Phenotyping for Abiotic Stress Tolerance in Maize

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Abstract

The ability to quickly develop germplasm having tolerance to several complex polygenic inherited abiotic and biotic stresses combined is critical to the resilience of cropping systems in the face of climate change. Molecular breeding offers the tools to accelerate cereal breeding; however, suitable phenotyping protocols are essential to ensure that the much-anticipated benefits of molecular breeding can be realized. To facilitate the full potential of molecular tools, greater emphasis needs to be given to reducing the within-experimental site variability, application of stress and characterization of the environment and appropriate phenotyping tools. Yield is a function of many processes throughout the plant cycle, and thus integrative traits that encompass crop performance over time or organization level (i.e. canopy level) will provide a better alternative to instantaneous measurements which provide only a snapshot of a given plant process. Many new phenotyping tools based on remote sensing are now available including non-destructive measurements of growth-related parameters based on spectral reflectance and infrared thermometry to estimate plant water status. Here we describe key field phenotyping protocols for maize with emphasis on tolerance to drought and low nitrogen.

Keywords: Drought; low nitrogen tolerance; maize; phenotyping; spatial variability.

Introduction

Population growth and climate change, combined with the degradation and scarcity of natural resources and recurrent food price crises, threaten food security and the livelihoods of millions of resource-poor people. In many regions of sub-Saharan Africa, maize is the principal crop accounting for up to 51% of consumed calories. Yield levels in SSA remain low and highly variable across years at less than 2 t/ha in most countries (Food and Agriculture Organization of the United Nations (FAO), 2011), which is insufficient to meet future demands. Although maize yield levels in Asia are much higher, with average yields in China and Indonesia at 5.2 and 4.2 t/ha, (FAO 2011), demand for maize within Asia is expected to rise sharply with increased demand for animal feed (Msangi and Rosegrant 2011). In 2011, the severe drought in Eastern Africa resulted in over 10 million people reliant on food aid, at an estimated 477 M USD (World Food Program 2011). Increasing temperatures, changing precipitation patterns, and extreme weather events are likely to further exacerbate the ability to meet future demands within maize growing regions (IPCC 2007). Meanwhile rising fertilizer prices, poor infrastructure and population growth are placing ever greater pressure on existing arable lands throughout Sub-Saharan Africa. Poor and depleted soil fertility remains a primary constraint to agricultural productivity in tropical regions, forcing farmers into marginal
lands and forested areas, thus contributing to environmental degradation and CO₂ emissions (Pingali and Pandey 2000). After water, nitrogen (N) is the single most important input for maize production, and lack of N is the principal constraint to cereal yields in areas with more than 400 mm average annual rainfall in sub-Saharan Africa (Buerkert et al. 2001). Fertilizer application in sub-Saharan Africa is negligible, accounting for less than 1% of the global N fertilizer application (Leff et al. 2004). The development of improved maize germplasm with tolerance to low N will increase yields and have a major impact on livelihoods and food security in sub-Saharan Africa.

While the ability to meet the needs of future generations is daunting, cereal breeding has an impressive history. The Green Revolution in combination with improved agronomic practices increased yields by up to 40% (Evanson and Gollin 2003). Maize yields within the top five maize producers in the world (USA, China, Brazil, Mexico and Indonesia) have increased by over threefold since 1961 (FAO 2011). Under drought stress, conventional breeding for drought tolerance has resulted in gains of up to 144 kg/ha per year in tropical maize when stress was imposed at flowering (Edmeades et al. 1999). In temperate maize, the rate of breeding progress has been estimated at 73 kg ha/yr for mild stress (Duvick 1997), 146 kg/ha per year when stress was imposed at the flowering stage, and 76 kg/ha per year when the stress was imposed during mid-grain filling stage (Campos et al. 2004). While improvements under both optimal and drought stress are notable, in the face of climate change and population growth, greater gains in the development of improved germplasm with tolerance to abiotic and biotic stresses are vital.

