# 3. Submergence tolerance

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## 3.1 Introduction

Flooding affects vast rainfed lowland rice areas in Asia during the monsoon season. In deep water and floating rice areas, water stagnates for longer duration, commonly more than a month, and genotypes adapt to these conditions through faster shoot elongation to avoid complete inundation. Transient submergence for periods of upto two weeks can also occur in some areas, at any time and mostly more than once during the growing season because of flash floods caused by either heavy rains or outflow of nearby rivers. This type of flooding affects over 22 Mha of rainfed lowlands in South and Southeast Asia, of which, over 6 Mha are in India (Sarkar *et al.*, 2006; Das *et al.*, 2009). Modern high-yielding rice varieties are essentially sensitive to complete submergence; however, numerous tolerant landraces have been identified earlier (Sarkar *et al.*, 2014). Physiological mechanisms associated with tolerance to flash-flooding during vegetative stage are extensively studied (Sarkar *et al.*, 2014).

The Indian cultivar FR13A is the most widely studied and used as a source of submergence tolerance in rice breeding, and a major QTL, designated *SUB1*, was identified that imparts submergence tolerance of this genotype (Xu and Mackill, 1996). *SUB1* was subsequently fine-mapped and cloned, and three genes encoding putative ethylene responsive factors (ERF), *Sub1A*, *Sub1B*, and *Sub1C*, were identified, with *Sub1A* recognized as the primary determinant of submergence tolerance (Xu *et al.*, 2006). Cloning of *Sub1A* provided opportunities to gain more insight into the molecular mechanisms involved and to unravel the pathways underlying the submergence tolerance conferred by this gene (Fukao and Bailey-Serres, 2008). Moreover, precise gene-based markers were designed for its successful introgression into popular high-yielding rice varieties (Neeraja *et al.*, 2007; Septiningsih *et al.*, 2009). Subsequent testing of the introgression lines in the field showed substantial enhancement in survival after submergence for 12 to 17 days (Sarkar *et al.*, 2009). The success of marker added backcrossing/selection and identification of suitable donors tolerant to submergence depends on proper phenotyping. National Rice Research Institute (NRRI) has excellent field screening facilities for submergence and stagnant flooding tolerance for rice since 1978-79 (Paul and Bhattacharya, 1980). The widely used *SUB1* gene came from the cultivar (*e.g.* FR13A) identified by NRRI has immensely contributed to yield enhancement in rainfed lowland flood prone ecosystem. The protocol for submergence tolerance screening is also described in this chapter. Adequate care should be taken during the experimentation to distinguish between susceptible and tolerant cultivars. Flood-water quality impacts greatly in survival under submergence (Das *et al.*, 2009). The cultivars which survive less than 14 days may perish within 7 days under turbid water (Panda *et al.*, 2006; Das *et al.*, 2009). However, sensitivity to submergence does not change. All the factors need to be considered while adopting the screening techniques.

## 3.2 Submergence tolerance

### 3.2.1 Under field tanks

The mechanisms of survival under flash flooding and stagnant water conditions are different. Screening for submergence tolerance plants are raised under direct seeded condition. Generally, 18-21 days old seedlings are completely submerged under 70-80 cm of water. Plant height is taken before and after submergence to know the elongation ability which may give an idea about the suitability of plants for flash flood or stagnant water conditions. The cultivar showing greater elongation and pushing their leaf tip above the water surface should be discarded before screening for submergence tolerance (Plate 3.1-3.4). Finally, number of survivors is counted after 10 days of desubmergence.

$$PS\ (\%) = \frac{N_{10d}}{N_{BS}} \times 100$$

Where, PS: plant survival, $N_{10d}$: number of plants after 10 days of desubmergence, $N_{BS}$: number of plants before submergence.

*Plate 3.1: Field screening facilities at NRRI for submergence tolerance, stagnant flooding tolerance (medium to semi-deep conditions (0 – 70) cm depth of water and germination stage oxygen deficiency stress (GSOD)*

*Plate 3.2: Submergence imposed to cultivars with and without* SUB1 QTL

*Advantage:* Both submergence tolerace and stagnant flooding tolerance screening are possible in a single experiment. Seeds can be harvested from the survived plants. In the same growing season hybridization programme can be initiated with survival plants.

