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Standard Guide for Conducting Terrestrial Plant Toxicity Tests[1]

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1. Scope

1.1 This guide covers practices for conducting plant toxicity tests using terrestrial plant species to determine effects of test substances on plant growth and development. Specific test procedures are presented in accompanying annexes.

1.2 Terrestrial plants are vital components of ecological landscapes. The populations and communities of plants influence the distribution and abundance of wildlife. Obviously, plants are the central focus of agriculture, forestry, and rangelands. Toxicity tests conducted under the guidelines and annexes presented herein can provide critical information regarding the effects of chemicals on the establishment and maintenance of terrestrial plant communities.

1.3 Toxic substances that prevent or reduce seed germination can have immediate and large impacts to crops. In natural systems, many desired species may be sensitive, while other species are tolerant. Such selective pressure can result in changes in species diversity, population dynamics, and community structure that may be considered undesirable. Similarly, toxic substances may impair the growth and development of seedlings resulting in decreased plant populations, decreased competitive abilities, reduced reproductive capacity, and lowered crop yield. For the purposes of this guide, test substances include pesticides, industrial chemicals, sludges, metals or metalloids, and hazardous wastes that could be added to soil. It also includes environmental samples that may have had any of these test substances incorporated into soil.

1.4 Terrestrial plants range from annuals, capable of completing a life-cycle in as little as a few weeks, to long-lived perennials that grow and reproduce for several hundreds of years. Procedures to evaluate chemical effects on plants range from short-term measures of physiological responses (for example, chlorophyll fluorescence) to field studies of trees over several years. Research and development of standardized plant tests have emphasized three categories of tests: (*1*) short-term, physiological endpoints (that is, biomarkers); (*2*) short-term tests conducted during the early stages of plant growth with several endpoints related to survival, growth, and development; and (*3*) life-cycle toxicity tests that emphasize reproductive success.

1.5 This guide is arranged by sections as follows:

Section	Title
1	Scope
2	Referenced Documents
3	Terminology
4	Summary of Phytotoxicity Tests
5	Significance and Use
6	Apparatus
7	Test Material
8	Hazards
9	Test Organisms
10	Sample Handling and Storage
11	Calibration and Standardization
12	Test Conditions
13	Interference and Limitations
14	Quality Assurance and Quality Control
15	Calculations and Interpretation of Results
16	Precision and Bias

1.6 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.7 *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 8.

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

D4547 Guide for Sampling Waste and Soils for Volatile Organic Compounds

D5633 Practice for Sampling with a Scoop

E1598 Practice for Conducting Early Seedling Growth Tests (Withdrawn 2003)[3]

E1733 Guide for Use of Lighting in Laboratory Testing

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] The last approved version of this historical standard is referenced on www.astm.org.

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2.2 *Code of Federal Regulations Standard:*
CFR 49 [4]

2.3 *Other useful references have described phytotoxicity test procedures*(**1-11**) .[5]

3. Terminology

3.1 *General Terminology—*The words "must," "should," "may,"" can," and "might" have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see Section 14). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, violation of several will often render the results questionable. Terms such as "is desirable," "is often desirable," and "might be desirable" are used in connection with less important factors. "May" is used to mean "is (are) allowed to," "can" is used to mean "is (are) able to," and "might" is used to mean "could possibly." Thus the classic distinction between "may" and "can" is preserved, and "might" is never used as a synonym for either "may" or "can."

3.2 *Definitions:*

3.2.1 *control, n—*the treatment group in a toxicity test consisting of reference soil or artificial soil that duplicates all the conditions of the exposure treatments, but contains no test substance. The control is used to determine if there are any statistical differences in endpoints related to the test substance.

3.2.2 *eluate, n—*solution obtained from washing a solid with a solvent to remove adsorbed material.

3.2.3 *hazardous substance, n—*a material that can cause deleterious effects to plants, microbes, or animals. (A hazardous substance does not, in itself, present a risk unless an exposure potential exists.)

3.2.4 *inhibition, n—*a statistically lower value of any endpoint compared to the control values that is related to environmental concentration or application rate.

3.2.5 *leachate, n—*water plus solutes that has percolated through a column of soil or waste.

3.2.6 *test material, n—*any formulation, dilution, etc. of a test substance.

3.2.7 *test substance, n—*a chemical, formulation, eluate, sludge, or other agent or substance that is the target of the investigation in a toxicity test.

3.2.8 *toxicant, n—*an agent or material capable of producing an adverse response (effect) in a biological system, adversely impacting structure or function or producing death.

3.2.9 *toxicity endpoints, n—*measurements of organism response such as death, growth, developmental, or physiological parameters resulting from exposure to toxic substances.

3.3 *Definitions of Terms Specific to This Standard:*

3.3.1 *chlorotic, adj—*the discoloration of shoots that occurs as chlorophyll is degraded as a result of disease, toxic substances, nutrient deficiencies, or senescence.

3.3.2 *coleoptile, n—*the protective tissues surrounding the growing shoot in a monocotyledonous plant.

3.3.3 *cotyledon, n—*a primary leaf of the embryo in seeds, only one in the monocotyledons, two in dicotyledons. In many of the latter, such as the bean, they emerge above ground and appear as the first leaves.

3.3.4 *cutting, n—*a vegetative segment of a plant, usually a stem that contains several nodes and associated buds, that can be used to regenerate an entire plant.

3.3.5 *dead test plants, n—*those individuals that expired during the test observation period as indicated by severe desiccation, withering, chlorosis, necrosis, or other symptoms that indicate non-viability.

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

3.3.7 *development, n—*the series of steps involving cell division and cell differentiation into various tissues and organs.

3.3.8 *dicotyledon, n—*in the classification of plants, those having two seed leaves.

3.3.9 *dormancy, n—*a special condition of arrested growth in which buds, embryos, or entire plants survive at lowered metabolic activity levels. Special environmental cues such as particular temperature regimes or photoperiods are required to activate metabolic processes and resume growth. Seeds that require additional treatment besides adequate moisture and moderate temperature to germinate are said to be dormant. (See *quiescence*.)

3.3.10 *emergence, n—*following germination of a plant, the early growth of a seedling that pushes the epicotyl through the soil surface.

3.3.11 *enhanced growth and yield, n—*when a treated plant exhibits shoot growth, root elongation, lateral root growth, or yield significantly greater than the control values, the plant is "enhanced" or "stimulated."

3.3.12 *epicotyl, n—*that portion of an embryo or seedling containing the shoot. It is delineated anatomically by the transition zone which separates the epicotyl from the hypocotyl.

3.3.13 *fruits, n—*the reproductive tissues derived from the ovary in the case of epigenous flowers or the ovary and accessory tissues in the case of hypigenous flowers.

3.3.14 *germination, n—*the physiological events associated with re-initiation of embryo growth and mobilization of reserve nutrients in seeds. The emergence of the seedling radicle from the seed coat defines the end of germination and the beginning of early seedling growth.

[4] Available from Standardization Documents Order Desk, DODSSP, Bldg. 4, Section D, 700 Robbins Ave., Philadelphia, PA 19111-5098, http://www.dodssp.daps.mil.

[5] The boldface numbers in parentheses refer to the list of references at the end of this guide.

3.3.15 *growth, n*—a change in size or mass measured by length, height, volume, or mass.

3.3.16 *hypocotyl, n*—that portion of an embryo or seedling containing the root or radicle. It is delineated anatomically by the transition zone which separates the epicotyl from the hypocotyl.

3.3.17 *inhibited plant growth and yield, n*— plant growth, root length and lateral root growth, or yield are "inhibited" when their measurements are significantly less than the control values.

3.3.18 *lateral roots, n*—roots growing off the primary roots, also referred to as secondary roots.

3.3.19 *monocotyledon, n— in the classification of plants*, those having a single seed leaf.

3.3.20 *mottled, adj*—marked with lesions, spots or streaks of different colors. This includes the discoloration of leaf margins.

3.3.21 *phytotoxicity, n*—a lethal or sub-lethal response of plants to a toxicant.

3.3.22 *quiescence, n*—a condition in buds, embryos, or entire plants characterized by lowered metabolic rates and limited or no growth. Seeds that germinate when supplied with adequate moisture and moderate temperature are said to be quiescient. (See *dormancy*.)

3.3.23 *radicle, n*—the emerging root of an embryo during germination.

3.3.24 *seed, n*—the propagule of a plant derived from an ovule. It consists of an embryo, a protective covering (seed coat), and may have storage tissue (endosperm).

3.3.25 *shoot, n*—the above-ground portion of a plant consisting of stems, leaves, as well as any reproductive parts that may be attached.

3.3.26 *surviving plants, n*—test plants that are alive at the time observations are recorded.

3.3.27 *viable, adj*—plants capable of resuming metabolic functions and growth are considered "viable." Buds, embryos, or entire plants may be dormant or quiescient and therefore exhibit no growth during the period of observation. Distinguishing dead plants from viable plants with certainty is difficult without special training and sophisticated measures of metabolic function.

3.3.28 *withering, v*—becoming limp or desiccated, deprived of moisture; often the result of root damage.

4. Summary of Phytotoxicity Tests

4.1 The terrestrial phytotoxicity tests covered under this guide apply to a range of test conditions and test species that can be adapted to meet project-specific objectives. Test organisms are maintained either as seeds or as cuttings until a particular test is to be conducted. A prescribed number of individual plants are introduced into test treatments that include a negative control, a series of positive controls, and one or more test-substance treatment concentrations. The treatment concentrations may be known or unknown; nominal or measured, depending on the nature of the investigation. In the case where the test substance is evaluated as an additive to soil, a range of concentrations is recommended. In tests of environmental samples that already contain a putative phytotoxic substance, the tests may be conducted with either the test soil as collected from the field, or as diluted with a suitable reference soil. Another variant of the tests allows for amendments, or spikes, of selected toxic substances to be added to environmental samples. Finally, in the case of the root elongation assay, eluates, effluents, or other aqueous derivatives of a soil sample are tested.

4.2 Plants are exposed to the test substances in the form described in the specific variations of the tests for a discrete period of time that ranges from 96 h to several months. For short tests, no nutrient additions or amendments are needed or recommended as the amendments may interact with the toxicant and alter the toxicity response. For tests lasting more than two weeks, nutrient additives may be warranted, depending on the test objectives, in order to maximize the potential for plant growth and development. Thinning, culling, or replacing individual plants must not be done once exposure of plants to a test substance has begun as such actions invalidate the test through the introduction of bias or variable test duration among test organisms. At intermediate times, and at the conclusion of the exposure period, tallies of survival and measures of shoot growth and development are made.

4.3 For phytotoxicity tests, 100 to 200 µmol m^{-2} s^{-1} of visible light (or photosynthetically active radiation, 400 to 700 ηm) has been found to be a broadly applicable fluence rate. In some cases, different light levels or spectral ranges (for example, solar ultraviolet) may be required. Guide E1733.

