PHENOTYPING FOR ABIOTIC STRESS TOLERANCE IN MAIZE:

WATERLOGGING STRESS

P.H. Zaidi, M.T. Vinayan and K. Seetharam
CIMMYT Asia Maize Program, Hyderabad, India

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WATERLOGGING STRESS

A field manual

Authors:
P.H. Zaidi, M.T. Vinayan and K. Seetharam

CIMMYT Asia Maize Program
Hyderabad, India

CIMMYT[MR]
International Maize and Wheat Improvement Center

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Work on waterlogging stress was scaled-up and expanded to South and Southeast Asia with continued support from GTZ-Germany through another project, titled "Abiotic stress tolerant maize for increasing income and food security among the poor in South and Southeast Asia". During the course of these projects, extensive work on developing and refining protocols for waterlogging stress screening and identifying traits associated with waterlogging tolerance were identified, which are compiled in this manual.

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CIMMYT – the International Maize and Wheat Improvement Center – is the global leader in publicly-funded maize and wheat research-for-development. Headquartered near Mexico City, CIMMYT works with hundreds of partners worldwide to sustainably increase the productivity of maize and wheat cropping systems, thus improving global food security and reducing poverty. CIMMYT is a member of the CGIAR Consortium and leads the CGIAR Research Programs on MAIZE and WHEAT. The Center receives support from national governments, foundations, development banks and other public and private agencies.

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Preface

Maize crops grown during the summer rainy season in the tropics occasionally face extreme weather conditions that limit crop establishment and yield potential. Among various abiotic stresses, contingent/intermittent soil waterlogging is one of the important constraints for maize production in the Asian tropics and many other maize growing areas around the world. Rainfed maize crops grown during the monsoon season in the Asian tropics occasionally face temporarily waterlogged soils and anaerobic conditions, even in well drained fields. These waterlogged soils adversely affect various crop growth stages, overall plant stand and final grain yield. Moreover, the increasing demand for maize in Asia is rapidly transforming cropping systems in the region from rice monoculture to more profitable rice-maize systems. However, maize production in rice-maize systems frequently faces the problem of early stage excessive soil moisture, as the soils of paddy fields are often saturated due to late monsoon rains.

This manual was developed for maize breeders and field technicians who manage screening/phenotyping for waterlogging tolerance with the purpose of identifying genotypic variability in maize for use in breeding programs targeting waterlogging tolerant maize. It will enable them to:

- Develop a suitable phenotyping system for various stages of waterlogging stress, including pre-screening of large sets of genotypes in a semi-controlled pot culture system and evaluation under field conditions.
- Phenotype for waterlogging tolerance using suitable secondary traits in addition to grain yield.
- Reduce experimental error and improve heritability of the experiment through proper stress management.

Waterlogging stress in tropical maize

When the amount of water in the soil exceeds field capacity, this results in waterlogging or excessive soil moisture. Excessive moisture in the root zone in the form of free water (not bound to soil particles) fills the air spaces in the soil, results in poor soil aeration and eventually affects plant growth and development. Waterlogging causes major changes in soil physical and chemical properties, which results in multiple crop stresses, including oxygen stress caused by low/no oxygen in the root zone, nutrient imbalance and biotic stress due anaerobic conditions.

Most crop plants are obligate aerobes and therefore require well-drained soils for optimal growth and development. Not being a wetland crop species, maize is highly susceptible to excess moisture/waterlogging stress. Unlike wetland crops such as rice, maize plants do not have a ventilation system for gaseous exchange between above-ground plant parts and inundated roots. Therefore, maize roots suffer as oxygen in the root zone progressively decreases, which results in oxygen stress, nutrient imbalance and, eventually, irreversible plant damage and yield losses.

Waterlogging may affect maize crops grown in the tropics at different crop stages, starting from pre-emergence until harvest.

1. **Pre-emergence stage waterlogging:** This is a common problem in tropical maize, especially if the maize seeds are planted in the following situations:
   - At the onset of the monsoon rains, and the rains continue for a few days after planting.
   - In a poorly drained field with heavy textured soil-type that was over-irrigated after sowing.

- Low-lying areas/patches in a poorly levelled field.
- Under saturated field conditions immediately after rice harvest (rice-maize cropping system in Asia) or in *char* or *diara* land (areas located near river banks that are vacated after the river water recedes after the rainy season).

