PHENOTYPING FOR ABIOTIC STRESS TOLERANCE IN MAIZE

LOW NITROGEN STRESS

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CIMMYT MR

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A field manual

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Acknowledgements

Breeding for low nitrogen stress tolerance is among the priorities for CIMMYT's Global Maize Program in sub-Saharan Africa. CIMMYT has been a pioneer in developing and deploying protocols for low nitrogen stress phenotyping, selection strategy and breeding for low nitrogen (N) stress tolerance (Bänziger *et al*., 2000). The present field phenotyping manual builds on the work undertaken by several scientists and experience gained over a decade at CIMMYT. The information presented in this manual is based on the phenotyping and breeding work on low nitrogen stress tolerance that received strong and consistent support from several donor agencies, especially the USAID, BMGF, DFID, GIZ, and the MAIZE CGIAR Research Program, as well as public and private sector partners in sub-Saharan Africa.

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.

Correct citation: M. Zaman-Allah, B. Das, J.E. Cairns, M.T. Vinayan, A. Tarekegne, C. Magorokosho, P.H. Zaidi, and K. Seetharam, 2018 Phenotyping for abiotic stress tolerance in maize – Low nitrogen stress. A field manual. CIMMYT, Zimbabwe.

Contents

Preface

Poor soil fertility is one of the primary constraints to maize production in Sub-Saharan Africa (SSA) where most of the farming land is heavily depleted of nitrogen (N) as a result of repeated crop cultivation over several decades without replenishment. This is largely associated with the high fertilizer cost and dependence on rain-fed agriculture that translates into most smallholder farmers being reluctant to invest in expensive inputs such as fertilizer.

One approach to addressing the poor soil fertility problem is to exploit natural genetic variation in maize to develop improved varieties that respond better to the small amounts of fertilizer applied by small-scale farmers in SSA. Research has shown that there is substantial genetic variation for maize yield under nitrogen stress.

Maize varietal performance under optimal fertilization is not always positively correlated, or predictive, of performance under the N stress conditions that prevail in SSA. Therefore, the most effective way to develop maize varieties that are better suited to low levels of nitrogen fertilization is to select directly under nitrogen stress through the establishement of well-managed, low N phenotyping sites.

This manual is designed for maize breeders and field technicians in sub-Saharan Africa, South and Southeast Asia and Latin America working on improving the tolerance of maize to low N stress. It covers aspects related to:

- Selection and development of fields suitable for low N stress phenotyping
- Factors that affect the quality of low N stress phenotyping
- Managing uniform stress in low N stress experiments
- Effective and timely phenotypic data collection

Nitrogen stress in tropical maize

Nitrogen is an essential component of all enzymes and therefore necessary for plant growth and development. It constitutes about one-sixth of the weight of proteins (many are enzymes), and is a basic element of nucleic acids. Nitrogen is especially plentiful in leaves, mainly in photosynthetic enzymes, where it may account for up to 4% of the dry weight. Because N uptake, biomass production, and grain yield are strongly correlated, N requirement of a maize crop can be related to grain yield (*Bänziger et al*., 2000).

Unlike drought, the pattern of N stress through the season is usually very similar from location to location. At the beginning of the season, due to fertilizer application, N supply usually exceeds crop demand, however as the season progresses, the available N in soil diminishes. Soil N mineralization is usually less than 1 kg N/ha/day, whereas a healthy maize crop can take up and assimilate 4 to 5 kg N/ ha/day, leading to soil N depletion and N stress in the plants as the season progresses (Bänziger *et al*., 2000). Depending on the timing of N stress in growing plant parts, different yield-determining factors are affected.

During the vegetative stage: Nitrogen stress before flowering reduces leaf area development, photosynthesis rate, and the number of ear spikelets (potential grains). About 50% of leaf N is directly involved in photosynthesis either as enzymes or as chlorophyll. Light-saturated photosynthetic rates show a strong dependence on leaf N content ($r > 0.75$), resulting in a curvilinear relationship between resource use efficiency and leaf N content that shows a saturation for maize at about 2% leaf N content. When N becomes scarce, plants adjust to some extent by

remobilizing N from older tissue, (leaves, stalk) to younger tissue (leaves, grains), leading to early senescence of the older leaf tissue. In addition, plants favor root growth over shoot growth under N stress and the root/shoot ratio increases. The absolute amount of roots, however, is usually less for plants grown under N stress than under optimal N fertilization.

During the reproductive stage: Nitrogen stress during flowering results in kernel and ear abortion, whereas stress during grain-filling accelerates leaf senescence; leading to reduced crop photosynthesis and kernel weight. Relatively little is known about the effects of N stress on reproductive development. Initiation and development of reproductive structures occur in distinct phases, each of which can be affected by N stress. The number of potential kernel ovules is established early in plant development. The kernel row number is set by the time most tropical maize plants have 12-14 visible leaves and the number of kernels per row by the time 16-18 leaves are visible (Kiesselbach 1949). The number of ovules that ultimately develop into mature kernels is affected by the extent of kernel abortion in the two weeks bracketing flowering (Below 1997). Severe N stress delays both pollen shed and silking, but the delay in silking is relatively higher, so that the anthesis-silking interval (ASI) becomes greater under low N stress that occurs at flowering. As with drought stress, delay in days to silking is positively correlated with kernel and ear abortion.