4.4 Measured endpoints and other observational data are used to calculate response levels in terms of ECxx or ICxx (where xx refers to a specified percentage response), or categorical descriptions of phytotoxic effects (for example, proportion of plants exhibiting abnormal development or other symptomatic indices that might be scored in qualitative terms) relative to controls. These are interpreted to characterize phytotoxic effects attributed to test substances.

5. Significance and Use

5.1 Terrestrial phytotoxicity tests are useful in assessing the effects of environmental samples or specific chemicals as a part of an ecological risk assessment (**3-6**, **12**, **13**).

5.2 Though inferences regarding higher-order ecological effects (population, community, or landscape) may be made from the results, these tests evaluate responses of individuals of one or more plant species to the test substance.

5.3 This guide is applicable for: (*a*) establishing phytotoxicity of organic and inorganic substances; (*b*) determining the phytotoxicity of environmental samples; (*c*) determining the phytotoxicity of sludges and hazardous wastes, (*d*) assessing the impact of discharge of toxicants to land, and (*e*) assessing the effectiveness of remediation efforts.

6. Apparatus

6.1 *Facilities*—The preparation of the test, test soil medium, storage of soil and seeds, and all stages of a test procedure must

take place in an atmosphere free from toxic contamination and vapors. The facility, whether a glasshouse or a growth chamber, should have reasonable temperature control and monitoring, as well as supplemental lighting. In general, the facility should be capable of maintaining uniform temperatures in the 20 to 30°C range. Lighting should provide at least 100 µmol $m^{-2}s^{-1}$ Photosynthetically Active Radiation (PAR) controlled on a clock timer to maintain a specified diurnal cycle. See appropriate annex for any specific requirements of a given test.

6.2 *Equipment and Supplies:*

6.2.1 *Plant Pots*—Containers should be chosen to be inert to test and control substances. The test or control substances should not adhere to or react in any way with the container. Glass, stainless steel, or paper containers with drainage holes can be used as plant pots. Paper or other natural fiber materials may absorb test substances. If pots with drainage holes are used, then a secondary container or shallow dish should be used to prevent cross-contamination among test units. 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 for the duration of the test. It is suggested that the selection of growth containers not be arbitrary, and that the appropriate size, shape, color, and composition of the container be considered for each plant species and toxicity test undertaken.

6.2.2 *Balance*—Sensitivity to 0.001 g.

6.2.3 *pH Meter*—Sensitivity to 0.1 units.

6.2.4 *Photometer (Radiometer)*—Capable of measuring the photosynthetically active range. Fluence rate of incident light should be expressed as µmol m^{-2} s^{-1}.

6.2.5 *Thermometer*—A continuous recording thermometer or a maximum-minimum thermometer that is checked daily. Many continuous recording units also record humidity.

6.2.6 *Industrial Mixer or Cement Mixer*—A revolving or rotating mixer is recommended for combining test substances or test soils with large volumes of control or reference soil medium.

6.2.7 *Reagent Water*—Unless otherwise indicated, references to water shall be understood to mean reagent water conforming to Specification D1193, Type III. Type III water may be prepared by distillation, ion exchange, reverse osmosis, or a combination of methods.

6.2.8 *Equipment Care*—Clean the test equipment after each use. Wash all new containers with a detergent and rinse thoroughly with water, pesticide-free acetone, dilute acid (such as 5 % hydrochloric acid), and at least twice with tap or clean water. Final rinses with Specification D1193 Type III water or equivalent is recommended. Clean equipment, such as the mixer and mixer blades by a procedure known to remove constituents of the test substance. Paper and plastic plant pots should be disposed after one use.

7. Test Material

7.1 *Chemical Substance:*

7.1.1 *General*—The test substance should be reagent-grade or better, unless a test on a formulation, commercial product, or technical-grade or use-grade substance is specifically needed. Before a test is initiated, the following information should be obtained about the test substance: identities and concentration of major ingredients and major impurities, for example, impurities constituting more than about 1 % of substance; solubility and stability in dilution water; an estimate of toxicity to the test species (a range-finding study may be required); precision and bias of the analytical method at the planned concentration(s) of the test substance; and an estimate of toxicity to humans and other potentially exposed organisms.

7.1.2 *Test Concentrations*—Chemical concentrations in soils are expressed as dry weight to dry weight. It is preferable to add the test substance directly to the test medium, however, a stock solution may be prepared and aliquots added to each test solution or test chamber. Special considerations regarding chemical degradation, complexing, and volatilization and other factors that might influence bioavailability should be evaluated to determine the appropriate mixing, handling, and storage procedures to be used. The number of selected test concentrations should be based on the goal of the study. Multiple concentrations can be used to calculate ICxx values, whereas, testing at a single concentration can be used to obtain rapid, simple answers. When the interest is (*a*) in the effect of a specific concentration of test substance on the growth of the test species or (*b*) whether or not the ICxx value is above or below a specific concentration, only one concentration and the controls need to be tested.

7.1.3 *Stock Solution*— For compounds with low water solubility, a solvent can be used to make a stock solution. If a stock solution is used, the concentration and stability of the test substance in the stock should be determined before the beginning of the test. If the test substance is subject to photolysis or other photo-reactive processes, the stock solution should be shielded from light. If a solvent is necessary, its concentration in test solutions should be kept to a minimum (not greater than 1 % [volume to volume or weight to volume]), and should be low enough that it does not affect either survival or growth of the test organisms. (These limitations do not apply to any ingredients of a mixture, formulation, or commercial product unless an extra amount of solvent is used in the preparation of the stock solution.) If the concentration of solvent is not the same in all test solutions that contain test substance, either (*a*) a solvent test must be conducted to determine whether either survival, or growth of the test species is related to the concentration of solvent over the range used in the phytotoxicity test or (*b*) such a solvent test must have already been conducted using the same dilution water and test species. If either survival or growth is found to be related to the concentration of solvent, a test would be unacceptable if any treatment contained a concentration of solvent in the response range. If neither survival, or growth is found to be related to the concentration of solvent, a toxicity test with that same species in the same water may contain solvent concentrations within the tested range, but the solvent control must contain the highest concentration of solvent present in any of the other treatments.

7.1.4 *Soil Medium*— Natural soil (free of chemical contamination), commercial potting soil, synthetic soil mixes, or washed quartz sand may be used as the "soil medium." Each

choice has substantive limitations for various phytotoxicity investigations. Natural soils are not easily demonstrated to be free of toxic substances. Some commercial potting soils may adversely affect growth and survival of some plants. Synthetic mixes may not be representative of real world conditions. Quartz sand or glass beads offer only a physical matrix; and therefore do not provide a realistic soil condition with regard to binding and exchange sites. It may be especially important to consider soil texture, pH, organic matter or other physical-chemical properties before embarking on a test. Preliminary trials are often valuable to ascertain the suitability of a particular soil medium for the test species and conditions to be investigated.

7.2 *Environmental Sample:*

7.2.1 *Liquid, Sludge, or Slurry*—These environmental samples may be handled as chemical additives described above. As complex mixtures, however, the test concentrations will most likely be handled as percentage dilutions of the 100 % sample concentration. In some cases, selected chemical analyses may be warranted as a means of expressing concentrations of selected constituents in ppm or molar values. All of the provisions described for single chemicals apply.

7.2.2 *Soil*—Site soils may be collected as cores or as bulk samples from specified soil depths (for example, 0 to 15 cm depth). Sampling and handling procedures may be found in Practices D4547 and D5633. The soil samples may be tested directly (that is, 100 % site soil) or diluted with an appropriate reference soil or a synthetic soil mixture to achieve specified relative concentrations. In some cases, selected chemical analyses may be warranted as a means of expressing concentrations of selected constituents in ppm (dry weight basis) or molar values.

7.2.3 *Eluates*—Aqueous extracts of soils are sometimes desired to evaluate the phytotoxicity of water-soluble soil constituents. The eluates are used in the same manner as liquid environmental samples described above.

8. Hazards

8.1 Many materials can adversely affect humans if safety 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, 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 or dust containing hazardous substances. Respirators may be warranted. Information on toxicity to humans (**14-18**), recommended handling procedures (**19-22**), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radio-labeled test materials (**23, 24**) and with test materials that are, or are suspected of being, carcinogenic (**25**).

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

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

9. Test Organisms

9.1 *Test Species*— The majority of species routinely used in phytotoxicity tests has been limited to agronomic plants. Under FIFRA guidelines (**4, 5**), ten species belonging to eight families are listed for toxicity testing (see Table 1). The United States Food and Drug Administration (**11, 26**), has relied on plant tests similar to those for FIFRA (see Table 1). International guidance (**10**) uses agronomic species, but has a broader selection of plants compared to United States guidance. CERCLA offers limited guidance with respect to plant testing. General methods recommended for the Remedial Investigation Baseline Risk Assessment portion of work listed by name only the seed germination and root elongation assays (**3, 6**). Only lettuce (*Lactuca sativa*) is listed as the standard species of the test, although "other (taxa) can be used." The Department of Interior in developing rules for Natural Resource Damage Assessment (**27**) referred to "economically important plant species." Thirty-one plant taxa are explicitly identified in federal and international test guidelines and standard test procedures (see Table 1). Many additional plant taxa including aquatic taxa were reported in phytotoxicity literature (see Table 2). Nearly a hundred plant taxa (see Table 2) have been used routinely to study phytotoxicity. In an early version of PHYTOTOX (**28**), 1569 plant species from 682 genera in 147 families were reported in the records. However, 42 % of the records referred to only 20 species.

9.2 *Purchase*—Seeds of the most commonly used taxa identified in FIFRA guidelines may be purchased from commercial seed companies. Many of the less common taxa are available from specialty seed companies, especially those that service landscaping and restoration activities. When purchasing seeds, it is best to talk to technical staff of the supplier to gather important information regarding the seed lot, collection, handling and storage practices of the seed company, germination percentage expected, and any special conditions affecting germination. Generally it is preferable to use untreated seeds (that is, not treated with fungicide, repellents, or other chemical agents) in phytotoxicity tests, however, specific test objectives may permit use of treated seeds. The principal investigator should detail the rationale for using treated seeds. Seeds should be acquired at least annually. At a minimum, a sufficient quantity of seeds should be acquired to allow tests of all treatments (including controls) to be conducted with seeds from the same batch.