*Plate 3.3: Cultivars with and without* SUB1 QTL *(5 days post submergence)*

*Plate 3.4: Submergence screening under pot conditions*

### 3.2.2 Under net house condition

Under net house condition, seeds are directly sown in small trays. After 10 days of sowing, the trays along with seedlings are submerged in small concrete tanks under 60 - 70 cm depth of water for 10 days. Plant survival is counted after 10 days of drainage of water. Plant height is taken immediately after drainage. This technique is highly useful for transgenic plant as well as for genetical studies. This saves time, needs limited resources and can be used to distinguish between tolerant and susceptible types. This requires less area and space. The demerit of this method is loosing of plant materials suitable for stagnant flooding for medium-depth condition.

***Precaution to be taken:***

Under clear water, submergence stress is given for 12-15 days depending upon the conditions of susceptible check. Depending on the quality of the food water, extreme yellowing of leaves and softening of base, decision may be taken about the duration of submergence. The thumb rule is that the mortality of the susceptible check should be close to 100%.

## 3.3 Phenotypic observations

- *Vegetative vigor:* Seedlings should have higher vigor to compete with weeds in both submergence and deepwater areas.
- *High tillering ability:* Cultivars should have higher tillering ability because some of the tillers may serve as energy tanks for survival and ultimately productivity.
- *Erect leaves, longer, wide and thick leaves:* Plant should have erect, longer and thick leaves to efficient utilization of light for better carbon assimilation.
- *Height and lodging:* Cultivars should have intermediate in height and resistance to lodging.
- *Length and weight of panicles:* Longer and heavy panicles should be preferred and it may have better culm strength.
- *Photoperiod sensitivity:* Cultivars must be photoperiod insensitive and tolerant to low light intensity.
- *Grain dormancy:* Grain must survive even under dormant condition and it is advantageous when water lodging or high humidity prevail just before harvesting.
- *Shoot elongation:* Extent of elongation of the plant shoots is determined by subtracting plant height before submergence (BS) from that after desubmergence (AS) and expressing it as percentage of plant height before submergence.
- *Plant survival:* Plant survival is determined by counting the numbers of plants that are able to produce at least one new leaf after 7 days of desubmergence and expressed as percentage of the initial number before submergence.
- *Leaf senescence:* Leaf senescence is assessed immediately after desubmergence on hill basis using a visual scale of 1 to 10. This visual score is based on the proportion of leaves that are yellow: 1= all leaves green; 10= all leaves completely yellow or degenerated.

## 3.4 Biochemical observation

- *Ethylene concentration:* Ethylene is measured according to procedure described by Kende and Hanson (1976). The internodes (2 cm long) of plant (2 from each treatment) are placed in 30 mL test tubes with 2 mL of water or test solution. The tubes should be stoppered with serum vial caps and kept horizontally. Ethylene is sampled by first injecting 1 mL of air into each tube with a tuberculin syringe, pumping the syringe several times, and then withdrawing 1 mL for analysis. Ethylene is determined by gas chromatography (GC) equipped with Porapak-Q column (6 feet long, 1/8 inch outer diameter, 80/100 mesh size, stainless steel column) and flame ionization detector. The oven, injector and detector temperatures should be set at 100, 300 and 150°C, respectively and the flow of carrier $N_2$ gas, air and $H_2$ are maintained at 15, 285, 30 mL per minute respectively. The amount of ethylene produced from samples is expressed by comparing with the standard curve of pure ethylene standard gas (9.12 ppm in $N_2$, Matheson Tri Gas) and under aforementioned GC conditions, ethylene is detected at retention time of 2.247 minutes.

- *Chlorophyll content:* Chlorophyll concentration is determined before and after submergence colorimetrically following the procedure of Porra (2002). Chopped fresh leaf tissue of 0.1 g is transferred to a capped measuring tube containing 25 mL of 80% acetone and kept inside a refrigerator (4°C) for 48 h before measurements is made using a spectrophotometer. The Chlorophyll a and b concentrations are calculated using the following equations:

  Chlorophyll a (µg $mL^{-1}$) = 12.25 ($A_{663.6}$) – 2.55 ($A_{646.6}$)

  Chlorophyll b (µg $mL^{-1}$) = 20.31 ($A_{646.6}$) – 2.55 ($A_{663.6}$).