2. Early seedling stage waterlogging:

- Heavy rainfall early in the season coupled with poor drainage.
- Over-irrigation coupled with poor drainage.
- Poorly levelled field with low lying patches where water stagnates after rain or irrigation, even if the field is well drained.

3. Vegetative growth stage waterlogging:

- Heavy and concentrated rainfall coupled with poor drainage.
- Over-irrigation coupled with poor drainage.
- Poorly levelled field with low-lying patches where water stagnates after rain or irrigation, even if the field is well drained.

4. Flowering/grain filling stage waterlogging:

- Late planted spring season maize crops in eastern India and Bangladesh (and similar ecologies elsewhere) are exposed to early monsoon rains in low-lying areas.

The response of a maize crop varies significantly when it is exposed to waterlogging stress at various growth stages. Therefore, depending on the type of waterlogging stress in a particular target environment, crop-stage specific screening/phenotyping for waterlogging tolerance should be conducted.

Phenotyping for waterlogging stress tolerance

1. Suitable soil type at the phenotyping site:

Waterlogging tolerance is a function of tolerance to oxygen stress in soil (hypoxia/anoxia) and nutrient imbalances; therefore, soil type may significantly affect the results of waterlogging trials. It is not uncommon for a genotype to be waterlogging tolerant at one location and turn out to be susceptible at another location that has a different soil type. This may be because different types of soil have different type of nutrient imbalances, even if the oxygen stress is the same at both locations. Also, in areas with duplex soil type (top soil is different from the subsoil), the stability of a genotype's performance may be significantly affected.

In view of the above, waterlogging screening/phenotyping needs to be done under distinctly different soil types to achieve stability in the performance of genotypes across locations.

- Top priority should be given to the soil type of the target population environment (TPE) where the waterlogging tolerant product will eventually be deployed.
- In addition to the TPE, other locations with distinctly different soil types should also be used for a more stable performance. For example, under Indian conditions, alluvial soils, black soils (*vertisols*) and red soils (*alfisols*) are the three major soil types where maize is usually grown; therefore, for wider adaptability, waterlogging phenotyping should be conducted in these three soil types.

- When doing pot-culture screening under semi-controlled conditions, a mix of distinctly different soil types should be used. For example, a mixture of alluvial, black and red soils might be a good option for screening maize germplasm for waterlogging tolerance under pot screening.

2. Grouping test entries by maturity groups

This is one of the basic requirements of phenotyping for most abiotic stresses, including waterlogging stress. Test entries should be grouped based on their anthesis date (preferably using growing degree days, GDD units). This is important for avoiding different levels of stress within a trial, as entries at different physiological stages may respond differently to the stress treatment. Ideally, all entries within a trial should have comparable anthesis days. Though a range of 4-5 days (40-50°C GDD units) is acceptable; more than 5 days' variation in anthesis days within a trial should be strictly avoided. After grouping the entries by their anthesis time, trials are constituted by keeping entries with comparable anthesis days in one trial. Formula for calculating GDD is given below. It is calculated on per day basis from planting up to anthesis and total (accumulated) GDD during this period is noted as GDD for anthesis for a genotype.

$$GDD = ((Tmax+Tmin)/2) - \text{Base temperature } (8°C)$$

3. Crop management

Except for the waterlogging stress treatment, which is applied at a targeted growth stage, all other recommended crop management practices should be followed in waterlogging phenotyping trials. Adequate crop management, including timely application of recommended agronomic practices and inputs, is a pre-requisite for quality phenotyping. The following aspects of crop management need due attention in order to generate quality phenotyping data:

3.1. Planting time

Genotype x environment interaction significantly affects the response of maize genotypes to various abiotic stresses, including waterlogging. Therefore, it is essential to conduct screening/phenotyping experiments during the appropriate season. Prevailing weather conditions, including temperature (both maximum and minimum), relative humidity, hours of sunshine, as well as soil temperature and temperature of stagnant water, can significantly change the response of genotypes to waterlogging stress. Therefore, waterlogging trials conducted during the dry summer or cool winter months may not truly represent waterlogging stress during the rainy season. Except for anaerobic germination/early seedling stage waterlogging stress in rice-maize cropping systems, waterlogging is a problem mainly for rainy season maize crops; therefore, screening/phenotyping for waterlogging tolerance should be conducted during rainy season. Planting time needs to be carefully selected so that there is a high probability of natural waterlogging with monsoon rains occurring at the targeted crop stage.

