PROTOCOL: Salt tolerance screening in rice using hydroponics

Contents

1. Abstract

A hydroponics screen for salt tolerance in rice is described. The screen is used to test tolerance to salt at the seedling stage. The seedling test is simple and rapid (4 - 6 weeks) and efficient and allows the screening of several hundred seedlings. The test can be adapted to screen M2 populations or M3/advanced generations. A list of the required equipment is given, along with set up procedures for hydroponics hardware and solutions. Tolerance is determined by performance comparisons against: 1) known salt tolerant genotypes and 2) each line tested. Control tests (no added salt) can also be performed if required as an indicator that the system is working and in comparing salt and non-salt growth. Indicators of tolerance are leaf colour, leaf rolling, leaf tip dying and seedling death. Root damage (growth and browning) and biomass can also be observed. The protocol is designed primarily to screen

rice mutant populations for salinity, but may be adapted to any segregating population of rice and to other small grain cereals.

2. Background

Salinity is a major abiotic stress limiting yield in many parts of the world. World-wide rice is grown on some 400 million hectares of salt affected land. Breeding for salt tolerance is a major goal for rice breeders for which screens are required to select out tolerant lines for breeding. Screening for salt tolerance in the field is difficult as soil salinity is dynamic: the level of salt varies both horizontally and vertically in the soil profile and changes with time. These environmental perturbations are overcome by testing in a hydroponics system in a stable environment.

Abiotic stress tolerance, especially salinity stress is complex because of variation in sensitivity at various stages in the life cycle. Rice is comparatively tolerant of salt stress during germination, active tillering (vegetative growth), and during the later stages of maturity. It is most sensitive during seedling establishment and reproductive stages (Lafitte, et al., 2004). Screening at an early growth stage (2 to 4 week old seedlings) is more convenient than at flowering as it is quick, seedlings take up less space, tolerant seedlings may be recovered for seed production and seedling tests are more efficient in terms of time and costs. Seedling screening offers the possibility of pre-selection of putative mutants, mutant populations, breeding lines and progeny, and cultivars before field evaluation.

The seedling test described is an adaptation of that originally devised in collaboration with the International Rice Research Institute (IRRI). The current system however does not use a floating support and the current system is designed to be flexible; it can be adapted to evaluate individual genotypes or large mutant populations. The hydroponics set up uses plastic tanks with tight fitting polyvinylchloride (PVC) support plates (platforms). Seeds are placed directly onto mesh compartments set into the PVC platforms and germinate *in situ*. Previous systems used bulky styrofoam supports, but these are difficult to maintain and become brittle and contaminated with algae and other microbes. The current system is more robust, easily cleaned and can be used repeatedly. The PVC platforms are also strong enough to support several hundred seedlings. The test is rapid (4 to 6 weeks) and for simplicity no aeration system is used as regular changes of the hydroponic solutions preclude forced air aeration.

3. Objective

The aim is to screen rice seedlings and classify their tolerance to salinity. Extensive tests have been carried out at the IAEA's Plant Breeding and Genetics Laboratory (PBGL) using rice genotypes with known susceptibility/tolerance to saline field conditions. Correlations have been established between seedling hydroponics responses and field salinity tolerance. Thus the seedling screen described here can be used to select plants that may be expected to perform well in saline fields.

4. Equipment

All equipment (tanks, trays, containers, drums and platforms) is dark coloured to minimize light penetration into the culture solution, thus reducing algal growth.

- Test tanks: these are made of plastic and have outside dimensions of 60 x 40 x 12 cm and contain approximately 24 litres each when full (Photo 1). The size of tank can be changed to suit local conditions.
- Support platforms: two formats are used:
  1) M2 test platforms: PVC support platforms are made up with the dimensions: 56 x 36 x 1.2 cm to fit inside the top of a test tank. These platforms overlap the top of the test tank by 2 cm by gluing an additional sheet of PVC (5 x 36 x 1.2 cm) at both ends (see Photo 1). M2 screening platforms contain 24 rectangular compartments (6 x 7 cm) cut at regular intervals with a spacing of 1.2 cm. Each compartment can accommodate 100-200 seed (useful for M2 screening). Nylon mesh (fly netting) is cut to fit the PVC platforms (56 x 36 cm) and glued to the underside using PVC-V glue (Photos 1 and 2).
  2) M3 and other advanced generation/line test platforms: these PVC support platforms are made up with the dimensions: 36.5 x 26.5 x 1.2 cm. These overlap the test tanks by 2 cm by fitting an addition sheet of PVC (5 x 36 x 1.2 cm) at both ends (see Photo 3). 100 round (2 cm diameter) holes are drilled out (at regular intervals, see Photo 3). Two of these support platforms can sit inside one test tank.

