Juvenile Seedling Growth Test: Oxic Conditions

Standard Operating Procedure for Test Methods using Wild Rice, *Zizania palustris*

1.1 Scope and Application

1.1.1 This method describes procedures to perform a toxicity test using wild rice in exposures of solutions containing elevated sulfate or cation concentrations under aerobic conditions.

1.1.2 This method consists of a test using a dilution series of at least four concentrations of a test chemical and a control.

1.2 Summary of Method

1.2.1 Germinated seeds of the aquatic macrophyte *Zizania palustris* are exposed in a static-renewal system to a dilution series of concentrations of sulfate or cations. The exposure duration is 10 days. The response of the germinated seeds is measured in terms of changes to growth in control plants vs. treatment as measured using biomass (weight) and measures of vegetative growth of the plant.

1.3 Quality Control Considerations

1.3.1 Toxic substances may be introduced by contaminants in dilution water, sampling hardware, or testing equipment.

1.3.2 Adverse effects of pH changes and cationic constituents in test media may augment or mask adverse effects of toxic substances.

1.3.3 Improper sampling of test solutions may adversely affect test results (see section 1.5, Standards and Reagents, and section 1.6, Toxicity Test Procedures)

1.3.4 Additional details are found in the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan"

1.4 Necessary Apparatus and Materials

1.4.1 Seeds of *Zizania palustris* are prepared in the laboratory for germination (see section 1.7, Wild Rice Seed Preparation). To initiate exposures of germinating seeds, sufficient numbers of conditioned seed must be available.

1.4.2 Environmental Growth Chamber: with lamps of maximum light intensity of 800 or greater μmol m^{-2} sec^{-1} (measured 6 inches below the lamps) produced by either fluorescent lamps or an LED light system and temperature control range of 15° C to 30° C ± 1°C).

1.4.3 Light meter: quantifies intensity as photosynthetic photon flux (PPF).

1.4.4 Test chambers: 70 mL Kimax (Kimble Chase Life Science and Research Products LLC) or Pyrex (Corning, Inc.) glass tubes with screw caps.

1.4.5 Meter: pH for routine physical measurements.

1.4.6 Volumetric flasks and graduated cylinders: class A, 10 – 2000 mL borosilicate glass for preparation of test solutions.

1.4.7 Volumetric pipets

1.4.8 Pipet bulbs and fillers

1.4.9 Balance: analytical, capable of accurately weighing 0.1 mg.

1.4.10 Magnetic stirrer and stir bars: for mixing test and growth media solutions

1.4.11 Filtering apparatus: for membrane and /or glass fiber filters

1.4.12 Tape: for labeling test chambers and containers for solutions

1.4.13 Water purification system: deionized water or equivalent

1.5 Standards and Reagents

1.5.1 Reagent-grade chemicals are used to prepare hydroponic growth media.

1.5.2 25 liters of a modified 1/5 strength Hoagland's stock solution (Table 1) is prepared using a ½ strength stock solution daily or more often as needed from 1.0 M stock solutions.

1.5.3 Stock SO_4 solution (3.200 g/L) is prepared daily as needed by adding 4.73 g anhydrous Na_2SO_4 (Fisher S421) or 8.22 g $MgSO_4*7H_2O$ (Fisher M63) to 800 mL deionized water and filling to 1 liter. Mixtures of Na_2SO_4 and $MgSO_4$ are determined by solving 2 equations with known Mg:Na ratios and known SO_4 final concentration.

1.5.4 Reagent water: defined as deionized water that does not contain substances that are toxic to the test organisms.

1.5.5 Appropriate amounts of each test solution (70 mL times number of replicates plus extra for analysis sample, i.e. 1600 mL for 20 replicates and ~200 mL sample) are made up immediately before use. Pre-determined amounts of 1/5 strength Hoagland's, PIPES buffer (Piperazine-N,N'-bis(2-ethanesulfonic acid) sesquisodium salt, Fisher Scientific/Acros Organics # AC32778-5000) , N, and P stock solution, and SO_4 stock solution are mixed and made to volume. The pH is adjusted to 6.8 +/- 0.2 with 1 M HCl.