3.2. Plant population

The number of plants/unit area is one of the components of final grain yield. Therefore, in field phenotyping, it is essential to make sure the trial has the recommended plant population before imposing stress. If seed is not a limitation, planting two seeds/hill (or double density) and thinning out the extra seedlings at V_{2-3} stage is advised.

3.3. Border and filler rows

It is essential to plant border rows (3.0-4.0 m in field trials and one row of pots in case of pot culture trials) all around phenotyping trials to avoid border effects and physical damage to test entries. Also, to maintain the same level of competition, no in-between space should be left unplanted. If there are free spaces on the trial map, they should be filled by planting bulk seed.

3.4. Moisture management

Except during the intended period of excess moisture/ waterlogging stress, the trials should be kept at optimal moisture by providing supplemental irrigation, if needed, and ensuring good drainage to quickly eliminate excess water in case of heavy rains.

3.5. Application of recommended inputs

Recommendations regarding inputs, including fertilizers (time of application and doses), as well as weed, insect pest and disease control measures, are usually location specific, depending on soil physical and chemical properties and common biotic stresses. It is therefore essential to have updated information on the recommended package of practices for the phenotyping site, and ensure that they are implemented on time in order keep the crop free from nutrient deficiencies and biotic stresses.

In essence, phenotyping trials should be grown under optimal conditions so that the crop is not exposed to any except the intended stress (i.e., waterlogging during a specific period of time). If these practices are applied with precision, and compound effects of other stresses with the intended stress are avoided, a high quality phenotyping data on genotypic variability for waterlogging stress tolerance could be gathered successfully.

4. Weather data

In field-based phenotyping trials, it is essential to record hourly weather data, including temperature (Tmax and Tmin) relative humidity, rainfall, dew and wind velocity, which could significantly alter the overall effects of actually experienced waterlogging stress. A portable weather data recorder should be installed within the phenotyping field to record daily weather parameters. The frequency of data recording should be every hour, so that all critical weather data are captured on an hourly basis.

This information will help determine the type of growing conditions, apart from waterlogging stress, at a particular site, and could be used for adjusting the data from different sites before performing across-site data analysis.

5. Managed waterlogging stress

In a waterlogging phenotyping trial, it is quite challenging to achieve uniform stress under field conditions in terms of both the space and duration of the stress. Variable water depth in different parts of the field and/or during the stress period may significantly affect stress intensity and uniformity and, consequently, trial repeatability. Therefore, when screening large numbers of untested genotypes, it is better to use pot-culture pre-screening where the intended level of stress can be achieved with good uniformity. Selected entries from the pre-screening experiment can then be evaluated under field conditions in smaller-sized plots where stress intensity/uniformity can be better managed.

5.1 Pre-screening using pot culture

Plastic pots of suitable size (depending on the targeted crop stage) are used for pre-screening large sets of genotypes for waterlogging stress. A mixture of major soil types (for example, *vertisols* and *alfisols*) and well decomposed farmyard manure at a 2:2:1 ratio is used when preparing soil for pot culture. Basal fertilizer dose is mixed into the pot culture soil based on soil weight. Soil, farmyard manure and basal fertilizer doses are thoroughly mixed before filling the pots. The filled pots are then placed in waterlogging pits (cement structures erected at ground level) in which water is maintained at the desired depth and then drained completely after the stress treatment is finished. We use pits of 12.0 x 4.0 x 0.5 m in size that can accommodate a total 300 pots 12.0″ size and 1,500 pots of 6.0″ size for waterlogging stress during the vegetative stage and the pre-emergence/seedling stage, respectively (Picture 1). After arranging the pots in pits and labelling each pot as per the experimental design, test entries are planted with replicates. To impose waterlogging stress, drainage knobs in the pits are properly closed and the pits are filled with water by opening the water-supply taps. The desired water depth is maintained in the trials by synchronizing the water supply with evaporative losses. In this way, a uniform level of stress intensity is achieved in the phenotyping trials, and genotypic variability among test genotype in a trial is clearly expressed.