Breeding strategy for N stressed environments

There are only a few breeding programs that have deliberately tried to increase the low N tolerance of maize, and most selections targeting N stressed environments have been conducted under well-fertilized conditions. Most often, as the severity of low N stress increases, the correlation between genotype performance under low N and well-fertilized conditions diminishes. If yields in the target environment are less than 40 % of the yields obtained under well-fertilized conditions (as observed in many tropical environments), germplasm should then be evaluated under severe N stress as part of selection (Bänziger *et al*. 1997).

In the case of low N stress, the breeding approach is simpler (compared to drought or heat stress) because the pattern of low N stress is very similar among low N fields: low N stress usually increases over time. Thus, one relatively severe low N stress regime should be sufficient to assess low N stress tolerance because, when combined with grain yield data from experiments under high N, it should allow prediction of genotype performance across a range of intermediate N levels (Bänziger et al. 1997).

Precision phenotyping for low nitrogen stress

Any breeding pipeline essentially involves the assessment of variability for a given trait in the available germplasm followed by selection for useful trait variants for use in breeding schemes. Progress in breeding is largely dependent on the quality of phenotypic data. Precision phenotyping enables breeders to: i) reliably identify and select superior progenies for targeted traits, ii) establish genotype-phenotype associations and iii) identify potential genomic regions for use in the breeding process. Therefore, precision phenotyping is indispensable for any successful crop improvement program.

Basic requirements for generating high-quality phenotypic data related to low N stress under field conditions are described below.

1. Understanding the target environment

Crop varieties are usually grown in a set of environments that vary from year-to-year and from field-to-field. A set of all environments, fields and seasons in which improved varieties are expected to do well is referred as the "target population of environments" (TPE) (Cooper and Byth,1996). Each breeding program must clearly define the TPE for which it is developing varieties. These environments may show variations, however, they must be sufficiently similar for one genotype to perform well in all of them. A clear understanding of the TPE is essential for planning and selecting the best selection environment where the phenotyping site should ideally be established. The phenotyping site does not necessarily have to be in TPE, but should have a good and relevant

relationship with the TPE. Therefore, a minimum amount of information about the TPE is highly required:

- Soil type, cropping season and cropping system, especially the window for planting maize.
- Historical weather data, preferably rainfall and its distribution pattern.
- Other relevant information, such as major biotic stresses and socio-economic constraints (for example access to fertilizer).

Analysing this information will help understanding the key requirements for establishing a phenotyping site that is significantly related to the TPE.

2. Establishing the phenotyping site

2.1 Site selection

Ideally, the low N stress phenotyping site should have the main characteristics of the TPE.

Developing a low N stress phenotyping site can take several seasons as the site will need to be depleted of naturally available soil nitrogen through repeated cultivation of a crop which removes the nitrogen from the soil. A low N site is therefore a long-term investment and needs careful consideration. Once the location is identified, it is important to ensure it satisfies the basic requirements of a phenotyping site such as:

- The size of a low N stress phenotyping site should typically be at least 1 hectare given the investment required to develop the site.
- The site should consist of fairly uniform soil that will deplete evenly. Different soil textures within a field can result in high levels of spatial field variation that will adversely affect trial results quality.

- Well-levelled field to facilitate uniform irrigation and avoid excess runoff and water stagnating in patches. A slope within the field can result in nutrient leaching and run-off to lower parts of the field creating field variation. In addition, the site should also not be located in a valley, where nutrients and rainwater may accumulate during the rainy season.

Besides, it is highly important to try as much as possible to avoid factors that can confound trial results. This can be achieved by:

- Choosing a field location away from trees or any physical structure that can create a shading effect. Shading can affect crop growth and the soil microenvironment leading to variation in rates of mineralization.
- Avoiding areas with leguminous trees that fix atmospheric N and increase soil N status.
- Ensuring the availability of a good irrigation facility to avoid unwanted random drought stress or excessive moisture/waterlogging.
- Avoiding locations where there is heavy incidence of pest and pathogen pressure such as termites, striga or maize lethal necrosis.
- Mapping spatial field variability to further improve the precision of field experiments: knowledge of soil physical and chemical properties that affects plant growth and stress development, and their uniformity within a field is essential in selection of a suitable phenotyping field. A spatial field variability map can help establish a suitable experimental design and trial layout, so that no part of the trial is located in a very bad patch.

  There are several options for mapping spatial field variability:

  (i) Ideally, it should be carried-out by growing a single crop variety (preferably maize because different crops may vary significantly in their sensitivity to soil physical and chemical problems) to be able to identify existing field variability and bad patches, if any.