Table 1. Composition of 1/5 Hoagland's Solution

Compound	Molar concentration in 1/5th strength growth solution
MgCl	0.4 mM
$CaCl_2 \cdot 2\ H_2O$	2 .0 mM
KCl	1.0 mM
NH_4Cl	0.08 mM
NaNO3	0.08 mM
KH_2PO_4	0.026 mM
H_3BO_3	22.5 µM
$MnCl \cdot 4\ H_2O$	4.5 µM
$ZnSO_4 \cdot 7\ H_2O$	0.5 µM
$CuSO_4 \cdot 5\ H_2O$	0.15 µM
MoO_3	0.07µM
Fe-EDTA	45.0 µM
$Na_2SiO_3*9H_2O$	1.5 mM
PIPES buffer	5.0 mM

1.6 Toxicity Test Procedures: Toxicant Exposures

1.6.1 Twenty 70 mL Kimax tubes are used for each test concentration prepared. Each tube is considered a replicate for the corresponding test concentration.

1.6.2 Each toxicity test will consist of at least four test concentrations of the toxicant (e.g., sodium sulfate) and a control (hydroponics medium).

1.6.3 Germinated wild rice seed (sprout) as described in section 1.7, Wild Rice Seed Preparation, are used to initiate the toxicity test.

1.6.4 Each Kimax tube is labeled with tape using a unique descriptor for the particular concentration of test solution and replicate for that tube. Each tube also is numbered from 1 to 120 and a table of these integers (1 – 120) randomized is prepared.

1.6.5 Each labeled tube is filled to the top with the particular solution as identified on its label.

1.6.6 Germinated seeds (sprouts) are removed from the pool of initial seeds (see section 1.8, Test Organisms) using a light forceps and put into the tube corresponding to the first integer read from the random integer table. This is done for all test tubes prepared for testing.

1.6.7. The filled tubes (solution and seed) are placed into every other opening in holding racks so that light can penetrate to all sides of each tube. A total of six 40-tube racks, each containing 20 tubes, are used to hold the test tubes. See Image 1.

1.6.8 Screw caps are placed loosely on the tubes.

1.6.9 All racks are placed in the growth chamber so that the spaces between the racks are the same as the spaces within the racks and the tops of the tubes are within 30 cm of the bottom of the lights. The location of each rack in the growth chamber remains the same for the test duration.

1.6.10 Test solutions in the tubes are renewed every two (2) days.

1.6.11 Solution renewals are accomplished by gently decanting or siphoning off the old solution leaving approximately one vertical cm of solution in the tube bottom. See Image 2.

1.6.12 New solutions are added by gently pouring into the tube until it reaches the top of the tube. The screw cap is then replaced on the tube.

1.6.13 Old solutions are retained for chemistry as described in the section 1.12, Analytical Chemistry.

1.6.14 Duration of the exposure is 10 days.

1.7 Wild Rice Seed Preparation

Wild rice seed must undergo a conditioning phase following its harvest from the field. In the wild, wild rice drops into the water after the seed has ripened, and sinks to the sediment. This seed, if left undisturbed, stays on or just below the surface of the sediment over the winter. This cold phase serves to condition the seed to enable it to germinate once water temperatures increase in the spring.

1.7.1 The following is a procedure that describes the method and handling of wild rice seed from initial harvest to its use in juvenile seedling toxicity tests.

1.7.2 Freshly harvested seed should be kept cool and moist and be placed into storage as soon as possible after field collection.

1.7.3 Harvested seed prepared for storage can be kept a) in plastic zip lock bags in a cooler set at just above freezing (4° C), or b) submerged in water just above freezing in the dark. Seed stored in either manner can have satisfactory germination rates for one to two years.

1.7.4 To begin the seed conditioning for germination, an aliquot of seed (approximately 2000 seeds) is removed from this ‘dry’ cold storage (as described in option (a) in 1.7.3) and placed into a container with water kept submerged at near freezing temperatures for at least one month. Following this time period, seed is ready (or conditioned) for germination for at least several months. For purposes of use in

laboratory testing, seed set in this conditioning phase is kept for up to two months before a fresh aliquot of seed is brought into the conditioning phase. Use of storage option (b) keeps the seed in this wet, cold conditioned phase until needed for testing. See Image 3.