The steps involved in pre-screening at different crop stages are as follows:

Pre-emergence stage waterlogging (anaerobic germination)

a. Pot size: diameter: 15 cm; height: 15 cm.

b. Fill the pots with prepared soil up to 12 cm, and place them in the waterlogging pit.

Picture 1

c. Prepare labels to identify entries and replicates and place them on the respective pots. Place the seed packets in the pots as per the experimental design and layout (Picture 2).

Picture 2

d. Plant 50 seeds per pot. Cover the seeds by adding 2.0 cm of soil above the seed, leaving about 1.0 cm free space at the top of the pots. Planting in this way ensures uniform seed depth in each pot, as seed depth is an important factor in terms of the time seedlings take to emerge.

e. Plant the entries in two sets, one for the pre-emergence waterlogging stress and the other one grown under optimal moisture conditions.

f. Once planting is completed and all the pots are properly labelled, close the drainage knobs of the pits and open the water-supply tap to fill the pits with water until all the pots are completely submerged, and the water level in the pit rises approximately 4-5 cm above the pot surface (Picture 3).

g. Since the dry soil will absorb water during the first filling, the water level in the pits may go down after the water tap is closed. The water level should be up to 4-5 cm above the pot surface by the second round of filling.

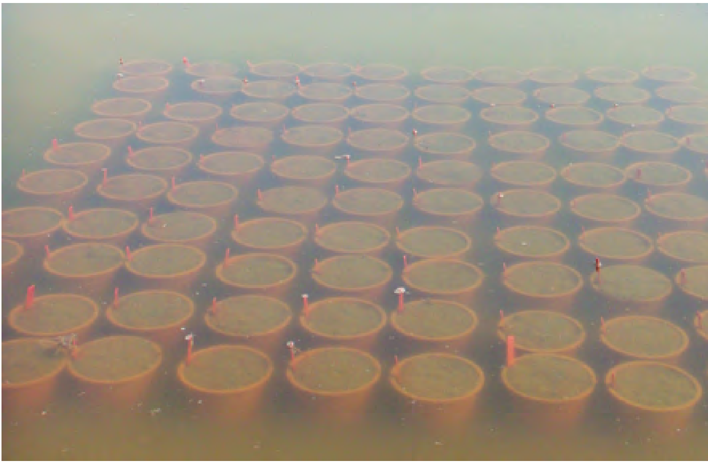
h. After adjusting water level couple of times on the day of planting, the water level is usually stable at the desired level, and any further decrease will occur only due to evaporative losses.

i. Decrease in water level due to evaporative loss should be monitored on a daily basis to ensure that the water is at the same level (4-5 cm above pot surface) throughout the stress period.

j. After 72 hours of submergence treatment, partially drain the pits so that the water depth comes down approximately to half of the height of the pots (about 6.0 cm) for another 24 hours (Picture 4). After completing the stress treatment, relieve the stress by draining out the pits completely.

k. After releasing the excess moisture stress, maintain optimal moisture conditions to allow seed germination and seedling emergence.

l. The second set should be maintained under optimal moisture conditions (stress-free) for proper germination, emergence and seedling growth.



Picture 3

Picture 4

Seedling stage waterlogging

a. Steps (a) to (c) are same as in pre-emergence stage waterlogging (as above).

b. Place the seed packets as per the experimental design and layout, and plant two seeds per pot.

c. Cover the seeds with 2.0 cm soil, leaving about 1.0 cm free space at the top of the pots.

d. Plant the entries in two sets, one for the seedling stage waterlogging stress treatment; the other is managed under optimal moisture conditions.

e. Apply irrigation for germination (avoid over-irrigation) and maintain adequate moisture for germination, seedling emergence and growth.

f. At V_{1-2} growth stage, waterlogging stress is applied to the first set by filling the pits with water. Maintain the water level in the pit at least 2-3 cm above the pot surface (Picture 5).

g. Any decrease in the water level due to evaporative loss should be monitored on a daily basis to ensure that water is at the desired level throughout the stress period.

h. After 120 hours, partially drain the pits so that water depth is at approximately half the height of the pots (about 6.0 cm) for another 24 hours. After completing the stress treatment, relieve the stress by draining the pits completely.

i. After relieving the excess moisture stress, maintain optimal moisture conditions to allow recovery and growth.