Biotechnology offers the ability to increase the speed and efficiency of plant breeding (Juma 2011). Molecular breeding is a general term used to describe modern breeding strategies where genotypic markers are used as a substitute for phenotypic selection (Whitford et al. 2010). Great advances in molecular biology have been made in the past 30 years from the identification of DNA markers ( Botstein et al. 1980), development of highly saturated molecular maps (McCouch et al. 1988; Causse et al. 1994; Kurata et al. 1994), to the availability of complete genome sequences within the public domain (for example, rice, Yu et al. 2002; maize, Schable et al. 2009; soybean, Schmutz et al. 2010). Many molecular breeding schemes, ranging from the selection and transfer of a small number of loci from one genetic background to another (marker assisted backcrossing, MABC) to the identification and selection of several genomic regions to increase the frequency of favorable alleles within a population (marker assisted recurrent selection, MARS), are widely used in the private sector (Ribaut et al. 2010). A vast amount of literature is available on putative genes and alleles associated with performance under abiotic stresses through QTL analysis, gene expression studies, transgenic research and TILLING, among other approaches (for example, Ribaut et al. 2009; Swarmy et al. 2011). However, despite the immense amount of information provided by molecular biology in the past few decades, the application of the outputs of these techniques in the development of improved germplasm have fallen short of expectations (Araus et al. 2008; Tuberosa 2011).

To allow information from molecular experiments to be connected to plant performance in farmers’ fields it is essential that suitable and reliable phenotyping methodologies are applied (Salekdeh et al. 2009). Carefully controlled environments (such as pots, soil-filled pipes and to a lesser extent now, hydroponics) are often favored by molecular-oriented researchers because unwanted environmental variation can be minimized (Liu et al. 1998; Hu et al. 2006; Karaba et al. 2007). However, controlled conditions tend to be very different to those prevailing in the target population of environments (TPE) and may limit the application of results in germplasm development. In the case of drought, pot studies greatly underrepresent changes in soil penetration resistance and are far removed from the dynamics of soil drying and the progression of drought stress in the field. While field experiments can offer a more appropriate phenotyping environment, care needs to be taken to reduce experimental noise and ensure results can be translated into germplasm improvement in the TPE. The aim of this methodology review is to highlight essential steps in the development of protocols for abiotic stress phenotyping, with emphasis on drought and low nitrogen stress in maize.

### Identification of abiotic stress phenotyping sites

The choice of managed stress environments (MSE) for phenotyping is essential for progress in abiotic stress breeding and careful selection will provide the cornerstone of success. Without an appropriate homogenous phenotyping site, the value of data acquired, regardless of cost and time, will be limited. Care needs to be taken to ensure an experimental site is representative of the TPE, and will allow the target stress to be imposed without interference from additional stresses, with minimal environmental heterogeneity to reduce experimental error.

### Drought phenotyping sites

For the selection of drought phenotyping sites, long-term daily climate data and soil data are required to ensure a site allows drought stress to be applied at the required growth stage, with minimum variation in soil properties. For drought breeding, knowledge of the types of drought environments encountered in the TPE is essential. Potential phenotyping sites should allow stress to be imposed at the critical stage(s) of the crop. Drought phenotyping is often conducted during the off (dry) season to control the timing, intensity and
duration of the period of water stress and avoid the climatic uncertainty associated with conducting drought trials during the main season. However conditions during the dry season are harsh for plants and generally do not reflect the environmental conditions plants will experience during a natural drought in the main (wet) season (Price et al. 2004). Temperatures and vapor pressure deficit (VPD) are generally higher during the dry season (Jagadish et al. 2011). An earlier study in maize suggested the combined effect of heat and drought stress was greater than the effect of each stress individually (Heyne and Brunson 1940). Care should be exercised when screening during the dry season to ensure performance under drought stress during this season is predictive of performance under drought stress in the target environment. Rainout shelters in the main season can be used as an alternative to screening in the dry season but cost and limited space are important considerations.