(ii) Direct assessment of soil variability can be made through soil sampling at 30 cm depth intervals (to a depth of 90 or 120 cm soil depth) and analysis of key soil physical and chemical properties, which can provide information on the suitability of a site for phenotyping. Ideally soil samples should be taken across the field using a square grid with a minimum of five sampling points per hectare (Masuka *et al*., 2012).

(iii) Many high-throughput techniques are available for mapping spatial field variability: soil electrical conductivity sensors, penetrometers, spectral reflectance, thermal imagery of plant canopies and measurements of plant growth as surrogates of variability (Prasanna *et al*., 2013).

2.2 Site development

There are a number of recommended steps that should be taken to develop a low N stress phenotyping site including mapping, plot demarcation and nitrogen depletion.

Preliminary Site Map

Once a low N stress phenotyping site has been selected, it should be clearly demarcated and sign posted as a low N site to prevent future mistakes in fertilization. The GPS coordinates of the site should be taken. A brief depletion history and map of the site should be recorded. The site should be cleared of all vegetation, free of termite mounds and deep ploughed to break any hard pans and facilitate drainage.

Baseline Soil Testing

Soil testing is important to obtain the baseline soil nutrient status and to allow the depletion of soil nitrogen to be tracked over years. A good method for soil sampling is called the grid sampling method. The field can be marked out using a grid of 20 meters by 20 meters and samples taken at the cross over of each grid. Separate soil samples should be taken at three soil depths (0-30 cm, 30-60 cm

and 60-90 cm) and all samples individually analysed. This will provide an overview of soil nutrient status at the start of site development also including information on the variability of soil chemical and physical properties.

Establishing permanent plots

Once a site has been selected it is important not to introduce field variability unknowingly. During experimentation, plants will remove more soil nitrogen from plots as compared to alleys and walkways. As a result, it is recommended to mark out permanent plots and alleyways (**Figure 1 a, b**). Permanent plots and alleys can be referenced from a single beacon or individually using permanent metal pegs set in concrete along the perimeter of the block. Typically, a low N field plot is 5 meters long with 75 cm interrow spacing. This will ensure that plots are consistent season after season and do not shift into parts

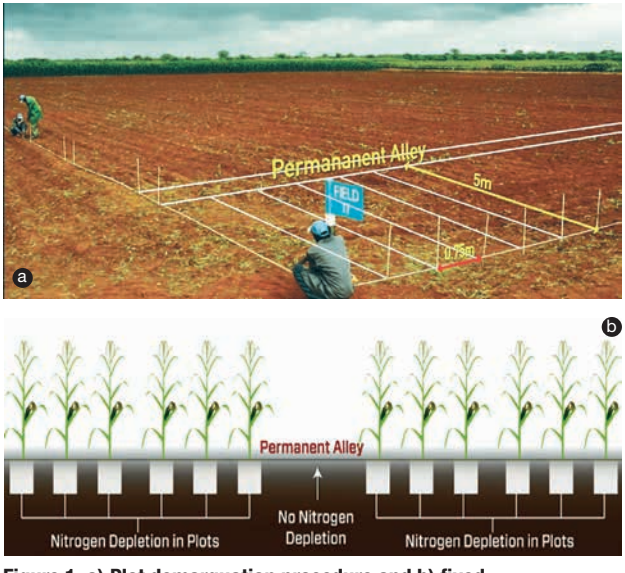

Figure 1: a) Plot demarquation procedure and b) fixed permanent plots

of the field that were previously used as alleys or borders. The fixed permanent plots also ensure that individual plot performance can be monitored season after season and that composite yield maps of the site can be generated to aid site management.

Site Depletion

Trial yields from a low N phenotyping site should be as representative of smallholder production as possible whilst enabling clear detection of genetic variation for low N performance. It has been shown that a 70% yield reduction, relative to optimal conditions, provides the best experimental conditions for detecting genetic differences amongst maize genotypes. This yield reduction relative to optimal potential typically translates to a yield of 2.5 to 3 tons per hectare in mid-elevation environments in Africa. In order to deplete a low N phenotyping site of residual nitrogen to achieve a 70% maize yield reduction, the site should be repeatedly sown to a single cereal variety. Maize, sorghum or wheat can be used. Leguminous crops will fix and increase the soil nitrogen pool rather than depleting it and should not be used. Sorghum is the most effective depletion crop as it develops biomass comparable to maize but can be planted at much higher densities, up to 400,000 plants per hectare without lodging. Sorghum is relatively drought tolerant which makes the depletion crop easier to manage off-season. In addition sorghum can be cut back at flowering stage (ratoon) allowing a second flush to develop, circumventing the need of a repeat planting for the next depletion cycle.

At the end of each depletion cycle, the entire biomass of the depletion crop should be removed from the low N phenotyping site including leaves, stems and if possible roots. Depletion will be apparent when a clear border effect is visible (Figure 2). Typically, two or three cycles of high-density depletion are required in the mid-elevation regions of southern and eastern Africa to adequately develop a low N stress site. However, it is important to keep in mind that the higher the clay content, the longer it takes to deplete nitrogen in a field.