Vegetative stage waterlogging

a. Pot size: diameter: 30 cm; height: 30 cm.

b. Fill the pots with prepared soil, and place them in the waterlogging pit.

c. Place the seed packets in pots as per the experimental design and layout, and plant two seeds per pot.

d. Apply irrigation for germination and maintain adequate moisture for germination, seedling emergence and growth.

e. At $V_{5\text{-}6}$ growth stage (approximately four weeks after planting), waterlogging stress is applied by filling the pits with water. Fill the pits until the water level is at least 2-3 cm above the pot surface (Picture 6, page 13).

f. Decrease in water level due to evaporative loss should be monitored on a daily basis to ensure that the water is almost at the same level throughout the stress period.

Picture 5

g. After 144 hours of stress treatment, partially drain the pits so that water depth in pits is at approximately half of the height of the pots (about 15.0 cm) for another 24 hrs. After completing the stress treatment, release the stress by draining the pits completely.

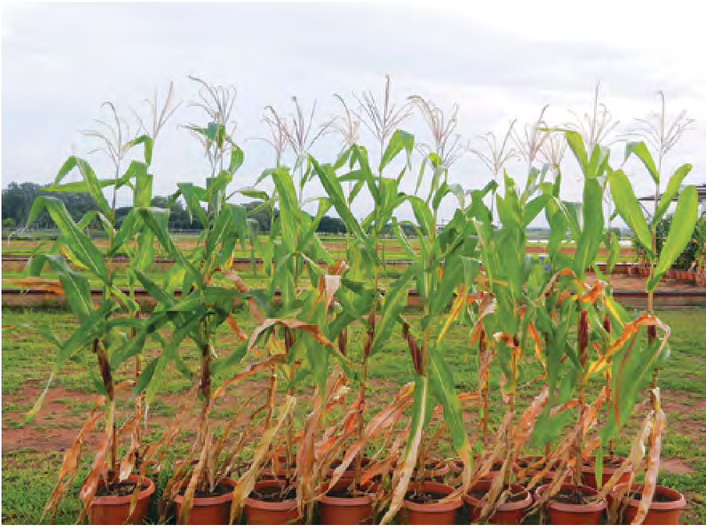
h. After releasing the stress, maintain optimal moisture conditions to allow recovery, and the life cycle to be completed. The pots may be shifted out of the pits, so that pits are available for pre-screening the next batch of entries for vegetative-stage waterlogging stress (Picture 7, page 15).

Picture 6

5.2. Phenotyping under field conditions:

To achieve uniform waterlogging stress for the intended period of time, standing water in the experimental plots needs to be uniformly maintained at a specific depth (for example, approximately 2-3 inches in case of vegetative stage waterlogging) in entire field throughout the stress treatment. A well-levelled field with zero slope is a prerequisite for managing waterlogging stress under field conditions. The key requirements for field phenotyping of maize genotypes for excessive moisture/waterlogging tolerance are as follows:

- A well-levelled field with good irrigation and drainage, so that it can be completely drained after the waterlogging stress treatment, and to avoid unintended excessive moisture due to heavy rains at post-stress stage.
- The field must be chosen based on soil type, as discussed in section 1 above.



Picture 7

- The field can be divided into micro-plots, as small plots help manage stress uniformly. For example, in a 20.0 x 25.0 m plot, 25 rows and 4 beds can be laid out (with a row length of 5.0 m and row-to-row space of 0.75 m).
- Planting should be done in a flat field (no ridge and furrow system), irrespective of the target crop growth stage for stress. After the waterlogging stress treatment, earthing-up can be done after inter-culture operations, once the field reaches suitable moisture for inter-culture operations.
- Plant the selected genotypes during pre-screening, along with check entries with known reaction to waterlogging stress, using standard crop management practices and planting geometry. Apply waterlogging stress at the targeted crop stage during the prescribed period, as follows:

  a. *Pre-emergence stage*: Immediately after planting, for 72 hrs.

  b. *Seedling stage*: At $V_{1\text{-}2}$ stage, for 120 hrs.

  c. *Vegetative stage*: $V_{5\text{-}6}$ stage, for 144 hrs.