9.3 *Collection*—If seeds are collected from the field, care must be taken to ensure that seeds from only a single species are obtained. The following minimum set of information should be recorded for each batch of seeds collected: the location of the collection site as precisely as practicable (for

TABLE 1 List of Plant Species Identified in Regulatory Documents and in Standard Test Procedures[A]

Family	Species	Common Name	FIFRA	TSCA	FDA	OECD	APHA AWWA	ASTM ESG
Chenopodiaceae	*Atriplex patula*	Seaside Greens					√	
Compositae	*Lactuca sativa*	Lettuce	√	√	√	√		√
Cruciferae	*Brassica alba*	Mustard				√		√
Cruciferae	*Brassica campestris var. chinensis*	Chinese Cabbage				√		√
Cruciferae	*Brassica napus*	Rape				√		√
Cruciferae	*Brassica oleracea*	Cabbage	√	√	√			√
Cruciferae	*Brassica rapa*	Turnip				√		√
Cruciferae	*Lepidium sativum*	Cress				√		√
Cruciferae	*Raphanus sativus*	Radish				√		√
Cruciferae	*Rorippa nasturtium-aquaticum*	Watercress					√	
Cucurbitaceae	*Cucumis sativus*	Cucumber	√	√	√			√
Leguminosae	*Glycine max*	Soybean	√	√	√			√
Leguminosae	*Phaseolus aureus*	Mungbean				√		√
Leguminosae	*Phaseolus vulgaris*	Bean			√			√
Leguminosae	*Trifolium ornithopodioides*	Fenugreek				√		√
Leguminosae	*Trifolium pratense*	Red Clover				√		√
Leguminosae	*Vicia sativa*	Vetch				√		√
Liliaceae	*Allium cepa*	Onion	√	√				√
Nymphaeceae	*Nelumbo lutea*	American Lotus					√	
Poaceae	*Avena sativa*	Oat	√	√	√	√		√
Poaceae	*Echinochloa cursgalli*	Japanese Millet					√	
Poaceae	*Leersia oryzoides*	Rice Cutgrass					√	
Poaceae	*Lolium perenne*	Perennial Ryegrass	√	√	√	√		√
Poaceae	*Oryza sativa*	Rice				√	√	√
Poaceae	*Sorghum bicolor*	Sorghum				√		√
Poaceae	*Spartina alterniflora*	Smooth Cordgrass					√	
Poaceae	*Triticum aestivum*	Wheat			√	√		√
Poaceae	*Zea mays*	Corn	√	√	√			√
Poaceae	*Zizania aquatica*	Wild Rice					√	
Solonaceae	*Lycopersicon esculentum*	Tomato	√	√	√			√
Umbelliferae	*Daucus carota*	Carrot	√	√	√			√

[A] FIFRA = Federal Insecticide, Fungicide, and Rodenticide Act **(4)** **(5)** ; TSCA = Toxic Substance Control Act **(2)** ; FDA = Federal Drug Administration **(11)** **(26)**; OECD = Organization for Economic Cooperation and Development **(10)**; APHA = American Public Health Association; AWWA = American Water Works Association **(1)**; and ASTM = American Society for Testing and Materials (Practice E1598).

example, section, township and range, county, state); the persons collecting the seeds; date of collection; description of noteworthy circumstances such as drought, flood, condition of surrounding landscape, and any indication of pesticide use in the vicinity; and quantity of seeds collected.

9.4 *Grading and Sizing Seeds:*

9.4.1 *Domestic Species—* Seeds of a given species vary in size, shape, and in some cases, color. These differences in external features of the seed are often associated with different rates of germination or even different germination requirements. To minimize the variance in test results the investigator should determine whether such variants in seed size, shape, or color are critical to the investigation. (For example, alfalfa seeds often come as a mixture of light-colored and dark-colored seeds. The dark-colored seeds have low percentage germination (~10 %), while the light-colored seeds have high percentage germination (~90 %).) Separation of broken or damaged seeds from the batch is important. Various sieves or screens may be useful in separating the seeds. Lettuce for example can be separated mechanically using wire mesh screens: 1⁄6 × 1⁄28 in.; 1⁄6 × 1⁄30 in.; 1⁄6 × 1⁄32 in.; 1⁄6 × 1⁄34 in. Red clover may be sized using perforated metal sheets with round holes of the following diameters: 1⁄19 in., 1⁄18 in., 1⁄17 in., 1⁄16 in.

9.4.2 *Native Species—* If this test uses native plant seeds rather than commercially selected plants, considerable care should be taken in sizing and sorting seeds collected. Numerous studies have shown that the variability in seed germination is not entirely random within a population of a particular species. The point during the growing season at which a lot of seeds are produced and collected will affect germination in many species. Also, the location within a particular inflorescence (for example, with composites) will also affect germination. There can also be considerable intra-species variation between remote populations. The test design becomes considerably more complicated to account for these and other potential sources of variation.

9.5 *Seed Storage and Maintenance*—Seeds should be stored in a desiccator and refrigerated until needed (preferably at 4 ± 2°C). It is recommended no disinfecting agent such as hypochlorite be used. Exceptions may be warranted for some investigations if gnotobiotic conditions are desired, however, such special cases must be described fully as exceptions to the guide described here. Examples of exceptions would include, but not be limited to, amendments with microbial inocula such as rhizobia for legumes, actinomycetes for actinorhizal species, or mycorrhizal fungi.

9.6 Seedlings or cuttings may be collected from the field, propagated by the investigator, or purchased from nurseries, horticulture supply houses, or research laboratories. As with seeds, it is important to document as much information as

reasonable for each batch of cuttings obtained. Care should be taken to limit the range of stem size, age, and developmental stage of the plant.

TABLE 2 Partial Listing of Plant Taxa studied for Toxicity Effects

Species	Common Name	Ref.
Agrostis alba	red top	(29)
Agrostis sp.	bentgrass	(29)
Apocynum sp.	milkweed	(30)
Arabidopsis thaliana	mouse-ear-cress	(30)
Arachis hypogaea	peanut	(29)
Avena sativa	oats	(29), (30), (31)
Beta vulgaris	beets	(29), (30), (31)
Beta vulgaris	chard	(29)
Beta vulgaris	sugarbeet	(30), (31)
Brassica campestris	kale	(29), (31)
Brassica nigra	mustard	(29), (30), (31)
Brassica oleracea	broccoli	(29)
Brassica oleracea	cauliflower	(29)
Brassica rapa	turnip	(29)
Bromus	smooth bromegrass	(29)
Bromus japonicus	Japanese bromegrass	(29)
Cenchrus ciliaris	buffelgrass	(29)
Chrysanthemum sp.	chrysanthemum	(31)
Citrus sinesnsis	orange	(31)
Cucumis sativa	cucumber	(29), (30)
Cyperus esculentus	yellow nutsedge	(32)
Dactylis glomerata	orchardgrass	(29)
Daucas carota	carrot	(29), (31)
Echinochloa crusgalli	barnyard grass	(33)
Elodea densa	elodea	(30)
Eragrostis curvula	weeping lovegrass	(29)
Eragrostis lehmanniana	Lehman lovegrass	(29)
Erysimum capitatum	wall flower	(31)
Fagopyrum esculentum	buckwheat	(31)
Festuca arundinacea	tall fescue	(29), (30)
Festuca pratensis	meadow fescue	(31)
Feasted rubber	red fescue	(29)
Forgery sp.	strawberry	(31)
Gladiolus sp.	gladioli	(31)
Glycine max	soybean	(29), (30)
Gossypium	cotton	(31)
Helianthus annuus	sunflower	(31)
Hordeum vulgare	barley	(30), (31)
Lactuca sativa	lettuce	(29), (30), (31)
Lemna gibba	duckweed	(30)
Lemna minor	duckweed	(30)
Lespedeza sp.	lespedeza	(29)
Lolium perenne	perennial rye	(29), (30)
Lotus corniculatus	birdsfoot trefoil	(29)
Ludwigia natans	floating loosestrife	(30)
Lupinus sp.	lupine	(29)
Lycopersicon esculentum	tomato	(29), (31)
Medicago sativa	alfalfa	(29), (30), (31)
Melilotus albal	white sweet clover	(29), (30)
Melilotus officinale	yellow sweet clover	(29)
Musa paradislaca	banana	(31)
Nicotiana tabaccum	tobacco	(31)
Oryza sativa	rice	(31)
Panicum miliaceum	millet	(30)
Panicum virgatum	switchgrass	(29)
Phaseolus sp.	beans	(30), (31)
Phaseolus vulgaris	pinto beans	(30)
Phleum pratense	Timothy grass	(29), (31)
Pinus talda	loblolly pine	(30)
Pistia statiotes	water lettuce	(30)
Pisum sativum	pea	(31)
Poa pratense	Kentucky bluegrass	(29)
Raphanus sativus	radish	(29), (30)
Rubus sp.	raspberry	(31)
Setaria italica	foxtail millet	(30)
Solanum tuberosum	potato	(30), (31)
Sorghum bicolor	sundangrass; sorghum	(29), (30), (31)
Spartina alterniflora	cordgrass	(33)
Spinacia oleracea	spinach	(29), (31)
Spirea alba	meadowsweet	(31)
Spirea alba	meadow sweet	(31)
Tagetes sp.	marigold	(29)
Thalassia testidinum	seagrass	(30)
Tradescantia paludosa	spiderwort	(30)
Trifolium pratense	clover	(30)
Triticum aestivum	wheat	(30), (31)
Vicia faba	broad bean	(30)
Vicia sp.	vetch	(29)
Zea mays	corn	(30), (31)

10. Sample Handling and Storage

10.1 The proper collection, packaging, and shipping of waste site samples is critical. Proper sampling and shipping ensures sample integrity, handling safety, and an adequate data base for sample processing and future sampling requirements. Local, state, and federal shipping regulations should be consulted regarding size and quantity restrictions, labeling, and documentation requirements. Sample packaging depends upon the type of sample. Double bagging is recommended. Soils and sediments may be stored in a plastic bag which is in turn placed in a second protective plastic bag before placing in a pail. The plastic bags as well as the pail should be sealed with tape.

10.2 Proper labeling should be placed inside and outside of all containers during the packaging process. All containers will be identified in accordance with specific requirements and sampling and shipping information recorded on a sample data sheet. The U.S. Department of Transportation regulations provide information governing shipping. Labeling must comply with Department of Transportation (DOT) CFR-49 specifications. These specifications are found in Section 172 of the DOT Hazardous Materials Shipping and Handling Regulations. These regulations can be found at the office of any carrier authorized to haul hazardous materials. If soils contain potential biohazards, special permits may be required to cross state lines or to be imported.

11. Calibration and Standardization

11.1 Calibration and standardization of routine laboratory equipment and growth chambers used in this toxicity test will follow manufacturers' recommended practices. In addition, any relevant ASTM methods to a particular procedure will also be followed.

12. Test Conditions

12.1 The annex for each specific test method should be consulted for detailed procedures. The investigator is urged to develop optimal test treatments to satisfy statistical demands of each study. In some cases it may be advisable to adjust the number of treatments and the number of replicates in order to increase the power of the test. (Refer to Section 15 for additional discussion of statistical issues related to test design.)

12.2 *Negative Control*— The negative control should consist of the identical solution (water, organic solvent, or nutrient solution) used to introduce the test substance into the soil medium.

12.3 *Positive Control*— Boron as boric acid may be used as the positive control (**34, 35, 36**). A watering solution of boric

acid at the desired concentrations is added to the test soil. A 0.5 dilution series (that is, 10, 20, 40, 80, 160, 320, and 640 mg kg^{-1} soil dry weight) brackets sensitivity of most plant species tested to date. Once the range of sensitivity is established for a species, fewer test concentrations are needed. However, different soils alter the bioavailable fraction and therefore, preliminary tests are recommended for each new soil medium tested. Alternative positive controls may be selected to meet the objectives of a specific investigation. In selecting alternative substances for use as positive controls, the investigator should consider potential health effects to workers, interference of test substance with soil constituents, known mode of action of the substance and therefore appropriateness for use with different plant species, and disposal restrictions.