- Germination lids: PVC covers are used to blank out light, these sit over the PVC support platforms to provide darkness during germination (not obligatory). Germination lid dimensions: 50 x 34 x 2 cm (Photo 4). The lids promote germination by helping to maintains humidity, temperature and cut out light.
- Recovery tanks: Recovery tanks: made of plastic with outside measurements of 40 x 30 x 17 cm. These hold approximately 20 litres (Photo 3).
- Support platforms for recovery tanks: PVC platforms are made up with the dimensions: 36.5 x 26.5 x 1.2 cm. These overlap the tanks by 2 cm by fitting an addition sheet of PVC (5 x 36 x 1.2 cm) at both ends (see Photo 3). 30 equidistant open holes (2.2 cm diameter) are drilled into the support platforms (without mesh).
- Storage containers for stock solutions: Stocks solution can be prepared in small amounts and stored in the glasshouse or at room temperature for 1 to 2 months, mineral precipitation or the change in the covalence such Fe or Cu in the solution is negligible over this period. The storage containers are air and light tight to allow long storage (1 – 2 months; Photo 5). Nutrients for rice hydroponics have been described by Yoshida (1976) and consist of six stock solutions (five for major elements and a 6th one for all micro-elements), for convenience we normally make these up in 5 litre amounts (see Table 1).
- pH meter.
- Electrical conductivity meter.

Table 1: Constitution of stock solutions

Stock No.	Chemical	Amounts (g or ml)/5 litres
1	NH_4NO_3	457.000
2	$NaH_2PO_4H_2O$	201.500
3	K_2SO_4	357.000
4	$CaCl_2$	443.000
5	$MgSO_4$ $7H_2O$	1,620.000
6	$MnCl_2$ $4H_2O$	7.500
	$(NH_4)_6$ Mo_7O_{24} $4H_2O$	0.370
	H_3BO_3	4.670
	$ZnSO_4$ $7H_2O$	0.175
	$CuSO_4$ $5H_2O$	0.155
	$FeCl_3$ $6H_2O$	38.500
	$C_6H_8O_7$ H_2O	59.500
	1M H_2SO_4	250 ml

- Storage containers for working Yoshida solution: The working solution is made up using the six stock solutions and then diluted with distilled water in large drums (Photo 6). For convenience the drum may be fitted with a submersible water pump to aid mixing, aeration and distribution into tanks. The solution may be prepared fresh or stored for incorporation in the next pH and volume adjustment (every 2 days). Large volumes of Yoshioda solution (up to 120 litres) may be stored in air thigh and light tight drums in the glasshouse for up to 1 week (Photo 6).

Note: distilled water is required in making up Yoshida solution as local tap water may result in precipitation of minerals and will alter mineral concentrations that may affect salt sensitivity.

5. Plant materials

Test materials should be compared against standard genotypes of known salt tolerance. At the PBGL we use:

Pokkali: salt tolerant wild type.

Nona Bokra: salt tolerant wild type.

Bicol: moderately salt tolerant.

STDV: moderately salt tolerant (induced mutant from IR29).

Taipei 309: salt susceptible.

IR29: salt susceptible.

The salt tolerance of the above standards in saline hydroponics has been correlated with field performance (Glenn et al 1997; Afza et al 1999). These standard materials can be requested free of charge from IRRI under a Standard Materials Transfer Agreement. Alternatively, local cultivars or breeding lines of known salt tolerance may be used as standards.

6. Setting up of hydroponics hardware

The screening is done in glasshouse conditions with day/night temperatures of 30/20ºC and relative humidity of at least 50% during the day. The glasshouse should be disease free and well lit by natural or artificial lighting. The tanks may be placed on the floor or on benching, but the surface should as level as possible or tank water levels adjusted using wedges.