The steps for imposing waterlogging stress are as follows:

- Close all the outlets and apply excess irrigation water, so that the soil profile is fully saturated and the water level rises above the soil surface.
- Ensure that water supply in the plots exceeds subsoil drainage/downward movement and the water level in the plots rises 2.0-3.0 inches above the soil surface.
- By regulating the rate of water supply, maintain the inundated water depth (at least 2.0 inches) in the plots continuously during the prescribed stress period.
- After completing the stress treatment, the field should be completely drained-out to ensure that no further waterlogging/excess water occurs in those plots, even in case of heavy/continuous rains, by maintaining good drainage.

6. Phenotyping traits

Yield is a trait of primary interest; however, dissecting it into its components (secondary traits associated with yield) gives a better understanding of the targeted trait, and helps to keep track with stress intensity for mid-term correction, if needed. Secondary traits could also be used as preliminary selection criteria when turnaround time between seasons is short.

Traits that are significantly affected by waterlogging stress and therefore should be recorded in waterlogging phenotyping trials are as follows:

Traits	How?	When?	Where?	Selection direction
Seed germination	- Count the number of coleoptiles that have emerged (visible tips) from the soil surface (Picture 8). - Calculate % germination in relation to the total number of seeds planted, as follows: $$\textit{Germination (\%)} = \frac{\textit{Number of coleoptiles emerged}}{\textit{Total number of seeds sown}}$$	Count the number of coleoptiles emerging from the soil surface by daily observation, starting from the 4^{th} day up to 15^{th} day after planting.	Pre-emergence waterlogging stress, under both pot and field conditions.	Maximum germination
Days to emergence	- Count the number of emerged seedlings on each day of observation. - Note the days it took to reach maximum emergence (90% to total emergence).	Count the number of coleoptiles emerging from the soil surface by daily observation, starting from the 4th day up to 15th day after planting.	Pre-emergence waterlogging stress, under both pot and field conditions.	Minimum (<5.0 days)' delay in seedling emergence
Seedling biomass	- Count the number of shoots per replication. - Cut the shoots at the base and record fresh weight (SWL). - Count the same number of seedlings under optimal moisture for the respective entry and record fresh weight (SNM). - Calculate loss of seedling biomass using the following formula: $$\textit{Loss in seedling biomass (\%)} = \frac{(SNM\text{-}SWL)}{SNM} \times 100$$	Two weeks after relieving the stress	- Pre-emergence waterlogging stress under pot conditions. - Seeding stage stress under pot conditions.	Minimum loss of seedling biomass

Picture 8

Traits	How?	When?	Where?	Selection direction
Seedling/plant mortality	- Count the number of seedlings/plants before applying waterlogging stress. - Count the number of dead plants one week after relieving the stress. - Calculate plant mortality using the following formula: $Mortality\ (\%) = \frac{No.of\ dead\ plants/\ seedlings \times 100}{Total\ number\ of\ plants}$	One week after relieving the stress	- Seeding stage stress (under both pot and field conditions) - Vegetative stage stress (under both pot and field conditions)	Minimum seedling/ plant mortality
Root/stem lodging	- Count the total number of lodged plants per plot, which are uprooted (root lodging) or broken at the stem nodes/inter-nodes (stem lodging) - Count the total number of plants in the plot. - Calculate root/stem lodging (%) as follows: $Loadging\ (\%) = \frac{No.of\ lodged\ plants \times 100}{Total\ number\ of\ plants/plot}$	2-3 weeks after relieving the stress	Vegetative stage stress (under both pot and field conditions)	Minimum lodging
Brace roots/ plant	- Count the number of brace roots at above-ground nodes in the plot (Picture-9). - Count the number of plants in the plot. - Calculate brace roots/plant as follows: $Brace\ per\ plants\ (\#) = \frac{Total\ no.of\ brace\ roots}{Total\ number\ of\ plants}$	2-3 week after relieving the stress	Vegetative stage stress (under both pot and field conditions)	Maximum stress-induced brace roots

