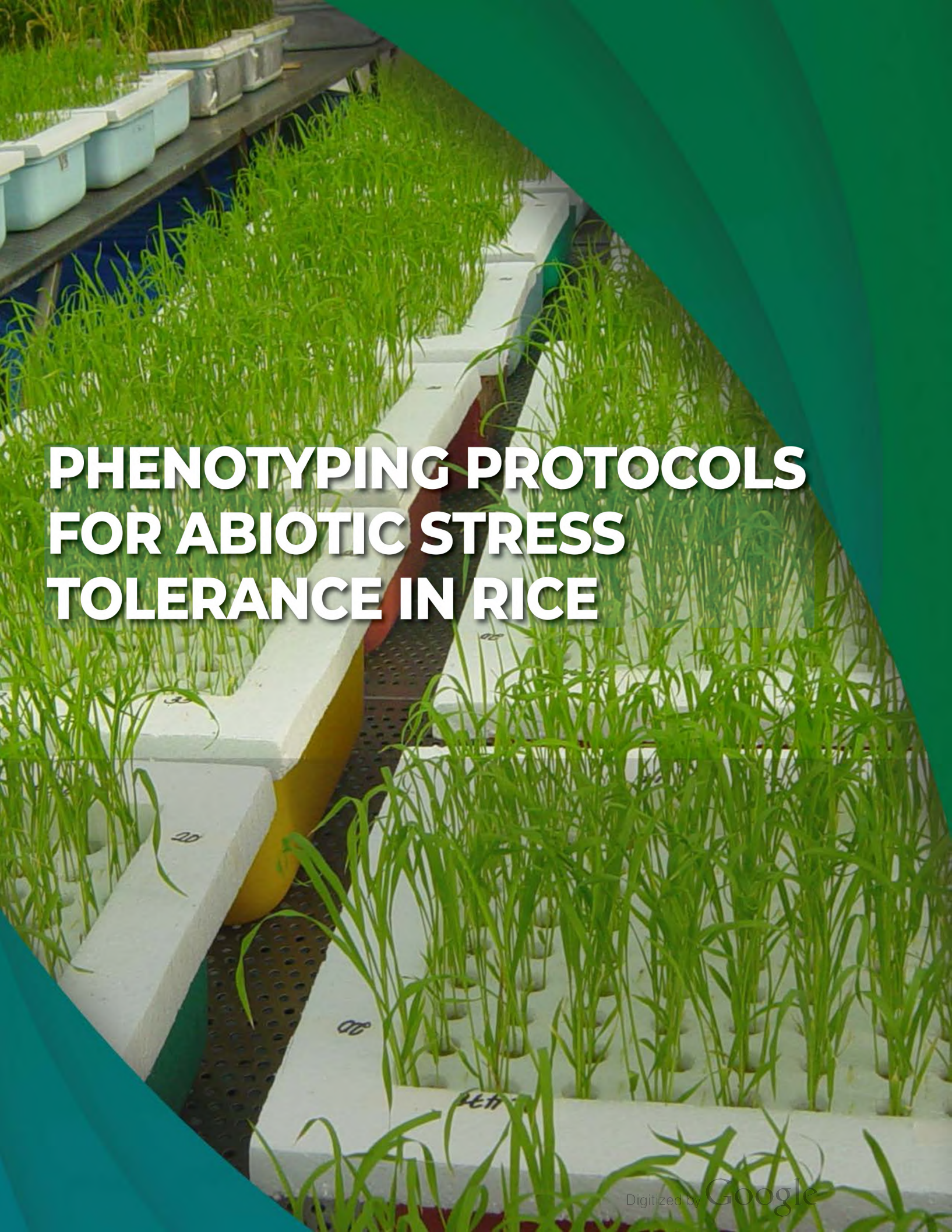

PHENOTYPING PROTOCOLS FOR ABIOTIC STRESS TOLERANCE IN RICE

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2021

The International Rice Research (IRRI) was established in 1960 by the Ford and Rockefeller Foundations with the help and approval of the Government of the Philippines. Today, IRRI is one of the nonprofit international research centers that are members of the CGIAR Consortium (www.cgiar.org). It is supported in part by government funding agencies, foundations, the private sector, and nongovernment organizations.

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Table of Contents

Preface

Rice production in rainfed lowland and upland areas is critical to global food supply, but is characterized by low and unstable yields due to a range of adverse climate and soil conditions. However, a large degree of genetic variation exists within *Oryza sativa*, and that variation can be exploited by identifying stress-tolerant genotypes and landraces for use in breeding to enable the development of new stress-tolerant high-yielding varieties. In order to screen accessions to identify stress-tolerant donors for breeding, and to further select breeding lines showing improved stress tolerance, robust phenotyping methods are necessary.

In this manual, we provide detailed protocols for screening rice under the major stresses affecting both rainfed and irrigated rice: submergence, salinity, drought, and the soil problems – excess iron and aluminum. We also include protocols for a new area of abiotic stress research since it is increasingly occurring in farmers' field due to climate change: combined stresses. Results from these protocols on combined drought + salinity and submergence + salinity indicate that the effects on the rice plant are sometimes different than under individual stresses.

The protocols in this manual have been developed and optimized over decades at IRRI. Numerous scientists, technical staff and students have been involved in the development and use of these protocols, and it is often their experiences that have led to improvements, all of which are highly acknowledged. Our objective with this compilation is to provide a resource for researchers around the globe who aim to improve the abiotic stress tolerance of rice as part of the effort to benefit rice farmers in both rainfed and irrigated areas affected by abiotic stresses.

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Screening for salinity tolerance

1. Nutrient solutions

Screening for salinity tolerance can be conducted in either solution culture or soil-based systems.

Screening for salinity tolerance at seedling stage is a commonly used method that is favored because it minimizes environmental heterogeneity compared to field conditions (Gregorio et al 1997).

The basis of all solution-culture salinity screening is the nutrient solution. Here we present three different nutrient solutions that can be used in salinity screening experiments.

1.1 YOSHIDA'S SOLUTION

The hydroponic solution described by Yoshida et al. (1976) is still the most widely used in salinity screening protocols. Its advantages are A) a well-established history of use, B) its precise, controlled composition, and C) it produces highly differentiating phenotypes between tolerant and sensitive lines. Its disadvantages are A) its slightly complicated preparation method, B) a lack of buffering, requiring constant monitoring and adjustment of pH, and C) a relatively short lifespan, requiring renewal of the solution (recommended every 5-7 days).

Table 1.1 Composition of Yoshida's solution

Stock solution	Common name	Formula	[Stock] (g/mol)	[Final] (M)	g/L	Element
N	Ammonium nitrate	NH4NO3	80.0434	1.1419	91.40	**N**
P	Sodium phosphate	NaH2PO4.H2O	137.9923	0.2580	35.60	**P**
K	Potassium sulphate	K2SO4	174.2602	0.4097	71.39	**K,S**
Ca	Calcium chloride, dihydrate	CaCl2.2H2O	147.0146	0.7982	117.35	**Ca**
Mg	Magnesium sulphate, 7-hydrate	MgSO4.7H2O	246.4756	1.3145	324.00	**Mg,S**
Micro-nutrients	Manganous chloride, 4-hydrate	MnCl3.4H2O	233.3582	6.4279	1.500	**Mn**
	Ammonium molybdate, 4-Hydrate	(NH4)6Mo7O24.4H2O	1235.8577	0.0599	0.074	**Mo**
	Zinc sulphate, 7-hydrate	ZnSO4.7H2O	287.5796	0.1217	0.035	**Zn**
	Boric acid	H3BO3	61.8330	15.1052	0.934	**B**
	Cupric sulphate, 5-Hydrate	CuSO4.5H2O	249.6860	0.1242	0.031	**Cu**
	Ferric chloride, 6-Hydrate	FeCl3.6H2O	270.2964	28.4872	7.700	**Fe**
	Citric acid, monohydrate	C6H8O7.H2O	210.1388	56.6292	11.900	
	Sulphuric Acid (concentrated; ~18.7M)	H2SO4	18.7000	935	50mL	**S**
Silicon	Sodium meta-silicate	Na2SiO3.9H2O	284.2000	15.8340	0.0045	**Si**

Note that each stock solution (except silicon, which is dissolved directly in the final solution) is prepared as an 800× stock.

Preparation of Yoshida's solution

Preparation of stock solutions

1. Prepare separate, labelled bottles for the solutions above (6 in total).
2. Macronutrients: for two litres of each stock solution, weigh the required amount of reagent and transfer to a 1000mL beaker

3. Dissolve each macronutrient in about 750mL distilled water. Transfer the mixture to a 2L volumetric flask, and add distilled water to 2 litres. Stir the mixture for 15 minutes using a magnetic stirrer then transfer to a stock solution bottle.
4. Micronutrients: Each reagent of the micronutrient listed should be dissolved separately. Use 50mL distilled water to dissolve each reagent except for ferric chloride, which must be dissolved in 100mL distilled water.
5. Add each solution to a 2L capacity volumetric flask containing 1L deionised water. Add the ferric chloride solution to the mixture just before addition of citric acid and stir the mixture for 15 minutes using a magnetic stirrer. Finally add 100mL sulphuric acid to the mixture and bring the volume to 2000mL. Stir for another 10 minutes and store in a dark glass bottle.

Preparation of working solution

6. To prepare, e.g. 100L of Yoshida's solution, add 90L of de-ionised (or tap) water to a container of appropriate capacity (e.g. 120L).
7. Add 1.25mL/L (125mL/100L) of each 800× stock solution (N, P, K, Ca, Mg, micronutrients).
8. Weigh out the appropriate quantity of sodium meta-silicate and dissolve in the solution. NOTE: not all researchers include silicon in the final Yoshida mix, especially for seedling stage screening. Silica typically improves seedling health and reduces lodging, but there is a possibility that it significantly affects the results, and differentially between some genotypes.
9. Make up to the required volume.
10. Adjust to pH 5.0 – 5.1. During the experiment, monitor the pH daily and re-adjust with 1M NaOH/ HNO_3.

1.2 SNAP SOLUTION

The SNAP solution is gaining favour due to its simplicity of preparation and maintenance. However a number of researchers have noted that it (along with Peters solution) does not produce as great a difference between tolerant and sensitive accessions, making moderately-tolerant lines difficult to distinguish. SNAP solution is composed of SNAP A and SNAP B that are available only at the Institute of Plant Breeding, Plant Physiology Division, University of the Philippines (Los Baños) for PhP50 (a little over one US$) per litre each. The SNAP solution can be used with tap water in its preparation rather than deionised or RO water. A one-time preparation of the SNAP solution is sufficient for the whole duration of screening, typically about 3 - 4 weeks. The pH of SNAP solution is monitored and adjusted if necessary 2 or 3 times per week, maintaining it within the range 5.0 to 5.1.

Materials

- SNAP A and B
- Ferrous sulphate ($FeSO_4.7H_2O$)

Preparation of SNAP solution

1. Stock solutions (SNAP-A and SNAP-B) are available as 100× concentrates. For every litre of solution, prepare 800mL water, add 10mL each of SNAP-A and SNAP-B, and make up to 1L.
2. Add ~2.5g/100L of ferrous sulphate to the solution.

1.3 PETERS SOLUTION

Materials

Peters (20-20-20) water soluble fertilizer
Ferrous sulphate ($FeSO_4.7H_2O$)

Preparation of Peters solution

1. Dissolve 1g Peters (20-20-20) water soluble fertilizer in one litre of tap water.
2. Add 200mg/L ferrous sulphate and adjust to pH 5.1.

2. Screening for seedling stage salinity tolerance

2.1 EXPERIMENTAL AIMS AND DESIGN

Due to its relative simplicity, ease of setup, short duration and modest space requirements, the seedling stage screen is the most commonly used salinity phenotyping method. The essential aim is to compare salinity responses of multiple entries (e.g. breeding lines, F_2 or later generation progenies, RILs, etc.) against at least one tolerant and one sensitive check. This may be as simple as recording the visual SES scores (Standard Evaluation System for Rice [SES], IRRI 2013), or often involves additional sampling of one or more tissues from each plant/entry for determination of intracellular ion contents, hormone levels, RNA responses etc. As with any experiment, the aims should be clearly articulated before designing the experiment.

The design of the experiment is largely dictated by the experimental setup (below). Specifically, as plants are grown in floats in individual trays, these trays typically form the fundamental unit of replication. Thus, an individual entry will typically have 3 or more plants per individual tray, and there will typically be 3 or more trays having that entry. The specific assignment of entries to each tray – and their positions within a tray – is best assigned according to a randomised complete block design (for screening at a single salinity level), or a split plot design (for two or more salinity treatments). However this design is not always followed as it results in complicated seeding patterns, making it prone to mistakes in the assignment of seed genotype to tray positions.

The major sources of variation tend to be: A) position within trays – plants in the outermost row are often exposed to higher light levels and temperatures, leading to increased transpiration and accelerated development of symptoms, particularly at high planting densities, and B) tray-to-tray variability, which is often attributable to preparation of nutrients/salinity treatments individually per tray or unfavourable tray positioning in the glasshouse. Steps can be taken to minimise these effects, such as rotating trays in the growth area. Provided there is sufficient replication within a tray, the planting density is kept constant, and sufficient replication of trays is carried out, results seem to be robust.

2.2 MATERIALS REQUIRED

General materials

- pH meter
- EC meter
- NaCl (AR grade)
- Graduated cylinder (1000mL capacity)
- Plastic Beaker (5000mL capacity)
- 8 L capacity rectangular plastic trays (dark colour trays are preferred to reduce algal growth). Those in use at IRRI have internal dimensions of approximately 34.5×25 cm at the lip.
- Styrofoam sheets at least 15 mm in thickness for seedling floats, or alternatively black polyethylene foam 3/8th inch thickness from McMaster-Carr (www.mcmaster.com)
- Hole punch ~15 mm diameter
- Standard shade cloth mesh (available from garden supply stores)
- Glue gun and glue sticks
- Standard black marker pens, permanent and preferably fade-proof

- Mixing containers (cylindrical rubber containers, 120 – 200 L capacity)
- Stirrer (wood paddle or fabricated stirrer) or submersible pump
- 1N NaOH or KOH, and 1N HNO_3 or HCl – for adjusting the pH of the solution

Materials for sample harvesting and analysis

General:

- 50mL Falcon tubes with caps
- Coin envelopes, labelled (to store samples)
- Ovens, for drying samples
- Acetic acid
- Adjustable pipettor, 50mL volume

For chlorophyll determination:

- Absolute ethanol
- Freeze-dryer

2.3 SCREENING SETUP AND MAINTENANCE

Preparation of seeds

1. Dormancy of seeds should be broken prior to germination. Incubate seeds at 50ºC for 3 – 5 days in a convection oven, depending on the level of dormancy expected. For example, freshly harvested seed will require about 5 days, irrespective of variety. Seed stored at room temperature for several months may require only 3 days (IR29, FL478) but some landraces (e.g. Hasawi) still require 5 days. If in doubt, incubate for 5 days.
2. ~2 days before the planned transplanting date, prepare clean petri dishes with a layer of paper towel on the bottom. Moisten the paper towel with water, and sprinkle seeds evenly over the area available (max. ~50 seeds per 90mm petri dish). Seed lots from different genotypes may be staggered according to the time required to achieve full germination. Tolerant and sensitive check varieties (e.g. FL478 and IR29, respectively) must be included in each experiment.

Preparation of seedling floats

Styrofoam floats

Styrofoam floats are the most commonly used seedling floats. They are cheap and easy to make, but are not (easily) re-usable.

1. Cut the Styrofoam sheets into sections (floats) that fit snugly ~5cm below the surface of the 8L plastic trays. A snug fit is required to prevent light entry and reduce algal growth.
2. Punch 15 mm diameter holes in the Styrofoam floats, spacing holes as evenly and regularly as possible across the length and width of the float, typically in a grid pattern of around 10 rows × 10 columns, 30mm between holes in a row and 20mm within a column (this can be adjusted to your experimental design).
3. Cut the shade cloth mesh to fit neatly under the Styrofoam float. Attach the shade cloth mesh securely to the float using the glue gun, and allow to set.
4. Label the columns and rows with the genotype/treatment/replicate information as appropriate for the experiment. It is sometimes easier to apply some masking tape over the Styrofoam and write the label on it. The seedling float is now ready for use. See Figure 2.1.