- *Non-structural carbohydrates:* Non-structural carbohydrate concentrations (NSC) of both roots and shoots should be determined in submerged plants, by following the procedure of Yoshida *et al.* (1976). Briefly, for each measurement, shoot samples should be dried and ground to a fine powder and extracted using 80% ethanol (v/v). The extract then used for soluble sugar analysis after addition of anthrone reagent, followed by a measurement of absorbance at 630 nm using a double beam spectrophotometer. The residue remaining after soluble sugar extraction is dried and extracted using perchloric acid and then it is analyzed for starch (as glucose equivalent) using the anthrone reagent as for soluble sugars.

- *Underwater PAR:* Light intensity should be taken under water using LICOR light meter and record at 12:00 h. It can be calculated as the percentage of total incidence irradiance above the water surface and below the water surface just above the plant canopy.

- *Measurement of photosynthetic rate:* Net photosynthetic rate and stomatal conductance of rice seedlings are measured 7 days after submergence with an infrared gas analyzer around 11:00 AM. The conditions in the assimilation chamber are kept as follows: air humidity, 70%; leaf temperature, 35°C; and light intensity (PAR), 1200 µmol $m^{-2}s^{-1}$. Measurement was carried out using middle portion (3 cm long) of the fully expanded and not senescent leaf blade. Net photosynthetic rate should take at the $CO_2$ concentration of 380 µmol $CO_2$ $mol^{-1}$.

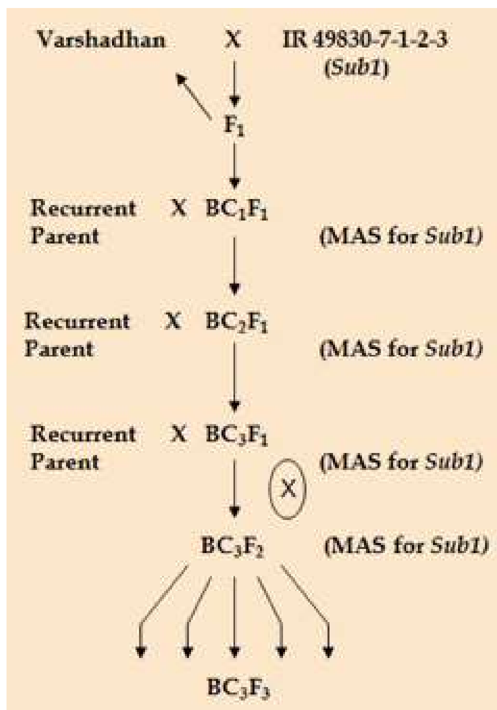
- *Extraction and assay of antioxidant enzymes:* A 500 mg sample of leaves is homogenized in 10 mL of grinding medium prepared for each enzyme, as mentioned below. The extract is centrifuged at 4°C at 15000 g for 20 min, and the supernatant is used for assays. All operations will perform under a dim green light.

- *Superoxide dismutase (SOD):* For the determination of SOD activity, the enzyme is extracted in 0.1 *M* potassium phosphate buffer (pH 7.8) containing 1 % (w/v) insoluble polyxeinyl poly pyrrolidone. The enzyme activity is determined by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) following Giannopolitis and Ries (1977) with modifications suggested by Choudhury and Choudhury (1985). The 3-mL reaction mixture contained 0.05 *M* $Na_2CO_3$, 0.1 m*M* EDTA, 63 µ*M* NBT, 13 µ*M* methionine, 0.2 mL enzyme extract and 1.3 µ*M* riboflavin. The riboflavin should be added last. The test-tubes should be placed under two 40-W fluorescent lamps at a distance of 30 cm at 25 °C. After 15 min, the light is switched off and the absorbance at 560 nm was noted. The non- irradiated sample served as a control and was deducted from $A_{560}$. The reaction mixture without the enzyme developed maximum color due to maximum photo-reduction of NBT. The reduction of NBT was inversely proportional to the enzyme activity. Thus, to obtain the activity, the $A_{560}$ of a particular set was deducted from the $A_{560}$ of the blank set (without enzyme).