Historical daily climate data can be used to identify potential drought phenotyping sites where the probability of encountering rainfall during a three to four month period is minimal, allowing drought stress to be carefully experienced through the withdrawal of irrigation. Figure 1 shows long-term rainfall patterns (1951 to 2000) at four key phenotyping sites in Mexico, Kenya, Zambia and Zimbabwe used for drought maize breeding at the International Maize and Wheat Improvement Center (CIMMYT). At these sites rainfall is minimal during a period of at least 3 months and drought stress can be reliably applied by adjusting planting to impose drought stress at the required phenology.

### Identification of low N phenotyping sites

It is important to allow genotypic yield potential to be separated from mechanisms associated with tolerance to low N stress, thereby avoiding complications in the separation of low N tolerant genotypes from yield potential. Thus low N stress screening sites should target a yield reduction of 60–75% relative to well-fertilised conditions at the same site (Bänziger et al. 2000). To attain this level of low N stress, phenotyping sites are normally depleted for several seasons by planting a non-leguminous crop with high biomass under no fertilisation to remove N from the soil. As the development of a low N phenotyping site takes a significant investment to sufficiently deplete the level of native N, the initial selection of a suitable site is essential. The development of N stress can be increased by the selection of a site with sandy soil as sandy soils generally tend to have low levels of mineral N and organic matter. Information on cropping history is important so fields which have previously had two distinct cropping systems on the field can be avoided. These different crop histories will leave a “shadow” that will persist for at least several seasons and be hard to remove through depletion.

### Field homogeneity in drought and low N phenotyping sites

Within field sites homogeneity of all factors affecting plant growth is essential (Blum 2011). Soil heterogeneity has the potential to become a significant source of experimental error within field experiments and undermine field screening. The effects of soil heterogeneity become more apparent under drought and low N, and thus great care should be taken to ensure a uniform field site is chosen for phenotyping (Bänziger et al. 2000). Soil characterisation of potential sites for drought or low N phenotyping, with emphasis on key soil parameters most likely to introduce variability in the development of stress, is important to avoid or reduce unwanted experimental error.

For drought experiments, differences in soil depth and the water holding capacity of the soil across a field can affect the imposition of stress across the field. Soil depth affects rooting volume and thus potential nutrient and water availability. Compaction, aluminium (Al) toxicity and soil acidity will also reduce the effective soil depth. Soil texture refers to the relative amount of sand, silt, and clay in a soil and is an important determinant of water holding capacity and water release characteristics of a soil (Marshall et al. 1996). Sandy soils have much lower water holding capacity and release more of their water at lower suctions compared to clayey soils. Furthermore, soil texture is closely associated with changes in soil penetration resistance under drought stress. Soil penetration resistance can restrict, or even halt, root growth thereby affecting root growth and the ability of a plant to access soil water during a drought period. As soil water content decreases, soil penetration resistance increases as a result of the increase in matric suction that acts as a force of attraction between soil particles and the strengthening of soil structural units caused by the reduction in water (Marshall et al. 1996). The relative increase in soil penetration resistance under drought stress is partly determined by soil texture. Clay particles play an important role in determining soil penetration resistance under drought stress. As soil dries, the water film between soil particles decreases increasing inter-particle attraction. In soils with high sand content, the increase in penetration resistance with concomitant drying is small compared to soils with a lower sand content (Cairns et al. 2004).