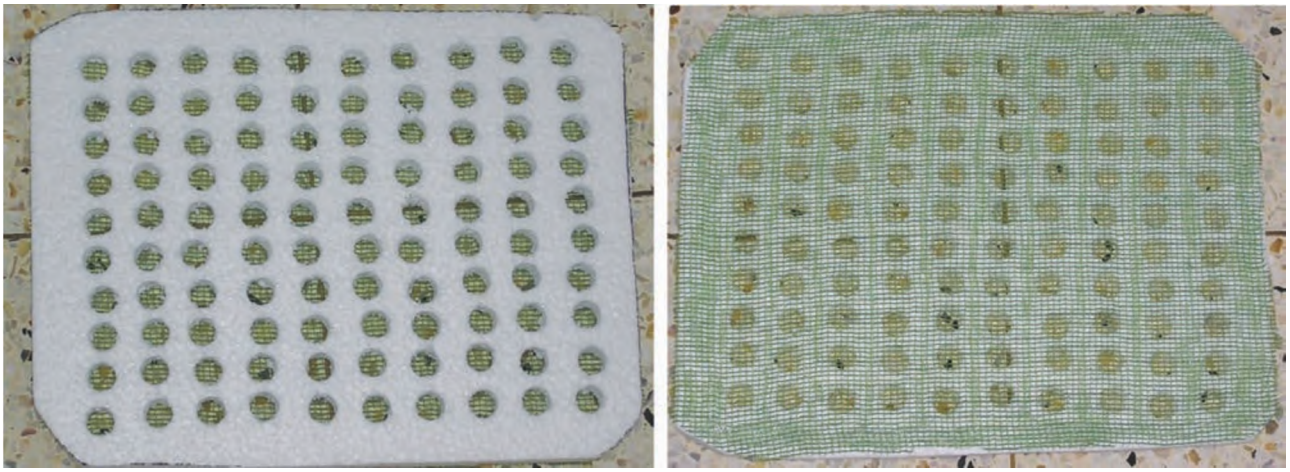


Figure 2.1. Seedling floats: top and bottom views. The arrangement of holes for seedlings, and nylon shade cloth used to support seedlings, can be seen.

Polyethylene floats

These floats have the advantage of allowing easy removal of intact plants for measurements; afterwards plants can simply be re-inserted into the float to continue growing, something not possible with Styrofoam floats. They are relatively expensive; the foam is available from McMaster-Carr (www.mcmaster.com/#8722k52/=eq3vs3) at a cost of US$26 per 36"×54" sheet and plants are typically at a lower density (requiring more replication), but they are re-usable for several screenings. Over time, however, they tend to shrink.

1. Cut the foam sheet into strips approximately 5cm wide and a length to fit the culture trays.
2. Along the length of the strip, make incisions ~25mm deep (i.e. halfway) and 30mm apart. Arrange strips in the culture tray.
3. Label strips with genotype; typically one genotype is used per strip.
4. Take pre-germinated seedlings, and gently insert them into the incisions, one plant per incision, with the shoot/root junction at the bottom edge of the foam.

Seeding of trays, and maintenance of plants

1. Prepare the appropriate number of plastic trays, filled with ~7L de-ionised water. Place the Styrofoam floats on the surface of the water.
2. Once seedlings have developed primary roots ~2 – 3cm long (typically ~36 hours after sowing in petri dishes), carefully transplant to the floats according to the experimental design. Roots should be carefully fed through a hole in the nylon mesh, and end up in good contact with the water. One to two seedlings should be transplanted per hole.

NOTE: Only good-quality seedlings, with a well-developed root and coleoptile, should be chosen.

3. Cover the trays with an opaque screen, and cultivate seedlings for 3 days on the de-ionised or good quality tap water under standard growth conditions. After this time, discard the de-ionised/tap water and replace with nutrient solution.
4. Monitor the pH and volume of the nutrient solution daily. Add de-ionised water to reconstitute 7L volume as required, and adjust to pH 5.0 – 5.1 as necessary with acid (1N HNO_3 or HCl) or base (1N NaOH or KOH) (Yoshida's solution typically requires addition of basic solution each day; SNAP is to some extent buffered, and may not require adjustment so frequently).
5. If using Yoshida's solution, replace weekly with fresh mix. SNAP solution can last up to 1 month.

6. One to two weeks after seeding, seedlings should be thinned to remove weak or unusual plants. Different researchers use different densities, ranging from >100 plants per float (one or two plants per hole) down to 50 (one every two holes). The critical factor is to be consistent between trays and experiments.

Imposition of salinity stress

1. Salinisation may commence once seedlings have 3 or more fully-expanded leaves, i.e. leaf 4 or above is emerging.
2. Apply salt preferably by exchanging the existing nutrient solution with fresh nutrients containing the appropriate concentration of salt.
3. Alternatively, salt can be weighed according to that required in each individual tray. Seedling floats are temporarily removed to a holding tray (with de-ionised/tap water or nutrient solution, to prevent root dehydration). The salt is then dissolved in the existing nutrient solution and mixed well, after which the seedling float is returned to the tray.
4. High levels of salinity should be applied in increments, separated by 1 – 2 days, to avoid severe osmotic shocks. For example, a salinity level of EC 18 might be applied in 2 increments, EC 0 → EC 10 (wait 2 days) → EC 18. Likewise a salinity level of 150mM might be applied in increments of 0mM → 75mM (wait 2 days) → 150mM.
5. Maintain growth as described above. Scoring and harvest typically commence once sensitive checks reach an average SES of 7; this is typically ~14 days after the start of salinisation.

2.4 SCORING, SAMPLE HARVESTING AND ANALYSIS

SES evaluation

Once symptoms have progressed to an appropriate extent (usually until the sensitive check reaches an average SES value of 7 – 8), visual injury scores may be assessed. These may be assessed per individual plant, or a single aggregate score per genotype. The SES should be evaluated according to the following criteria:

SES	Description	Tolerance
1	Normal growth, only the old leaves show white tips while no symptoms on young leaves	Very high
3	Near normal growth, but only leaf tips burn, few older leaves become whitish partially	High
5	Growth severely retarded; most old leaves severely injured, few young leaves elongating	Moderate
7	Complete cessation of growth; most leaves dried; only few young leaves still green	Susceptible
9	Almost all plants dead or dying	Highly susceptible

Note that the SES score is originally a 9-point scale, but many workers score a 5-point scale as above. It is essentially a continuous scale, and scores at intermediate points (SES 2, 4, 6, 8) are simply intermediate in symptoms between the points stated above. Photos of plants showing common SES symptoms can be seen in Figure 2.2; example screening data can be seen in Figure 2.3.

SES 3: Minor browing of tips, particularly older leaves

SES 5: Significant dying of most leaves, emerging leaf dying

SES 7: Most leaves dead or nearly so; growth ceased, main dying

Figure 2.2. Examples of salt-induced injuries, and appropriate SES scores for these individuals.

Sample harvesting

The harvesting design will vary depending on the type of analysis to be carried out. Some common examples follow.

Na^+ and K^+ determination:

1. Prepare at least two, preferably three, plastic trays, each containing several litres of de-ionised water, to wash the samples.
2. Identify the tissues/organs for which Na^+ and K^+ levels should be determined. Dissect these from the plant.
3. Wearing gloves and using forceps, wash the samples sequentially in the 2 – 3 trays of de-ionised water. Transfer sample to labelled coin envelope. If the fresh weight is required, this should be measured prior to placing the samples in envelopes.
4. Dry samples at 50°C or higher for several days, until completely dry. Some researchers homogenise their samples once dry.
5. Prepare labelled 50mL Falcon tubes. Determine the dry weight of the samples (either directly, or via difference).
6. Add ~30 – 40mL 0.1M acetic acid to each sample. The exact volume is not critical, but should be reproducible between samples (or measured per sample).

7. Digest samples for a few hours at 80ºC, or overnight/a couple of days at 60ºC in an oven.
8. Leaf samples will often float on the solution, due to air spaces in the tissue and the hydrophobic waxes on the leaf surface. If this is the case, samples should be vigorously shaken every few hours until they no longer float. Digestion is complete once the sample is fully hydrated and sinks in the solution. Often the solution becomes a pale yellow colour.
9. Na^+ and K^+ can be determined on a flame emission photometer, atomic emission spectrophotometer, or mass spectrometer. Calibration against a set of known standards (usually prepared in the same buffer system used for digestion) is usually required
10. Depending on the specifications of the instrument, the sample may need to be diluted. Highest Na^+ levels are usually seen in leaf sheath tissue.
11. Example Na^+ data (and correlation with SES score) can be seen in Figure 2.4.

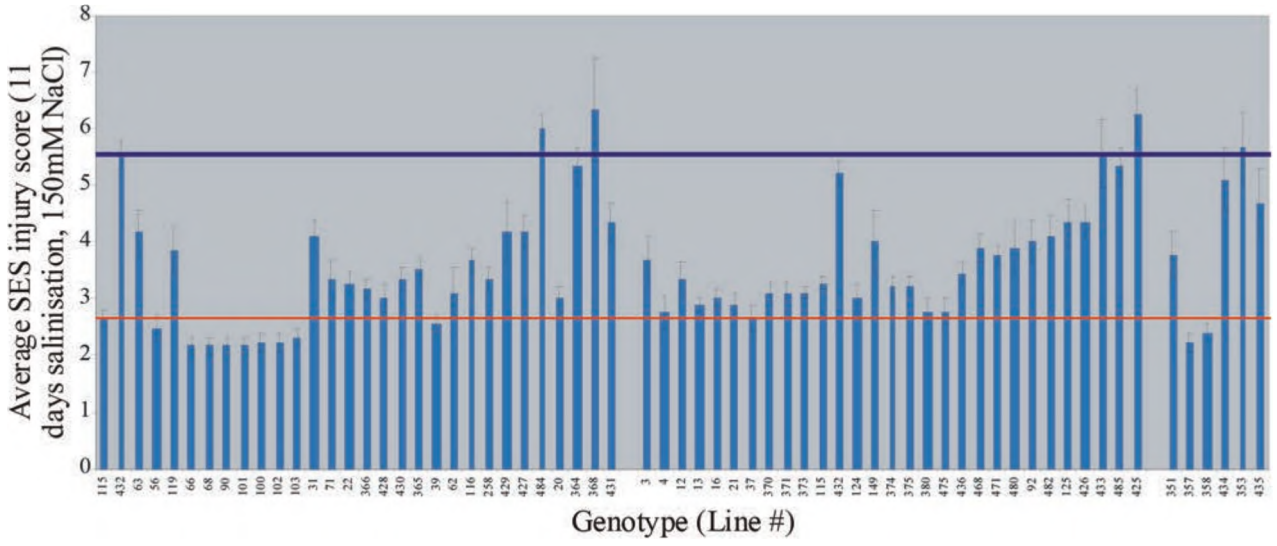


Figure 2.3. Example SES scores from a screening experiment (in this case involving an unusually high proportion of tolerant landraces). Average SES scores for FL478 and Nipponbare are indicated by the red and blue lines, respectively. An average SES score of 4 or below would indicate a tolerant line.

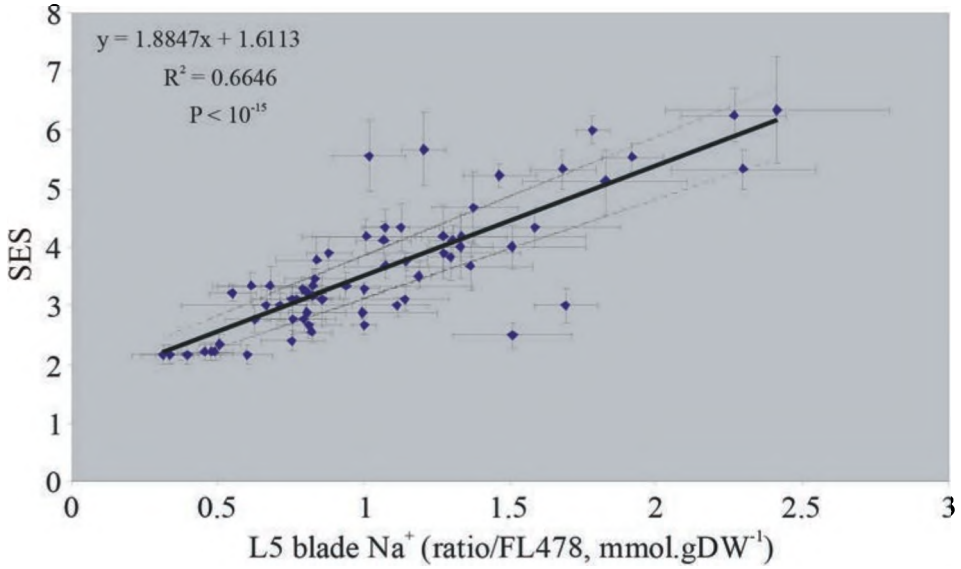


Figure 2.4. Correlation of SES score with leaf blade Na+ content across approximately 80 tolerant or moderately tolerant landraces.

Chlorophyll determination:

1. The harvesting procedure is performed in much the same way as for Na^+ determination (steps 1 – 3), except that the sample is flash-frozen in liquid N_2 immediately after placing it in the envelope. Samples are stored at -20°C.
2. Samples should be freeze-dried until fully dried. Dried samples are stored in air-tight bags in the dark at -20 – 4°C until ready to proceed.
3. Dry weights are recorded as described above. Add 20 – 40mL 95% ethanol to the dried sample (in a 50mL tube) and cover (but leave the lid loose to prevent explosion).
4. Boil samples at 80°C for 10 minutes, allow to cool to room temperature and reconstitute the required volume of ethanol with fresh 95% ethanol.
5. Record the absorbance at wavelengths of 470, 649 and 664nm. Chlorophyll concentrations may now be determined by the equations outlined in Lichtenthaler and Buschmann (2001), p F4.3.6. Concentration values (mg/L) may then be converted back to the quantity of chlorophyll per gram (dry weight) of tissue (mg/gDW).

3. Determination of tissue Na^+ tolerance

Rice salinity studies typically examine tissue Na^+ and/or K^+ concentrations, comparing these between tolerant and sensitive lines. This is ideal for identifying and characterising traits such as Na^+ exclusion, but does not give quantitative information about the level of Na^+ within the leaf that is toxic. The latter parameter is usually known as "tissue tolerance", and can be an indication of processes such as Na^+ sequestration in the vacuole and biochemical tolerance. The determination of tissue tolerance is significantly more complex than that of Na^+ exclusion, and has less frequently been the subject of salinity research in rice. The identification of lines showing high levels of tissue tolerance, particularly lines which use this method to produce salt tolerance, has great potential for pyramiding. The protocol outlined here is based on that originally described in Yeo and Flowers (1983).

The determination of tissue tolerance uses the same materials and approach as described in Chapter 2; the key difference is in experimental design. Whereas a screening for Na^+ exclusion may assess salinity responses at a single salinity level, or between salinised and control treatments, determination of tissue tolerance typically makes use of 5 or more salinity levels. The same lines are exposed to each salinity level, and treatment is carried out until each line reaches an average SES of 7 in the highest salinity treatment. Once an individual line reaches this level of injury, the youngest fully-expanded leaf is harvested from plants of that line only, from ALL the salinity levels. This continues for each line until all are harvested; note that the harvest time of each line will vary depending on its tolerance. These samples are then freeze-dried, and tissue chlorophyll and Na^+ contents measured. The regression of chlorophyll content vs. Na^+ content is then used to estimate the tissue Na^+ content at which a 50% reduction in chlorophyll occurs, which is then designated as the tissue tolerance of that line.

It should be noted that by harvesting each line when it reaches SES 7 at the highest salinity level, rather than after a set time, effects such as Na^+ exclusion are largely cancelled. That is, a line with high Na^+ exclusion may take a long time to reach SES 7, but when it does, the tissue Na^+ contents are often still low and thus the tissue tolerance score is low. Thus this method provides a way of discriminating between lines that have high tolerance due to Na^+ exclusion but low tissue tolerance, lines whose tolerance is due to high tolerance of tissue Na^+ content, and lines with low overall tolerance but high tolerance of internal Na^+ levels. One interesting observation is that many "sensitive" lines such as Azucena and IR29 score quite highly on this test.