Picture 9

Traits	How?	When?	Where?	Selection direction
Surface rooting	- Count the roots tips (white tips) emerged from soil around the stem in the plot (Picture-10). - Count the number of plants in the plot. - Calculate surface roots/plant as follows: $\textit{Surface root (\#)} = \frac{\textit{Number of surface roots per plot}}{\textit{Number of plants per plot}}$	2-3 week after relieving the stress	Vegetative stage stress (under both pot and field conditions)	Minimum surface rooting
Days to 50% anthesis (AD)	- Observe the plants that extruded anthers (anthesis). - Record the date when almost half (50%) of the plants reached anthesis. - Calculate the days to 50% anthesis (AD) by calculating the total number of days from planting to date of 50% anthesis.	During the reproductive phase, observe each plot on a daily basis starting from tassel emergence until all the entries in the trials have completed male flowering.	Vegetative stage stress (under both pot and field conditions)	Least change in days to anthesis
Days to 50% silking (SD)	- Observe the plants where silks have emerged from the tip of the ear (silking). - Record the date when almost half (50%) of the plants reached silking. - Note the days to 50% silking (SD) by calculating the total number of days from sowing to date of 50% silking.	During the reproductive phase, observe each plot on a daily basis starting from tassel emergence until two weeks after completion of anthesis in the trial.	Vegetative stage stress (under both pot and field conditions)	Least delay in days of silking
Anthesis-silking interval (ASI)	- Calculate the anthesis-silking interval (ASI) as follows: (ASI = SD – AD)	At the time of data analysis	Vegetative stage stress (under both pot and field conditions)	ASI <5.0 days

Traits	How?	When?	Where?	Selection direction
Ears per plant (EPP)	- At harvest, count the total number of ears/ plot. *(Note: A cob with at least one kernel is as considered one ear)* - Count the number of plants per plot $\text{Ears per plant (\#)} = \frac{\text{Number of ears per plot}}{\text{Number of plants per plot}}$ - The main purpose of making this observation is to assess stress-induced barrenness Barrenness = 1 - EPP	Immediately after harvest	Vegetative stage stress (under both pot and field conditions)	Maximum EPP (minimum barrenness)
Grain yield*	- Weigh total ears per plot using a suitable balance (with sensitivity of not less than 10 g). - Calculate grain yield in tons per hectare, after accounting for area, shelling percentage and grain moisture content at harvest.	Immediately after harvest	Vegetative stage stress (under both pot and field conditions)	High yield
Grain moisture	- Using a suitable grain moisture meter, record moisture content in at least 10% of the plots in a trial. - Use mean grain moisture content for calculating grain yield at 15% moisture.	Immediately after harvest	Vegetative stage stress (under both pot and field conditions)	-

* Though ear weight/plot may not give the exact value of grain yield, however, it is accurate enough for Stage 1 (early generation lines or 1st time testcross progenies with a large number of entries) and Stage 2 trials (2nd time testcross progenies with a high number of entries). However, direct grain yield of advanced stage lines, Stage 3 and Stage 4 trials should be recorded after shelling the ears from each plot. This will of course require collecting all the ears from each plot and carefully threshing them on a per-plot basis followed by weighing them separately.

Picture 10

7. Precautions/Points to note:

- Use the recommended crop management practices to promote proper plant growth and development before and after the waterlogging stress treatment.
- Ensure good drainage to strictly avoid any unintended excess moisture before or after the targeted stress treatment.
- Avoid any limitation/stress other than the intended waterlogging stress treatment.
- Avoid conducting excess moisture/waterlogging trials under cold (Tmin <10°C) or hot-dry (>35°C) weather conditions.
- Avoid using soils that have any type of soil-related stress, such as salt stress, low pH, or acute deficiency of essential elements.
- Ensure that the waterlogging treatment is uniform, with no significant water level variation with space and/or time.
- Ensure minimum disturbances in stagnant water during the stress treatment; for example, avoid walking in the field during the stress treatment, speedy water flow across the plot and water level fluctuations.
- Do not enter the in wet field after the stress treatment is completed, until the field is suitable for walking.

CIMMYT
International Maize and Wheat Improvement Center

India:
Asian Regional Maize Program, CIMMYT
C/o ICRISAT, Patancheru-502 324,
Greater Hyderabad, (A.P.), INDIA

Headquarters:
Apdo. Postal 6-641 Mexico D.F., Mexico 06600
Email: cimmyt@cgiar.org
www.cimmyt.org