7. Setting up the hydroponics solutions

The working solution is prepared as described by Yoshida et al 1976 with adaptations made by Glenn et al 1997 (Table 2), for example: each stock solution is shaken and 150 ml of each stock are mixed together and made up to 120 litres (drum capacity, Photo 6). The pH of the working solution is adjusted in the drum to 5.0 with 1N sodium hydroxide (NaOH) and 1N hydrochloric acid (HCl) with continuous stirring (the pump may be used) to insure the solution is homogenized, this simultaneously aerates the solution

Table 2. Stock solutions: labelling, main element and amounts required to make up the working hydroponics solution

<table>
<tr><th>Stock</th><th>Main element</th><th>Amounts of stock solutions needed for one (20 L) tank</th><th>Amounts of stock solutions needed for one (120 L) drum</th><th>Concentration of the elements in working solution (mg/l)</th></tr>
<tr><td>1</td><td>N</td><td>25</td><td>150</td><td>40.00</td></tr>
<tr><td>2</td><td>P</td><td>25</td><td>150</td><td>10.00</td></tr>
<tr><td>3</td><td>K</td><td>25</td><td>150</td><td>40.00</td></tr>
<tr><td>4</td><td>Ca</td><td>25</td><td>150</td><td>40.00</td></tr>
<tr><td>5</td><td>Mg</td><td>25</td><td>150</td><td>40.00</td></tr>
<tr><td rowspan="6">6</td><td>Mn</td><td rowspan="6">25</td><td rowspan="6">150</td><td>0.50</td></tr>
<tr><td>Mo</td><td>0.05</td></tr>
<tr><td>B</td><td>0.20</td></tr>
<tr><td>Zn</td><td>0.01</td></tr>
<tr><td>Cu</td><td>0.01</td></tr>
<tr><td>Fe</td><td>2.00</td></tr>
</table>

8. Seedling establishment in hydroponics

Test tanks are filled with distilled water until the water level is about 1mm above the mesh. The water level may be adjusted using wedges. Seed are then placed into the wet compartments. For M2 screening 30-50 seed from one panicle is placed into one compartment (6 x 7 cm); for M3 and advanced line testing 5 seeds are placed into each 2cm diameter compartment (Photo 7), lines may be replicated within and among tanks. The test platforms are then covered with a lid for one week to promote germination in the dark. At day three the water is replaced with half-strength Yoshida solution as vigorous seedlings require some nutrients. After one week the platform of germinated seed is transferred to a test tank containing full-strength Yoshida solution to establish healthy seedlings prior to salt treatment. Seedlings are grown on up to the two-leaf stage and should appear green and healthy.

Note: The test should not be carried out on unhealthy seedlings.

Note: If seed samples are not clean and rotting occurs during germination, these must be removed. Contaminated seed may be surface sterilized prior to germination by soaking in 20% chlorox solution for 20-30 minutes, followed by three rinses in distilled water. Chlorox treatment also helps to promote germination.

9. Care of plants in hydroponics

Due to evaporation and transpiration there will be loss of solution volume and pH change (algal growth may also contribute in pH fluctuation). Every two days (or thrice a week) the volume needs to be brought back to the level of touching the netting in the platform compartments and the pH adjusted to 5. We change solutions by lifting off the platforms and placing them temporarily onto empty tanks and pouring the hydroponics solutions back into a drum where the bulked solution is pH adjusted for the whole experiment in one step. Once adjusted the solution is re-distributed into the test tanks and the seedling platform returned. These operations also act to aerate the hydroponics solution. Alternatively, the pH can be adjusted on a tank basis and more working solution may be added to make up the volume in each tank.

Note: The loss of hydroponics solution due to evaporation and transpiration is dependent upon local conditions (temperature and humidity).

10. Salt treatment

Salt treatment is applied at the 2-3 leaf stage, after 1-2 weeks of seedling establishment in full-strength Yoshida solution (depending on the rate of seedling establishment, Photo 9). The salt treatment is applied in one go and not incrementally. The test salt concentration is 10 dS/m (10 dS/m corresponds to 6.4 g of NaCl in one litre). Salinization of the nutrient solution (working solution) is done for large volumes by adding dry NaCl in a drum containing Yoshida and mixed using a submersible water pump. Salt is added until the 10 dS/m is reached, electrical conductivity is measured using an EC metre.