12.4 *Seed Planting*— A template made of stainless steel or wood may be used to make holes approximately 2.5 to 4.0 cm deep in the soil for large seeds, (for example, corn and beans), and 1.0 to 1.5 cm deep for smaller seeds. Templates only help standardize planting in large scale testing; for most purposes manual planting will suffice. Seeds should be planted at a soil depth 1.5 to 2 times the seed diameter. It is suggested that a minimum of 25 seeds be planted per concentration (for example, five replicates of five or more seeds each). Increasing the number of seeds or plants per treatment improves the ability to distinguish treatment effects. There may be instances that a single seed would be placed in a test container. After the seeds have been placed in the holes in the soil, tap the pots lightly to cover the seeds. Additional soil may be required to fill the pots once they have settled. The plant pots that contain the test substance mixed throughout the soil medium should be watered to bring them to field moisture capacity. Sub-irrigation is preferred, as this minimizes disturbance to the planted seeds. Those pots that will be exposed via sub-irrigation can be hydrated at this time. Excess water should be allowed to drain from the pots that are sub-irrigated before placing them in an environmental chamber or greenhouse.

12.5 *Soil Water Holding Capacity*—In some testing situations, it is desirable to know the quantity of water that can be stored in a soil. For some species, germination is improved if the soil is maintained at approximately 85 % water holding capacity. Whether test soils are saturated or maintained at less than saturation (for example, 85 %), all treatments and replicates should be handled similarly. Water holding capacity is expressed as a percentage of soil dry weight. To determine the water holding capacity of a soil, saturate a volume of soil with water and allow to drain for one hour. After the excess water has drained from the soil, measure the weight of the saturated soil. The soil is then dried in an oven (105°C) until constant weight is achieved. The water held by the soil is determined as the difference in saturated weight and the dry weight.

12.6 *Test Condition Monitoring:*

12.6.1 The light irradiance level (fluence rate) should be determined at the start and conclusion of a test with the radiometer or quantum sensor that detects PAR. Light measurements should be repeated anytime during the test if events that potentially affect the light sources occur (for example, light bulb replacement). Adjustments to supplement lighting may be necessary. In some cases full spectrum (PAR plus Ultraviolet) light may be required (see Practice E1733).

12.6.2 Air temperature should be monitored at least daily. It is recommended that the air temperature and relative humidity be monitored continuously and recorded with the use of a seven-day recorder. A thermal probe can be used to measure soil temperature of representative plant pots.

12.6.3 The relative humidity may be monitored continuously and recorded using a seven-day recorder or an instrument equipped with an electronic datalogger. Relative humidity generally should be maintained above 30 % (recommended approximately 50 %). It may be necessary to increase the relative humidity in the growth chamber or the greenhouse if the soil dries rapidly.

12.6.4 Soil pH (or pH in water) should be checked the day the test soil medium is prepared, and again at the end of the study. The soil pH is determined by placing 100 g of soil in a 250-mL flask containing 100 mL of distilled water. The resulting slurry is mixed for 30 s to 1 min, left to stand for 1 h, then measured with the appropriate pH electrodes and meter (**37**). The pH of a soil may require adjusting if outside the optimum growing range from 6.0 to 7.5. The pH of an acid soil can be raised by the addition of calcium carbonate. By adding an acid, such as sulfuric acid, gypsum, or ammonium sulfate to a soil, the pH can be lowered (see Note 1). The addition of calcium carbonate, gypsum, ammonium sulfate, sulfuric acid, or other additives to change soil pH should be selected so that they do not interfere with the test/control substances.

NOTE 1—**Caution:** Caution should be used when working with an acid.

13. Interference and Limitations

13.1 Toxic substances can be introduced as contaminants in dilution water, glassware, sample hardware, and testing equipment. In addition, high concentrations of suspended dissolved solids, or both, can mask the presence of toxic substances. Improper hazardous waste sampling and eluate preparation also can affect test results adversely. Pathogenic or herbivorous organisms, or both, in the dilution water and test samples can affect test organism survival, thereby confounding test results.

13.2 Several potential matrix interference problems can limit bioavailability of toxic substances. This includes, but is not limited to: differential solubility across a range of pH values; precipitation as sulfides or oxides with several cations; and covalent bonding of organic substances with humic acid. Matrix attributes such as soil texture, soil structure, aeration, and soil-borne pathogens can limit seedling emergence. Caution must be used in all interpretations of causality to ensure that the measured differences in endpoint response are attributable to toxic materials and not merely matrix interference problems.

13.3 Volatile substances are readily lost from the soil medium resulting in a rapidly changing exposure concentration.

13.4 Environmental samples may contain a few to many viable seeds. During the test, the seedlings emerging from this seed bank must not be misinterpreted as emergence of test species seedlings.

13.5 Interpretation of phytotoxicity from tests with seeds must be tempered to reflect ecological aspects regarding ecophysiology of seeds. First, the seed has evolved to protect the embryo of adverse environmental conditions. Physical, chemical, and physiological barriers characteristic of many species, especially seeds of nondomesticated species, limit exposure of the embryo to environmental conditions, including toxic chemicals. Second, except for annual species, many species effectively reproduce vegetatively. For those species, impaired germination may not pose a substantive ecological problem.

14. Quality Assurance and Quality Control

14.1 Quality assurance (QA) practices include all aspects of the test that affect the accuracy and precision of the data, such as: sampling and handling, source and condition of the test organisms, condition of equipment, test conditions, instrument calibration, use of reference toxicants, and record keeping.

14.2 The test may be conditionally acceptable if temperature and other specified conditions fall outside specifications, depending on the degree of the departure and the objectives of the test. The acceptability of the test depends on the best professional judgment and experience of the investigator. Any deviation from test specifications is noted when reporting data from the test.

14.3 Temperature must be maintained within the limits specified for the test. Soil pH will be checked using a standard method (**37**) at the beginning of the test and, if necessary, at the end of the test period.

14.4 *Test Acceptability:*

14.4.1 Test results are considered acceptable for the individual plant species if the following are fulfilled: the mean control seedling growth does not exhibit phytotoxicity or developmental effects, and survival through the duration of the exposure period meets minimum standards for that species. The USDA established the following percentage germination standards: field corn (85 %), popcorn (75 %) sweet corn (75 %), carrot (55 %), onion (70 %), tomato (75 %), field-garden bean (70 %), pea (80 %), pepper (55 %), beet (65 %), buckwheat (60 %), cabbage (75 %), lettuce (55 %), mustard (75 %), soybean (75 %), sugarbeet (55 %), wheat (80 %), oats (80 %), barley (80 %), rice (80 %), ryegrass (75 %), vetch (75 %), alfalfa (70 %), clover (70 %), and rape (75 %) (**38**). Alternatively, the criterion for acceptance of control seedling emergence may be established statistically as within ±2 S. D. of mean for the species. The test should be repeated for those plant species for which the criterion is not met. Seeds that fail to germinate at the stated response shall be discarded and new seeds purchased.

14.4.2 Contamination of the test substance, or soil medium, or other laboratory accidents, have not occurred such that the integrity of the test might have been affected.

14.4.3 The results of the reference toxicant tests are unacceptable if mean control survival is less than 80 %. The results of the definitive toxicity tests are also unacceptable if control survival is less than 80 %, unless a lower criterion value was established for the species.

15. Calculations and Interpretation of Results

15.1 Test data are presented in tabular form. Data are presented for each species tested. Where suitable, appropriate statistical analysis is carried out. At a minimum, the means, with 95 % confidence limits, and standard deviations for each of the quantitative sets of data are presented. Summary data may also be reported as EC50 values, (for example, concentrations which inhibit emergence, root elongation, or other suitable endpoint by 50 % relative to the negative control data). Analysis of variance (ANOVA) can be computed using each set of data collected on the last day of the test. All data is used in these calculations, unless justification can be given for excluding outliers. Ease of data management, calculation, charting, and reporting may be aided through the use of spreadsheets such as Excel, Lotus, or equivalent software systems. Data analysis may be performed with suitable software programs to calculate descriptive statistics and median effect values. Please note that in some instances data may not be distributed normally, may have unequal variances, and transformations may not correct the situation. In such cases, non-parametric tests are warranted.

15.2 The mean and standard deviation of the biological effects (for example, number emerged) are calculated for each replicate test concentration. The percent effect is then calculated using the following formula:

$$\text{percent effect} = \frac{(\text{control endpoint mean} - \text{treatment endpoint value}) \times 100}{\text{control endpoint mean}} \quad (1)$$

15.3 Percentage difference between treatment seedlings and the control seedlings that are less than 10 % typically are not considered biologically relevant even if statistical significance is demonstrated. Additional statistical analysis that may be appropriate for the data include: linear regression, multiple range test, Dunnett's, Scheffe's Test; one-way ANOVA; Levene's Test for Equal Variances; and Power Calculations for the ANOVA.

15.4 Linear or non-linear regression analysis can be used to obtain point estimates of concentrations which cause specified toxicity effects (that is, EC50). Several methods of regression analysis for quantal data (for example, percentage of seeds germinated) are commonly used, including logit, probit, moving average, trimmed Spearman-Karber, and Litchfield-Wilcoxin. For continuously distributed endpoints (for example, height, length, mass) regression of raw data or of transformed data may be performed if the statistical assumptions are met. Please note that the power of the regression analysis may be enhanced substantially by increasing the number of treatments and the number of replicates per treatment. This may be particularly useful in characterizing hormesis responses at low concentrations.

15.5 Prior to regression analysis, scatter plots of the percent effect (*y*-axis) should be plotted against site sample concentration (*x*-axis). The coverage of the regression model should be restricted to an appropriate region of values of the independent variable (percent site sample concentration.) An outlier may be discarded ". . . only if there is direct evidence that it represents

an error in recording, a miscalculation, a malfunctioning of equipment, or a similar type of circumstance" (**39**). It is recommended that a statistician be consulted if it is desired to apply statistical tests to aid in evaluating outliers. Asymptotic portions of the plot may need to be discarded since they can significantly pull the line away from its correct position.

15.6 Plant tests often exhibit hormesis effects (apparent stimulation) near to "no effect" level concentrations. There is disagreement in the technical community as to whether stimulatory responses should be considered adverse or deleterious. Graphical representation of the response versus concentration may be helpful. Methods for calculating regressions may require selection of linear portions of the response range. When data are used in the linear regression which do not fall along the linear portion of the line, the quality of the goodness of fit and confidence levels suffer. Three data points are the absolute minimum that can be used to perform a linear regression of the data. (Depending on method used: Spearman-Karber, Probit, etc. Some require partial effects or two concentrations with no effects.)

15.7 As seeds may fail to emerge because of a lack of germination, death, or slowed growth rate, it may be necessary to uncover planted seeds, seedlings, or remains carefully in order to determine or explain apparently anomalous results. If so, laboratory worker safety procedures need to be adhered to due to the nature of the test samples being studied.