In low N experiments, variation in soil N supply will increase spatial variability in plant growth. Soil N is mainly found in forms that are unavailable for plant uptake. In the process of N mineralization, organic N is converted into plant usable organic forms (ammonium, NH$_4^+$ and nitrate, NO$_3^-$). Soil texture and organic matter affect the inherent soil N supply and mineralization rate. The amount of clay in a soil also affects N mineralization reactions. Differences in N mineralization rate will lead to differences in N availability across a low N phenotyping site. Soil variability in low N phenotypes sites can
Figure 1. Monthly rainfall in various regions.


represent a significant source of error. Bänziger et al. (1995) showed the residual variance of genotypes in small, single row plots was less than when grown in larger 4-row plots under low N, suggesting soil variation is a major source of experimental error within low N experiments. After the selection of a uniform site, careful management is required to prevent introducing variability. Gaps in the field through poor stand establishment or in alleys between ranges will have higher plant available N from nitrate that is mineralized and not taken up by plants. These shadows can introduce variability which will be visible over several years. Maintaining alley width and position from season to season and ensuring good stands through planting at twice to normal density and thinning to the desired density at establishment will prevent the introduction of unwanted variation in plant available soil N.

Knowledge of soil physical and chemical properties that will affect plant growth and stress development, and their uniformity within a site is essential in the selection of suitable phenotyping sites. Any site with significant heterogeneity must be eliminated as a potential phenotyping site to avoid introducing unwanted experimental error. Direct assessment of soil variability within a field site can be made through destructive soil sampling. Destructive soil sampling at 30 cm depth intervals (to a depth of 90 or 120 cm soil depth) for key soil physical and chemical properties can provide information on the suitability of a site for phenotyping. Ideally soil samples should be taken across a field using a square grid basis with a minimum of 5 sampling points per hectare. The location of soil samples should be positioned by GPS to allow the test results to be mapped to the exact location (Campos et al. 2011). Soil samples should be analyzed for texture (particle size analysis), pH, macro and micro nutrients at minimum.

While initial site characterisation of potential phenotyping sites increases phenotypic accuracy by eliminating sites with high unwanted variability and confounding factors, soil mapping can be used to further improve the precision of field experiments. Mapping soil spatial variability is commonly used within precision agriculture to identify within-field management options but is rarely used to improve phenotyping precision within molecular breeding programs. Knowledge of soil variability at the plot level can be incorporated into field layouts to ensure planting within areas of the least spatial variability to further
Figure 2. Field variability mapping data.

Examples of field variability maps produced using (A) visual scores to estimate plant biomass and (B) normalized differential vegetation index (NDVI) to estimate plant biomass.

The units are a) “visual biomass” and b) Electrical conductivity (mS/m)

reduce unwanted experimental error (Cairns et al. 2004; Cairns et al. 2009). Many high-throughput techniques are now available for mapping variability within field sites based on soil electrical conductivity sensors (Sudduth et al. 1997; Cairns et al. 2012), penetrometers (Cairns et al. 2011), spectral reflectance (Rossel et al. 2006; Dang et al. 2011), thermal imagery of plant canopies (Campos et al. 2011) and measurements of plant growth as surrogates of variability (Prasanna et al. 2012) (Figure 2). Soil mapping can be used to identify gradients of variability which can be incorporated into experimental designs to reduce within replicate environmental error (Cairns et al. 2004).

Application of drought stress and soil moisture monitoring

Different traits will confer adaption to different types of drought stress, thus drought experiments should aim to impose a similar water stress (in terms of the timing, frequency and intensity of drought) as experienced in the TPE. Tolerance to vegetative drought stress does not necessarily confer tolerance to reproductive stage drought stress. Drought stress negatively affects all stages of maize growth and production; however the reproductive stage, particularly between tassel emergence and early grain filling is the most sensitive to drought stress (Grant et al. 1989). To ensure drought is imposed at the correct phenological stage, irrigation should be withheld prior to this stage. For flowering stage drought stress, irrigation scheduling should be designed to result in silking delay and cause ear abortion. In this case the anthesis-silking interval (ASI) is a good indicator of the level of drought stress. For grain filling stage drought stress, drought should develop directly after flowering resulting in increased leaf senescence (Bänziger et al. 2000). A crop water balance can be used to determine the last date of irrigation to ensure plants experience drought stress at the target stage (Bänziger et al. 2000). A crop water balance estimates the last day of irrigation based on the average anthesis date within the trial, estimated daily water consumption, soil texture, plant-available water and estimated maximum rooting depth. Crop simulation models can more accurately estimate the crop water balance to determine the last date of irrigation. While drought stress should be imposed at the same time across all genotypes within an experiment, frequently there is substantial variation in phenology across genotypes. To palliate that problem, drip irrigation can be used to allow plot level control of irrigation or genotypes can be grouped into subsets of similar maturity and planted at different times to ensure phenological synchronisation across genotypes at the crucial stage when drought stress is imposed. This is particularly important to avoid differences in stress development due to climatic conditions. A pre-study can be used to determine the phenology of genotypes prior to drought experiments. Information on phenology (number of days or accumulated degree-days to anthesis) can be used as a covariate adjustment in instances where uniform stress may not have been applied due to lack of prior phenology information.