3.1 EXPERIMENTAL DESIGN

A critical feature of the experimental design is the use of multiple (5 or more) salinity treatments. A typical design would be as follows:

Salinity treatments: 5 (EC 0, 6, 12, 18, 24 or 0mM, 60mM, 90mM, 120mM, 150mM, 200mM)
Genotypes (entries): 5: IR29 (sensitive check), FL478 (tolerant check), 3 test entries
Replication: 2 lanes (10 plants each) per entry per tray
1 or more trays per salinity treatment

It is important to include an unsalinised control treatment to allow determination of maximum chlorophyll content in the absence of salt stress.

3.2 MATERIALS

Materials are essentially as described in Chapter 2. Note that the materials for both Na^+ and chlorophyll extraction are required.

3.3 PROTOCOL

1. Seedling germination, float preparation and nutrient solutions are as described in Sections 1 – 2. The critical difference with this protocol is that one is comparing both genotypic and salinity treatment effects.
2. Once plants reach an appropriate age, salinisation may commence. It is critically important to salinise in increments, particularly for the higher salinity levels, as applying these levels in a single dose will kill all plants simply due to the extreme osmotic shock. An example salinisation schedule may be as follows:

Final concentration	Day 1	Day 3	Day 5	Day 7
EC 0	No change			
EC 6	EC 6	No change		
EC 12	EC 6	EC 12	No change	
EC 18	EC 6	EC 12	EC 18	No change
EC 24	EC 6	EC 12	EC 18	EC 24

3. Monitor plants daily. Adjustment of pH and water levels, and refreshment of solution, is as described in Chapter 2. It is advisable to refresh the solution weekly to ensure salinity levels are maintained; these will vary over time, and differentially between varieties, as some varieties take up more Na^+ than others.
4. Harvesting commences once a particular variety reaches an average SES of 7 in the highest salinity treatment. At this time, the youngest fully-expanded leaf is harvested from all plants of that variety, from all salinity treatments. Typically the date of harvest and SES scores (per individual plant) may also be recorded.
5. Harvested samples are freeze-dried and stored at -20ºC until ready to extract.
6. Chlorophyll extraction is done in batches. Record the dry weight of each sample, and add 30mL 95% ethanol.
7. Extract at 80ºC for 10 mins in a water bath. Tubes should not be sealed tight, to avoid explosion.
8. Cool to room temperature and immediately record the absorption at wavelengths of 470, 649 and 664nm. It may be necessary to reconstitute the volume of ethanol to replace that which boils off. After measurement it is important to return the aliquot to the sample tube.
9. Add $1/10^{th}$ volume 1M acetic acid (to 0.1M final concentration) and re-incubate at >70ºC for a few hours to extract Na^+ and K^+. Determine contents on a flame photometer/atomic emission spectrophotometer.

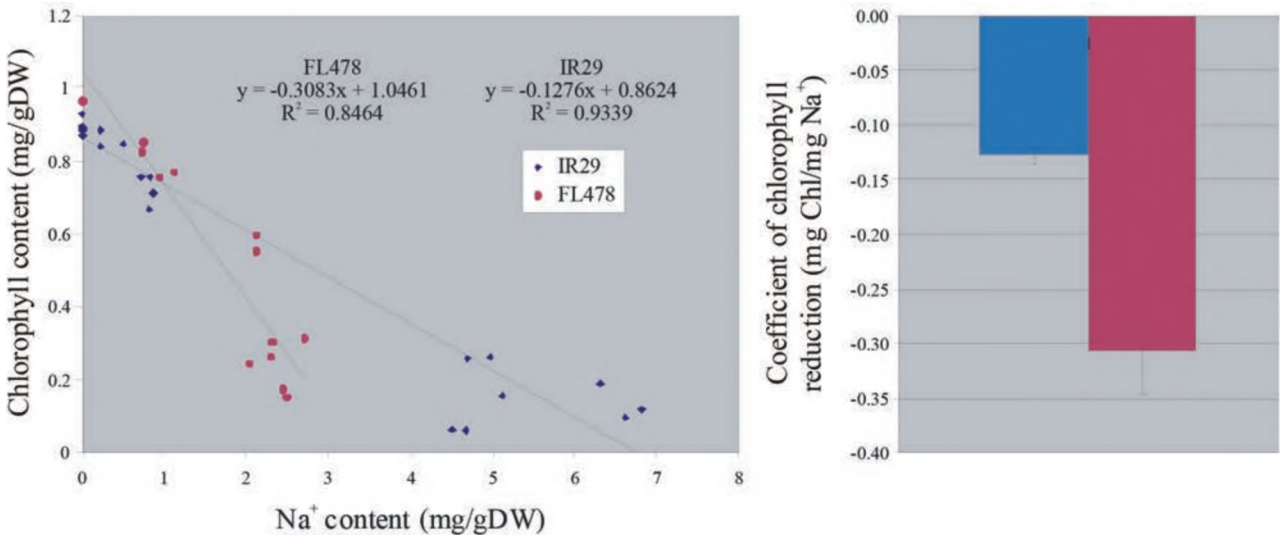


Figure 3.1. An example of tissue tolerance data. FL478 and IR29 are contrasting in their tissue tolerance, but IR29 has high tissue tolerance and FL478 low tolerance. This is indicated by the greater negative slope of the FL478 regression line. From this data, the LC50 for FL478 is (0.5*0.645-1.046)/-0.308 = 1.829, while for IR29 LC50 = (0.5*0.929-0.862)/-0.128 = 3.120.

3.4 ANALYSIS

1. Chlorophyll contents of extracts are determined as described in Lichtenthaler and Buschmann (2001) p. F4.3.6. Concentration values are multiplied by the volume of extract (30mL) and divided by the dry weight to give a value of mg chlorophyll per gram tissue.
2. The tissue chlorophyll and Na^+ contents (mg/g DW and mmol/g DW respectively) for each line are plotted against each other. Example data can be seen in Figure 3.1.
3. A linear regression line is used to determine the relationship between tissue chlorophyll and Na^+ contents. From the coefficients of this equation it is simple to calculate the Na^+ content at which the chlorophyll content is half its maximum level (in the EC 0/untreated control plants). This Na^+ content is designated the tissue tolerance of that line (LC_{50} in the notation of Yeo and Flowers 1983).

4. Glasshouse screening for reproductive stage salinity tolerance

Genotypic variation in stress response may vary with the growth stage at which the stress is applied; genotypes tolerant to seedling stage salinity may differ from those tolerant to reproductive stage salinity (Moradi et al. 2003). In this chapter we describe a protocol for reproductive stage salinity screening in the greenhouse. Since the timing for moving each plant into the salinity treatment is based on its individual phenology, this protocol allows for uniform targeting of reproductive stage even among diverse accessions. Two options are presented here: a gravel-based supported hydroponics method, and a soil-based method. The gravel-based method can facilitate root sampling. The soil-based method is preferred where nutrients can be added to the soil in the pots, whereas for the first, a nutrient solution is required.

4.1 EXPERIMENTAL DESIGN

1. The test entries should be grouped according to plant height and duration to have uniform conditions from flowering to ripening and to minimize shading effects. i.e. group into tall and semidwarf for height, and early (100 ± 10d), medium (125 ±10d), and late (150 ± 10d) for duration, resulting into 6 groups: early-tall, early-semidwarf, medium-tall, medium-semidwarf, late-tall, and late-semidwarf.
2. Tolerant and sensitive checks must be used for comparison with the test entries. It is advisable to include both checks in every tray. Suggested check varieties for every entry group are shown below:

Varietal Group		Tolerant check	Sensitive check
Early	Tall	TCCP266-1-3B-10-3-1	PP2462-11
	Semi-dwarf	IR45427-2B-2-2B-1-1	IR58
Medium	Tall	Pokkali 108921	FR13A
	Semi-dwarf	IR63731-1-1-4-3-2	IR29
Late	Tall	SR26B	Wagwag
	Semi-dwarf	IR52724-2B-6-2B-1-1	Bg 407

3. Either a completely randomized (CRD) or randomized complete block design could be used. Since only one test entry is planted in a pot, each pot could be considered as one experimental unit.
4. Include two treatments - saline and non-saline conditions - to estimate the effect of salinity on growth and reproduction.

4.2 MATERIALS REQUIRED

General materials

- Plastic pots (~1 L capacity)
- Plastic trays (60L capacity)
- Graduated cylinder (1000mL capacity)
- Plastic Beaker (5000mL capacity)
- Mixing containers (cylindrical rubber made containers: 60-200L capacity)
- Stirrer (wood paddle or fabricated stirrer)/water pump

- NaCl (rock salt or Laboratory reagent-LR grade)
- EC meter

Gravel-based supported hydroponics method

- Volcanic gravel (coarse sand or other inert media may possibly be substituted)
- pH meter
- SNAP solutions A and B (Institute of Plant Breeding, University of the Philippines, Los Baños)

Soil-based method

- 100% cotton cloth bags or nylon mesh
- Well-ground soil, preferably from a rice paddy
- Standard NPK rice fertiliser

4.3 SETUP AND MAINTENANCE

Prepare perforated plastic pots. Take a plastic pot ~20cm in diameter. Drill 3 – 4mm holes in 4 rows down its sides, 2cm between holes. The topmost circle of holes should be at least 3cm below the rim of the pot. Stitch bags from the cotton cloth that fit well inside the plastic pot.

Gravel-based supported hydroponics method:

1. Prepare seedlings and floats as described in Chapter 2. Sow pre-germinated seeds two per hole on seedling floats using SNAP or Yoshida solution.
2. Replant 14-day-old seedlings in perforated plastic pots (one seedling per pot) filled with volcanic gravel and keep in trays with SNAP/Yoshida culture solution (Figure 4.1).
3. Plants should be sown in two batches. Plant the first batch 2 weeks prior to test entries (2nd batch). These will serve as an "indicator" of booting, to determine when to apply salt stress to the test entries.
4. Into perforated plastic pots, fill the cloth bag/nylon mesh (inside pot) with fertilised soil [50N-25P-25K (or 0.1 g urea, 0.14g P2O5, and 0.04g K2O) per kilogram of soil] to ~1cm above the top row of holes. Level the soil.
5. Place 4 – 5 pre-germinated seeds of the test entries on the soil surface, and raise the water level to ~1 cm above soil level. Extra soil can be added if necessary. Thin seedlings to 3 per pot after 14 days. Tap water (not SNAP solution) is used for this protocol.

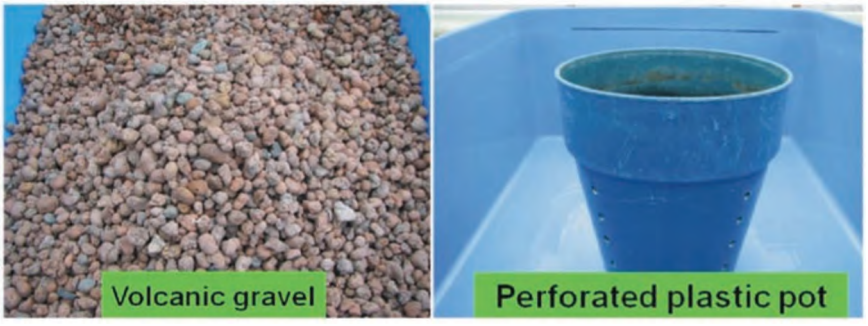


Figure 4.1. Materials for setting up the gravel-based supported hydroponics screening method.

6. Raise the level of solution to about the gravel/soil surface.
7. Due to evaporation and transpiration there will be loss of solution volume in the trays. Reconstitute the volume daily with tap water.

8. Maintain the pH of the SNAP solution at pH 5 by adding 1 N nitric acid every 2 days (Figure 4.2).

Figure 4.2. Adjustment of solution pH levels.

9. Application of salt stress should be done when the test entries are at panicle initiation stage; this is judged as the time when indicator plants are about to enter booting stage. Dissecting the growing point of a few main tillers will help more accurately determine when transition started. Apply NaCl to the nutrient solution/water to reach a final EC of 6 dS m-1. For the soil method, drain all water in the water bath and allow soil water to drain. Replace water with salinised solution.
10. After de-salinization, maintain water levels daily with tap water (NOT salinised water).

Soil method using large trays (60L capacity)

1. Prepare pots with soil fertilized with 50N, 25P and 25K (or 0.1 g urea, 0.14g P2O5, and 0.04g K2O) mg kg-1 of soil.
2. Place 5 pre-germinated seeds of the test entries on the soil surface, and raise the water level to ~1 cm above soil level. Extra soil can be added if necessary. Thin seedlings to 1 per pot after 14 days.
3. Raise the level of solution to about the soil surface.
4. Due to evaporation and transpiration there will be loss of solution volume in the trays. Reconstitute the volume daily with tap water.
5. At the onset of flag leaf appearance (booting stage), plants can be trimmed leaving the top 3 leaves per tiller, however this step is not usually necessary. Salt stress equivalent to EC 10 dS m-1 will then be imposed up to 20 days.
6. After 20 days in salinized water, plants are returned to non-salinized water until harvest.

4.4 SCORING

1. Test entries can be scored as soon as the susceptible check expresses stress symptoms (leaf rolling and whitening), at about 3 weeks after salinisation.
2. Percent green leaf area of the flag leaf is convenient and reliable to use as a measure of tolerance.
3. Grain yield and yield component data such as plant height, number of panicles per plant, straw yield per plant, number of filled grains per plant, number of unfilled grains per plant, spikelet fertility, pollen fertility (see Chapter 6) and Na/K ratio of flag leaf under both normal and salinised conditions could then be collected. (NOTE: it is impossible to get a good estimate of the total number of spikelets under reproductive stage salinity, due to malformed and aborted spikelets, and sometimes the spikelets drop off.)

5. Field screening for reproductive stage salinity tolerance

Field screening for any type of abiotic stress tolerance is a prerequisite for moving breeding lines towards release of new varieties. Although field-based salinity screening is considerably more challenging than screening in a greenhouse in solution culture, it is achievable with sufficient addition of salt/saline water and frequent monitoring of the EC of the water in the field. Note that a field dedicated to salinity screening is recommended as this treatment will affect the soil in subsequent seasons.

5.1 MATERIALS

- Plastic water tank (5000 L capacity)
- Water pump
- NaCl (rock salt)
- EC meter
- Piezometer

5.2 SETUP AND MAINTENANCE

1. Sow 100 seeds for each entry in soil-filled germination trays.
2. Transplant 21-day-old seedlings into two treatments: salinised and control. At IRRI we use concrete blocks to keep the treatments separated, but this can also be done in a bunded field with 10m between treatments to avoid seepage of saline water into the control treatment. A typical arrangement is 10 seedlings per row in 3 replications for each entry in both control and saline plots. Example setup is seen in Figures 5.1 – 5.2.
3. Apply fertilizers as usual.
4. Apply NaCl (EC 6 dS m^{-1}) to the saline plots after active tillering or 10 days before booting stage; use an EC meter to check the EC level of the water in each block.
5. Maintain the EC at 6 dS m^{-1} until maturity (dissolve NaCl in water in the tanks, and add to the saline

Concrete blocks for planting | Water tank and pump | EC meter

Figure 5.1. Materials for setting up a reproductive stage screening experiment in field conditions.

Figure 5.2. Transplanting in concrete blocks.

plot to maintain EC level, or add fresh water to dilute if the EC level is high).

6. At maturity, grain yield and yield component data such as plant height, heading date, number of panicles per plant, straw yield per plant, number of filled and unfilled spikelets per plant, spikelet fertility, pollen fertility (see Chapter 6) and Na/K ratio of the flag leaf under both normal and salinised conditions can be analysed.
7. Analysis: calculate the percent reduction for each trait in the saline treatment in comparison with the control treatment (e.g. Figure 5.3).