11. Scoring

Visual symptoms of salinity stress are: reduced leaf area; lower leaves become whitish, leaf tip death and leaf rolling. The technique for salinity screening is based on the ability of seedlings to grow in salinized nutrient solution. Standard genotypes are included in each test tank for comparison. Scoring is relative and carried out according to the standard evaluation system of IRRI with a score 1 for tolerant and 9 for sensitive. Scoring is carried out at or around day 12 of salt treatment. At this stage sensitive seedlings begin to die, whereas intermediate genotypes show varying degrees of tolerance. Table 1 gives classification criteria for salt tolerance based on known standards.

Note: Scoring may be carried out at each day of treatment if quantitative data are required. Growth curves may be plotted to study responses over time. The biomass of seedlings may be recorded for this purpose using shoot/root/whole plant weight (fresh and dry), plant height, and tillering can be scored during the qualitative evaluation over time. However, scoring should be carried out for longer than 12 days of salt treatment as it is at this point that growth reduction of susceptible seedling becomes most apparent, whereas tolerant seedlings show some growth increase (but reduced compared to control (0 NaCl) seedlings).

Table 1. Relative classification: scoring test genotypes/populations against known standards.

	Two standard genotypes used Pokkali and IR29	Three standard genotypes used Pokkali, Bicol and IR29
Salt tolerance classes	I: More susceptible than IR29 II: Equally susceptible as IR29 III: Moderately tolerant IV: Tolerant	I: More susceptible than IR29 II: Equally susceptible as IR29 III: Less moderately tolerant than Bicol IV: Moderately tolerant comparable to Bicol V: Less tolerant than Pokkali VI: Tolerant

At day 12 of salt treatment tolerant standards (Pokkali and Nona Bokra) show slight damage with leaf tips becoming brown; moderately tolerant standards (Bicol and STDV) exhibit more leaf damage with older leaves dead and younger leaves being green only at their leaf bases; susceptible standards (IR20 and Taipei) are dead.

Susceptible lines will die at the same time as, or before the sensitive standard IR29.

Moderately tolerant lines will respond in a similar manner to Bicol.

Tolerant lines can be selected when Bicol begins to die or has died, and may be removed to a recovery tank.

Symptoms on selected tolerant lines may be compared to Pokkali to estimate the degree of tolerance.

12. Recovery of salt tolerant lines

Selected tolerant seedlings are teased out of the test tanks with care taken to keep roots intact. The base of the aerial part of each selected seedling is then wrapped with a sponge strip (10 x 2 x 1 cm) and the seedlings inserted into a recovery tank (Photo 11).

Selected seedlings can be grown to maturity in these tanks filled with Yoshida solution changed every 2 weeks.

13. Examples

The following tables summarize salinity data from results of seedling hydroponics screening carried out at the PBGL on materials from Vietnam, Myanmar and Iran.

Table 2. Classification of salt tolerance in 41 Iranian rice cultivars.

More susceptible than IR29	Susceptible, equivalent to IR29	Moderately tolerant equivalent to Bicol
Anbarbo, Hazar, Hashemi, Sadri, Domsefid, Mehr, Nedah, Kadous Tarom Mahali, Daylaman, Hasan Sarsii, Saleh, Sangeh tarom, Amol-3, Ghil-1, Drafk, Salari, Bejar, Nikjou, Pooya, Sahel, Shafagh, Fajer, Tabesh, Shirodi, Line-147, Line-145, Line-54, Line-29.	Champabodar, Mazandaran, Shahpasand, Gharib, Hasan saraii atashgah, Dom siah, Ghashangeh, Line-144.	Neamat, Ghil-3, Binam, Ahlameytarom.

Data from training Fellowship (Mr. Masoud Rahimi) in Technical Cooperation Project IRA/04035: Developing salt-tolerant crops for sustainable food and feed production in saline Lands (INT5147).