Figure 2: Border effect in a low N maize field trial.

During the depletion phase, a variability map can be generated and used to block trials so that they are contained within areas of limited variation to provide the most uniform conditions for individual trials.

Site Characterisation

Field variability is always higher under abiotic stresses. Thus, even when the most uniform field sites are chosen, inherent field variability will still compromise the identification of nitrogen use efficient varieties. It is therefore recommended that low N phenotyping sites should be characterized prior to experimentation to better understand natural field variation in nutrient status. This information can then be used to guide trial layout and site management. It is recommended that the site characterization is undertaken prior to experimentation and then repeated after every 5 cycles of experimentation. This will enable detection of variation that might have been introduced through field management during experimentation such as irrigation or fertilizer application.

The most common way of characterizing a site is by soil sampling. However, due to the cost and effort involved, detailed soil sampling is not recommended. The grid sampling method will provide a useful overview of soil nutrient status in the low N phenotyping blocks.

Generally, nitrate level in the soil is considered deficient when it ranges between 7.5 to 15 parts per million (ppm). Soils are considered depleted when nitrate levels fall below 7.5 ppm. Values around 10 ppm provide good experimental conditions for detecting useful genetic variation.

Site characterization can also be performed using crop biomass vigour. Characterising a low N phenotyping block by crop biomass or vigour is generally the fastest and most economical method of characterizing a site. It is assumed that crop vigour or biomass are reflective of soil nutrient status. Plot biomass can be assessed using a variety of methods such as plant height, visual estimate of plot vigour or by measuring normalized differential vegetation index (NDVI) using a tool called GreenSeeker. All three methods are positively correlated. NDVI is an estimate of green biomass and offers the most objective and high resolution method of site characterization. A GreenSeeker is used to measure NDVI from each plot when the crop is knee high (6 to 8-leaf stage). It is at this stage that differences in plot vigour are clearly pronounced.

Site Monitoring

Once operational, the performance of a low N stress phenotyping site should be carefully monitored for the level of yield reduction and field variability. The average trial yield of the site should be determined to ensure that actual yields are close to the target yield reduction of 70% compared to well-fertilised trials. If yields are too high, then the site requires more depletion, while if yields are too low, small amounts of nitrogen fertilization may be required. Mapping trial yields and heritability across the site will provide a good understanding of the site variation.

Low N phenotyping blocks should have at least two rows of border planted on the periphery of the field to avoid the border effect as plants growing on the edges of the field perform better than those within the field due to reduced competition for light and nutrients.

The site should also be maintained free of weeds, both during and out of the cropping cycle as some weeds can be leguminous and add nitrogen to the soil introducing further unwanted field variability.

Type of material to be used (Hybrids and Inbreds)

Inbreds are smaller than hybrids and will therefore remove less nitrogen from the soil. Random placement of inbred trials within the screening field can introduce unwanted variation in soil nutrient status. Therefore, specific blocks should be demarcated for inbred and hybrid trial evaluation within the low N managed stress field.

3. Crop management

In order to promote early plant vigour and maintain targeted yield levels, limited amounts of fertilizer will need to be applied to low N stress blocks. Typically, 50 kg of diammonium phosphate is applied per hectare at planting and 75 kg of calcium ammonium nitrate is applied as top dressing when the crop is knee high (6 to 8-leaf stage). All other nutrients should be applied at the location specific recommended rate. Potassium availability in nitrogen depleted sites should be monitored closely in particular. After four of five cycles of experimentation, crop residue can be incorporated into the low N phenotyping blocks to ensure soil organic content and micronutrients do not become limiting factors to crop growth.

Striga can become a problem in low N screening blocks in regions where it is endemic. This is because maize is more susceptible to striga infestation under low fertility stress. Adding limited amounts of fertilizer to restore soil fertility is the most effective way to manage striga.

Alternatively, a volunteer crop such as sorghum may be sown in an effort to exhaust the striga seed bank. The crop will then need to be removed or ploughed under prior to striga flowering. However, it should be noted that under severe striga infestation, the striga seed bank may be active for up to twenty years.

4. Trial management

Adequate crop management, including timely application of recommended inputs and agronomic operations, is a pre-requisite for quality phenotyping. Field variation is the largest source of error when conducting low N stress phenotyping. This is because inherent field variation that is usually masked under optimal fertilization conditions is exposed when soils are depleted of nitrogen (Figure 3).

Whilst field variation cannot entirely be eliminated, low N stress trials can be managed using methods that can significantly reduce experimental error. Poorly managed trials often result in large experimental errors that limit the ability to detect genetic effects. Data from poorly managed trials is often unusable despite the effort and investment that was made in conducting the trials.

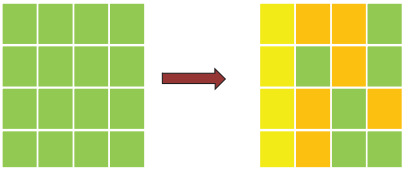

Figure 3: Inherent field variation as affected by management.