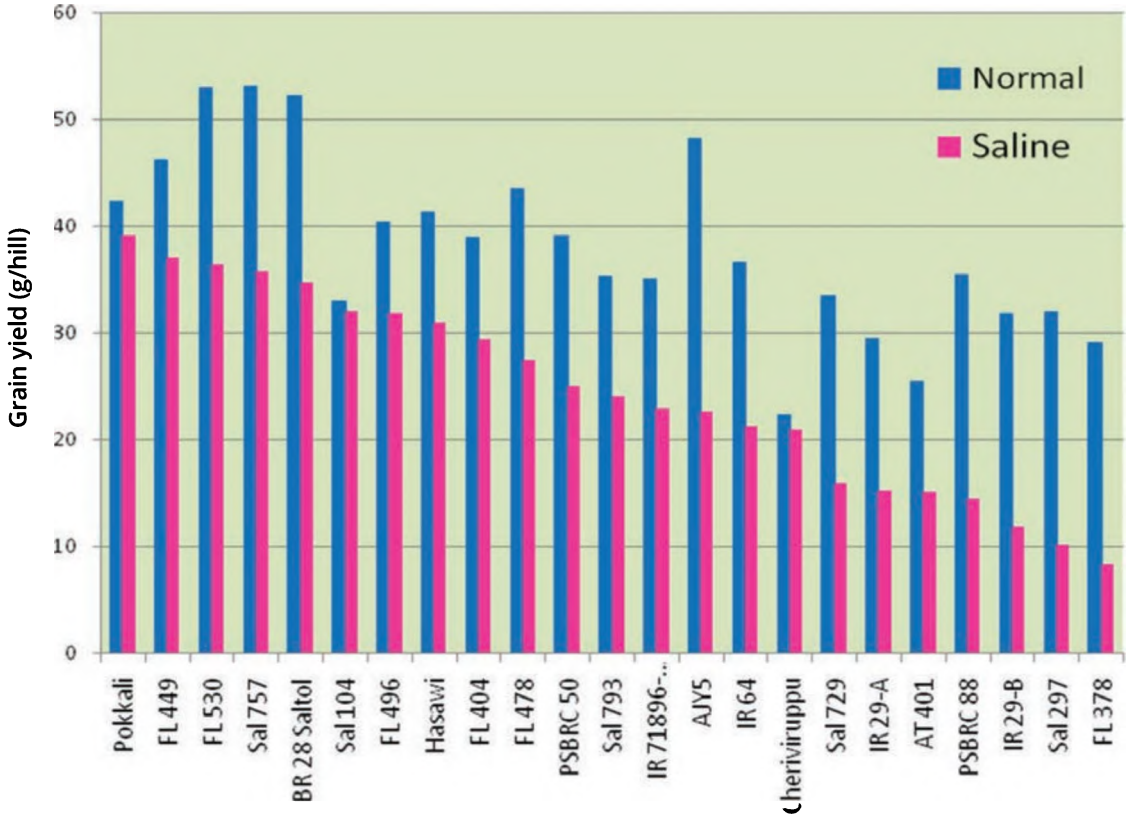


Figure 5.3. Grain yield of different genotypes in saline and control plots.

6. Reproductive-stage salinity tolerance phenotyping protocols

Mid-season phenotyping can be an important complement to yield-under-stress data, and is especially valuable for revealing genotypic differences in stress tolerance that become less apparent by harvest time in cases of severe stress. Here we describe three approaches for phenotyping salinity response - pollen fertility, plant height, and spikelet fertility - in addition to grain yield.

Source: International Network for Genetic Evaluation of Rice (INGER) (1996). Standard Evaluation System for Rice, 4th ed. International Rice Research Institute, Los Baños, Philippines.pp. 12-20

6.1 THE POLLEN FERTILITY TEST

Materials

- Microscope
- Glass slides and cover slips
- Forceps/flat-tip needles
- Dropper bottles
- Glass vials
- 70% ethanol
- 1% w/v KI (potassium iodide) solution
- Beaker of water

Methods

Preparation of 1% KI solution

1. Weigh out 10 g KI
2. Dissolve in 1 L Nanopure water.
3. Store in amber bottles, in the dark.

Determination of pollen fertility

4. Harvest spikelets from individual plants at growth stage 6 (Heading). At least 10 spikelets should be harvested per plant. After harvest, spikelets should be fixed and stored in 70% ethanol.
5. Extract 2 – 3 anthers from each of 5 florets per sample on a glass slide. Separate the lemma from the palea of a test spikelet by pulling on the palea.
6. Using a needle, scoop the anthers out of the palea and squash on a glass slide.
7. Add 1 drop of 1% KI and cover with a cover slip. Wash the forceps in water.
8. Examine the pollen under a dissecting microscope (25x magnification can be used). The iodine solution stains starch a dark purple. Viable pollen will therefore be dark, while sterile pollen (unfilled grains without starch) will be much lighter.
9. Make a count of the number of stained and unstained pollen grains in the sample (at least 100 grains per sample). At least three microscopic fields-of-view are used to count sterile pollens (unstained withered, unstained spherical and partially round), and fertile pollen grains (stained round).
10. This should then be replicated for multiple plants per genotype/treatment.

11. The percentage of fertile pollen is then given by:

$$\% \text{ filled} = \frac{\text{\# filled grains}}{\text{Total \# grains}} \times 100\%$$

Scale	Pollen fertility (%)
1	>90% fertile
3	80 – 89%
5	70 – 79%
7	60 – 69%
9	< 60%

6.2 PLANT HEIGHT

Record the total height from soil surface to the tip of the tallest leaf/panicle. Specify the growth stage at which height was measured. Record in whole numbers.

Scale

1 Semidwarf (lowland: less than 110 cm; upland; less than 90 cm)
5 Intermediate (lowland: 110-130cm; upland: 90-125 cm)
9 Tall (lowland: more than 130 cm; upland: more than 125 cm)

6.3 GRAIN YIELD

Report in terms of grams per plant.

6.4 SPIKELET FERTILITY

1. Identify filled grains by pressing spikelets between fingers.
2. Record the number of filled and unfilled spikelets (those with and without grains, respectively).
3. Calculate the % fertility:

$$\% \text{ fertility} = \frac{\text{\# filled spikelets}}{\text{Total \# spikelets}} \times 100\%$$

Rank	Fertility (%)	Interpretation
1	>90% fertile	Highly fertile
3	75 – 89%	Fertile
5	50 – 74%	Partly sterile
7	Trace – 50%	Highly sterile
9	0%	Full sterility

Screening for Flooding Tolerance

7. Screening for tolerance of complete submergence

There are a range of different types of flooding stress that can affect rice. This chapter describes the standard protocol for screening under submergence, which typically occurs in farmers' fields following intense rainfall leading to rapid rise of water levels that cover the plant for 1-2 weeks.

7.1 MATERIALS REQUIRED

- Greenhouse concrete tanks or field plots that allow maintenance of floodwater at >1m depth for a given duration. Field plots can be used with only minor modifications as appropriate.
- Garden soil.
- Pre-germinated rice seedlings:
  - Scatter dry seeds onto a tub of water. Discard seeds that float.
  - Soak the remaining seeds for 24 h at 30ºC in the dark inside an incubator.
  - Decant the water and transfer seeds in petri plates lined with moistened coarse filter paper at 30ºC.
  - Incubate for 2 more days under the same conditions inside the incubator.
- IR42 (susceptible check); FR13A, IR40931, or IR49830 (Sub1 donors as tolerant checks, use any of the three).

7.2 PROCEDURE

1. Place soil into the concrete bed, then label entries according to the layout. Maintain a row spacing of not less than 1cm.
2. Select well-germinated rice seeds and sow them 0.5cm below the soil surface. Record the number of seedlings per entry (minimum of 20 seeds per row, 2 rows per replication).
3. After sowing one row, cover the seedlings with sieved garden soil.
4. Grow the seedlings for 14 to 21 days after soaking. Water plants leaving 3-5 cm standing water and weed regularly. Do not fertilize with N if soil is fertile (but with 0.5 g of solophos and 0.5 g muriate of potash per L soil at sowing). A plant height (of 14 to 21-d-old plants) is usually 25 to 35 cm at the start of submergence.
5. Start the submergence treatment from 11:00 to 14:00 to allow the plants to photosynthesize before the treatment. Maintain a water flow to completely submerge the plants in a shortest possible time without disturbance; this is best if done within 3-5 hrs to avoid elongation with partial submergence.
6. Check the water depth daily and add water to completely submerge the materials, as leaves of sensitive genotypes will start to elongate. Each day, the greenhouse and floodwater temperatures and other floodwater conditions (dissolved O_2, light penetration, and pH) should be monitored in the morning (07:00-08:00) and in the afternoon (13:00-14:00) at various depths during submergence as needed. Plants that elongate fast and emerge above water level should be considered sensitive to submergence.
7. When 14-d-old seedlings are used before submergence, monitor the IR42 check plants daily starting from day 6 of submergence (or day 8 of submergence when older seedlings are used before submergence) and check when the plants become soft at the growing point (shoot-root junction) with a rotten smell. When this junction (in 5–8 out of 10 IR42 test plants) starts to become

soft, de-submerge the entire setup the following day. Include extra seedlings of IR42 in pots or around the experimental plots in the field for sampling, so as not to disturb the setup during this monitoring. Samples can be collected by pulling 5-10 plants of IR42 from the buffer area or by including extra pots for examination every 2-3 days. Fourteen-day-old seedlings of tolerant and intolerant rice genotypes can be easily visually distinguished in terms of survival after about 6–10 days, whereas 21-d-old seedlings require a longer duration of submergence treatment to be distinguished.

8. Open the water outlets/drains to de-submerge the plants. De-submergence is preferably started after 13:00, and should end in late afternoon (around 17:00) when light intensity is low, thus minimizing post-submergence injury. Also, to minimize lodging, slowly drain the tank when the water is at seedling height by closing one or more outlets at a time to slow down de-submergence (fast de-submergence will cause seedlings to lodge).

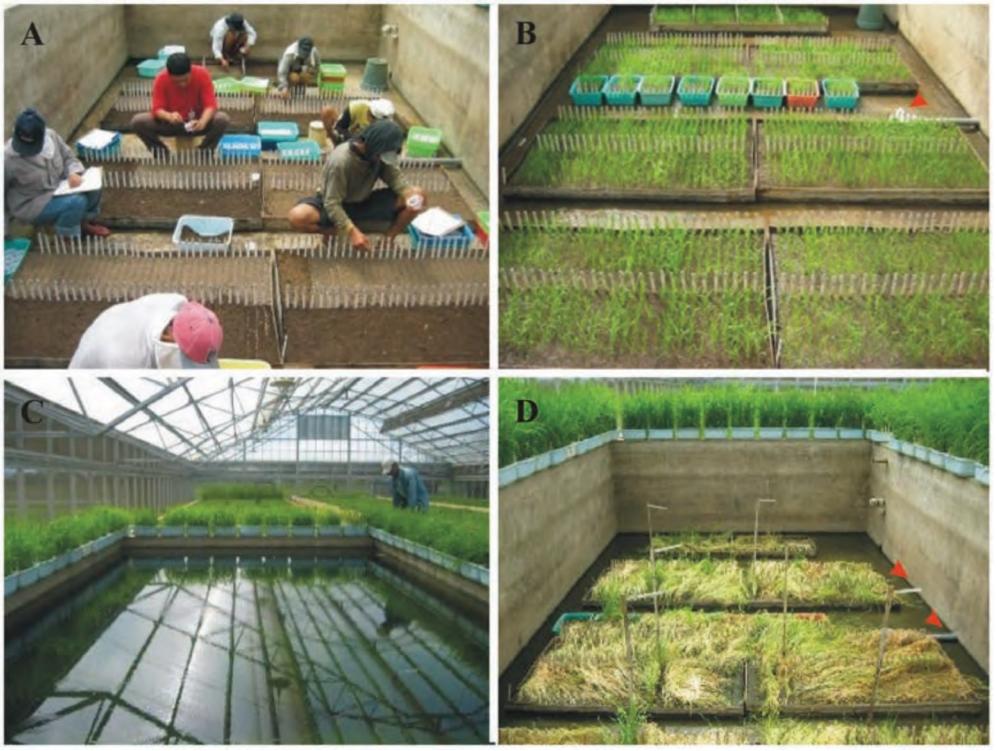


Figure 7.1. Flooding setup at vegetative stage. A. Sowing pre-germinated seeds. B. Normal growth of seedlings for 2 to 4 weeks. C. Flooding with tap water to submerge seedlings completely. D. Recovery after complete submergence. Water inlets are indicated with arrows.

7.3 SCORING

1. Per entry, record the number of seedlings before submergence (X) and also the number of survivors at 21 days after de-submergence (Y). A surviving seedling has a new leaf emerging 21 days after de-submergence.
2. Then calculate percentage survival. Divide Y by X and then multiply by 100.

Some additional notes:

- Scoring earlier than 21 days after de-submergence can overestimate survival (seedlings may appear green a few days after de-submergence but finally die later).
- A plexiglass aquarium can also be used for this purpose, but could only accommodate a few entries.

8. Screening for tolerance of long-term stagnant flooding

Stagnant flooding is distinguished from submergence stress by the fact that plants are not completely submerged, and the flood water under natural floods varies between 25 to 50 cm for most of the season. The upper portion of the plant is typically above the flood waters, and thus gas exchange, photosynthesis and respiration can continue. Nevertheless, stagnant flooding leads to significant yield losses in sensitive lines. It is notable that Sub1 confers no benefit under stagnant flooding. Thus stagnant flooding is considered a separate stress to submergence (Singh et al. 2011). Due to the nature of the stress, stagnant flooding tolerance is usually assessed in the field.

8.1 MATERIALS REQUIRED

- Deep pond field sites (or tanks) that allow maintenance of floodwater depth at 0.5m. Separate field sites for control (non-flooded) conditions. Separate RCBD experiments may be conducted for control and flooded sites.
- Germinated seeds (incubated in petri plates lined with moistened coarse filter paper at 30^{o}C for 3 days in the dark). Field nurseries can also be used to transplant uniform seedlings directly into the experimental field or concrete tanks for flooding treatment.
- Seeds of one tolerant check (IRRI 119 or IRRI 154), one intolerant check (Swarna-Sub1 or PSBRc18-Sub1), parental lines, and entries for screening.

8.2 PROCEDURE

1. Healthy pre-germinated seeds of all entries are sown in unfertilized normal soil in nursery trays using 3 seeds per hole (each hill measures 1.5 × 1.5 × 2.5 cm) or in seedboxes with a spacing of 4 cm or in seed bed with normal nursery raising practices.
2. Prepare the land in the field. Apply molluscicide after the first and second harrowing.
3. Apply nitrogen, phosphorus, potash and zinc at the rate of 30:30:30:5 kg.ha^{-1} as basal one day before transplanting.
4. Transplant 14-d old seedlings into the field at 20 × 20 cm^2 spacing, with 2 seedlings per hill. A certain area is to be designated at the center of the plot of each entry where survival will be scored. Ensure 100% plant population before submergence by transplanting in missing hills as needed.
5. Allow transplanted seedlings to grow normally in the field for another 14 days with 5-7 cm water depth. Count the number of plants in the designated area before submergence.
6. Start filling the ponds of the stagnant flooded setup with water gradually at a rate of 1.5 cm daily starting from day 14 after transplanting until a depth of 50 cm up to 4 weeks after transplanting. Ensure normal water depths of 5-7 cm in the control ponds. Maintain the water depths in both plots up to maturity. Observe and record the water depths in both stagnant flooded and control plots daily.
7. Monitor the floodwater conditions daily in the submerged field in the morning (07:00-08:00) and in the afternoon (13:00-14:00) at various depths during flooding.
8. Hand weeding, snail control and other plant protection measures should be adopted when needed.
9. Count the number of surviving plants in the designated area at maturity (after lowering the water depth).

Figure 8.1. Screening for tolerance to stagnant flooding. A. Sow pre-germinated seeds in beds. B. Cover seeds with soil and water. C, D. Remove seedlings from seed bed and transplant in deep ponds. E, F. Plants four weeks after transplanting under stagnant flooding (E) and control (F) conditions. G, H. Screening for stagnant flooding tolerance under 50 cm water. Two contrasting varieties are shown, a sensitive variety Swarna-Sub1 (G) and a tolerant variety IRRI 154 (H).

8.3 SCORING

1. Traits such as plant height and number of tillers can be monitored every two weeks without lowering the water level. Other traits like stem thickness, lodging, number of productive tillers, panicle size, etc., can be determined at harvest.
2. At harvest, determine the percentage of surviving plants, number of effective tillers, fertility and grain yield. Tolerance indicators are determined by comparing these parameters under stagnant flooding relative to that under control or shallow conditions for each entry.

9. Anaerobic germination

Rice usually has low germination under anaerobic conditions. This is particularly a concern in direct-seeded rice. In addition to improving direct seeded rice rice crop stands, improving the anaerobic germination ability of rice would allow the use of water as a method of weed control.