Table 3. Classification of salt tolerance in 50 Myanmar rice cultivars

More susceptible than IR29	Susceptibility equivalent to IR29	Moderately tolerant
Thone Hanan Pwa, Ye Baw Yin, Ekare, Pa Chee Phyu, Mya Sein, Shwe Kyi Nyo, Mwe Swe, Maung Tin Yway, Shwe Ta Soke, Zein Yin	Pin To Sein, Shwe Dinga, Mine Gauk 1, Kauk Thwe Phyu, Pa Che Mwe Swe, Lin Baw Chaw, Rakhaing Thu Ma, Emata Ama Gyi, Hnan War Mee Gauk, Imma Ye Baw, Ye Baw Latt, Ekarin, Ban Gauk, Pa Din Thu Ma, Bom Ma De Wa, Nga Kywe, Sein Kamakyi, Nga Kywe Taung Pyan, Kha Yan Gyar, Nga Kywe Yin, Paw San Bay Kyar, Kamar Kyi Saw, Saba Net, Bay Kyar Gyi, Paw San Yin, Pathein Nyunt, Nga Kyein Thee, Mee Don Hmwe, Byat, Law Thaw Gyi, Moke Soe Ma Kywe Pye, Taung Hti	Aung Ze Ya, Ekarin Kwa, Ye Baw Sein, Gauk Ya, Nga Shink Thway, Paw San Hmwe, Saba Net Taung Pyan, Sit Pwa

Data from Fellowship training (Mr Tet Htut Soe and Ms Nacy Chi Win) in Technical Cooperation Project MYA/06031.

Table 4. Summary of results after screening 370 Vietnamese aromatic and Basmati rice mutants of the cultivar TAM.

More susceptible than IR29	Equally susceptible than IR29	Moderately tolerant
TAM (parent)	QLT4	TDS1
HNPD103	T43	TDS3
BAS370 mutant	TDS4	TL2
	TDS5	HNPD101

Data from training Fellowship (Ms Doan Pham Ngoc Nga) in Technical Cooperation Project VIE/066011: Enchancement of quality and yield of rice mutants using nuclear and related techniques (VIE5015).

14. Benefits and drawbacks

Advantages	Drawbacks
• Cheap, fast and simple. • Clear classification into susceptible, moderate and tolerant types. • Tolerant seedlings may be recovered. • High throughput screen. • Pre-selection technique for putative mutants. • Equipment is re-usable.	• Requires continuous vigilance and maintenance (replenishment of test solution every 2 days) • Solutions need to be changed; therefore adequate stock chemicals are required. • Requires good quality growing conditions. • Homogenous, good seed quality required.

15. Alternative screens

Hydroponics may be technically demanding. A soil-based screen for salt tolerance in rice has also been developed by the IAEA. This is cheaper and easier than the hydroponics methods described here. Other tests commonly used in salinity research include carbon isotope discrimination and tissue ion composition (these require sophisticated analytical procedures).

16. References

Afza R., Zapata-arias F.J., Zwiletitsch F., Berthold G. and Gregorio G. (1999). Modification of a rapid screening method of rice mutants for NaCl tolerance using liquid culture. *Mutation Breeding Newsletter*, Issue No**. 44**: 25-28.

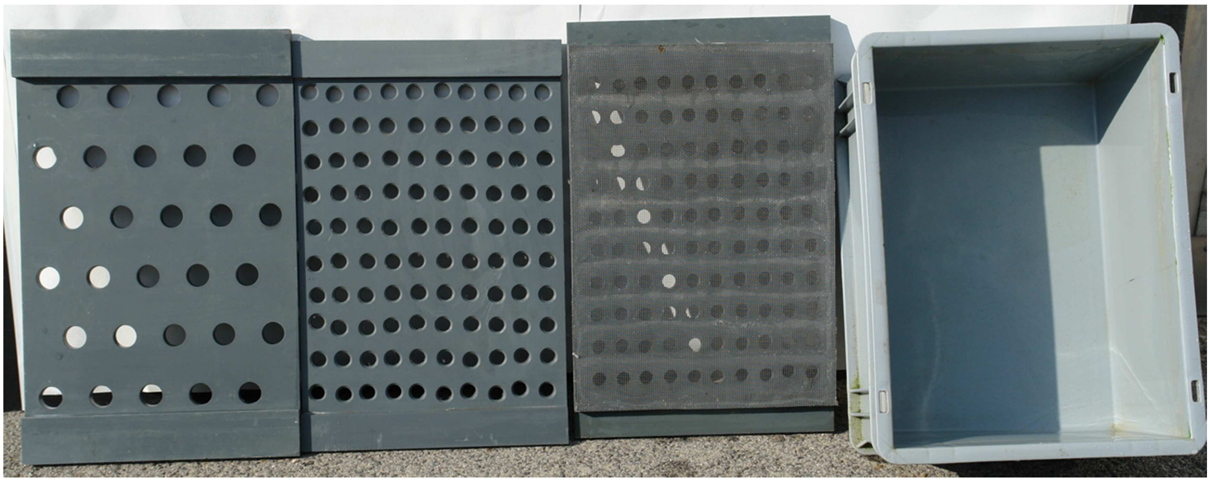
Gregorio G.B, Senadhira D, Mendoza R.T (1997). Screening rice for salinity tolerance. *IRRI Discussion Paper* series 22, IRRI, Manila. pp.30.