1.8 Test Organisms, Germinated Wild Rice Seed

1.8.1 As seed is needed for testing, a smaller aliquot from the wet-stored (conditioned seed) is removed and placed into a container with water and placed in the dark incubator at 20+/- 1C. Germination tests (see Germination Growth Tests: Oxic Conditions) are initiated using this conditioned seed and placed immediately from the cold, submerged condition onto an open tray (see Image 3) to be placed into the test chambers. Seed used for initiating a toxicity test is visually screened for viability based on the color of the seed coat and fullness of the seed body. Seeds that float, are misshaped, or are otherwise malformed are not used for testing.

1.8.2 For this method, the seed will begin to sprout in about 2 to 3 days. Germinated seeds (referred to as a sprout) are selected with 1-2 cm of mesocotyl growth. A germinated seed (sprout) is described as growth of the mesocotyl that is longer than the seed coat. See Image 4.

1.8.3 Sprouts are selected from the pool of available seeds and placed into a separate container with water. A total of 150 sprouts are selected with 120 sprouts used for initiating the toxicity tests, and 20 sprouts put aside to be dried and weighed to measure initial weight.

1.9 Light, Photoperiod, Temperature and Humidity Test Conditions

1.9.1 Tests are performed under a 16h:8h light:dark schedule.

1.9.2 Light intensity is 350 µmol m^{-2} sec^{-1} as measured at the mid-point of the exposure tube.

1.9.3 Temperature is maintained at 21^{o} C during lighted periods and 19^{o} C during dark period.

1.9.4 Test growth chamber is maintained at 85% humidity.

1.10 Phytotoxic Effects

1.10.1 Observations of sprouts should be made during test solution renewal every 2 days. All abnormalities should be recorded.

1.10.2 Observations should include the date, time, treatment level, and replicate number.

1.10.3 After the duration of the test, exposure tubes are kept in the random order as placed initially.

1.1.4 Solutions are decanted into labeled sample bottles in the same manner as described for solution renewal.

1.10.5 Once the solution is removed, the sprout is removed from the tube for subsequent measurements. The sprout has three distinct sections: stem/leaf, root and seed. See Image 5.

1.10.6 Measures of stem/leaf length (mesocotyl growth) is performed by placing the stem/leaf stretched out on a flat surface next to ruler with the zero mark aligned with the point of stem-root transition. The length from the stem-root transition to the tip of the stem/leaf is measured and recorded to the nearest millimeter. See Image 6.

1.10.7 The stem/leaf growth is cut from the plant at the point of the stem-root transition and placed into pre-weighed drying tin. See Image 7.

1.10.8 Plant biomass is measured as follows: Each seedling is placed into a numbered, weighed aluminum weighing dish (Fisher 08-732-101). The dishes are placed on trays, and the trays are put into a drying oven at 100° C for at least 48 hours. Each dry sample and dish is placed into a desiccator until room temperature, and is weighed to the nearest 0.1 mg.

1.10.9 Roots are separated from the remaining seed material, placed in a labeled plastic bag and refrigerated. See Image 8.

1.10.10 The remaining seed material is placed into a pre-weighed drying pan.

1.10.11 Pans containing stem/leaf, and seed material are placed in the drying oven at 100^{o} C for at least 48 hrs.

1.10.12 Length and area of root material is determined using a flatbed scanner and imaging software using the method titled: Determination of Wild Rice Total Root Length Using Scanning Hardware and Software found in Appendix C of the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan"

1.10.13 After all root samples are scanned, each root sample is removed from its storage bag and placed into separate pre-weighed pans and dried for at least at 100^{o} C for at least 48 hrs.

1.10.14 All dried plant materials are weighed on a Sartorius 2700 balance to 0.1 mg following the method titled "Total Plant Biomass (Dry Weight) Methods" found in Appendix C of the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan". See Image 9.

1.11 Acceptability of Test Results

1.11.1 At least 90% of control juvenile seedlings are living at test termination.

1.11.2 Mesocotyl length of juvenile seedlings from control exposures will be at least 5.0 cm at the end of the 10 d duration of growth.

1.1.3 Control juvenile seedlings should not indicate any visible phytotoxic or developmental symptoms at any time during the test.

1.12 Analytical Chemistry

1.12.1 Sampling and analysis of chemical solutions used for initiating and renewing test exposures will use the following procedures.

1.12.2 New test solutions –Immediately after adding the new test solution into the jars an aliquot (approximately 250 ml) of the remaining unused portion is poured directly into a pre-labeled sample bottle.