9.1 MATERIALS REQUIRED

- Seed boxes (38 x 53 cm, labelled according to layout) with 34 columns of grids (17 grids per column, each grid measures 1.5 × 1.5 × 2 cm). A separate experiment may be conducted for submerged and non-submerged (control) trials in a RCBD.
- Greenhouse concrete tables that would allow maintenance of floodwater depth of ≥10 cm.
- Garden soil
- Clean and healthy dry seeds of one tolerant check (Khao Hlan On, Khaiyan, Mazhan Red, or Kharsu 80A), one intolerant check (IR42, FR13A, or any Sub1 line), parental lines for mapping populations, and other entries for screening.

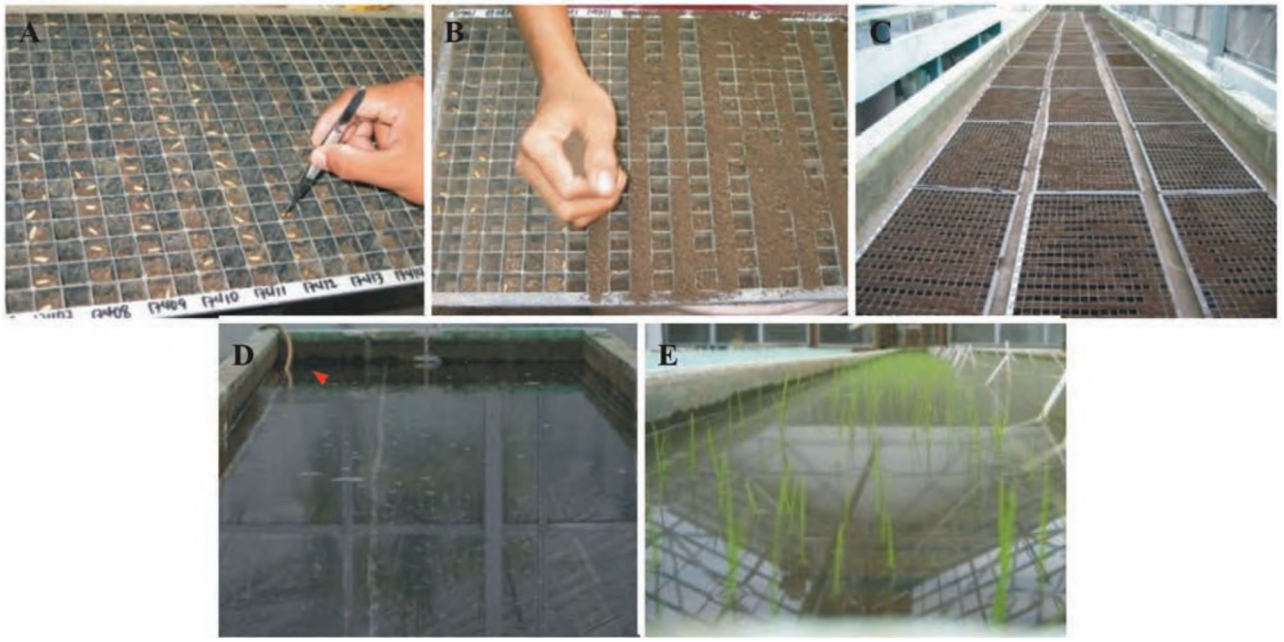


Figure 9.1. Flooding setup during germination and early seedling stage, for determination of anaerobic germination tolerance. A, B. Dry seeding at 0.5cm soil depth. C. Tray layout in concrete tanks. D. Flooding after dry seeding. The water inlet is indicated with an arrow. E. Surviving seedlings ready for scoring 2 weeks after sowing and flooding.

9.2 PROCEDURE

1. Half fill the grids of seedboxes with sieved garden soil.
2. Sow dry seeds according to the layout (one seed per grid) then cover with a 0.5 cm layer of the same garden soil. Record the number of seeds per entry. The minimum number of seeds typically sown is 17 per row and 2 rows per entry, with 2 – 3 replications. Maintain normal growth with a water depth of 3-5 cm in the non-flooded control setup to adjust for germination percentage. The flooded (anaerobic germination) setup is kept on a concrete table with 10-cm water depth.

3. Monitor the greenhouse and floodwater temperatures and other floodwater conditions (dissolved O_2, light penetration, and pH) daily in the morning (07:00-08:00) and in the afternoon (13:00-14:00) at various depths during flooding as needed, if the equipment is available.

9.3 SCORING

4. Per entry, record the number of seeds sown (X). Record the number of surviving seedlings at 3-day intervals starting at 9 days after sowing, and continuing up to day 21 of flooding (Y). A surviving seedling is able to reach the water surface. For large-scale phenotyping, one scoring at 3 weeks after sowing will suffice.
5. Percentage survival is then calculated. Divide (Y) with (X) then multiply by 100.

Toxic soils

10. Iron (Fe^{2+}) toxicity

The iron toxicity screening protocol is also based on a hydroponics system utilising Yoshida's nutrient solution. Preparation of seedling floats is as described in Chapter 2. The Yoshida's solution, while based on the same chemical composition, has been substantially modified to facilitate the imposition of Fe^{2+} stress. Certain chemicals, such as P, are present at lower concentrations to prevent precipitation of the iron, and several solutions including chelators are added.

10.1 MATERIALS REQUIRED

- Chemicals for nutrient solutions (Table 10.1)
- Storage bottles for nutrient solutions
- 5L beaker
- Materials for Styrofoam floats, as per Chapter 2
- UV-Visible spectrophotometer, capable of reading 562nm and 882nm
- 1L measuring cylinder(s)

Ferrozine assay:

- Ferrozine (PDT disulfonate; 3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4´-disulfonic acid.Na-salt) in 50 mM HEPES buffer pH 7.0 (store in the dark at room temperature, stable for at least a month)

P assay:

- Ammonium molybdate
- Antimony potassium tartrate
- L-Ascorbic acid
- Concentrated sulphuric acid (~18.7M)
- 1L volumetric flask

10.2 PREPARATION OF NUTRIENT SOLUTIONS

Table 10.1 Composition of modified Yoshida's solution

Stock solution	Common name	Formula	[Stock] (g/mol)	[Final] (M)	g/L	mL/L for final solution
N	Ammonium nitrate	NH4NO3	80.0434	1.428	114.3	1
P	Sodium phosphate	NaH2PO4.H2O	137.9923	0.045	6.210	1
K, S	Potassium sulphate	K2SO4	174.2602	0.28075	48.926	2
Ca	Calcium chloride, dihydrate	CaCl2.2H2O	147.0146	1.000	147.02	1
Mg, S	Magnesium sulphate, 7-hydrate	MgSO4.7H2O	246.4756	0.823	202.85	2
Fe3+ (nutrient)	Ferric chloride, 6-hydrate	FeCl3.6H2O	270.29	0.015401	4.1628	5
	HEDTA, trisodium salt		344.2	0.013525	4.6552	
Micro-nutrients	Manganous chloride, 4-hydrate	MnCl3.4H2O	233.3582	0.01184	2.34	1
	Sodium molybdate, 2-Hydrate	Na2MoO4.2H2O	241.9	0.00107	0.26	
	Zinc sulphate, 7-hydrate	ZnSO4.7H2O	287.5796	0.00306	0.88	
	Boric acid	H3BO3	61.8330	0.03301	2.04	
	Cupric sulphate, 5-Hydrate	CuSO4.5H2O	249.6860	0.0008	0.2	
Silicon	Sodium meta-silicate	Na2SiO3.9H2O	284.2000	0.04222	12	0.375
Fe2+ (stress)	Ferrous sulphate	FeSO4.7H2O	278.01	Varies		

Preparation of Fe-HEDTA solution

To make 5L of stock:

1. Weigh out 23.276g of HEDTA trisodium salt and transfer to a 5000mL beaker.
2. Add ~1500mL of de-ionised water to the beaker, and fully dissolve the HEDTA.
3. Weigh out 20.814g of $FeCl_3.2H_2O$ and dissolve in ~500mL of de-ionised water.
4. Calibrate pH meter and put the electrode in the 5000mL beaker with HEDTA.
5. SLOWLY (to prevent precipitation) add Fe solution to the HEDTA solution. Use a 10mL pipetter and add Fe in 10mL aliquots.
6. The pH should drop as you add the Fe. After you have added all the Fe, the pH should be ~2.15
7. Make 1L solution of 0.1M NaOH and slowly add to solution until pH is ~3.8. Again, this should be done slowly, but can be done faster than when adding the Fe.
8. When pH is ~3.8, add de-ionised water until the volume is 4800mL.
9. Add more NaOH until the pH reaches ~4.2 (between 4.1 and 4.3).
10. Complete the volume to 5L using graduated cylinder(s).

10.3 SCREENING PROTOCOL

1. Preparation and germination of seeds is as per Chapter 2. Suggested control lines are:

Variety	IRGC Accession	Fe^{2+} Tolerance
Azucena	IRGC 52992	Tolerant
Bao Thai	IRGC 61181	Sensitive
Suakoko 8	IRGC 50405	Moderately tolerant

2. Grow seedlings as per the protocol in Chapter 2, using normal Yoshida's nutrient solution.
3. Stress is imposed beginning approximately 7 days after sowing. The regular Yoshida's solution (Table 1.1) is replaced with the modified Yoshida's solution (Table 10.1), supplemented with $FeSO_4$ to the desired level of stress:

FeSO4.7H2O (278.01g/mol) stress:

Stress level (ppm = mg Fe/L working solution)	Stock concentration (mM)	g/L solution
50	0.898	0.2497g
100	1.795	0.4990g
200	3.591	0.9983g
500	8.977	2.4957g
1000	17.953	4.9911g

The optimum concentration is between 200 – 300ppm.

4. Solutions are monitored and adjusted daily for pH, phosphorous (P assay) and Fe (Ferrozine assay). Details of assays follow.
5. Seedlings are scored for visual injury symptoms at 7 and 14 days (or as appropriate) after imposition of $FeSO_4$. A similar SES evaluation is used to that outlined in Chapter 2, but symptoms are typically bronzing and dying of leaves (rather than bleaching, as in salinity stress), and stunting/die-back of root growth.

10.4 THE FERROZINE ASSAY

This assay is based on the chelation of ferrous iron (Fe^{2+}) by ferrozine (Figure 10.1). Ferrozine forms a complex with Fe^{2+} that has an intense magenta colour, that is easily assayed on a spectrophotometer; when ferrozine is in excess, the amount of colour will be dependent on the quantity of Fe^{2+} in solution. Because only solubilised Fe^{2+} is available to complex with ferrozine, it is possible to distinguish between ferrous iron in solution (and thus available to stress the plant) versus that in precipitate (which will not stress the plant) simply by assaying the mixture before and after acidification with HCl. Ferrozine will NOT bind Fe^{3+}, the predominant species used by plants for Fe nutrition. The assay is valid in the range 0.1 – 5mM Fe^{2+}.

As ferrous iron is highly sensitive to oxidation at neutral pH, it is advisable to either work anaerobically (e.g. under N_2), work very quickly (this will give an approximate value), or work under acidic conditions. As ferrozine will not bind Fe^{3+}, it is possible to distinguish the quantity of ferrous (Fe^{2+}) versus ferric (Fe^{3+}) iron by measuring first the straight ferrozine assay, followed by reducing all iron to Fe^{2+} with hydroxylamine and measuring again to read the total iron content. The ferric iron content is then simply the difference between the two measurements.

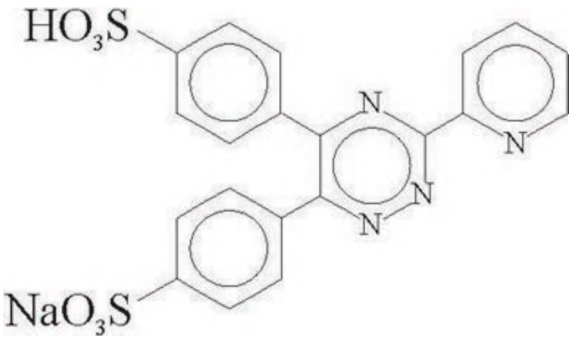


Figure 10.1. Structure of ferrozine.

1. Prepare a 1mM stock of ferrozine. This can be prepared ahead of time and is stable, but should be protected from light.
2. Take a sample (1 – 2mL) of nutrient solution from the top of the growth tray. Centrifuge at 3000g for 5 minutes. Remove the supernatant to a new tube; this will be used in the ferrozine assay.
3. Set up the spectrophotometer to read at a fixed wavelength of 562nm.
4. Prepare the following ferrozine reactions. Note that the dilution factor is different for different levels of Fe^{2+} treatments; these dilutions should give levels within the effective range of the assay (0.1 – 5mM Fe^{2+}), but can be varied if necessary. NOTE: Add water first, then ferrozine, then sample.

Fe treatment	Dilution factor	Sample volume (µl)	H2O (µl)	Ferrozine (µl)	Total volume (mL)
50 ppm	25	120	2280	600	3
100 ppm	50	60	2340	600	3
150 ppm	75	40	2360	600	3
200 ppm	100	30	2370	600	3
300 ppm	150	20	2380	600	3
450 ppm	250	24	4776	1200	6

5. Gently mix tubes by swirling. Measure the A_{562} and record. Standards are not necessary, but a blank solution (containing water, ferrozine and no sample) is required.
6. The concentration of Fe^{2+} is then given by:

$$Fe^{2+}\,(mM) = \frac{A_{562}}{27.900} \times \text{Dilution factor}$$

10.5 THE P ASSAY

This assay is derived from that used to assay tissue P contents. Imposition of high levels of Fe^{2+} will promote the formation of iron-phosphate salts, which remove both Fe^{2+} and phosphate from the solution, making them unavailable to plants. This assay is used to determine the amount of P present in solution, and thus available to the plant.

Preparation of standards

Standards need to be prepared very carefully!

1. Weigh 137.99mg (0.13799g) $NaH_2PO_4.H_2O$. Dissolve and make to exactly 1L in a volumetric flask. This is the 1mM P stock.
2. P standards must be made in a background solution the same as the nutrient solution being used. Prepare a matrix solution as follows:

Modified Yoshida's Fe stock solution	mL/L Matrix solution
Mg	2
Ca	1
K	2
N	1
Fe-HEDTA	5
Micronutrients	1

3. Prepare working P standards as follows:

Standard ID	P Std (µM P)	#mL 1mM P stock	#mL Matrix solution	Total volume (mL)
1	0.0	0.0	100.0	100.00
2	10.0	1.0	99.0	100.00
3	20.0	2.0	98.0	100.00
4	40.0	4.0	96.0	100.00
5	60.0	6.0	94.0	100.00
6	80.0	8.0	92.0	100.00
7	100.0	10.0	90.0	100.00

Preparation of reagents

Stock A can be made ahead of time and stored at 4°C in the dark. Stock B and working colouring stocks must be made fresh for each assay.

Stock A: (Sulfuric-Ammonium molybdate Antimony Potassium tartrate dye stock)

1. Dissolve 12g ammonium molybdate in 500mL water.
2. Dissolve 0.2908g antimony potassium tartrate in the ammonium molybdate solution.

3. Slowly add 148mL concentrated (~18.7M) sulphuric acid, stirring while adding. Mix well.
4. Let the solution cool, and make up to 2L. Store at 4°C in the dark.

Stock B: (Sulfuric-Ammonium molybdate Antimony Potassium tartrate - Ascorbic acid dye stock)

5. Make fresh. Dissolve 0.269g L-ascorbic acid in 50mL Stock A (ammonium molybdate – antimony potassium tartrate dye stock).

Working colouring stock:

6. Make fresh just prior to use. You will need 2.5mL colouring stock per sample.
7. For 100mL colouring stock, dilute 32mL Stock B with 68mL de-ionised water.

Colour reaction and reading

4. Take 5mL from the top of the culture solution and centrifuge as in the ferrozine assay.
5. Add 2.5mL sample to 2.5mL working colouring stock. React at room temperature for 1 hour.
6. Repeat this also for each standard solution.
7. Read the absorption at 882nm on the spectrophotometer.
8. Plot a standard curve from the standards measured (A_{882} vs. µM P of standard solution). Fit a line to the data points. The standards should give you a linear slope with $R^2 > 0.99$ (Figure 10.2). Extract the slope (m) and intercept (c) values.
9. P contents of samples are then given by P (µM) = (A_{882} – c)/m Note that c should be 0.