- **Trial Design:** Effective trial management begins with sound experimental design. Due to the high levels of field variation, replication both within trials and across sites is important for trials conducted under low N stress. In preliminary hybrid testing, trials should consist of a minimum of two replications and may be evaluated in three to five nitrogen stressed locations. In advanced hybrid testing, trials should consist of three replications and should be evaluated in as many nitrogen stressed locations as possible. Blocking, or the arrangement of experimental plots, into homogenous subgroups is important to reduce the effects of field variability. Research has demonstrated that alpha lattice or incomplete block designs are more effective than

randomized block designs in controlling field variation. Alpha-lattice designs are replicated designs that divide each replicate into incomplete blocks. Each incomplete block contains a fraction of the total number of entries. Genotypes are distributed among the blocks so that all pairs occur in the same incomplete-block in nearly equal frequency. The design maximizes the use of comparisons between genotypes in the same incomplete-block. Therefore, it is strongly recommended that incomplete block designs are used in trial design. Due to reduced crop vigour under nitrogen stress, inter row competition for sunlight is not as important as in optimal trials. As a result, single row plots are often used to screen preliminary hybrid trials. It is recommended that two row plots may be used in advanced hybrid screening.

- **Trial Layout:** Site characterization can effectively guide trial layout in the field. Trials should be laid out so that they do not cut across highly variable parts of the field. Generally, the most uniform, reliable parts of the low N block should be devoted to the most important trials such as genetic marker studies or advanced testing.
- **Planting:** As described in the site development section, planting trials using marked permanent plots and alleys will ensure that plots are maintained every year with minimum introduction of variation in the field. A low N stress phenotyping block should be surrounded by at least two border rows to ensure uniform competition amongst trial plots.

  Wherever possible, it is recommended that two seeds are planted per planting station and later thinned to one plant per planting station post emergence. This is to ensure a good stand in the low N stress phenotyping block. Good stands are critical in low N stress blocks, more so than in optimal trials, as gaps in the stand will result in uneven competition within and amongst rows. Poor stands will also contribute to field variation through uneven depletion of the phenotyping block. A standard maize trial row in eastern and southern Africa is 5 meters long with 25 cm between planting stations and 75 cm

between rows. A single row therefore has 21 planting stations. It is therefore recommended that 42 seeds be packed for each experimental row. Where sufficient seed is not available, it is strongly recommended that at least two seeds are planted every alternate planting station.

- **Fertilizer application:** Good low N sites should consistently yield between 2 and 3 t/ha. To maintain these yield levels, 50 kg of diammonium phosphate per hectare should be applied at planting. It is important that nitrogen is the only limiting factor within trials so additional phosphate and potassium fertilizer should be applied at the recommended rate for the location to prevent confounding the effects of low N stress with other stresses. Once the crop is knee high (6 to 8-leaf stage), 75 kg of calcium ammonium nitrate should be applied per hectare as top dressing. Calcium ammonium nitrate has a lower nitrogen content than Urea and will also not contribute to soil acidification. In some instances, specific clusters of plots in a trial that are highly depleted relative to the other parts of the field can be observed. This is normal and arises from natural field variation. If the stress is excessive, spot applications of foliar or granular fertilizer to rescue the trial can be an option. These plots should be noted and longer term efforts such as residue incorporation should be implemented to improve plot performance.
- **Plant population:** Any gaps in a low N field will add variation to the field as a result of uneven removal of soil nitrogen by plants. Ten days post emergence, stands should be thinned to leave one plant per planting station and ensure an even stand. During thinning, extra plants should be used to fill gaps where seed did not germinate or where seedlings died (**Figure 4**). This process is called gap filling and will promote the establishment of a complete stand. Occasionally, stand establishment is inadequate due to poor seed germination and it may not be possible to establish a complete stand even after gap filling. A few planting stations may be empty or an entire row may be blank due to poor seed quality. In these cases it is recommended that sorghum be used

Figure 4: Gap filling procedure after emergence.

to fill gaps or rows in a trial. As sorghum is similar to maize in basic plant structure, it will ensure competition amongst plants within and amongst rows. It is also easy to distinguish the sorghum from maize during data collection and harvest. Maize plants in low N stress trials are already severely stressed and all efforts should be made to reduce further competition for light and nutrients by weeds. It is therefore imperative that low N stress trials are maintained weed free using either herbicides or by manual weeding.

- **Irrigation:** Trials are generally conducted under rain fed conditions during the main cropping seasons. It is highly recommended, however that supplimentary irrigation facilities are available to avoid unwanted drought stress. This is because drought will confound the effects of low N stress and in some cases may result in the complete loss of a trial.
- **Interactions with other stresses:** The presence of other biotic agents or abiotic factors that influence plant growth and functions can limit the accuracy of low N stress tolerance evaluation. These agents/factors can cause mechanical damage to roots (e.g., nematodes, root-worms),

impairment of root growth (e.g., soil acidity, boron toxicity, salinity) and/or reduce water availability to the crop (e.g., presence of weeds) and source capacity (e.g., foliar diseases, insect damage to the canopy). Standard pest and disease control measures should be taken to ensure that trials are free of pests such as termites, stem borers and armyworms.