Knowledge of soil moisture conditions during a period of water deficit is necessary to define the drought environment plants experience. In addition, most experiments require repetition in subsequent years to confirm initial results and in many molecular biology applications to confirm marker-trait associations. Knowledge of soil moisture profiles will allow the
repetition of an experiment under similar drought conditions. Jones (2007) highlighted the lack of characterization of soil water status in the majority of molecular studies on drought. In a review of molecular papers focusing on the effects of drought on gene expression or transgenes under drought stress, over half of the papers had no measure of water status (plant or soil). This lack of understanding of the drought environment experienced by plants not only hinders the ability to replicate experiments but also reduces the ability to interpret results.

Plant available water largely depends on the amount of water stored in the soil and its relationship to soil water potential. Many different methods are available to measure the amount of water stored in the soil. The majority of measurements use thermogravimetric methods, neutron techniques and measurement of soil dielectric properties/capacitance sensors (Gardener et al. 2000). Thermogravimetric methods are the only direct measurement of soil water content with all other measurements relying on the physical properties of the soil that depend on water content. The gravimetric method ($\Theta_m$) relies on the amount of water that can be lost from the soil after heating to 105 °C until there is no further weight loss. If the volume of the soil sample is known then the gravimetric water content can be converted into the volumetric water content ($\Theta_v$) by dividing by the volume of the sample. Both gravimetric and volumetric measurements involve destructive sampling which is slow and labor intensive, and cannot be measured again in the same place nor used to follow changes in soil moisture content over the growing period.

To overcome the problems associated with gravimetric and volumetric methods, many alternative methods have been developed which rely on a surrogate to estimate volumetric water content. Calibration at each location is essential to establish the relationship between the surrogate and soil water content to allow the former to be determined. Indirect measurements can be calibrated by simultaneously taking direct (thermogravimetric) measurements at field capacity and permanent wilting point. The main advantage of these methods is they are non-destructive and can provide regular measurements of soil water content in the same location over time. Neutron probes have been used extensively in the field to estimate soil water status since the 1970’s (Hignett and Evett 2005). The majority of hydrogen (H) in soil is present within water molecules, thus changes in soil H concentration are primarily related to moisture content. The neutron probe contains both a radioactive source, which emits fast neutrons, and a slow neutron detector. As the fast neutrons collide with other atoms, particularly H ions, they slow down. The concentration of slow neutrons provides the surrogate for the estimation of soil water content. The choice of methodology used for monitoring soil water content will depend on many factors including the cost, degree of drought, field variability, accuracy and precision required.

**Advances in high-throughput phenotyping for drought and low N**

Phenotyping remains as one of the bottlenecks for crop breeding, even more so when molecular breeding is reaching its maturity, with capacity of genotyping platforms strongly increasing at the same time as cost of analyses are decreasing and outsourcing allows easier access to these techniques. Phenotyping is time-consuming and labor-intensive and still keeps a high degree of empiricism in its assumptions, particularly for quantitative traits. For a secondary (i.e. other than yield itself) trait to be useful in a program, it must comply with several requirements revised elsewhere (e.g. Bänziger et al. 2000; Araus et al. 2008). Moreover the selection strategy, which includes traits and tools, will depend on the target environment for selection. Many new promising tools for evaluating physiological traits are now available. Below we discuss current physiological tools used for high throughput phenotyping with focus on field-based methodologies with emphasis on remote sensing techniques designed to assess plant performance in a fast and non-destructive manner.