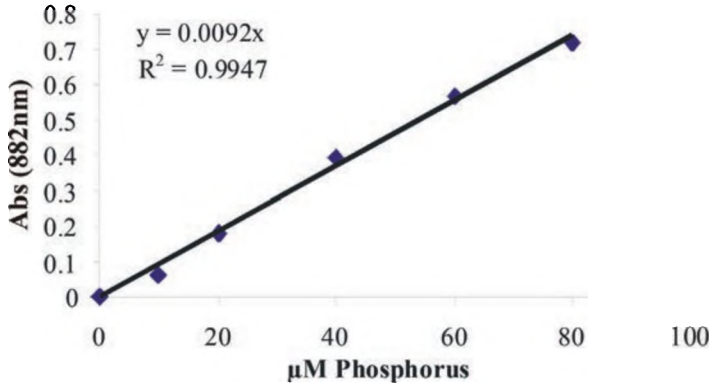


Figure 10.2. Example standard curve for the P assay.

11. Aluminium toxicity

Aluminum toxicity in rice crops is particularly a problem in upland soils and causes inhibition of root and leaf elongation. In this protocol, aluminum toxicity tolerance is assessed by scoring leaf and root elongation rates.

11. 1 MATERIALS REQUIRED

- Materials for preparation of Magnafaca's nutrient solution (see 11.2)
- Styrofoam trays as described in Section 2.3.
- Chemicals for nutrient solutions
- Storage bottles for nutrient solutions
- 5L beaker
- Root scanner (e.g. model STD 4800 dual-lens system)
- Aquarium aerator

Stock solution	Element	Formula	Formula weight (g/mol)	[Stock] (M)	g/L stock	mL stock per L working solution
1	**K**	KCl	74.55	1	74.55	1
2	**N**	NH4NO3	80.04	1.5	120.06	1
3	**Ca**	CaCl2.2H2O	147.02	1	147.02	1
4	**P**	K2HPO4	174.18	0.045	7.838	1
5	**Mg**	MgSO4.7H2O	246.36	0.2	49.272	1
		Mg(NO3)2.6H2O	256.41	0.5	128.205	
		MgCl2	95.211	0.155	14.758	
6	**Fe**	Fe-HEDTA		See below		5
7	**Micro**	MnCl2.4H2O	197.7	0.01184	2.341	1
		H3BO3	61.8	0.03301	2.040	
		ZnSO4.7H2O	287.4	0.00306	0.879	
		CuSO4.5H2O	249.5	0.0008	0.200	
		Na2MoO4.2H2O	241.9	0.00107	0.259	
8	**Al**	AlCl3	133.341	0.54	72.004	1

11.2 PREPARATION OF MEDIA

1. Prepare Magnafaca's nutrient solution according to the following table (adapted from Famoso et al. 2010):
2. Preparation of Fe-HEDTA:

- Weigh out 23.276g Na_3HEDTA and transfer to a 4000mL beaker.
- Dissolve fully in approximately 1.5L de-ionised water.
- Weigh out 20.814g $FeCl_3$ and dissolve in approximately 500mL water.
- SLOWLY add the Fe solution to the HEDTA (e.g. in 10mL increments using a 10mL pipette), mixing continuously to prevent precipitation of the Fe. Monitor the pH; the pH should drop as Fe is added, to a final value of approximately 2.15.
- Slowly add 0.1N KOH until the pH reaches 3.8. In total about 1L 0.1N KOH may be required.

- Make up the solution to 4.8L.
- Add more 0.1N KOH until the final pH is between 4.2 and 4.3.
- Complete the volume to 5L.

3. Preparation of Magnafaca's solution:
- Prepare a container containing about 80% the final volume of de-ionised water (e.g. for 10L solution, add 8L de-ionised water).
- Add the appropriate quantities of stock solutions 1 – 7 above (i.e. excluding the $AlCl_3$), stirring thoroughly between each solution.
- Check the pH. This will probably be around 6. Adjust to pH 7.8 with KOH.
- Add the $AlCl_3$ (solution 8) whilst stirring thoroughly. This will reduce the pH to less than 4.
- Adjust the pH back to 4.0 with KOH.

11.3 SEEDLING PREPARATION AND TREATMENT

1. Break the dormancy at 50°C for 5 days. Be sure to include sufficient seeds for both control and Al+ treatments. Suggested control lines are:

Variety	IRGC Accession	Al^{3+} Tolerance
Moroberekan	IRGC 12048	Tolerant
Pokkali	IRGC 108921	Sensitive

2. Sterilise seed by soaking in 15% household bleach, and rinse well with de-ionised water.
3. Soak seeds in de-ionised water for 48h at 32°C.
4. Germinate seeds on wet paper towel rolls at 32°C for 48h.
- Lay paper towel flat on a tray. Pour de-ionised water over the paper towel until soaked but without excess water.
- Arrange seeds along one edge of the paper towel, as shown in Figure 11.1. For optimum results, orient the long axis of the seed vertically, with the embryo at the bottom and the tip of the seed just poking above the edge of the paper towel.
- Carefully roll up the paper towel, so that seedlings are at one end of the resulting paper roll.
- Label the paper rolls with the genotype they contain. Gather the paper rolls together and stand them upright in a container of de-ionised water (bundling several paper rolls together with a tie gives added structural support).
- Incubate at 32°C, replacing water as necessary to prevent desiccation.
5. After 2 days, transfer seedlings to the glasshouse (still in the paper rolls). Allow seedlings to acclimatise for a further 1 – 2 days.
6. Transplant seedlings to hydroponic trays containing the Magnafaca's solution as described above (with added aluminium, in the treated trays). Select seedlings with uniform root lengths, as shown in Figure 11.2. Record the RL0 and SL0 as below. Put an aquarium aerator in the solution and aerate to prevent complex formation and precipitation by the aluminium.
7. Monitor the nutrient solution every day, maintaining the pH at 4.00±0.25 with HCl or KOH. Replace the nutrient solution every 2 – 4 days.

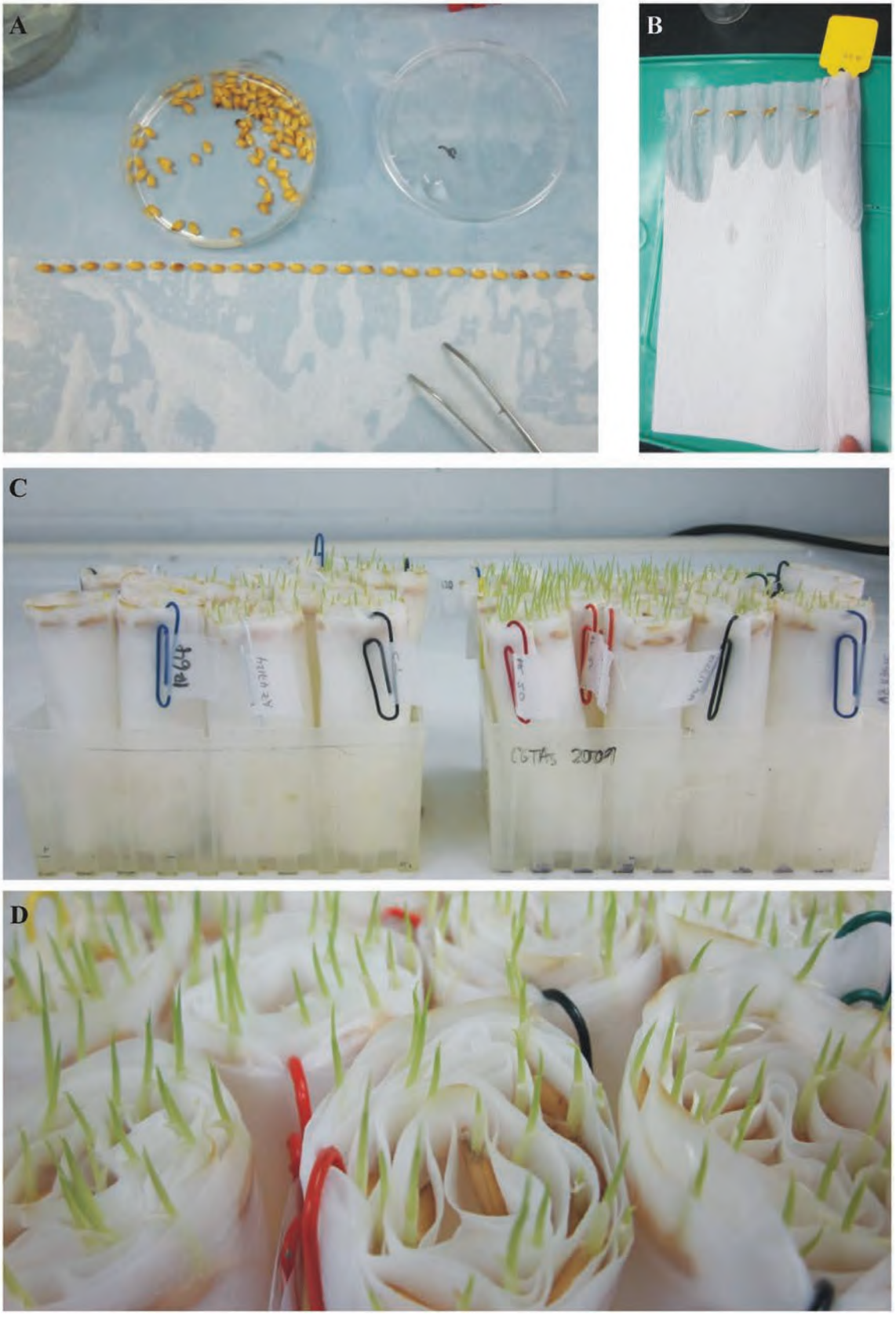


Figure 11.1. Germination for the aluminium toxicity assay. A. Arrangement of pre-soaked seeds along the edge of a paper towel. This is then rolled as shown in B. C. Labelled rolls of paper towel are stood upright in a container of water. After 2 days they are transferred to the glasshouse to acclimatise. D. After acclimatisation the seedlings are ready for transplanting.

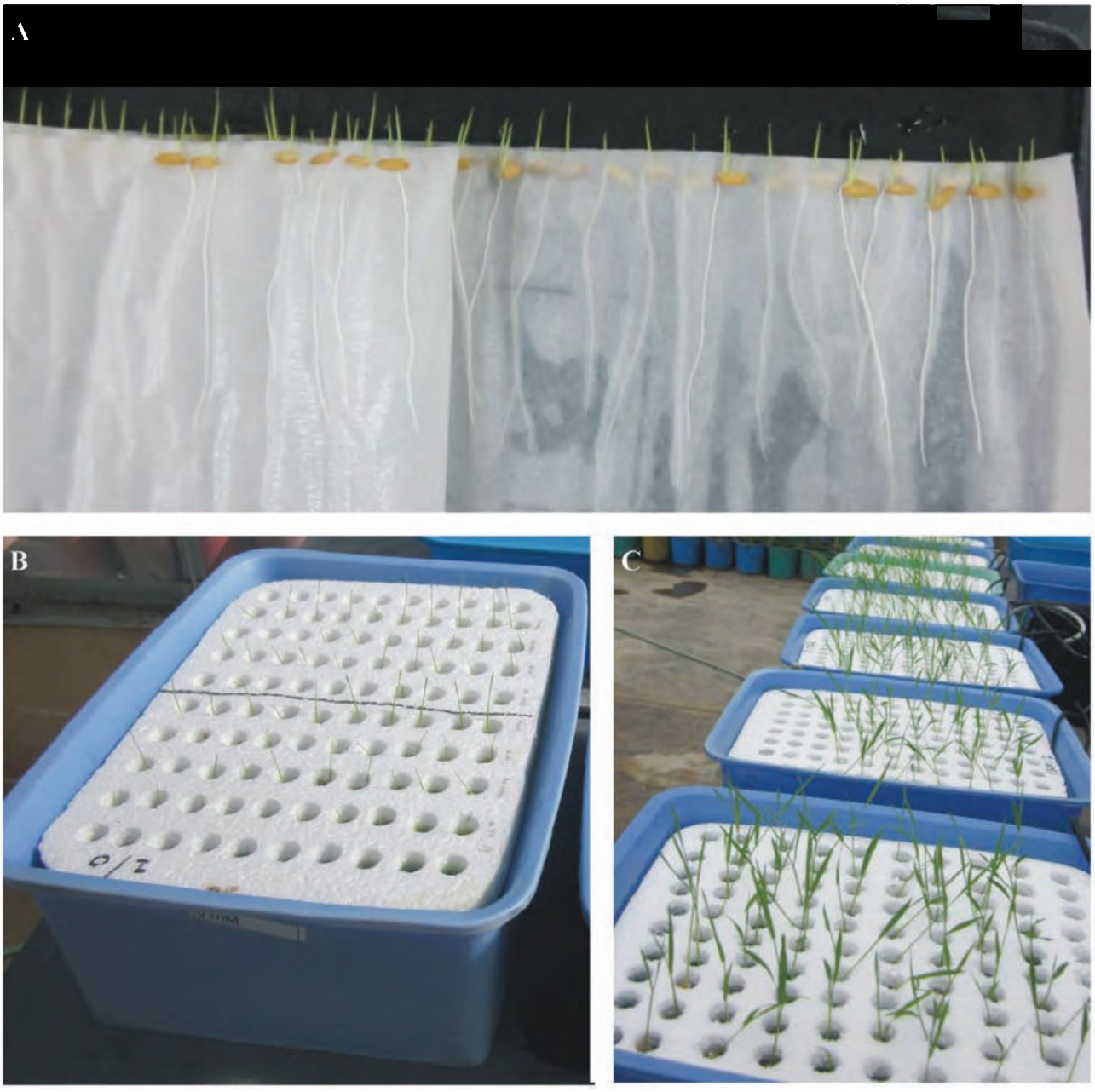


Figure 11.2. Transplanting of seedlings for the aluminium toxicity assay. A. Unroll the paper rolls containing the seedlings. Select seedlings with uniform root lengths. Measure the root and shoot lengths at time of transplant. B. Transplant seedlings to Styrofoam floats. C. After five days' growth the plants are ready for final measurements.

11.4 SCORING

1. Observations are made before and after the treatment. Measure the longest root and shoot lengths at the time of transplanting from paper rolls to hydroponic trays (before treatment; RL0 and SL0 respectively).
2. Repeat these measurements after 5 days of treatment (RL5 and SL5; see Figure 11.3).
3. Carefully remove seedlings from the Styrofoam trays. Measure the total root length per seedling with a root scanner (e.g. model STD 4800 dual-lens system).