1.12.3 Old test solutions – When exchanging solution or before decanting the final solution the Kimax tube is inverted once to mix the solution and is poured directly into a pre-labeled sample bottle. Depending on the analyses required, it may be necessary to make a composite of the solution in multiple replicate tubes from a given treatment.

1.12.4 Sulfate concentration is measured following the method titled, "Determination of Sulfate By Flow Injection Analysis" found in Appendix C of the document titled: "Hydroponic Experiment on Response of Wild Rice to Sulfate - Quality Assurance Project Plan"

References

U.S. EPA. 2012. Ecological Effects Test Guidelines. OCSPP 850.4230: Early Seedling Growth Toxicity Test. EPA 712-C-010.

U.S. EPA. 2012. Ecological Effects Test Guidelines. OCSPP 850.4100: Seedling Emergence and Seedling Growth. EPA 712-C-012.

U.S. EPA 2012. Ecological Effects Test Guidelines. OCSPP 850.4400: Aquatic Plant Toxicity Test Using *Lemna* spp. EPA 712-C-008.

U.S. EPA. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, 4th ed. EPA-821-R-02-013.

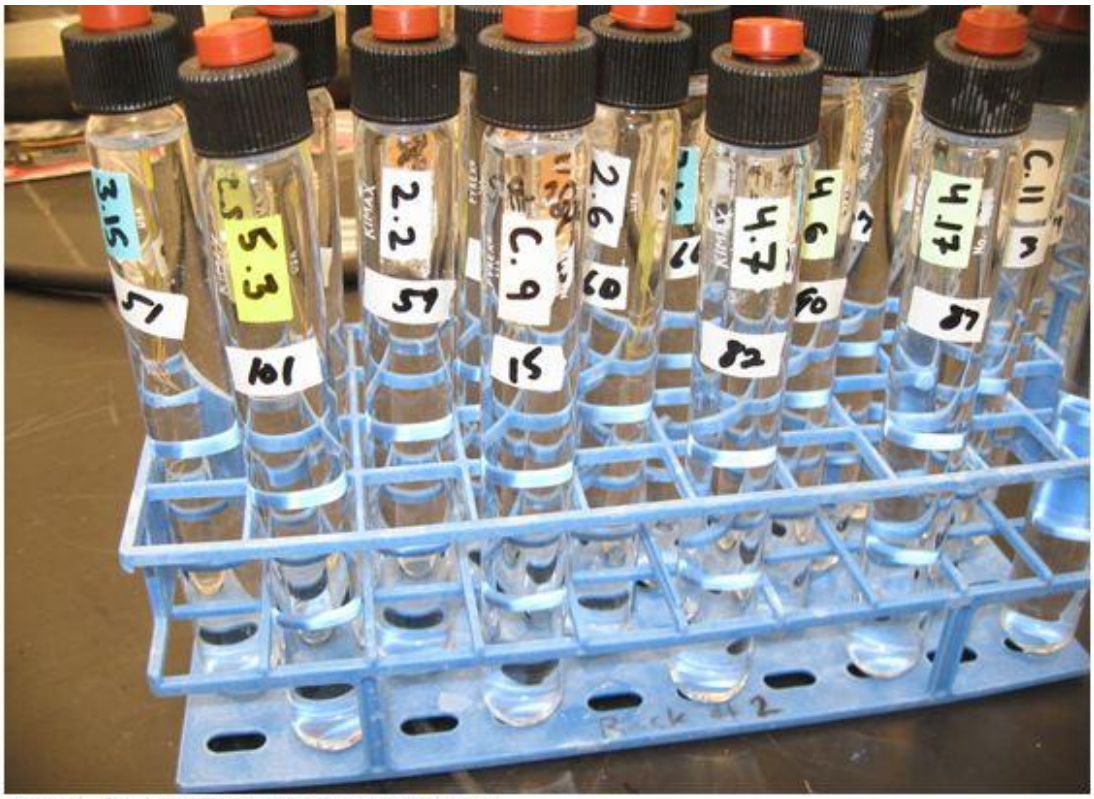

Image 1. Random placement of exposure tubes


Image2. Exposure solution renewal

Image3. Wild rice seed conditioned for germination.

Image 4. Wild rice sprout selection; sprouts on the left have mesocotyl growth of 1-2 mm long, sprouts on the right have mesocotyl growth either too long or too short to be acceptable for test initiation.