Lafitte H.R., Ismail A., Bennett J. (2004). Abiotic stress tolerance in rice for Asia: progress and the future. "New directions for a diverse planet" *Proceeding of 4th International crop Science Congress*, 26 Sep. - 1 Oct., 2004, Brisbane, Australia. Published on CDROM. Web site www. Cropscience.org.au.

Yoshida S, Forno D. A., Cock J. H., Gomez K. A. (1976). *Laboratory Manual for Physiological Studies of Rice*. IRRI, Las Banos, Laguna. pp.83.

Photograph 1. Test tank (right); support platform (left).

Photograph 2. Support platform sitting on top of test tank.

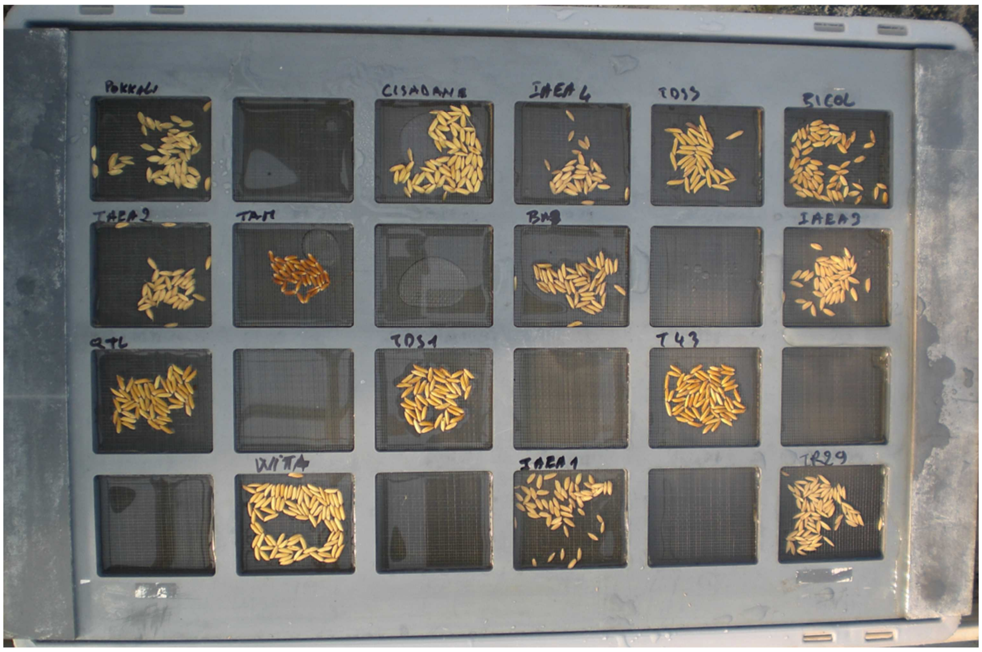


Photograph 3. From left to right: recovery platform; M3/advanced line screening platform; reverse side of M3/advanced line platform showing attached mesh; test tank.