4. The relative elongation for roots and shoots (RRE and RSE) are given by:

$$RRE = \frac{RL5 - RL0\ (Al+\ treatment)}{RL5 - RL0\ (control)} \times 100\%$$

Likewise:

$$RSE = \frac{SL5 - SL0\ (Al+\ treatment)}{SL5 - SL0\ (control)} \times 100\%$$

The relative root growth (RRG) is given by:

$$RRG = \frac{Total\ root\ length\ (Al+\ treatment)}{Total\ root\ length\ (control)} \times 100\%$$

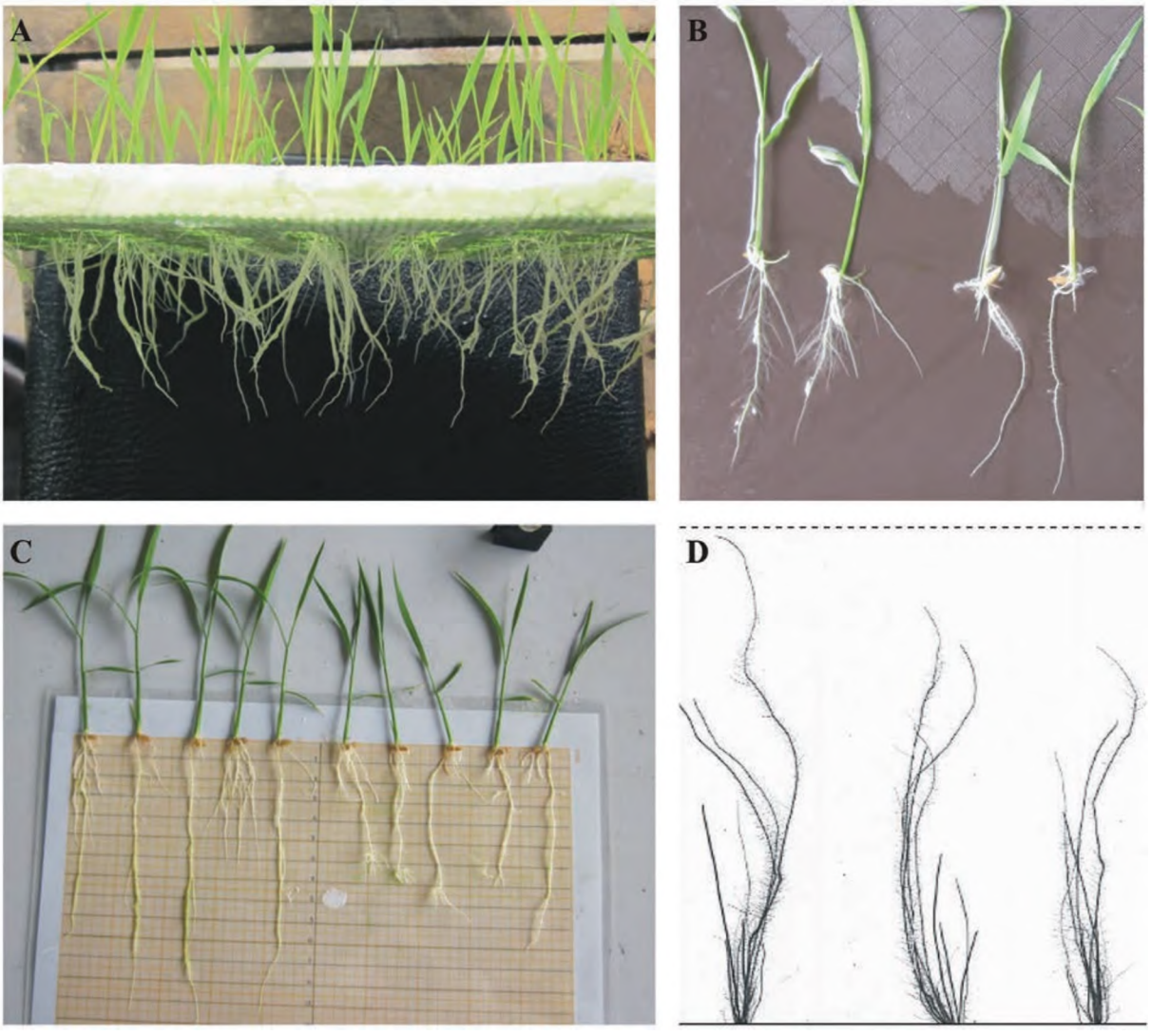
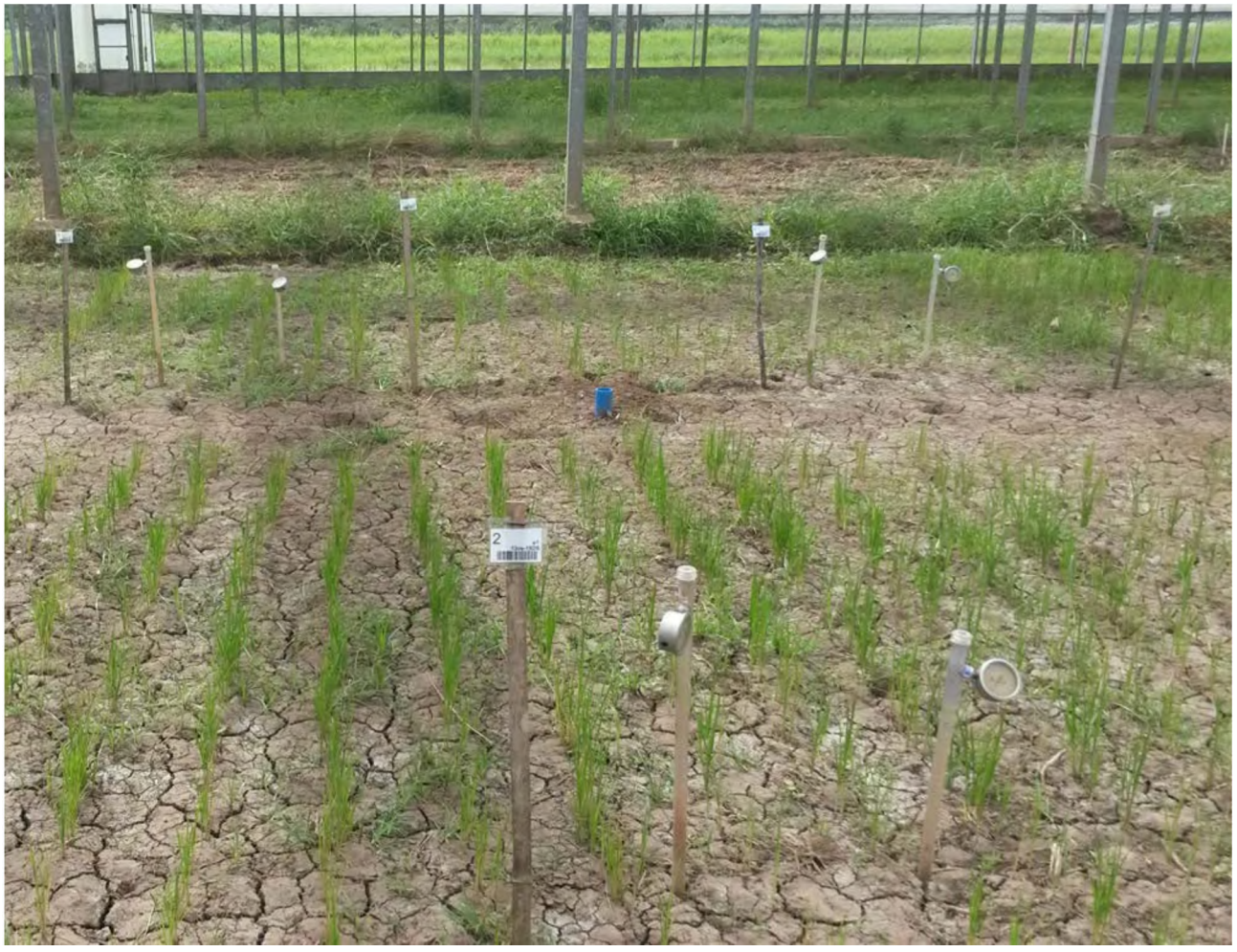


Figure 11.3. Scoring of seedlings from the aluminium toxicity assay. A, B. Seedlings after 5 days of treatment. Note differences in longest root and total root length in B. C. Measurement of longest root length. D. Measurement of total root length on a root scanner.

Drought Stress

12. Managing seedling stage drought stress experiments

Drought is one of the major factors contributing to low rice crop production worldwide. It can occur at any growing stage of rice. This chapter presents protocols for both dry-direct seeded (upland) and lowland seedling stage stress screening in the field. For both types of experiments, the soil should be levelled during land preparation to achieve uniform drydown after irrigation, and footprints in the soil should be avoided. Basal fertilizer is applied to the soil either before or one week after planting. Soil moisture is monitored by installing tensiometers, volumetric soil moisture sensors, and water table tubes. Protocols can be found in "General guidelines for monitoring soil moisture in drought screening trials": https://excellenceinbreeding.org/sites/default/files/manual/Soil%20moisture%20monitoring%20booklet.pdf

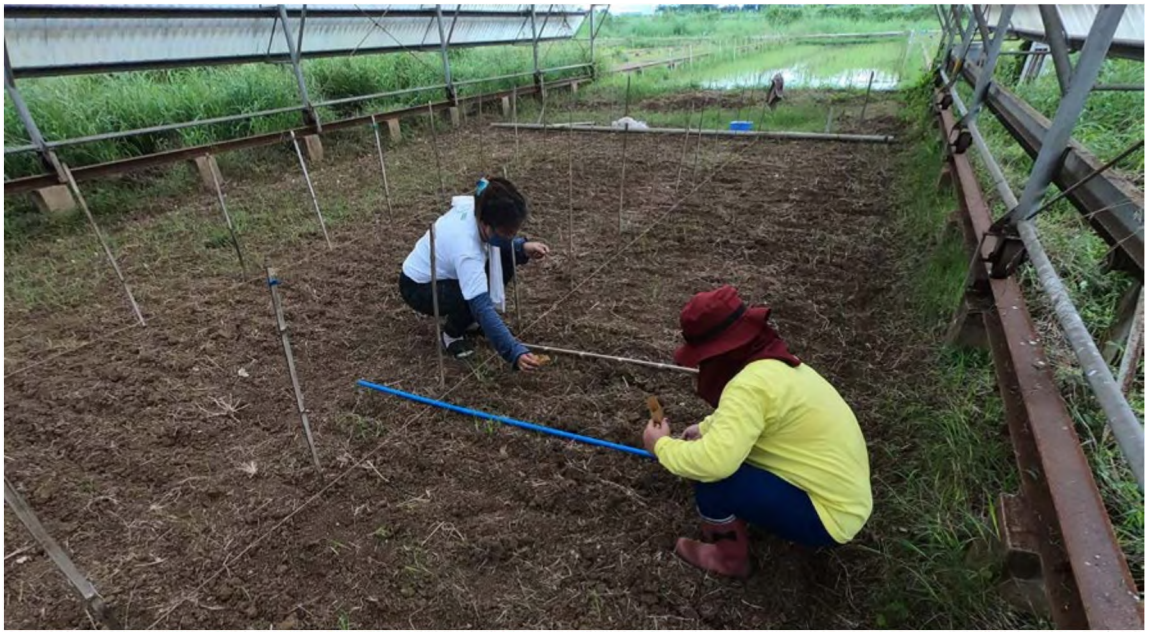


12.1. DRY DIRECT SEEDED SEEDLING STAGE DROUGHT STRESS

1. Plow, rotovate, and furrow the field before sowing. To maximize weed control, stale seedbed technique is preferred: After tillage operations, irrigate the soil using a sprinkler and till at 1-3 cm depth to allow weed germination. Apply non-selective herbicide to kill emerged weeds. Allow the soil to dry without further tillage operations.

2. Construct furrows 2-3 cm deep and 25 cm apart before sowing.

3. Sow 3-5 seeds directly into the furrow with 10 cm distance or continuously spread 2 grams per linear meter. Cover the sown seeds with dry, fine soil. Then install soil moisture sensors.



4. After sowing, irrigate the surface at least the top 15 cm of the soil using sprinkler irrigation. Careful and uniform irrigation must be applied to avoid the sown seeds from being washed out or having non-uniform germination. Irrigate the field 3x per week to keep the soil around field capacity. To prevent the seeds from being eaten by birds, use nets to cover the plot for small experiment and for big experiments, a person can be assigned to watch out and scare off the birds.

5. Impose drought stress by withholding irrigation 14 days after sowing for three weeks or until 35 days after sowing depending on the desired severity of drought. Physiological measurements can be done during the drought stress period.

12.2. TRANSPLANTED VEGETATIVE STAGE DROUGHT STRESS

1. Fill seedling trays with dry, sieved soil and water them before sowing. Sow one pregerminated seed into each soil-filled hole and cover with fine soil. Keep the seedling trays well-watered until pulling. Alternatively, plants can be established in a field seed bed nursery.

2. Create a bund surrounding the whole experimental field to keep the water inside. Puddle the soil several times and keep it soaked until transplanting.

3. Pull the seedling from the seedling trays 2-3 weeks after sowing and distribute into its designated plots. Transplant one plant into each hill at 20-cm intervals in rows with 25 cm spacing. Keep the plots flooded until drought imposition.

4. One week after transplanting, impose drought stress treatment by draining the soil and let it dry for 2 weeks. Install soil moisture sensors and conduct the physiological measurements.

13. Cylinder Preparation Protocol for Greenhouse Drought Studies

The advantages of using containers (pots, tubes, cylinders, lysimeters, etc) for drought studies is that the exact amount of water accessible to the plant can be controlled, and that the entire root system can be collected for measurement. The protocol described here is for plants grown in soil-filled cylinders to evaluate root growth and performance under a controlled dry down. Different physiological measurements can be done in this type of experiment. It is also important for all cylinders to have a uniform amount of dry soil and degree of compaction, resulting in the same volume. Cylinders are weighed at regular intervals and rewatered as necessary with defined amounts of water. Water uptake is normalized for plant size by shoot imaging at each cylinder weighing time.

Here we present protocols for two sizes of cylinders: large diameter (19 cm) and small diameter (5 cm) which can be used to target different growth stages.

13.1. LYSIMETERS/CYLINDERS

Materials

19 cm dia. x 40 cm or 95 cm ht PVC Cylinders
Rubber stoppers
Compaction plunger
Elastomeric sealant
Plastic liners
50-ml falcon tubes
Masking tape
Soil (dried, sieved, fertilized)

Preparation

1. Gather all cylinders needed for the experiment and check for leaks. Use cylinders sealed at the bottom but with a hole plugged with a removable rubber stopper in case draining is desired. Note that for well-watered treatments it is better not to drill holes in the cylinder since they are prone to leakage. Use epoxy or elastomeric sealants to seal the crevices. Make sure that the rubber

stoppers are tightly plugged into the hole during soaking. Fill the empty cylinders with water to test for leaks.


2. Record the weight of each empty cylinder. If they will be used for a root study, a plastic liner can be inserted inside the cylinder, which should also be weighed. A plastic liner is made by vertically cutting a 25-in x 50-in. plastic sack and sealing each piece to fit the inside wall of the PVC cylinder.


3. If fertilizer application is needed (such as when the plant will be grown up to about flowering stage or the soil to be used has inherently low nutrient content), mix the fertilizer thoroughly with the soil before filling them into the cylinder. A fertilizer rate of 50-50-50 can be applied using "Complete Fertilizer". The amount of fertilizer material per cylinder can be computed from the nutrient rate per hectare using the proportion of soil weight in the cylinder and the theoretical soil weight (2,000,000 kg) per hectare furrow slice. Additional 50N can be top-dressed using ammonium sulfate at least 4 days before draining the drought-stressed treatment if plants will be grown until maturity. A 19 cm diameter x 40 cm height PVC cylinder and 19 cm dia. x 95 cm height PVC cylinder has an approximately 8.5 kg and 25 kg, respectively, soil capacity.

4. Use dry soil that passed through 3.5 mesh (or about 5.6 mm opening) sieve. Fill the cylinder with the same amount and compaction of soil to about a 5-6 cm distance from the top edge. A plunger can be used to obtain similar compaction among cylinders.

5. Fill the cylinders with water until there are no bubbles coming out. Let the water stand for at least 2 hours then loosen the plugs to gradually drain water out. When water flow recedes to very slow drips, remove the plugs carefully to protect the soil from being washed out through the holes. Let the remaining water drip out overnight. In the morning, put the plugs back and weigh the cylinders in order to determine its weight at field capacity.

6. Label the cylinders before sowing. Bar/QR coded labels are preferred to facilitate faster and more error-free data collection.

7. At the time of draining the drought stress treatment, cover the cylinders with plastic to minimize evaporation. This is done by cutting a piece of plastic into circles of approximately 30 cm diameter, sealed to the PVC cylinder with masking tape. Install a sealable tube to facilitate watering the covered cylinder.