**Spectral reflectance**

Spectral reflectance normally refers to the fraction of incident radiation reflected by a surface. Many phenotyping tools based on spectral reflectance indices are used to provide fast, non-destructive measurements of green biomass, canopy chlorophyll content, leaf and canopy senescence (or stay green) and plant water status. Earlier studies used portable leaf chlorophyll meters such as the SPAD meter of Minolta, which measures optical density differences between two wavelengths within the red and the near-infrared regions (650 and 940 nm) and have been used as an indirect measurement of leaf N and chlorophyll content (Dwyer et al. 1991). However SPAD measurements are only taken on individual leaves, with a very small measurement area (2 mm x 3 mm). At the canopy level, the normalized differential vegetation index (NDVI) of the light reflected by the canopy (measured with a field spectroradiometer) has been used to quantitatively assess plant growth and senescence in wheat and barley (Aparicio et al. 2000; Bort et al. 2005; Marti et al. 2007). In maize, NDVI has been used for different purposes such as site specific nutrient management (Inman et al. 2005), evaluating crop management practices (Velhurst et al. 2010), and yield predictions (Mkhabela et al. 2005), to assess the effect of heterosis (Araus et al. 2010) and growth maintenance under drought (Lu et al. 2011). Fast measurements of NDVI may be performed using spectroradiometers provided with active (i.e. with their own source of radiation) sensors. This is the case of the GreenSeeker (from N-Technologies) which is a relatively low cost spectroradiometer designed to allow fast measurements of NDVI. However, NDVI and other
vegetation indices frequently reach saturation at anthesis which prevents their use to assess genotypic differences in biomass at the critical stage. Other field spectroradiometers allow the measurement of hundreds of wavelengths in the visible and near-infrared range of the spectrum, which allows the calculation of a large set of different reflectance indices that may provide information on plant performance characteristics (such as photosynthesis, water status) other than green biomass, chlorophyll content or senescence provided by the vegetation indices (such as actual biomass or senescence) (Weber et al. 2011). However, the cost of using these reflectance-measuring tools is relatively high, which among other reasons, prevents their wide adoption by breeding programs.

Digital Imagery

Digital imagery can be used for estimating leaf nitrogen content, early biomass and response to water-limited conditions. Advantages of digital imagery include low cost, the small amount of technical experience required and a form of image processing that can be centralized to standardize analysis (Rorie et al. 2011a). The use of digital imagery in phenotyping has mainly focused on individual properties of pictures or the development of indices combining digital values. Casadesus et al. (2007) investigated the use of individual digital picture properties (picture-derived vegetation indices, picVIs) as a phenotyping tool for durum wheat breeding under well-watered and rainfed conditions. Digital pictures taken approximately two weeks prior to anthesis showed hue and brightness were highly correlated with measurements of NDVI. Under rainfed conditions, picVIs had a similar or higher estimation of grain yield than NDVI. As in the case of NDVI (see above) high biomass under well-watered conditions saturated vegetation indices, preventing application of picVIs to estimate grain yield when measured prior to anthesis. Karcher and Richardson (2003) combined hue, saturation and brightness values from digital images to determine the dark green color index (DGCI). DGCI was used to quantify turfgrass color under different N treatments. The same technique has been successfully applied in maize to quantify leaf color and N content (Rorie et al. 2011b). DGCI was found to be closely associated with leaf SPAD and N concentration across years. A further study comparing different models of digital cameras showed standardized results across cameras (Rorie et al. 2011a). Hue, saturation and brightness values have also been combined to determine digital ground cover in bread wheat (Mullan and Reynolds 2010).