15.8 At the beginning of each project, the principal investigator should determine how data will be collected and handled for plants that die during the test period. An operational definition of what constitutes "death" should be stated. Decision rules regarding proper analysis of the data should consider the assumptions and limitations of the statistical models to be used. For example, analysis of variance techniques are normally used in order to estimate a NOEC or LOEC. If one or more of the treatment groups at the highest concentrations have many dead plants, either treating the dead plants as missing data or as zero can have a negative effect on the statistical analysis. Very unequal *n*'s may result from omitting the plants entirely and unequal within-treatment variances may result from substituting zeros (or other low values). Therefore, a survival analysis is recommended as the first step. If a treatment group is identified as an effect level from the survival analysis, it may be appropriate to omit those data from the analysis of variance on the growth parameters as the omitted groups have already been identified as effect levels. No further statistical testing of them would be required. Moreover, including these data may distort the observed significance levels (*P* values) for the other groups. If there are only a few dead plants in the other treatment groups, they may be treated as missing data for the analysis of variance.

16. Precision and Bias

16.1 Precision describes the degree to which data generated from replicate measures differ. It is the quantitative measure of the variability of a group of measurements compared to their average value. The precision of toxicity tests is determined by replicating the treatments. Comparable procedures for field measurements provide precision estimates derived from statistical distributions of values. Variance, standard deviation, standard error terms, or a combination of these, are reported in defining precision.

16.2 Bias is defined as the bias in a measurement system and is the difference between the value of the measured data and the true value. Determining the bias of the toxicity tests for environmental samples is not possible since the true values cannot be known; no methods directly measure the accuracy of the toxicity tests. Therefore, bias is estimated indirectly by testing the sensitivity of organisms used in the toxicity tests with reference toxicants and by use of toxicity test control blanks.

16.3 *Documentation/Data Management:*

16.3.1 The final submittal contains: the name and address of the testing facility; dates of the study; names of the persons conducting the test; detailed information about the test species, including the scientific name, the source, germination rate if applicable, and lot number; protocol used; number of test species used per concentration or material; a description of detrimental effects determined during the course of the study and at study termination; number and percentage of control organisms that exhibit abnormal growth.

16.3.2 Photographs may be taken of various stages during the study, or to document abnormal growth, where appropriate. Any amendments or deviations from the method described herein, and any other relevant information, are included.

ANNEXES

(Mandatory Information)

A1. SEEDLING EMERGENCE

A1.1 Scope and Application

A1.1.1 This test evaluates the inhibitory potential of toxic materials regarding germination of terrestrial plant seeds under laboratory conditions. This guide is applicable for: (*a*) establishing phytotoxicity of herbicides and other pesticides; (*b*) determining the phytotoxicity of sludges, and (*c*) assessing the impact of discharge of toxicants or other amendments to land. This test is most useful as a screening tool to examine the likelihood of adverse effects of soil contaminants or soil amendments on potential establishment of vegetation via germination. Phytotoxic effects that occur as a consequence of impairment of photosynthetic systems, flower development, or even early growth of shoots or roots might be underestimated by this test method.

A1.2 Method Summary

A1.2.1 Seeds are germinated in a test matrix which may be a natural soil (free of chemical contamination), commercial potting soil, synthetic soil mixes, or washed quartz sand. (**40, 41, 42**) and Practice E1598). Chemical additives or amendments may be added to formulated or reference soil media at various concentrations. Contaminated soils may be tested at full-strength (that is, 100 %) or diluted with a suitable reference or formulated soil. A positive control (for example, boron as boric acid), and a negative control (deionized water and reference or formulated soil) should be included in the test. The test duration should be approximately twice the time required for normal germination of the test species. The duration of the study may be increased to enable evaluation of seedling growth. The number of emerged seedlings out of total plantings is scored at the termination of the test. Additional metrics and observations regarding shoot and root growth and development are encouraged.

A1.3 Safety

A1.3.1 See Section 8.

A1.4 Apparatus and Equipment

A1.4.1 The preparation of the test soil medium, the test substance, the storage of soil and seeds, and all stages of the test procedure must take place in an atmosphere free from contamination. The growth area should have reasonable temperature control and adequate lighting, with a photoperiod of 16 h on, 8 h off. Equipment includes:

A1.4.1.1 Disposable, sterilized, petri plates or plastic pots (for example, 4 × 4 in. though other sizes may be used).

A1.4.1.2 *Balance*—sensitivity to 0.001 g.

A1.4.1.3 *pH meter*—sensitivity to 0.1 units.

A1.4.1.4 *Photometer (Radiometer)*—Capable of measuring the photosynthetically active range. Fluence rate of incident light should be expressed as μmol m^{-2} s^{-1}.

A1.4.1.5 A continuous recording thermometer or a maximum-minimum thermometer that is checked daily. Many continuous recording units also record humidity.

A1.4.1.6 *Industrial Mixer or Cement Mixer*—A revolving or rotating mixer is recommended for combining test substances or test soils with large volumes of control or reference soil medium.

A1.4.1.7 *Routine laboratory glassware and materials*—funnels, graduated cylinders, beakers, stainless steel spatulas and scoops, filter paper, laboratory sealant film, marking pens, disposable latex gloves, notebooks, resealable bags, laboratory coats, and certified respirators.

A1.5 Procedure

A1.5.1 *Pretest Documentation:*

A1.5.1.1 Petri plates or pots are labeled with project identification, test sample identification, species, and replicate number.

A1.5.2 *Test Procedure:*

A1.5.2.1 Approximately 100 to 300 g (nominal dry weight) of test soil medium is placed in each petri plate or pot. Five replicates of each soil sample, additive, or amendment treatment, a positive control, and a negative control are tested. Each replicate container should be planted with 5 or 20 seeds depending on the size of the seed and seedling and test requirements. Fewer seeds may be used in specialized cases such as large seeds or very rare species, however, use of fewer seeds reduces the precision of the test. Seeds should be planted at a soil depth 1.5 to 2 times the seed diameter. Deionized water should be used to bring the pots to water holding capacity, unless experience with a given species indicates using less water. Containers should be placed at previously determined random test areas in the test facility.

A1.5.2.1.1 *Range-finding or Screening Test*—Often it is desirable to identify the approximate response range of endpoints for a test substance. Though statistical analyses are possible, these exploratory tests are intended to provide qualitative information at relatively low cost, with the information used to design subsequent tests with more statistical power (for example, Definitive Tests). As a screening test, it is suggested that two or more concentrations at decade levels be tested. All test concentrations are nominal unless specific analysis of stock solutions or treated soil is determined.

A1.5.2.1.2 *Limit Test*—Use of limit tests for plants generally is restricted to substances that are suspected to exhibit relatively little phytotoxicity (for example, PCBs or iron). A single high concentration is administered to the test matrix and results are compared to those from the negative control. Such tests are used to establish the safety of a substance at the highest expected concentrations based on physical properties of the test substance or maximum observed levels.

A1.5.2.1.3 *Maximum Challenge or Maximum Dose Test*—Maximum Challenge tests are similar to Limit Tests. The single test concentration of the substance (typically a pesticide) is equal to the maximum label rate for that substance.

A1.5.2.1.4 Test substances with low aqueous solubility might require being dissolved in an organic solvent, such as acetone. The solvent/chemical substance stock solution can be added to quartz sand or glass beads and allowed to dry. The sand or glass beads, or both, can then be mixed with soil for testing, or seeds can be placed in the sand or glass beads and exposed by adding the proper nutrient solution. (As noted in 5.2.2, the use of sand or glass beads most likely will not be representative of a natural soil.) If an organic solvent is used in this test, solvent controls also should be used.

A1.5.2.2 For each species tested, the normal time required to achieve acceptable percentage germination levels should be determined. The test duration should be approximately twice that length of time. It is recommended for improved laboratory management, that test duration be adjusted to the nearest whole week. In other words, if for lettuce, 90 % germination is attained after four days, the test duration would be seven days; for a range grass that reaches its normal maximum of 80 % germination after seven or eight days, the test duration would be two weeks. As the seeds are placed below the soil surface, there is no need to store the containers in the dark as done in some tests conducted on filter paper or in quartz sand. However, during the pre-germination period, containers should be covered so that evaporation is minimized. Upon emergence, the container covers should be removed. Once the covers have been removed, a regular watering schedule (at least daily) must be followed to ensure that adequate moisture is maintained for the duration of the test.

A1.5.2.3 When watering, care must be taken to minimize disturbance of the soil surface. Careless, forceful application of water can uncover some seeds while burying others to undesirable depths. If watering from the surface, slow rates of application are needed. Sub-irrigation is recommended except when using coarse sands that do not provide adequate capillary movement of water to reach the soil surface. In most cases, the volume of water to be added will vary in accordance with evaporative conditions of the test area. Water additions to saturation or less (for example, 85 % water holding capacity) should be provided at least once each day. The investigator should determine if more frequent watering is needed.

A1.5.2.4 Unless specific test objectives impose different requirements, lighting from fluorescent/incandescent lamps that provide 16 h of light per day is recommended. For most tests, 100 to 200 µmol m^{-1} s^{-1} of visible light (or photosynthetically active radiation, 400 to 700 ηm) has been found to be a broadly applicable fluence rate. In some cases, different light levels or spectral ranges (for example, solar ultraviolet) may be required. Guide E1733 should be consulted.

A1.5.2.5 Air temperature, relative humidity, and barometric pressure are monitored at least for daily minimum and maximum values, but it is preferably to monitor continuously with a recording device or an electronic datalogger. Temperature should be maintained between 20°C and 30°C, unless special requirements exist for a particular test species. Relative humidity is generally maintained above 30 % (recommend ≥50 %), though it may be advisable to increase the relative humidity in the growth area if the soil dries rapidly.

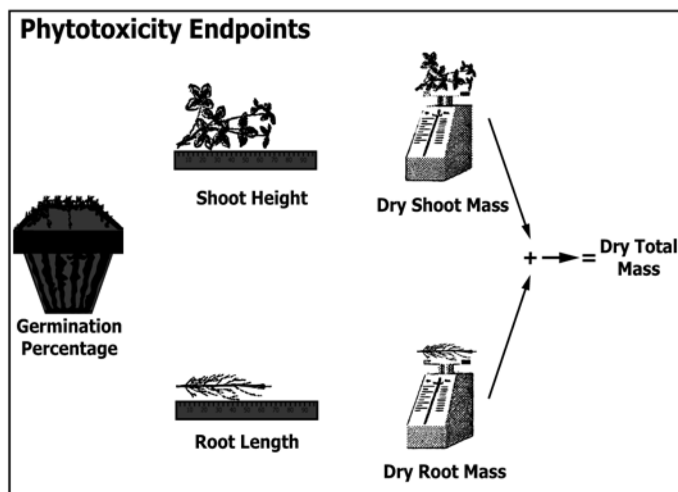

FIG. A1.1 Array of Measurement Endpoints

A1.5.3 *Termination of the Test*—The primary data collected from this study is the number of seedlings out of total planted that emerge above the soil. Abnormal patterns in growth and development, or abnormal plant morphology as compared to untreated controls, should be noted and presented in the report in narrative or tabular form. Optional measures include shoot and root growth (See Fig. A1.1). Shoot measurements are made from the transition point between the hypocotyl and root to the tallest point on the shoot. Root measurements are made from the transition point between the hypocotyl and root to the tip of the root. At the transition point between the hypocotyl and the primary root, the axis may be slightly swollen, contain a slight crook, or change noticeably in size. Measurements should be taken to the nearest mm. For some species, there may be sufficient growth to obtain dry weight measurements. The harvested material should be placed in a pre-weighed drying vessel and placed in a drying oven set at 70°C until constant weight is achieved (recommended time is 24 h). The weights should be measured to the nearest 0.001 g. For small plants it may be appropriate to combine all plants of a replicate for a species at a given contaminant concentration to determine growth.