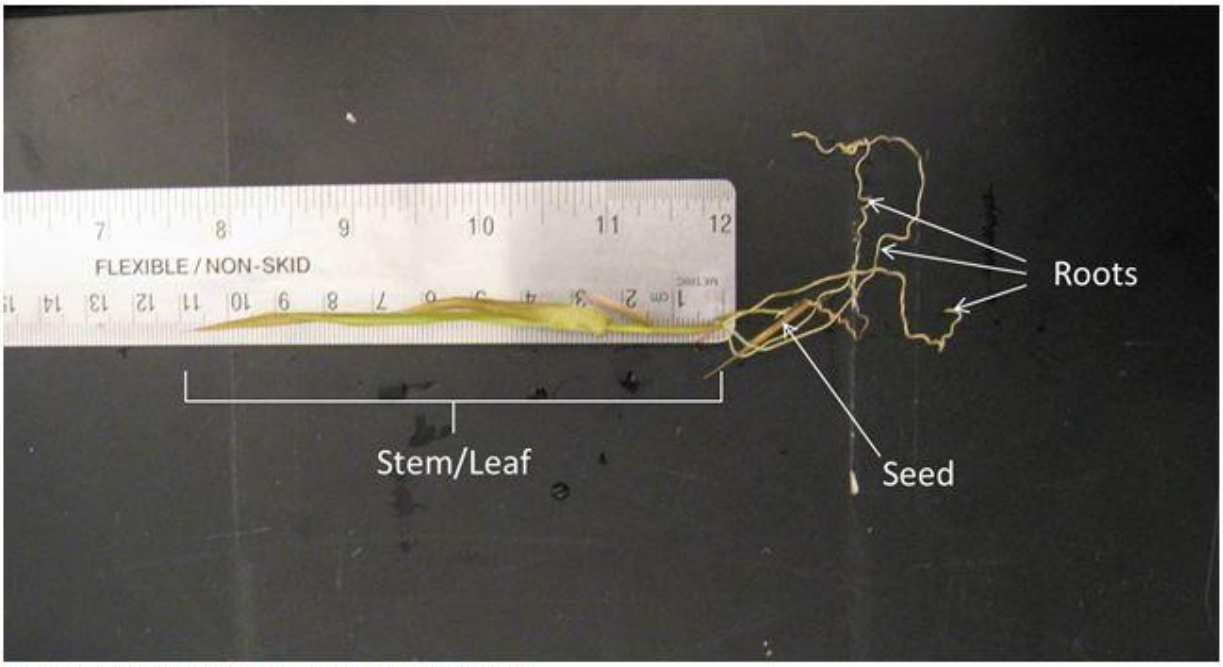

Image 5. Identification of sections of wild rice sprout.