Thermal Imagery

During transpiration, a substantial amount of energy is required to convert each molecule of water from liquid to vapour. This energy is then taken away from the leaf in the evaporating water thus cooling it (Jones et al. 2009). Thus, for a given environmental condition, leaf transpiration is an important determinant of leaf temperature. During periods of water deficiency, an immediate plant response is a reduction in transpiration to reduce water loss, increasing leaf (and canopy) temperature. Infrared (IR) thermography has been used to determine leaf and canopy temperature and as an indirect estimate of plant water status (Garrity and O’Toole 1994). Maize, classified as isohydric, has been noted to react rapidly to developing water stress such that its response to drought can be readily visualized. This makes maize ideal for thermographic monitoring. Thus genotypes with a higher resistance to drought stress, based for example in a greater access and/or uptake of soil water, will generally maintain a higher stomatal conductance and therefore can be identified as plants with cooler leaves (Berger et al. 2010). IR thermometers have been successfully deployed in wheat breeding for both drought and heat screening (Fischer et al. 1998; Reynolds et al. 1998; Brennan et al. 2007). In maize this approach has been used to assess the association between leaf temperature differences and relative biomass accumulation in response to drought at the seedling stage (Liu et al. 2010). However for large-leaf species with non homogenous canopies such as maize, there is a basic problem in recording a truly representative ground-level canopy temperature since dark (cooler) spaces between the large leaves might bias the reading (Jones et al. 2009). Therefore IR thermometry in maize is usually applied to measure individual leaves instead of the whole canopy as is usually the case in small grain cereals such as wheat, rice or barley which are characterized by relatively small leaves and homogenous canopies.

While the comparatively low cost of IR thermometers (≥150 USD) and ease of use make them an attractive methodology to measure plant temperature, to obtain an accurate representation of plot temperature, a large number of leaves must be measured. Phenotyping large breeding populations would be time-consuming, resulting in interacting problems of daily patterns of radiation and temperature, and moreover of those associated with the occurrence of wind and clouds. Thermal imaging offers a promising high-throughput alternative, allowing information on canopy temperature to be captured simultaneously across many plots. In a study conducted by Romano et al. (2011), an infrared camera was used to take images at canopy level of maize under reproductive stage drought stress between anthesis and blister stage (Figure 3). IR thermal imaging was identified as a potential tool that can accelerate phenotyping and screening in maize water stress (drought) breeding programs. Leaf or canopy temperature can be used directly as the absolute value or, more frequently, as the difference from air temperature (for example canopy temperature depression, CTD) which takes into consideration daily changes in air temperature. Plant temperature also allows the calculation of the crop water stress index (CWSI) which gives an indication of the degree of stress in a crop (Romano et al. 2011). Water stress and CWSI have been linked to soil.
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water availability, leaf water potential, stomatal conductance and yield. In a recent study, IR thermography (using an infrared camera) was proved to be related to soil- and plant-based measures of water stress. It was also observed that IR thermography can be potentially used for identifying differences between genotypes, and CSWI generated could be used for irrigation scheduling (Zia et al. 2011). Thermal imagery of a single cover crop under drought stress has also been used to identify within-field variability for phenotyping site selection (Campos et al. 2011).

**Stable isotopes**

The signature of stable isotopes in plant matter has been proposed as a powerful selection trait in the sense that they may integrate in time (i.e. along the crop cycle) and level or organization (whole plant – canopy level) the behavior of the crop under stress. This has been the case in carbon isotope discrimination ($\Delta^{13}C$) which remains as one of the few examples of indirect analysis (i.e. based in a physiological trait other than yield, the primary or target trait) which has been successfully deployed in a breeding program, in this case to produce wheat with higher water use efficiency (WUE) and which is better adapted to the Mediterranean drought of Australia (Richards 2006). In contrast, the use of $\Delta^{13}C$ in C4 crops like maize is not encouraging (Monneveux et al. 2008; Cabrera-Bosquet et al. 2009c) mainly because of the nature of C4 photosynthesis. Moreover while much attention has focused on improving WUE it would now appear that, except in severe drought conditions, the amount of water effectively used by the crop is a more important adaptive trait when breeding for drought adaptation (Araus et al. 2008; Blum 2009). The effective use of water is related to the genotypic capacity to use available water and, therefore, to sustain transpiration under unfavorable environmental conditions (Blum 2009).