A1.6 Interference and Limitations

A1.6.1 See Section 13.

A1.7 Calculations

A1.7.1 See Section 15.

A1.8 Quality Assurance and Quality Control

A1.8.1 See Section 14.

A2. ROOT ELONGATION

A2.1 Scope and Application

A2.1.1 The root elongation assay (modified from Porcella (**43**), Ratsch (**44**), and Gorsuch et al. (**45**) estimates the acute toxicity of liquid hazardous wastes and hazardous waste eluates in a 120-h static test. Though butter crunch lettuce (*Lactuca sativa* L.) is often used in this test, the method may be used with any plant species that germinates uniformly and quickly and produces a linear root during the first several days of growth. The measured response integrates synergistic, antagonistic, and additive effects of all the chemical, physical, and biological components of the test conditions that adversely affect plant root growth. Detection limits of the toxicity of a hazardous waste solution or pure substance are organism-dependent. This method should be performed by, or under the supervision of, professionals experienced in environmental toxicity testing.

A2.2 Method Summary

A2.2.1 Root elongation is a key component of the early stages of plant growth and development. It has been used for determining selective toxicity of herbicides (**45**), (**46**, **47**), screening for heavy metals (**48**, **49**), and evaluating toxic chemicals (**50**, **51**) and allelopathic substances (**52**, **53**).

A2.2.2 Several species have been used for root elongation tests including cucumber, wheat, alfalfa, radish, red clover, rye, rape seed, and lettuce. Cucumber and wheat seeds generally grow longer roots in the five day incubation period, offering greater length differentials to interpret response to test substances. Generally, wheat and cucumber varieties are less sensitive. Butter crunch lettuce is a commonly used test species for the root elongation bioassay as it is easily handled in the laboratory, root growth is sufficient to give good measurement precision within the 120-h incubation period, and lettuce is among the most sensitive of species. Investigators are, however, encouraged to expand the range of test species. In using non-conventional taxa, the investigator should document and establish control charting of typical elongation growth rates under control conditions.

A2.2.3 A dilution series of test product or eluate and five replicates per treatment are recommended. Deionized water is used as a negative control. If a solvent or some other solution is used to introduce a test substance, an additional control having all the ingredients but the test substance should be added to the testing scheme. If eluates of site soils are tested, then an eluate of a reference soil should be tested. Boron as boric acid is used as a positive control.

A2.2.4 Seeds are incubated in either growth pouches, or petri dishes with filter paper, or seed trays (**54**) saturated with the appropriate treatment, for 120 h. Root lengths are measured and compared among treatments and controls.

A2.3 Safety

A2.3.1 See Section 8.

A2.4 Apparatus

A2.4.1 *Facilities*—The preparation of the test, test substance, 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 area should also be protected from rodents, insects, and other vermin.

A2.4.2 *Equipment and Supplies:*

A2.4.2.1 Screen sized and separated seeds;

A2.4.2.2 Calibrated pH meter;

A2.4.2.3 Filter paper;

A2.4.2.4 Growth pouches or disposable, sterilized, polystyrene petri dishes, 100 mm × 15 mm (glass petri dishes should be used if suspected or known chemical constituents that would react with the plastic exist in the toxicant sample);

A2.4.2.5 33 gallon black plastic garbage-bag to line the cardboard box; pipettes; metric ruler;

A2.4.2.6 25 mL volumetric flask;

A2.4.2.7 Forceps (for seed handling and placement); glass plate (for measuring root length);

A2.4.2.8 Sample containers (for shipment and storage);

A2.4.2.9 Controlled test area capable of maintaining a uniform temperature of 24 ± 2°C;

A2.4.2.10 Water purification system;

A2.4.2.11 Top loading balance capable of weighing soil samples to 0.1g;

A2.4.2.12 Reference weights (for checking performance of balance);

A2.4.2.13 Electronic thermometer (for continuous monitoring of temperature);

A2.4.2.14 National Bureau of Standards certified thermometer;

A2.4.2.15 Routine laboratory glassware and materials: funnels, graduated cylinders, beakers, stainless steel spatulas and scoops, filter paper, laboratory sealant, marking pens, disposable latex gloves, notebooks, re-sealable bags, lab coats; and

A2.4.2.16 Certified respirators are required to conduct this test.

A2.5 Procedure

A2.5.1 *Preparation:*

A2.5.1.1 *Test Solutions:*

A2.5.1.1.1 The dilution series to be used is generally a 0.5 level dilution (for example: 3.125 %, 6.25 %, 12.5 %, 25 %, 50 %, and 100 %). Deionized water is used as the diluent.

A2.5.1.1.2 Boron as boric acid solutions for 40 ppm, 80 ppm, 160 ppm, 320 ppm, and 640 ppm are used for the positive control.

A2.5.1.1.3 The volume of test solution should be determined for each type container. The volume of test solution should be sufficient to saturate the paper.

A2.5.1.2 *Seeds:*

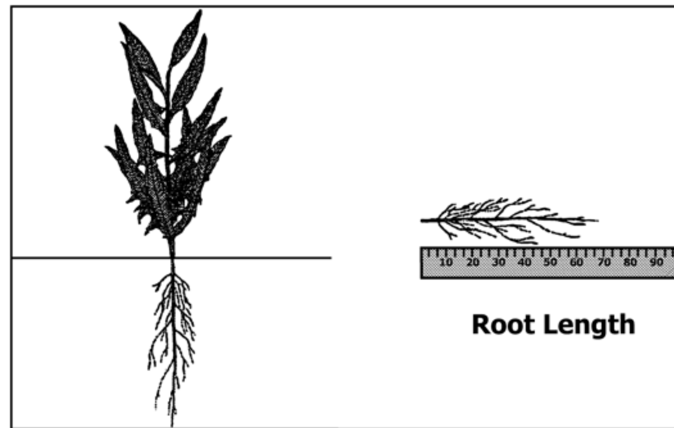

FIG. A2.1 Illustration of Root Length Measurement

A2.5.1.2.1 Seeds may be size-graded using four nested wire mesh screens or otherwise sorted to improve uniformity in germination and root growth rates.

A2.5.1.2.2 The seeds are stored in resealable bags; labeled with seed type, date screened, and fraction size; in a 4°C refrigerator until used for testing.

A2.5.1.3 *Pretest Documentation*—Growth pouches, petri dishes, or seed trays are labeled with test concentrations, species, and replicate number. Pre-test checklists may be helpful to verify all scheduling and material needs.

A2.5.2 *Test Procedure:*

A2.5.2.1 The root elongation bioassay is comprised of five replicates at each test concentration, a negative control using deionized water, and a positive control using boron as boric acid. Other controls may be added as appropriate (for example, eluate of reference soil; solvents). Each test container receives 10 seeds equally spaced in the test container. A total of 50 seeds is used per concentration and 50 seeds used for each of the negative and positive control.

A2.5.2.2 The pH of the test solution is taken and recorded prior to making dilutions.

A2.5.2.3 Dilutions may be made in the same 25 mL volumetric flask, beginning with the lowest concentration first.

A2.5.2.4 Each test concentration is prepared using deionized water (or a suitable solvent amendment as required) to dilute the test substance to the appropriate test concentrations. The quantity of treatment as determined in 6.1.1.3 should be administered to each replicate.

A2.5.2.5 The negative controls are prepared by dispensing deionized water to each of the replicate test apparatus. The positive controls are prepared by dispensing the appropriate quantity of boric acid solution to each of the replicate test containers.

A2.5.2.6 The growth pouches are either placed in racks or stacked in a dark growth chamber, with damp laboratory towels placed in the bottom, for 120 h at 24 ± 2°C. For the petri dish methods (either filter paper or germination trays), damp towels are placed between layers of petri dishes to maintain the filter paper moisture throughout the incubation period. (Darkness is not a requirement for this test, however, in some cases algal contaminant growth may interfere with test results.)

A2.5.3 *Termination of the Test:*

A2.5.3.1 Root lengths are measured (after 120 h of dark incubation) on a glass plate or other firm flat surface. Measurements are made from the transition point between the hypocotyl and root to the end of the root tip (see Fig. A2.1). For plants with a fibrous root mass, the investigator should specify whether the longest root or the primary root was measured. A minimum of 80 % of the negative control seeds must have germinated for the test to be valid. Alternatively, the criterion for acceptance of control seed germination may be established statistically through control charting as within ± 2 S. D. of mean for the species. The seeds are removed from the filter paper and placed on the glass work surface. The distance from the transition point between the hypocotyl and root to the tip of the root is measured. At the transition point between the hypocotyl and the primary root, the axis may be slightly swollen, contain a slight crook, or change noticeably in size. All roots are measured to the nearest millimeter and recorded on a data sheet.

A2.5.3.2 As an optional or additional endpoint, roots may be harvested, placed in preweighed drying pans, and dried to constant weight (recommend using 70°C for 24 h). The oven dry weight of all roots from a treatment-replicate is then measured to the nearest 0.001 g.

A2.5.3.3 Final pH readings are obtained after the test is completed. Taking final pH readings when using the petri dish technique may be very difficult since the vast majority of liquid is retained in the filter paper.

A2.6 Interference and Limitations

A2.6.1 In many instances, direct tests are preferred over indirect tests because the exposure to the test organism more closely simulates environmental conditions. Non-water soluble constituents bound or tightly adhered to soil particles will not be contained in an eluate, whereas unbound water soluble constituents would. If the principal question relates to surface water or groundwater runoff into streams, lakes, or wetlands; or groundwater flow off site (for example, off site well water contamination), it may be most appropriate to test derivatives of soils. In addition, the test assumes the primary influence on plant root elongation is the contaminant of interest. Other

potential toxicants (metals, other pesticides, and naturally occurring growth regulators) can alter and/or counteract this assumption.

A2.6.2 The choice of test apparatus can alter the measured response. Filter paper or toweling in growth pouches may bind certain test substances, especially metal cations, and render them unavailable (**54**).

A2.6.3 Toxic substances can be introduced by contaminants in water, glassware, sample hardware, artificial soil, and testing equipment.

A2.6.4 Improper hazardous waste sampling and handling can adversely affect test results. The test is not generally suited to evaluate phytotoxicity of volatile substances.