- **Harvest:** Low N trials should be harvested following crop physiological maturity when grain moisture content is below 20 percent. It is recommended that at least one day prior to harvest, the edge plant on either end of a trial row is removed and not included in yield assessment due to the border effect.

5. Weather data

For field-based phenotyping trials, it is essential to record weather parameters (including max and min temperature, relative humidity, rainfall, dew, and wind velocity), which could significantly alter the overall effects of low N stress experienced by the crop. A portable weather data recorder should be installed within the phenotyping field for recording these weather parameters. The frequency of data recording is set at one-hour intervals, so that all critical weather data are captured on an hourly basis.

6. Data collection

Good phenotyping is critical for any kind of experimental activity. The challenges in collecting good phenotypic data are often due to difficulties in standardizing, controlling and monitoring the environmental conditions under which plants are grown and the data are collected, especially in the field. The basic attributes of good phenotyping carried out with appropriate genetic materials are accuracy and precision of measurements, coupled with relevant experimental conditions that are representative of the TPE. Accuracy involves the degree of closeness of a measured or calculated quantity to its actual (true) value. Precision, also termed reproducibility or

repeatability, is the degree to which further measurements or calculations show the same or similar results.

Yield is a trait of primary interest; however, dissecting it into its components (secondary traits associated with yield) can be helpful, especially in molecular breeding. Recording key secondary traits along with yield helps to keep track of stress intensity for mid-term correction, if needed. Secondary traits can also be used as preliminary selection criteria when the turnaround time between seasons is short.

Traits that are significantly affected by low N stress under field conditions and thus should be recorded in low N stress phenotyping trials are detailed in the table below.

(A) Priority traits (must record)	
1	Days to 50% anthesis and silking
2	Anthesis-silking interval (ASI)
3	Plant and ear height
4	Root and stem lodging
5	Leaf senescence
6	Plant population
7	Ear per plant
8	Ear weight (field weight)
9	Grain yield (grain weight)
10	Grain moisture content
(B) Additional traits	
11	Days to emergence
12	Seedling vigour
13	Pollen shedding (duration)
14	Physiological maturity

Descriptions of each trait, along with the suitable stage and method of observation are given below.

(A) Priority traits

1. **Days to 50% anthesis and silking:** The male (anthesis) and female (silking) flowering traits can be affected by low N stress especially regarding the reproductive success.

   When to record: Each plot should be monitored daily from the first tassel emergence in the field until all the entries in the trial have completed anthesis and silking. If entries are properly grouped by maturity, it should take approximately one week to finish recording flowering data, except in case of highly susceptible entries where anthesis or silking may be delayed due to stress.

   How to record: Both anthesis and silking dates are recorded on a plot basis (not on the basis of just a few plants in the plot). Record the date when at least half of the plants in a plot *extruded the first anther (pollen shedding begins)* as 50% anthesis, and when the first silk is visible on at least half the plants in the plot as 50% silking (Figure 5 a & b). Convert them into days after planting date, which indicates how many days it took to reach 50% anthesis or silking. Both days to anthesis and days to silking can be expressed in growing degree days (GDD) if daily maximum and minimum temperatures are recorded from planting date.

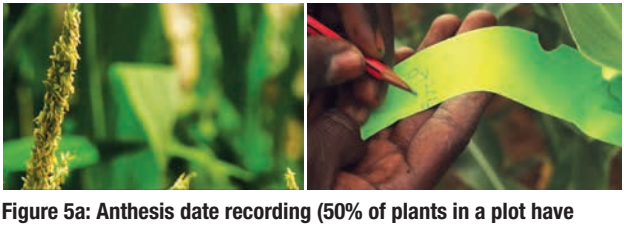

Figure 5a: Anthesis date recording (50% of plants in a plot have started shedding pollen).

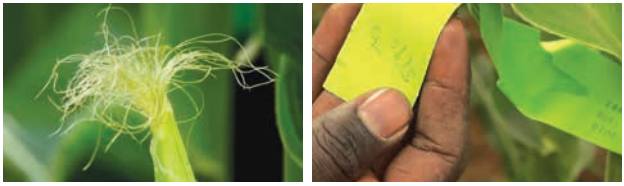

Figure 5b: Silking date recording (50% of plants in a plot have visible silk).

In some genotypes, pollen shedding may start when the tassel is still in the leaf whorl (tassel not visible outside). In such rare cases, the leaf whorl can be partially opened manually, so that the tassel is visible for recording days to anthesis.

What to select: Genotypes with no significant change in anthesis or silking under low N stress compared to optimal conditions.