8. Weigh cylinders at regular intervals to monitor water uptake, and add water to reach target weights according to the experimental protocol and desired rate of drydown. Below is an example of the target weight computation.

		cylinder+ dry soil		cylinder+ wet soil			Target weight (g)		
Pot #	Empty pot weight (g)	Dry weight (g)	Soil weight (g)	Saturated weight (g)	Water content (g)	Total dry weight (g)	91% FC weight (g)	82% FC weight (g)	73% FC weight (g)
1	1812	10311	8500	13580	3269	10351	13326	13032	12738
2	2171	10671	8500	13924	3253	10711	13671	13378	13086
3	2413	10914	8501	14100	3186	10954	13853	13567	13280
4	2313	10814	8501	14048	3234	10854	13797	13506	13215
5	2272	10772	8500	13842	3070	10812	13606	13329	13053

Note that we have found that grain yield measurements using this system generally do not correlate well with grain yield measurements in field drought studies. For a protocol on field drought screening, please see the following chapter:

Torres et al. 2012. Methodologies for managed drought stress experiments in the field. In: Methodologies for root drought studies in rice. IRRI, Philippines. https://books.google.com.ph/books/about/Methodologies_for_Root_Drought_Studies_i html?id=1kkAHUiBJGsC&printsec=frontcover&source=kp_read_button&redir_ esc=y#v=onepage&q&f=false

13.2. TUBES

Materials

5.5 cm dia. x 40 cm ht PVC tube
5 cm dia. x 50 cm ht mylar tubes
Elastomeric sealant
Cheesecloth-like fabric
High performance duct tape (preferably transparent)
Soil (dried, sieved, fertilized)

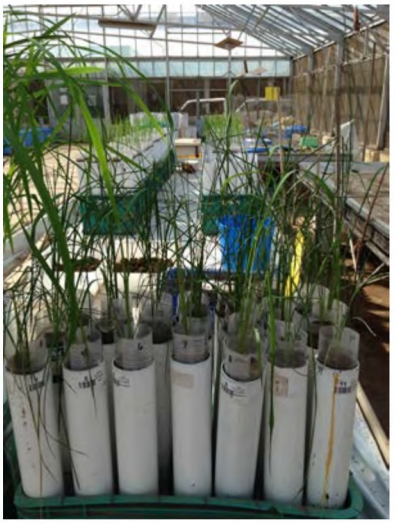
Preparation

1. Gather all PVC tubes sealed at the bottom and check for leaks by filling them with water. Use epoxy or elastomeric sealants to seal the crevices.
2. Cut the mylar plastic and assemble into 50 cm long x 5 cm diameter mylar tubes sealed with duct tape on the side, and close the bottom with cheesecloth-like fabric (attached with duct tape) to allow absorption of water at the bottom of the PVC tube. Record the empty PVC tube weight with the mylar tubes inside. Fill the mylar tubes with ~950 g of dried and sieved soil to a height of 40 cm to achieve a uniform bulk density in each tube, and insert each tube inside a PVC cylinder.

3. Label the PVC tubes and mark the corresponding mylar tubes before soaking. Bar/QR coded labels are preferred to facilitate faster and more accurate data collection.

4. For the control treatment set up, soak the soil-filled mylar tubes in a large container filled with water until there are no bubbles coming out. Let it soak for at least two hours and then remove the mylar tubes from the container to let the water drain out. Allow the remaining water to drip out overnight. In the morning, put the mylar tubes back into the PVC cylinders and weigh the tubes in order to determine their weight at field capacity. For the dry down set up, add an equal amount of water on top of the mylar tube and at the bottom of the PVC tube to achieve a continuous column of water. For drought treatments, we prefer to sow the seeds into soil at 75% of field capacity, to give the drydown process a head-start and ensure an effective drought treatment during the short timeframe of the seedling stage studies conducted in this system.

5. Weigh tubes 3x per week to monitor the dry down rate, and, if necessary, add water to reach the target weights (as described above) until the end of the study.



6. This system allows convenient sampling of the root system since the entire soil column can be accessed by removing the duct tape and unrolling the mylar.

Combined stress screening protocols

14. Combined drought + salinity screening at seedling stage

The case of combined drought and salinity stress can occur in rice fields, especially those located in coastal areas and river deltas. This protocol allows for a controlled screening under combined drought+salinity stress applied to soil-grown plants at seedling stage.

14.1 MATERIALS REQUIRED

- EC meter
- NaCl (table salt)
- Plastic Beaker (5000mL capacity)
- Plastic trays of 56.5 cm x 36.5 cm x 15 cm
- Wooden planks fitted with handles
- Seed boxes (38 x 53 cm, labelled according to layout) with 34 columns of grids (17 grids per column, each grid measures 1.5 × 1.5 × 2 cm.
- Greenhouse concrete tables that would allow seedling establishments and maintenance
- garden soil, sieved and sterilized
- Mixing containers (cylindrical rubber made containers: 60-200L capacity)
- Weighing scale (Kern FKB 65k1A)
- Stirrer (wooden paddle or fabricated stirrer)

14.2. SCREENING SETUP AND MAINTENANCE

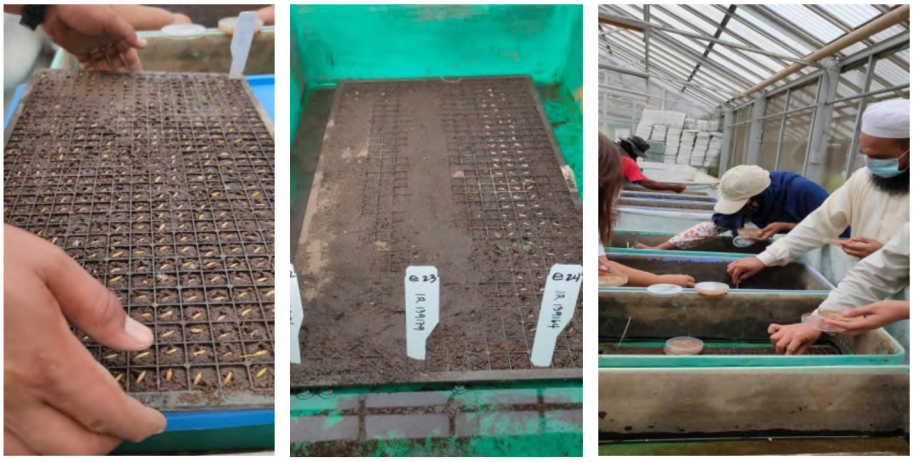
Preparation of Trays with soil

1. Prepare 56.5 cm x 36.5 cm x 15 cm seed storage trays by filling with ~25 kg of sieved, dry soil. The same amount of soil should be used in each tray, filled to the same height.
2. The amount of water to achieve field capacity of the soil should be determined before the start of the experiment as follows: Seed storage trays with 3 drainage holes on each side of the trays plugged with a rubber stopper are used for the sole purpose of the field capacity measurement. Weigh each empty tray. Plug the 3 holes with a rubber stopper and fill the trays with soil (~25 kg of soil); record the weight of the trays. Submerge the trays in a larger tray filled with tap water; de submerge the trays out of the water when you observe that no bubbles are coming out of the tray; remove or unplug the rubber stopper to drain excess water from trays for 12 hours or overnight. The following day, take the weight of the trays. Calculate the Field Capacity (100%):

Vol (liter)$_{100FC}$ = (wt of tray with soil after de submerged overnight - wt of tray with soil before submerging in water)

14.3 SEED PREPARATION AND PRE GERMINATION

1. Break the dormancy of seeds as described in section 2.3.
2. Soak the seeds 2 days before the planned incubation date. Seed lots from different genotypes may be staggered according to the time required to fully imbibed the seeds.
3. After soaking the seeds, transfer them into clean petri dishes with a layer of paper towel on the bottom. Moisten paper towel with tap water, and sprinkle seeds evenly over the area available (max. ~50 seeds per 90mm petri dish)
4. Germinate the seeds on petri dishes at 32ºC for 24h.
5. Half fill the grids of seedboxes with sieved and sterilized garden soil.
6. Sow the pre germinated seeds based on the layout (one seed per grid) then cover with the same garden soil. Record the number of seeds per entry. The minimum number of seeds typically sown is 30 seeds per entry.



7. Monitor daily and add water as needed to the seedling trays for 5 days or until most of the seedlings reach the 2 to 3- leaf stage.

14.4 PREPARATION AND SCREENING

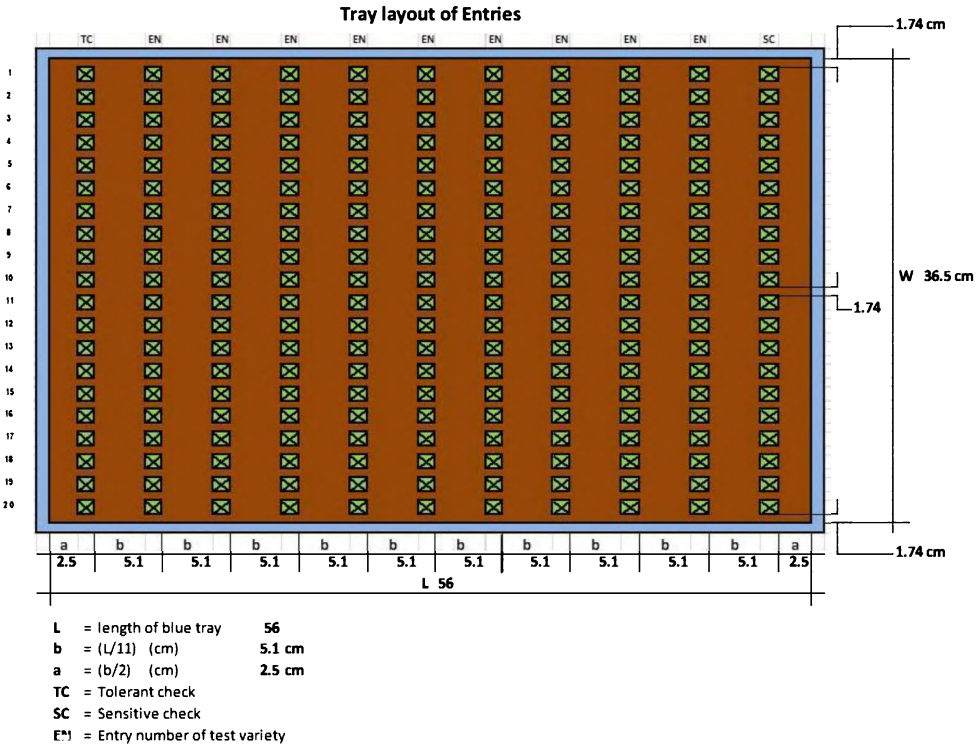
1. Based on germination of seeds, prepare the required labels and the required volume of saline water and prepare the experimental design, randomisation and tray layout of the experiment. The saline water should be prepared according to the amount of salt needed to reach EC10 at FC, dissolved in the amount of water for 75% of field capacity (or 75% FC [EC10 dSm^{-1}@FC]).
2. Weigh the required number of trays (use 56.5 cm x 36.5 cm x 15 cm seed storage trays
3. Fill the trays with soil (25 kg of soil); record the weight of trays

4. Add the required volume of saline (75% FC [EC10 dSm^{-1}@FC]) solution into each tray to achieve the target 75% field capacity

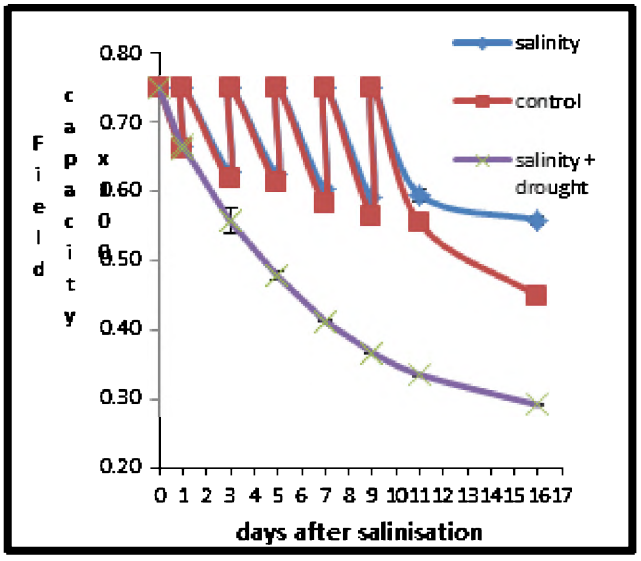
$Vol\ (liter)_{75FC} = 75 \times Vol(liter)_{100FC} / 100$

5. Using a planting guide, punch holes in the surface of soil to facilitate the transplanting of seedlings

6. Transplant the 1-2 leaf stage seedlings at 20 seedlings per row. A sensitive check variety (e.g. IR29) and parental lines or recurrent parent must be included in each experimental tray



7. Drought+salinity stress treatments are allowed to dry down until the end of the experiment (about 2 weeks after transplanting) or until the soil moisture level reaches 30% of field capacity, at which time tap water can be added to maintain the soil moisture at 30% of field capacity.
8. Monitor the soil moisture by weighing (Kern FKB 65k1A) every other day to maintain the targeted soil water status treatment. To reduce cracking of the soil that could occur while moving the experimental trays, wooden planks fitted with handles can be kept under each tray during the course of the screening to carry each tray to the balance



9. At the end of experiment, count and score the surviving plants (SES scoring as described in sec 2.4)

15. Combined salinity and submergence screening at seedling stage

In coastal areas, ideal rice genotypes need to have dual tolerance of salinity and submergence for better adaptability and stable production, because young seedlings are sometimes submerged with brackish water. Breeding strategies to achieve this include transferring Saltol and SUB1 to a single background using marker-assisted backcrossing (MABC) to develop varieties that can tolerate both saline and submerged conditions. In other cases, SUB1 is transferred into a salt tolerant genotype.

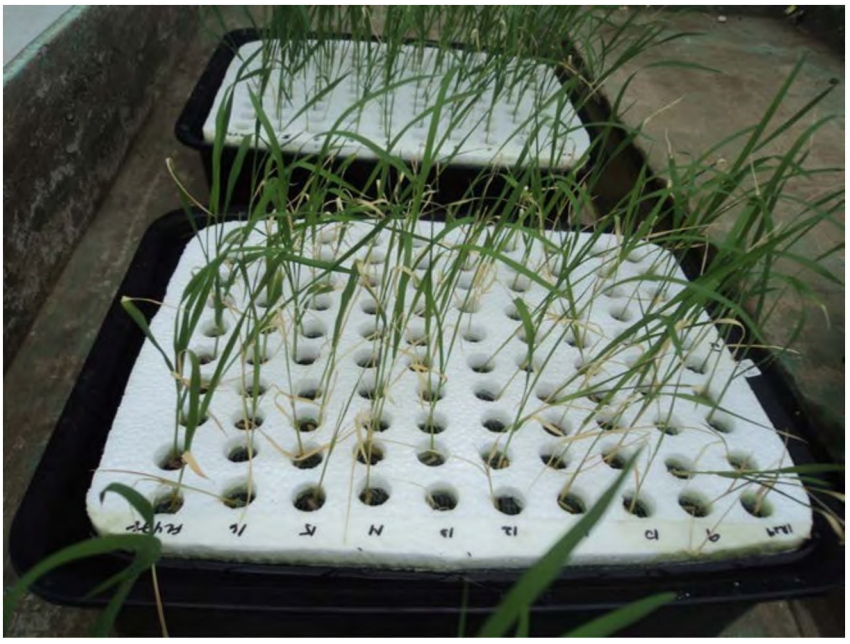
15.1. MATERIALS NEEDED

- EC meter
- NaCl (table salt)
- Plastic trays (8L capacity)
- Greenhouse concrete tanks that would allow seedling establishments and maintenance
- garden soil, sieved and sterilized

15.2. SCREENING SETUP AND MAINTENANCE

Before subjecting the test genotypes to combined salinity and submergence, the entry list can be narrowed down by first screening under each individual stress:

1. Evaluation of progenies for seedling stage salinity tolerance - See Section 2 (2.1 to 2.4).



2. Evaluation of progenies for submergence tolerance - See Section 7.

15.3 COMBINED SALINITY AND SUBMERGENCE SCREENING

1. Surviving plants from salinity and submergence screening can be rescued for subsequent screening under combined salinity and submergence.
2. The rescued plants may be genotyped with Saltol and Sub1 specific markers. Sometimes only SUB1 markers are used. Genotypes having Saltol (or are salt tolerant) and the SUB1 allele can be selected for selfing, fixing and generation advancement.
3. Pre-germinate the seeds in clean petri dishes with a layer of paper towel on the bottom. Moisten the paper towel with water and sprinkle seeds evenly over the area. Incubate for 48-72 hrs at 32°C.
4. Sow the pre germinated seeds in 8L plastic trays filled with garden soil.
5. After 14 days of seedling establishment, submerge the plants in a concrete tank with water salinized at an EC of 6-8 dS m^{-1}.The salt should be dissolved in the water in a separate tank before applying it to the experimental treatment.
6. Desubmerge the plants after 10 days and wash with fresh water to remove excess salts.
7. Count the number of surviving plants to determine percentage survival. SES scores can also be used to score genotypic performance
8. Plant height can be measured after 21 days of desubmergence.

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