A2.6.5 Pathogenic organisms in test materials can affect test organism survival, and also confound test results.

A2.6.6 See Section 13 for additional details.

A2.7 Calculations

A2.7.1 See Section 15.

A2.8 Quality Assurance and Quality Control

A2.8.1 See Section 14.

A3. BRASSICA LIFE CYCLE

A3.1 Scope and Application

A3.1.1 This test evaluates the inhibitory effects of test materials regarding germination, growth of shoots or roots, photosynthetic systems, flower development, and reproductive capabilities of *Brassica rapa* plants under laboratory conditions. This practice is applicable for establishing phytotoxicity of herbicides and other pesticides, determining the phytotoxicity of sludges, and assessing the impact of discharge of toxicants or other amendments to land. Addition of contaminants at varying temporal intervals can be used to simulate application of contaminants to existing site vegetation at different points of the growth cycle. The primary use of this test is to assess full life cycle effects of toxicants. It can also be used as a screening tool to examine adverse effects of soil contaminants or soil amendments on germination and shoot and root development. If alternative species are to be used, the test procedures must be modified to conform with growth requirements phenology of the particular species. This test may be combined with other tests such as the seed germination test to extend the suite of endpoints to include germination and early growth of seeds produced by the test plants.

A3.2 Method Summary

A3.2.1 Seeds are germinated in a test medium which may be a formulated soil, a reference soil, or a contaminated soil (**55-57**). Replicate seedlings are allowed to develop fully through maturation of siliques (**58**). Chemical additives or amendments may be added to formulated or reference soil media either pre-emergence or post-emergence, and at various temporal spacings or concentrations. Contaminated soils may be tested at full-strength (that is, 100 %) or diluted with a suitable reference or formulated soil. A positive control (for example, boron as boric acid), and a negative control (deionized water or specified nutrient solution added to reference or formulated soil) should be included in the test. The test duration should be 36 to 42 days, the approximate time required for life cycle completion of *Brassica rapa* under normal and test conditions (**58**). Numerous quantitative data points may be obtained throughout the testing period, with additional information being obtained through generational tests on seeds produced.

A3.3 Safety

A3.3.1 See Section 8.

A3.4 Equipment and Apparatus

A3.4.1 The preparation of the test, the test soil medium, the test substance, the storage of soil, and all stages of the test procedure should take place in a setting free from contamination. The growth area should have reasonable temperature control, relative humidity control, and adequate lighting. The recommended photoperiod is 16 h on, 8 h off. Equipment includes:

A3.4.1.1 Disposable, sterilized, plastic pots or containers (for example, 7.5 × 15 cm, although other sizes may be used).

A3.4.1.2 Disposable plastic petri dishes for use as pot or container covers.

A3.4.1.3 Stainless steel rods, bamboo skewers or equivalent inert support; approximately 30 cm in length.

A3.4.1.4 Balance—sensitivity to 0.01 g.

A3.4.1.5 pH meter—sensitivity to 0.1 units.

A3.4.1.6 *Photometer (Radiometer)*—Capable of measuring the photosynthetically active range. Fluence rate of incident light should be expressed as µmol $m^{-2}s^{-1}$.

A3.4.1.7 A continuous recording thermometer or a maximum-minimum thermometer that is checked daily. Many continuous recording units also record humidity.

A3.4.1.8 *Industrial Mixer or Cement Mixer*—A revolving or rotating mixer is recommended for combining test substances or test soils with large volumes of control or reference soil medium.

A3.4.1.9 Drying oven capable of maintaining 85° C.

A3.4.1.10 Routine laboratory glassware and materials including: funnels, graduated cylinders, beakers, magnetic stir plate, stainless steel spatulas and scoops, filter paper, tissue

paper; laboratory sealant film, marking pens, disposable latex gloves, notebooks, re-sealable bags, lab coats, and certified respirators.

A3.5 Procedure

A3.5.1 *Preparation:*

A3.5.1.1 Label Petri plates and pots or containers with project identification, test sample identification, and replicate number.

A3.5.1.2 If the investigator has decided to utilize a soil dilution series or artificial soil for this procedure, complete all soil assembly or mixing prior to test initiation.

A3.5.1.3 Test aliquots of each soil, soil dilution, or artificial soil to be utilized for pH.

A3.5.2 *Test Procedure:*

A3.5.2.1 Place test soil medium in each pot or container so as to bring matrix to 1 cm from rim; amount needed will vary with type of container and soils used. Test at least five replicates of each soil sample, additive, or amendment treatment, a positive control, and a negative control. Plant each replicate container with 1 or 5 *Brassica rapa seeds*, depending on the investigators need for statistical germination data. For very sandy reference or artificial soils, it may be advisable to place each seed in a folded 1 cm^2 piece of laboratory tissue to avoid dislodging the seed during watering. Plant seeds at a depth of 1 cm. Use deionized water or treatment solution to bring the pots to water holding capacity. Place clear plastic petri dishes on top of each replicate container to minimize evaporation during germination. Place containers at previously determined random test positions in the test facility.

A3.5.2.2 *Brassica rapa* has been shown to complete a life cycle in as little as 36 days under ideal laboratory conditions; studies have shown that cycle times may increase to 42 days when testing toxicants (**58**). The test duration should be approximately that length of time. It is recommended for scheduling management that test duration be adjusted to the nearest whole week. Upon seedling emergence, remove container covers. Once the covers have been removed, a regular watering schedule (at least daily) must be followed to ensure that adequate moisture is maintained for the duration of the test. When watering, care must be taken to minimize disturbance of the soil surface. Careless, forceful application of water or treatment solution can uncover some seeds while burying others to undesirable depths. If watering from the surface, slow rates of application are needed. In most cases, the volume of water to be added will vary according to evaporative conditions of the test area. Provide water or treatment solution additions to saturation or less (for example, 85 % water holding capacity) at least once each day. The investigator should determine if more frequent watering is needed.

A3.5.2.3 Unless specific test objectives impose different requirements, lighting from fluorescent/incandescent lamps that provide 16 h of light per day is recommended. For most tests, 100 to 200 µmol $m^{-2}s^{-1}$ of visible light (or Photosynthetically Active Radiation, 400 to 700 ηm) has been found to be a broadly applicable fluence rate. In some cases, different light levels or spectral ranges (for example, solar ultraviolet) may be required. Guide E1733 should be consulted.

A3.5.2.4 Air temperature, relative humidity, and barometric pressure are monitored at least for daily minimum and maximum values, but it is preferable to monitor continuously with a recording device or an electronic datalogger. Maintain temperature between 20 and 30° C. Relative humidity is generally maintained above 30 % (recommend ≥ 50 %), though it may be advisable to increase the relative humidity in the growth area if the soil dries rapidly.

A3.5.2.5 After cover removal, seedlings are allowed to emerge until development of the first true leaves occurs, usually four to eight days. At this point, insert an inert support rod into the soil medium. Measurements of morphological, phenological, and other endpoints are taken periodically throughout the experiment. These include, but are not limited to the following: germination, emergence, foliar height, stem diameter, internode length, leaf length and width, branching morphology, bolt timing, initial flowering date, silique development, chlorosis, stunting, and survival. Foliar height may be measured daily or weekly by gently straightening the foliage and measuring between the cotyledons and the uppermost point of the foliage. Special care is needed to avoid injuring the plants during measurement. Replicates are allowed to continue development until seed is set or the life cycle is complete, or both.

A3.5.3 *Termination of the Test:*

A3.5.3.1 At takedown, invert the pots or containers and gently tap to remove the plants and matrix together. If post-test concentration of toxicants in the matrix are to be determined, take aliquots of the matrix before washing the roots. Place the roots and adhering matrix in a tub of deionized water and gently wash to remove the matrix from the roots. The plants should be washed individually, although it may be acceptable to wash all replicates of a given treatment at once. If replicates are washed as a batch, broken roots cannot be assigned to individual plants and therefore constrains statistical descriptions of treatment variability. Once washed, separate shoots from the roots by cutting with a razor blade at the cotyledons. Take takedown measurements at this time. These include, but are not limited to, the following: wet and dry foliar and root weights, maximum foliar height, stem diameter, number and length of axillary stems, number of siliques, and number and size of seeds. Place the harvested foliar and root material in pre-weighed and labeled drying vessels and place in a drying oven set at 85° C until constant weight is achieved (recommended time is 24 h). Measure the wet and dry weights to the nearest 0.01 g.

A3.6 Interference and Limitations

A3.6.1 See Section 13.

A3.7 Calculation

A3.7.1 See Section 15.

A3.8 Quality Assurance and Quality Control

A3.8.1 See Section 14.

A4. WOODY PLANT SPECIES GROWTH & DEVELOPMENT

A4.1 Scope and Application

A4.1.1 This test evaluates the inhibitory effects of test materials on growth and development of woody plant species under laboratory conditions. This practice is applicable for establishing phytotoxicity of herbicides and pesticides, determining the phytotoxicity of soils, sediments, and sludges, and assessing the impact of discharge of toxicants or other amendments to land on species that occur naturally or are being considered as cover for contaminated and remediated areas. This test is most useful as a screening tool to examine the likelihood of adverse effects of soil contaminants or soil amendments on potential regeneration of plant communities in areas of past disturbance or contamination. The test may also be used to conduct definitive tests that estimate concentration related effects levels (for example, ECxx). Phytotoxic effects that occur as a consequence of impairment of photosynthetic systems, shoot growth, or root development can be assessed.

A4.2 Method Summary

A4.2.1 Woody plants are grown in a test medium which may be silica sand, a formulated soil, a reference soil, or a contaminated soil (**56, 57**). Chemical additives or amendments may be added to silica sand, formulated, or reference soil media at various concentrations. Contaminated soils may be tested at full-strength (i.e., 100 %) or diluted with a suitable reference or formulated soil. A positive control (e.g., boron as boric acid), and a negative control (deionized water or specified nutrient solution added to reference or formulated soil) should be included in the test. The test duration should be approximately twice the time required for significant shoot and root development of the test species. The duration of the study may be increased to enable evaluation of plant growth under longer exposure conditions. The wet and dry shoot mass, number of new shoots or leaves, and total plant weight changes are scored at the termination of the test. Additional metrics and observations regarding shoot and root growth and development are encouraged.

A4.3 Safety

A4.3.1 See Section 8.

A4.4 Facilities and Equipment

A4.4.1 *Facilities*—The preparation of the test, test soil medium, test substance, storage of soil, pretest plant care, and all stages of the test procedure must take place in an atmosphere free from contamination. The growth area should have reasonable temperature control, and adequate lighting. The recommended photoperiod is 16 h on, 8 h off, unless a given test species requires special photoperiod conditions for the objectives of the test.