2. **Anthesis-silking interval (ASI):** This is a key secondary trait that is significantly affected by most abiotic stresses, including low N stress. It is the difference between the number of days to anthesis and the number of days to silking, and illustrates the synchrony between male and female flowering, essential for reproductive success. Under optimal conditions, male and female flowering is usually well synchronized (occurring within 2 or 3 days). However, under stressful conditions ASI may be prolonged, mainly due to a delay in days to silking (and, in some cases, due to a delay in days to anthesis as well), which results in poor synchrony and poor seed set.

   How to record: ASI is calculated as the difference between anthesis date (AD) and silking date (SD), as follows: ASI = SD – AD.

   What to select: Genotypes with the shortest ASI (less than five days in the case of low N stress).

Note: *In general, maize is a protandrous crop (male flowering happens first); therefore, ASI is mostly a positive value. However, in a few genotypes, ASI may be negative, given that some genotypes show protogyny (female flowering happens first).*

3. Plant and ear heights:

When to record: Any time after anthesis and before harvest.

How to record: Plant height should be measured from the soil surface to the base of the tassel (excluding tassel length); ear height should be measured from the soil surface to the base of the ear, i.e. the node bearing the uppermost ear (Figure 6 a & b). Observations should be recorded on five representative plants within each plot, avoiding the plants near the alley, and noted as average.

Figure 6: (a) Plant height and (b) ear height measurements.

What to select: Genotypes with the lowest reduction in plant and ear height due to low N stress compared to plant and ear height in an optimal N fertilization.

4. **Stem and root lodging:** Due to competition for assimilates and nutrients in favor of ear growth and grain development, there is a tendency towards increased plant lodging (stem or root lodging) under most abiotic stresses, including low N stress.

   When to record: Between physiological maturity (when most of the husk cover has dried) and harvest.

   How to record: Count the number of plants in a plot that lodged due to stem bending/break at an internode above the ground (*stem lodging*, Fig. 7a) or from the base (*root lodging*, Fig. 7b). Calculate stem and root lodging percentage separately in relation to the total number of plants in the plot.

   What to select: Genotypes with no plant lodging under drought stress.

5. **Leaf Senescence:** The speed in which a plant responds to low N stress by entering senescence reflects the ability of a plant to tolerate low N stress. This is particularly important during grain filling when resources are re-allocated within the plant. Initiation and rate of senescence are often correlated with yield under stress.

Figure 7: (a) Stem and (b) root lodging in maize plants.

Both the rate and degree are assessed by visual scoring with heavy reliance on staff training and uniformity of application.

When to record: 2-3 weeks after flowering on a weekly basis.

How to record: Score using a scale from 0 to 10 on a plot basis, dividing the percentage of estimated total leaf area that is dead by 10: 1 = 10% dead leaf area; 2 = 20% dead leaf area; 3 = 30% dead leaf area; 4 = 40% dead leaf area; 5 = 50% dead leaf area; 6 = 60% dead leaf area; 7 = 70% dead leaf area; 8 = 80% dead leaf area; 9 = 90% dead leaf area; 0= 100% dead leaf area (Figure 8).

What to select: Genotypes with delayed senescence, having in mind that this can also result in poor nutrient remobilization.

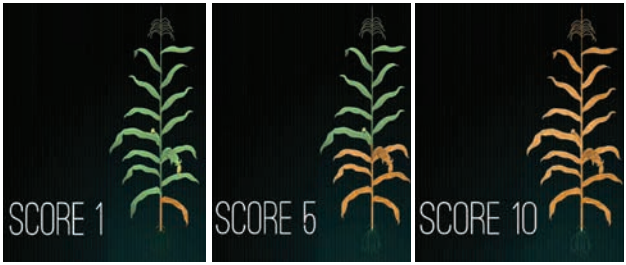

Figure 8: Leaf senescence scoring on a scale of 0 (no deaf leaf) to 10 (all leaves are dead).

6. **Plant population:** Though plant population may not be directly affected by low N stress, it is an important trait that should be recorded under various abiotic stresses and even in un-stressed trials. Plant population is directly related to yield per unit area, and also used in calculating various other stress related traits, such as plant lodging and ears per plant. Plots with more than 10% missing plants should be discarded from analysis to increase data quality.

When to record: Between physiological maturity (when most of the husk cover has dried) and harvest.

How to record: Count the total number of plants in a plot (excluding one plant on each side of the alley), including both lodged and standing plants.

7. **Ears per plant:** This is a key trait, as it is a yield attribute under both stress and non-stress conditions. It indicates the extent of barrenness among the genotypes under stress conditions.

   When to record: In the field, immediately after harvest.

   How to record: Count the total number of ears harvested in each plot (Figure 9). If an ear has at least one grain, it should be counted as one ear. Calculate the number of ears per plant (EPP) using the formula given below. The purpose of making this observation is to assess stress-induced barrenness in the plot.

$$EPP = \frac{Number\ of\ ears\ per\ plot}{Total\ number\ of\ plants\ per\ plot}$$

Figure 9 : Recording numbers of ears per plot.

8. **Ear weight (kg/plot):** Ear weight is recorded in terms of ear weight per plot (also called field weight per plot) immediately after crop harvest.

   When to record: In the field, immediately after harvest.