Photograph 4. Lid being placed over seed for germination

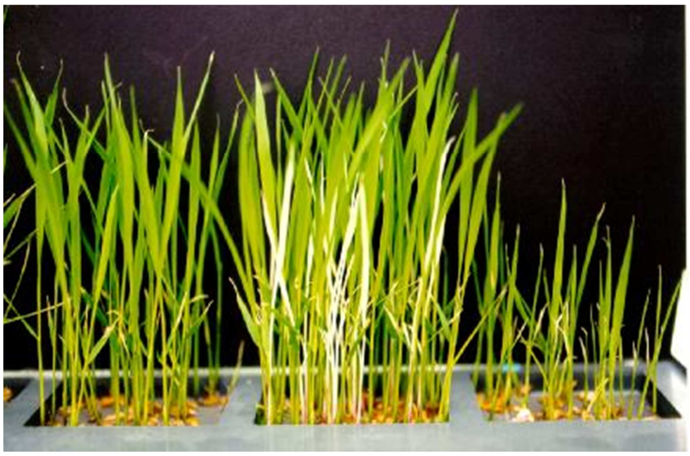
Photograph 5. Six containers (5 litre) for Yoshida stock solutions.

Photograph 6. Drum for preparation, aeration and storage of Yoshida working solution.

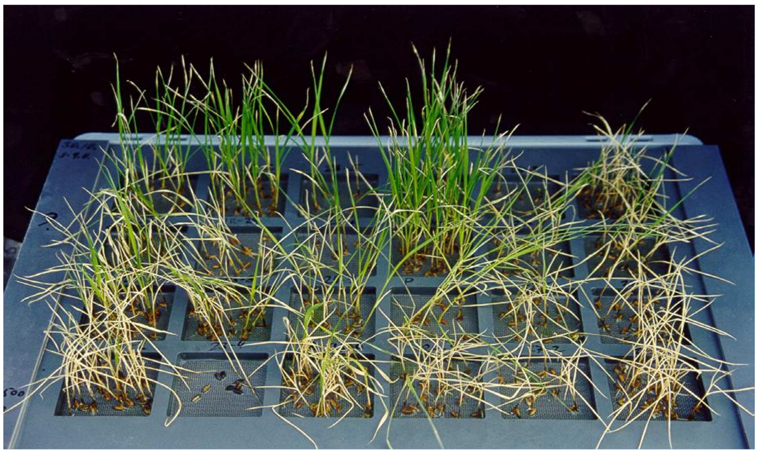


Photograph 7. M2 platform with seeds ready for germination, each compartment contains seed from one panicle per M1 plant.

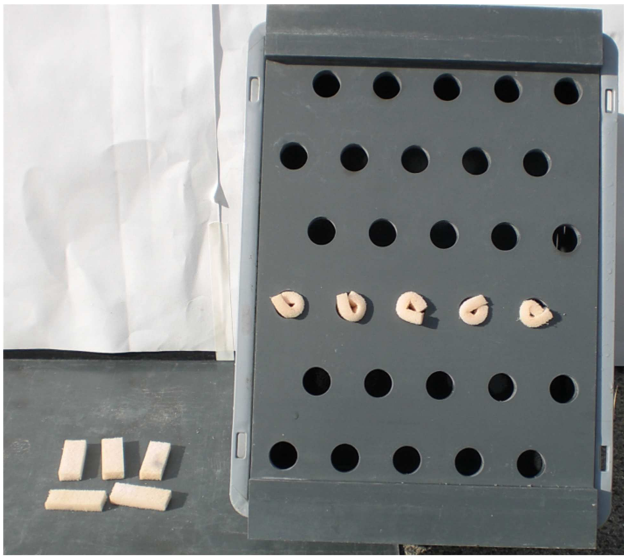
Photograph 8. M3/advanced line platform showing seed ready for germination, each compartment contains 3-5 seed from each line.



Photograph 9. Healthy M2 seedlings ready for salt treatment. Note: M2 seedlings often segregate for colour variation (pale green, yellow and albinos).



Photograph 10. Comparison of tolerant and susceptible seedlings at day 12 of salt treatment. The tolerant standard Pokkali (central position) shows little damage (with slight browning of leaf tips); the moderately tolerant standard Bicol (top left) shows greater leaf damage and the tolerant standard Nona Bokra (next to Bicol) remains green; the susceptible standard IR29 (bottom right) is completely dead. Other test M2 populations show varying degrees of tolerance from tolerance with some segregation for moderately tolerant seedlings in some compartments.



Photograph 11. Placement of selected salt tolerant seedlings in recovery tanks.