- *Catalase (CAT):* The CAT activity is measured in a reaction mixture containing 25 m*M* phosphate buffer (pH 7.0), 10 *mM* $H_2O_2$ and the enzyme extract. The degradation of $H_2O_2$ was followed at 240 nm (Cakmak and Marschner 1992).
- *Peroxidase (PER):* The PER activity is measured in a reaction mixture consisted of 0.2 mL of enzyme, 5 mL phosphate buffer (0.05 *M*, pH 6.0), 1 mL $H_2O_2$ (46.9 *mM*) and 1 mL catechol (0.5 %). PER was assayed by the method of Chance and Maehly (1955), whereby colorimetric determination of the change in the colour intensity of oxidized catechol at 420 nm should be recorded.
- *Estimation of malondialdehyde (MDA):* Lipid peroxidation is measured as the amount of MDA produced by thiobarbituric acid (TBA) reaction, as described by Heath and Packer (1968). A 500-mg sample of leaves is extracted with 1 % (w/v) trichloroacetic acid (TCA) and MDA content is determined by adding an equal aliquot of 0.5 % TBA in 20 % TCA to an aliquot of the extract. The solution should be heated at 95 °C for 25 min. Absorbance should measure at 532 nm, corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The amount of MDA is calculated by using an extinction coefficient of 155 $mM^{-1}$ $cm^{-1}$.

## 3.6 Marker assisted breeding

Release of Swarna *Sub1* introgression of submergence tolerance gene "*SUB1*" into popular lowland rice varieties *viz.*, Gayatri, Sarala, Varshadhan is completed. The improved lines are in the process of testing for yield and submergence tolerance. Work on introgression of "*SUB1*" gene into Pooja and Pratikshya varieties is in progress. A schematic diagram has been provided here in developing submergence tolerant Varshadhan *i.e.* Varshadhan *Sub1* (Fig. 3.1 and 3.2).




Select lins with *Sub 1* gene having all the characters of recurrent parent will be filed tested for yield and tolerance

*Fig. 3.1: Schematic diagram of development of Varshadhan Sub1*

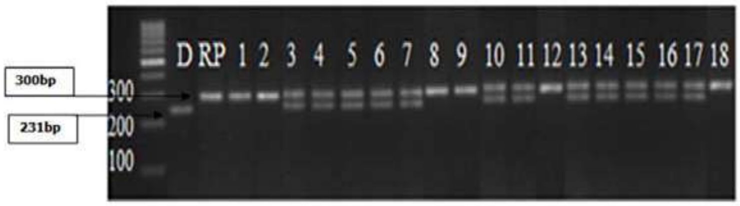



*Fig 3.2: Foreground selection of $BC_1F_1$ (Varshadhan*2/IR 49830-7) for Sub 1 loci with gene specific marker Sub1BC2; Donor (IR 49830-7); RP- Recurrent Parent (Varshadhan); Lane 1-18 $BC_1F_1$ plants*

## 3.7 Genotypes and varieties for submergence tolerance

- *Numbers of germplasm screened:* 7085
- *Numbers of tolerant germplasms (survival % >80%):* 99 (Kalaputia, Dhulia, Gangasiuli, Khoda, Khadara, Kusuma, Kanta Kunga, Atirang, Kalaketaki, Bhundi, AC1303, AC 1017, AC43307, AC43378, AC43386, AC43359, AC43351, AC43360, AC43328, AC43364, AC43393, AC43341, AC43326, AC43336, AC43352, AC43338, AC43340, AC43349, AC43395, AC43390, IR85212-186-1-1-1, IR84649-303-10-1-1-B *etc.*)
- *Numbers of highly submergence tolerant germplasms (survive up to 3 weeks):* 12 (AC1303B, INGR04001, INGR08110, AC38575, AC37887, IC258990, AC258830, AC42087, AC20431, INGR08113, INGR08109, AC42091)
- *Numbers of medium-tolerant germplasms (survival % 60-79%):* 124 (AC 45865, AC 45881, AC46096, IR76509-1-CN8-3-1, AC 10205 *etc.*)
- *Numbers of susceptible germplasms:* 6852 (Swarna, Savitri, Sarala, Gayatri, Varshadhan *etc.*).
- *Varieties:* Swarna-*Sub1* (India, Bangladesh, Nepal), IR64-*Sub1* (All Asia), Samba Mahsuri-*Sub1* (India, Bangladesh, Nepal), TDK 1-*Sub1* (Laos), BR11 *Sub1* (Bangladesh), CR1009-*Sub1* (India), PSBRc82 *Sub1* (Philippines), Ciherang-*Sub1* (Indonesia), and PSBRc18 *Sub1* *11* (Philippines).

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