *desiccated*—the plant, or portion of plant, that is dried in comparison to the control plant.

2.1.6 *dwarfed plants or leaves*—those plants or plant parts that are abnormally small, when compared to the control test system.

2.1.7 *emergence*—the hypocotyl hook or first true leaves (coleoptile) are observed above soil medium surface.

2.1.8 *enhanced plant growth, root length or lateral root growth, and yield*—when a treated plant exhibits plant growth, root length, lateral root growth, or yield significantly greater than the control values, plant growth is "enhanced".

2.1.9 *inhibited plant growth, root or lateral root growth, yield*—plant growth, root length and lateral root growth, and

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yield are "inhibited" when their measurements are significantly less than the control values.

2.1.10 *lateral roots*—those roots growing off the primary roots, also referred to as secondary roots.

2.1.11 *limit test*—plants are exposed to one concentration, such as 1000 mg/kg or 100 mg/kg.

2.1.12 *mottled*—marked with spots or streaks of different colors, blotched. This includes the discoloration of leaf margins.

2.1.13 *necrotic*—the presence of dead tissue.

2.1.14 *normal*—the measurements and observations of treated test organisms do not differ from the control test systems.

2.1.15 *severed*—the abnormal separation of plant parts, for example, the roots being separated from the plant stalk. The plant parts being severed appear to be pinched apart.

2.1.16 *staining*—the discoloration of plant parts (roots, vegetative growth) caused by the test substance.

2.1.17 *wilting*—becoming limp, deprived of moisture.

2.1.18 *withering*—the process of drying; frequently the result of root damage.

3. Summary of Practice

3.1 Separate groups of seeds/seedlings are exposed to different concentrations/percents of test substances for up to 28 days (at least 21 days post-emergence). Dilution water containing no potential toxicants is used as a control to provide a measure of acceptability of the test. After 28 days, or earlier termination of the study, seedling heights, above ground dry weights, or root lengths, or a combination thereof, are determined. Visual assessment of overall damage, carried out by an experienced assessor, may be appropriate for determining phytotoxic effects. Test results are then analyzed statistically for determination of a no observed effect concentration (NOEC).

4. Significance and Use

4.1 The results of this practice provide information on the effects of potentially toxic materials on the growth of terrestrial plants under laboratory conditions.

4.2 This practice is applicable for: establishing phytotoxicity of organic and inorganic substances, measuring the temporal effects of wastewater and landfill leachates, determining the phytotoxicity of sludges, and assessing the impact of discharge of toxicants to land.

4.3 The phytotoxicity test's major advantage is providing an evaluation that can be used to predict the likelihood of adverse effects from seedling exposure to chemicals, wastewaters, leachates, or sludges.

5. Apparatus

5.1 *Facilities*—The preparation of the test, test soil medium, storage of soil and seeds, and all stages of the test procedure must take place in an atmosphere free from toxic contamination and vapors. The facility, whether a greenhouse or a growth chamber, should have reasonable temperature control, relative humidity control, and supplemental lighting. Temperature, humidity, and light control may require monitoring to satisfy regulatory Good Laboratory Practice (GLP) requirements. If

so, equipment needs to be calibrated prior to and periodically during use to ensure that critical ranges are maintained.

5.2 Equipment and Supplies:

5.2.1 *Plant Pots*—Containers should be chosen to be inert to test/control substances. Test/control substances should not adhere to or react in any way with the container or should materials from the container interfere with the test/control substances. Glass, stainless steel, or paper containers with drainage holes can be used as plant pots. Polyethylene pots or other containers may be used, provided they are free of toxic materials. The volume of the pot container should be large enough so as not to restrict seedling growth. It is suggested that the selection of growth containers not be arbitrary, and that the appropriate size, shape, color, etc., of containers be considered for each plant species and toxicity test.

5.2.2 *Soil Medium*—Washed quartz sand, glass beads, natural soil (free of chemical contamination), or a commercial potting soil, may be used as plant support medium. (One artificial soil is known as the "Cornell Mix" and contains sphagnum peat moss, No. 3 grade vermiculite, granular dolomite and sand, major nutrients, some micro-nutrients, and a wetting agent. Major and minor nutrients include nitrate, phosphate, iron, potassium, calcium, sulfur, boron, copper, manganese, molybdenum, and zinc.) A nutrient solution, such as the Hoagland Solution, (13-15), would be used if quartz sand or glass beads serve as the soil medium. (It should be noted that exposures in sand and glass beads will most likely be different than exposures in soil.) Artificial soil, however, contains essential nutrients for growth up to four or five weeks. Artificial soil contains vermiculite and peat moss; this may not be appropriate for some tests where the organic material would bind with the test chemical. For GLP compliance, composition of the soil medium, if commercially obtained, needs to be known. It may be necessary to retain documentation on composition or at least batch/lot identification so that the supplier can provide the information. The same is true for any added nutrients. (The following caveat may be appropriate in some circumstances: A "standard" soil is preferable as it allows interpretation to the field, however organic matter of <5 % should be stipulated to avoid loss of chemical activity due to adsorption. It is recommended that the soil used be characterized at least for organic matter, pH_w, soil texture and type, cation exchange capacity (CEC), and major nutrients.)

5.2.3 *Balance*—Sensitivity to 0.001g.

5.2.4 *pH Meter*—Sensitivity to 0.1 units.

5.2.5 *Photometer*—Capable of measuring and recording both the visible and ultraviolet (UV) spectrum, if necessary. Light intensity should be expressed as $\mu\text{mol}/\text{m}^2/\text{s}$ or W/m^2 , or $\text{mE}/\text{m}^2/\text{s}$, or equivalent.