   How to record: Measure total ear weight per plot using a suitable digital balance with a sensitivity of not less than 10 g (Figure 10). Please take the following precautions when taking ear weight in the field.

Figure 10: Ear weight (field weight) per plot measurement after harvest.

- Calibrate the balance using a reference weight at the beginning of the harvest.
- When weighing in the field, confirm the accuracy of the values displayed by the balance at regular intervals (at least after every 100 plots) using a reference weight.
- Avoid using a hanging balance in windy conditions.

  Note: Though ear weight/plot may not give the exact grain yield, it is accurate enough for Stage 1 (early generation/1st time testcross progenies with a large number of entries) and Stage 2 trials (advanced generation/2nd time testcross progenies with a high number of entries). However, direct

grain yield of 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.

9. **Grain weight (kg/plot):** Grain weight is recorded after ear shelling for each plot (also called *grain yield per plot*).

   When to record: After shelling the ears for each plot

   How to record: Measure total grain weight per plot using a suitable digital balance with a sensitivity of not less than 10 g (Figure 11). Please take the following precautions when taking ear weight in the field.

   Calibrate the balance using a reference weight at the beginning of the shelling.

   - Avoid using a hanging balance in windy conditions.

Figure 11: Grain weight per plot measurement after ear shelling.

10. **Grain moisture content:** In general, grain moisture content is high (about >20%) at the time of harvest. It may also vary significantly among the different entries in the trial. Therefore, it is important to record grain moisture content in order to calculate final grain yield at a uniform (12.5%) grain moisture content.

When to record: Moisture content should be recorded immediately after shelling the ears.

How to record: Prepare 2 to 3 bulk samples of grains per plot. Using a grain moisture meter, record moisture content for the plot (Figure 12).

Figure 12: Grain moisture measurement.

(B) Additional traits

11. **Days to seedling emergence:** This is a key trait often associated with seedling vigor. Significant genotypic variability is observed for this trait even under optimal conditions. It is measured as the number of days required for at least 50% seedlings per plot to emerge from the soil surface from date of sowing (commonly referred to as *germination*), which indicates genotypic variability for seed germination and coleoptile elongation during the autotrophic phase of seedling establishment.

    When to record: Start from the 3rd day after the effective planting date until at least 50% of all seedlings have emerged above soil surface. The (effective) planting date is the day when the 1st irrigation after

planting is applied for germination, if sowing is done in dry soil. Otherwise, it is the sowing date, if planting is done in a pre-irrigated field with enough moisture for germination.

How to record: Count the number of coleoptiles visible above the soil surface in a plot. Note the date when at least 50% of the coleoptiles (of all seeds planted per plot) have emerged, and calculate the total number of days it took to reach 50% emergence.

What to select: Genotypes with faster seedling emergence.

12. **Seedling vigor:** Early plant vigor is an important trait, as it contributes to the overall performance of a genotype, especially under stress conditions.

    When to record: During the second and third week after seedling emergence.

    How to record: Record NDVI (Normalized Difference Vegetation Index) data using a GreenSeeker. If there is no GreenSeeker available, seedling vigor can be scored visually on a 1-5 scale (1 = high, 5 = low).

13. **Pollen shedding duration:** On average, pollen shedding in maize usually lasts 5-7 days under optimal conditions. Stress significantly affects pollen shedding duration and, therefore, how long pollen is available for pollination, which eventually affects reproductive success.

    When to record: Pollen shedding is recorded simultaneously with days to anthesis and days to silking, but continues until each genotype in the trial is finished shedding pollen.

    How to record: Once days to 50% anthesis in a plot are noted, continue monitoring until the anthers on the lower branches of the tassel stop shedding pollen.

Note the date when most of the plants in a plot have stopped shedding pollen. Pollen shedding duration can be calculated using the following formula:

Pollen shedding duration (d) = Days to end of pollen shedding (E) – Days to 50% anthesis (A)

14. **Physiological maturity:** At physiological maturity, as all grains have achieved their maximum dry matter weight and become disconnected (though not detached) from the ear and an abscission layer (called black layer) is formed. At this stage, the crop can be harvested, as grains have fully matured (could be used as seed). However, at physiological maturity, grain moisture content is usually high (30-40%); therefore, the crop is left in field for a few more days so that grain moisture content will decrease to 20-25%, which is called *harvest maturity*. The period from physiological maturity to harvest maturity is the dry-down phase.

    When to record: About 4-6 weeks after anthesis, when the crop is heading towards maturity.

    How to record: Physiological maturity is recorded on a plot basis (not just on a few plants per plot). It is difficult to record accurately. The date when >70% of the husk cover leaves on the ears have dried can be used as a proxy.

    Note: *Before proceeding to the final harvest, please ensure that:*

    - *Data on plant and ear height, root and stem lodging, and plant population per plot are recorded.*
    - *If flowering data are recorded at the field level, they are transferred in a data-sheet.*
    - *The first plant (along with the ear) in each row next to the alley is removed.*

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Notes:

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