A4.4.2 *Equipment and Supplies:*

A4.4.2.1 Disposable, sterilized, plastic pots or containers (for example, 7.5 by 20 cm, though other sizes may be used);

A4.4.2.2 Metal or plastic plant identification labeling tags;

A4.4.2.3 *Balance*—sensitivity to 0.01 g;

A4.4.2.4 *pH Meter*—sensitivity to 0.1 units;

A4.4.2.5 *Photometer (Radiometer)*—Capable of measuring the photosynthetically active range. Fluence rate of incident light should be expressed as μmol m^{-2} s^{-1};

A4.4.2.6 A continuous recording thermometer or a max.-min. thermometer that is checked daily. Many continuous recording units also record humidity;

A4.4.2.7 *Industrial Mixer or Cement Mixer*—A revolving or rotating mixer is recommended for combining test substances or test soils with large volumes of control or reference soil medium;

A4.4.2.8 Drying oven capable of maintaining 85°C; and

A4.4.2.9 Routine laboratory glassware and materials including: funnels, graduated cylinders, beakers, magnetic stir plate, stainless steel spatulas and scoops, filter paper, laboratory sealant film, marking pens, disposable latex gloves, notebooks, re-sealable bags, lab coats, and certified respirators.

A4.5 Procedure

A4.5.1 *Pretest Plant Preparation:*

A4.5.1.1 Obtain more plant material than will be needed to conduct the test (including all test treatments and controls). If the plants have been grown in a soil medium, wash the roots gently by dipping the root mass into deionized water. This allows the soil particles to settle from the roots. While plants are being handled, it is very important to maintain moist conditions for the roots. Wet paper or cloth toweling may be used. Alternatively, the roots may be placed in trays with sufficient deionized water depth to submerge all roots.

A4.5.1.2 Pre-sort the plants according to size and stage of development. The intent here is to get relatively uniform sized plants in similar stages of root and shoot growth. Cull spindly and extremely robust plants, relative to the average plant in the lot. Plants should be small enough to permit substantial growth (both root and shoot) in the container and head space available for the test. Species that readily form lateral sprouts may be pruned to achieve uniform size. Root mass may also be trimmed, particularly if the plants were grown in tubes and have become root bound. If the root mass has become bound, considerable damage to the roots may occur that will likely influence the test results. Small, sharp scissors, pruning shears, scalpels, or razor blades may be used to trim the shoots and roots. An acclimation period following re-potting may be warranted. Advice on pruning shoots and reducing root mass may be obtained from horticulturist or nursery staff. The investigator should become familiar with the characteristics of the species used before conducting a test.

A4.5.1.3 Several endpoints are measured in the conduct of the test. Potential quantitative endpoints could include shoot height, root length, number of shoots, number of leaves, number of root initiation points, and measures of mass.

Potential qualitative endpoints could include observations on general plant condition, leaf malformations or death, new root growth, and general treatment cohort condition. With the woody plants, the test begins with a substantial amount of shoot and root material. Therefore, it is important to quantify the starting point in order to interpret the effects of the test substance. Two methods may be used:

A4.5.1.4 *Average Starting Conditions Using Test Plant Population Mean:*

A4.5.1.5 Randomly assign pre-sorted plant specimens into treatment groups. The groups should include all treatments and controls as well as a group designated as the starting condition group.

A4.5.1.6 The starting condition group is measured for all endpoints described for the test, (that is, shoot height, root length, shoot mass, root mass, etc.).

A4.5.1.7 The statistical description (mean, standard deviation, UCL, etc.) of data for measured endpoints of each parameter of this starting condition group becomes the baseline for comparisons among treatments.

A4.5.1.8 *Individual Starting Conditions:*

A4.5.1.9 Each pre-sorted specimen is given a unique, waterproof identification label.

A4.5.1.10 Each labeled specimen is measured for each non-destructive quantitative (total plant weight, shoot number, etc.) and qualitative (shoot description, root description, etc.) parameter.

A4.5.1.11 The labeled specimens are assigned randomly to treatment groups.

A4.5.1.12 At the conclusion of the test, specimens are measured for each parameter. The difference between post-test and pre-test non-destructive endpoint measurements is the magnitude of growth achieved during the test. Measures of shoot or root dry-weight mass can only be interpreted relative to values for negative controls.

A4.5.1.13 There are substantive trade-off considerations distinguishing these two methods. In using the average method, one must have a sufficiently large number of plants to harvest for the initial conditions. Also, the magnitude of variation among specimens should be relatively small so that random sampling differences among treatment groups are inconsequential compared to expected treatment effects. Specific data quality objectives could be developed to anticipate the magnitude of permissible variation, but in general this method is probably a poor choice if the coefficient of variation (standard deviation/mean) is greater than 20 %. The major disadvantages with the individual starting conditions are the labeling requirements and the inability to obtain starting values for dry weight or other destructive measurement parameters.

A4.5.1.14 As part of the test procedure, the investigator should stipulate which method was chosen and the rationale for selecting the method. Also, as this method describes only general steps, the test documentation should provide additional pertinent detail to describe what was done and as appropriate indicate why certain steps were performed as they were.

A4.5.1.15 Pots or containers are labeled with project identification, test sample identification, test species, and replicate number.

A4.5.2 *Test Procedure:*

A4.5.2.1 Place at least 5 cm of test medium in the bottom of the labeled pot or container. These should be of sufficient dimension to contain test medium to a depth of approximately two times the root length of the test plants to allow unrestricted root growth during the test. Medium quantities will vary dependent on test species and medium type. Each replicate container should be planted with one test replicate. Five replicates of each soil sample, sample dilution, additive, or amendment treatment, a positive control, and a negative control are tested. Each plant should be placed in the pot or container with the roots just touching the medium in the container. Additional medium should be added gently to cover the roots completely and to bring the medium level within 1 to 2 cm of the top of the pot or container. The stem of the replicate should be covered to a depth sufficient to support the replicate during the testing period. Medium may be gently packed by hand to assist with this, but care must be taken (especially with clay soils) to not compact so as to restrict root development or moisture transfer. Deionized water should be used to bring the pots to water holding capacity. Containers should be placed at previously determined random test areas in the test facility.

A4.5.2.2 Growth rates among species vary widely; actively growing poplar shoots may produce 5 cm or more growth per week, whereas conifers may have 1 cm growth per week. The normal time required to achieve amounts of shoot and root growth acceptable for statistical characterization of the test species should be determined. The test duration should be approximately twice that length of time. It is recommended for improved laboratory management that test duration be adjusted to the nearest whole week. In other words, if for poplar shoot and root development sufficient for quantitative comparisons occurs in ten to fourteen days, the test duration would be twenty eight days.

A4.5.2.3 A regular deionized water or treatment application schedule should be followed to ensure that adequate moisture is maintained for the duration of the test. In most cases, the volume of deionized water or treatment solution to be added will vary according to evaporative conditions of the test area and plant transpiration rates. Additions to saturation or less (for example, 85 % water holding capacity) should be provided at least once each day. The investigator should determine if more frequent application is needed.

A4.5.2.4 Unless specific test objectives impose different requirements, lighting from fluorescent/incandescent lamps that provide 16 h of light per day is recommended. For most tests, 100 to 200 µmol m^{-2} s^{-1} of visible light (or photosynthetically active radiation, 400 to 700 ηm) has been found to be a broadly applicable fluence rate. In some cases, different light levels or spectral ranges (for example, solar ultraviolet) may be required. Guide E1733 should be consulted.

A4.5.2.5 Air temperature, relative humidity, and barometric pressure are monitored at least for daily minimum and maximum values, but it is preferable to monitor continuously with a recording device or an electronic datalogger. Temperature should be maintained between 20 and 30° C, unless special requirements exist for a particular test species. Relative humidity is generally maintained above 30 % (recommend ≥ 50 %),

though it may be advisable to increase the relative humidity in the growth area if the soil dries rapidly.

A4.5.3 *Termination of the Test:*

A4.5.3.1 The primary data collected from this study include quantitative changes (total plant weight, number of shoots or leaves, etc.) in the test replicates as compared to the controls and will vary dependent upon initial data collected prior to test initiation. Abnormal patterns in growth and development, or abnormal plant morphology as compared to untreated controls, should be noted and presented in the report in narrative or tabular form. Optional endpoint measures include qualitative observational data endpoints (scored values for shoot condition, root condition, etc.) outlined prior to test initiation. At takedown, the pots or containers should be inverted and gently tapped to remove the replicate and medium together. If post-test determination of toxicants in the medium are to be determined, aliquots of the medium should be taken before washing the roots. The roots and adhering medium should be placed in a tub of deionized water and gently washed to remove the medium from the roots. The plants should be washed individually, although it may be acceptable to wash all replicates of a given treatment at once. If replicates are washed as a batch, broken roots cannot be assigned to individual plants and therefore constrains statistical descriptions of treatment variability. Total replicate weight can then be obtained and should be measured to the nearest 0.01 g. Shoots and roots should be collected by cutting from the main stem as closely as possible with a razor blade. The harvested material should be placed in a pre-weighed and labeled drying vessel and placed in a drying oven set at 85°C until constant weight is achieved (recommended time is 24 h). The wet and dry weights should be measured to the nearest 0.01 g. For small plants it may be appropriate to combine all replicates for a species of a given treatment to determine growth.

A4.6 Interference and Limitations

A4.6.1 Interpretation of phytotoxicity from this test must be tempered to reflect ecological aspects regarding test species utilized. Determinant growth plants such as conifers have limited periods of growth. Indeterminant growth plants such as many angiosperm tree species exhibit extended periods of growth under favorable conditions. Desired data applicability must be addressed early in the test planning stage to eliminate misinterpretation of data that is determined by species characteristics rather than phytotoxic effects. If determinant growth species are to be used the plants should be near the beginning of their growth cycle. Indeterminant growth species offer greater temporal latitude for conducting the tests.

A4.6.2 Tests using woody plants require special steps to enhance the value of the data. Woody plants may come in one of three forms: seedlings (that is, young plants grown from seeds), older rooted plants having a substantial stem and root mass (the plant may have been started from seed or from stem cuttings), or bare stem cuttings that can be nurtured to regenerate roots and shoots. Each form of plant requires somewhat different steps to prepare the plants for testing. Care must be taken to minimize root and shoot damage during handling, extraction, root cleaning, and replanting prior to test setup in order to preclude phytotoxic effects being masked by physical damage. If the investigator has limited knowledge of a species, experiments to characterize effects of handling the plants should be conducted as a companion set of tests.

A4.6.3 Phytotoxic effects will often be expressed differently at differing stages of plant growth. For this reason, careful consideration should be given the decision to utilize seedlings, rooted plants, or cuttings of a potential test species. Seedlings are normally at an early stage in the life cycle and may exhibit differing chemical uptake and phytotoxic effects compared to a mature specimen. Rooted plants often vary widely in beginning endpoint characteristics such as total plant weight, number of shoots, number and size of leaves, and root development while offering mature plant physiological processes for testing. Sprouted cuttings can be processed to give the most standardized starting data endpoints while exhibiting earlier life cycle physiological characteristics. The study design must reflect consideration of these differing developmental characteristics in selecting the plants to use for phytotoxicity testing.

A4.6.4 See Section 13.

A4.7 Calculation

A4.7.1 See Section 15.

A4.8 Quality Assurance and Quality Control

A4.8.1 See Section 14.

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