5.2.6 *Thermometer*—A recording thermometer, that records temperature and relative humidity on a seven-day basis, is recommended. A thermal probe can be used to record soil temperatures.

5.2.7 *Propagating Mats*—To be used to maintain soil temperature in the event that temperatures fall below 20°C during the germination period.

5.2.8 *Industrial Mixer or Cement Mixer*—May be used to mix large volumes of soil medium and to homogenize test

substances into large volumes of soil.

5.3 *Equipment Care*—Clean the test equipment after each use. Wash all new equipment with a detergent and rinse thoroughly with water, pesticide-free acetone, water acid (such as 5 %, hydrochloric acid), and twice with tap or clean water. Clean the equipment, such as the mixer and mixer blades by a procedure for removing known constituents of the test material. For GLP compliance, documentation of cleaning procedures may be required. Paper and plastic plant pots should be disposed and not reused.

6. Hazards

6.1 Many materials can adversely affect humans if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment, putting hands in test solutions or treated soil medium, or handling treated plant material), laboratory coats, aprons, and glasses. Special precautions, such as ventilating the area surrounding the flats, should be taken when conducting tests on volatile materials. Information on toxicity to humans (1-5),² recommended handling procedures (6-9), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radiolabeled test materials (10, 11) and with test materials that are, or are suspected of being, carcinogenic (12).

6.2 Although disposal of stock solutions, test solutions, test soil, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions. Hazardous materials must be disposed of in accordance with state and federal regulations.

6.3 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a well-ventilated area in which no smoking is allowed and no open flame, such as a pilot light, is present.

6.4 An acidic solution should not be mixed with a hypochlorite solution because hazardous fumes might be produced.

6.5 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a well-ventilated area.

6.6 Because water is a good conductor of electricity, use of ground fault systems and leak detectors should be considered to help avoid electrical shocks.

7. Test Organisms

7.1 *Test Species*—The seeds used in this study should be from the same batch/lot for each of the individual plant species. The identity of the seeds is obtained from the container in which they are received. For this test the plants chosen represent two groups. One group is dicotyledons (plants with two seed leaves); three species of at least two families, one of

which is a legume and a second is a root crop, are recommended from this group. The second group is monocotyledons (plants with one embryonic seed leaf, and a leaf sheath referred to as a coleoptile); two species from at least one family, including corn, are suggested from this group. Other species from either group can be used provided the guidance in 1.3 is considered. It is recommended that certified seeds be used when available. Seeds of uniform size should be used to limit variability. Information that is necessary for compliance with Good Laboratory Practice (GLP) standards includes the packet size (ounce-pounds), percent germination rating, the batch or lot number, the date of the germination rating, variety, and the name of the supplier. The information that should be placed in a logbook and on the packets includes the date of purchase and the date that the pack is opened.

7.2 The test is conducted on at least five species of plants, see Table 1, or as needed, other species of required significance can be used.

7.3 *Seed Storage and Maintenance*—The seeds are stored in a refrigerator until needed (preferably at $4 \pm 2^{\circ}\text{C}$). Seeds should be purchased at least annually, preferably within one year of the date of seed germination response. It is recommended that the purchase of a sufficient amount of seeds be made to run an entire test. This will ensure that the certified seeds are from the same batch/lot. See criteria for test acceptability (Section 12). Some researchers prefer to treat seeds with a chlorine dip; however, others recommend against this practice (16). The chlorine treatment is a 10 to 15-min dip in 5 % hypochlorite, followed by 5 to 10 min of rinsing with water, then air dried. It is recommended that this practice not be carried out since the natural microbial population on seeds helps to resist the attack of foreign microbes in the soil medium

TABLE 1 Recommended Species of Plants

Dicotyledons		
Family	Species	Common Name
Compositae	<i>Lactuca sativa</i>	Lettuce
Cruciferae	<i>Brassica alba</i>	Mustard
Cruciferae	<i>Brassica campestris</i> var. <i>chinensis</i>	Chinese Cabbage
Cruciferae	<i>Brassica napus</i>	Rape
Cruciferae	<i>Brassica oleracea</i>	Cabbage
Cruciferae	<i>Brassica rapa</i>	Turnip
Cruciferae	<i>Lepidium sativum</i>	Garden Cress
Cruciferae	<i>Raphanus sativus</i>	Radish
Cucurbitaceae	<i>Cucumis sativa</i>	Cucumber
Leguminosae	<i>Glycine max</i>	Soybean
Leguminosae	<i>Phaseolus vulgaris</i>	Pinto Bean
Leguminosae	<i>Phaseolus aureus</i>	Mung Bean
Leguminosae	<i>Trifolium pratense</i>	Red Clover
Leguminosae	<i>Trifolium ornithopodioides</i>	Fenugreek
Leguminosae	<i>Vicia sativa</i>	Vetch
Solanaceae	<i>Lycopersicon esculentum</i>	Tomato
Umbelliferae	<i>Daucus carota</i>	Carrot
Monocotyledons		
Family	Species	Common Name
Amaryllidaceae	<i>Allium cepa</i>	Onion
Gramineae	<i>Avena sativa</i>	Oat
Gramineae	<i>Lolium perenne</i>	Perennial Ryegrass
Gramineae	<i>Zea mays</i>	Corn
Gramineae	<i>Oryza sativa</i>	Rice
Gramineae	<i>Triticum aestivum</i>	Wheat
Gramineae	<i>Sorghum bicolor</i>	Sorghum

² The boldface numbers in parentheses refer to the list of references at the end of this practice.