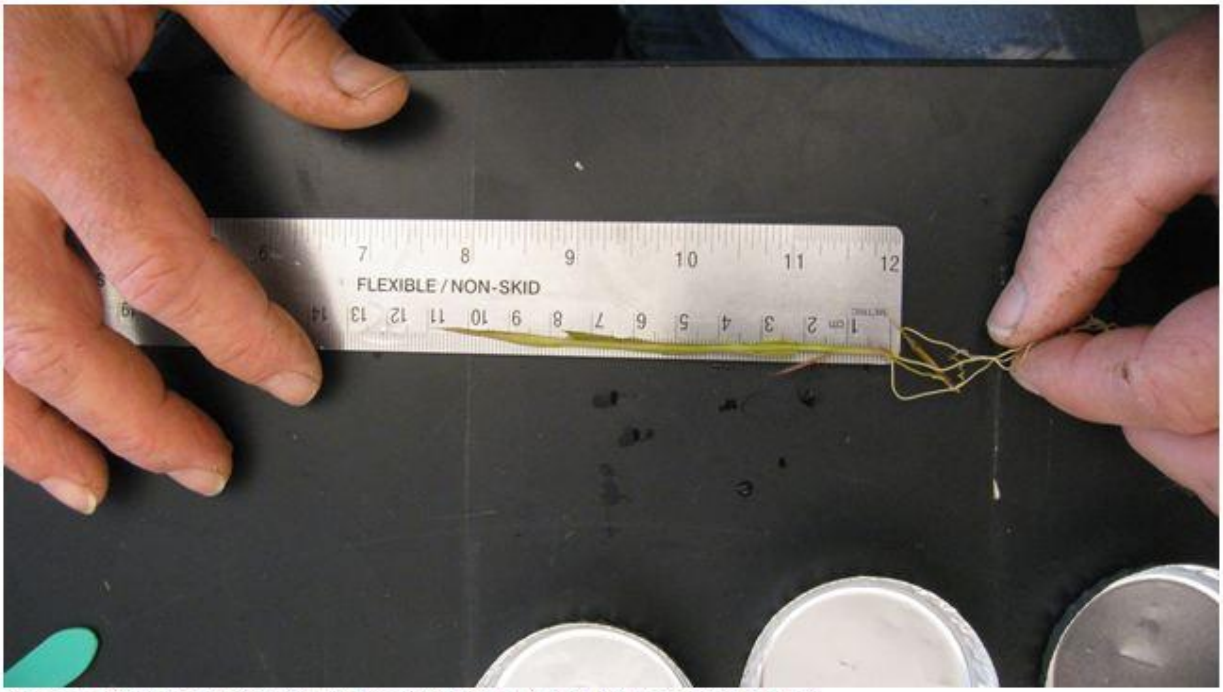

Image 6. Wild rice seedling length measurement following 10 d exposure period.

Image 7. Wild rice seedling showing removal of stem portion in preparation for drying.

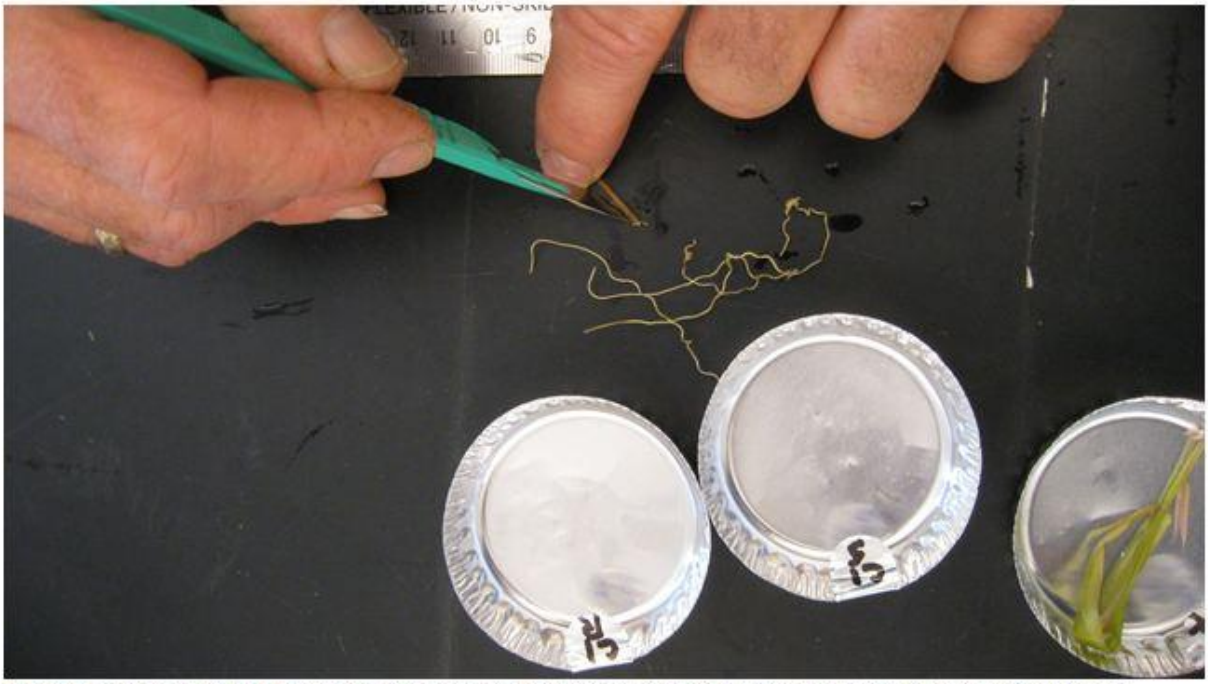

Image 8. Wild rice seedling root removal showing roots being cut from remaining seed material in preparation for measuring root length and area; stem portion of seedling is shown in a drying pan.

Image 9. Wild rice seedling showing stem, root and remaining seed portions in preparation for drying.