In this sense, oxygen isotope enrichment ($\Delta^{18}O$) or oxygen isotope composition ($\delta^{18}O$) directly have therefore been proposed as “time-integrative” indicators of transpiration and the effective use of water in different plant species (Barbour 2007; Farquhar et al. 2007; Cabrera-Bosquet et al. 2009a, 2011a).
including maize (Cabrera-Bosquet et al. 2009b; Araus et al. 2010). Moreover, since $^{18}$O signature in plants is independent of the photosynthetic metabolism, it may be in selecting for drought adaptation in both C$_3$ and C$_4$ grasses. Thus Cabrera–Bosquet et al. (2009b) propose that within a given environmental growing condition, and providing there is water available in the soil profile along all the crop cycle, the maize genotypes with higher yield potential and better adaptation to moderate water stress will exhibit lower $^{18}$O signatures, indicating that they transpire more.

In the same sense, maize hybrids have lower $^{18}$O values compared with the corresponding inbred lines regardless of the growing conditions (Araus et al. 2010) which suggest yield potential and drought adaptation associated to heterosis is caused by a higher transpiration and a better water status. However, since $^{18}$O enrichment in leaves is directly dependent on relative humidity, differences between samples can only be interpreted in terms of genetic differences within a given site. Thus, for a given transpiration rate an increase in relative humidity may cause a decrease in $^{18}$O enrichment. In addition, as for $\Delta^{13}$C samples, sampling must be from tissues formed over the same period of time. Nevertheless, this is not a major problem if kernels are used for analysis, since this plant part is formed during grain filling and for maize, the contribution of pre-flowering reserves to filling grains are minor.

The natural variation in plant N isotope composition ($\delta^{15}$N) is potentially useful for genotypic screening under drought because it is linked to N metabolism, even though a complete knowledge of the underlying biochemical mechanisms is lacking (Cernusak et al. 2009; Tcherkez 2011; Yousfi et al. 2010, 2012). Isotope fractionation may occur during enzymatic assimilation of nitrate or ammonium into other N forms. Further fractionation may take place due to N recycling in the plant or through translocation, exudation, or volatilization (Evans 2001; Tcherkez et al. 2011). Moreover, the $\delta^{15}$N in dry matter also reflects the isotopic signature of the N source used by the plant. Since the $\delta^{15}$N of the chemical fertilizers used to be lower than that of the soil N, the $\delta^{15}$N of the plant in its natural abundance may be used as a proxy to assess what genotypes exhibit a higher efficiency in using N fertilizer (Serret al. 2008), providing the plants grow in absence of other stress factors such as drought.

For a large-scale phenotyping associated with, for example, marker assisted selection, the above isotopic signatures may be estimated in a fast and low-cost (even if less precise) manner by near-infrared reflectance spectroscopy (NIRS) (Ferrio et al. 2001; Cabrera-Bosquet et al. 2011b). Moreover, NIRS is routinely used to analyze leaves and kernels other chemical traits, such as ash and N content, which beside their usefulness for food or feed quality, such evaluation may also be applied to select genotypes with better performance under drought (Cabrera-Bosquet et al. 2011b).

**Conclusions**

Molecular biology will play an important role in optimising breeding pipelines and ensuring the development of improved germplasm for future climates. However insufficient attention is often given to improved phenotyping methodologies within molecular biology, thereby reducing the potential transfer of results to product development and, ultimately, farmers’ fields. Variation in soil properties within field sites is often not known and reported even less. Soil variability will introduce unwanted experimental error and has implications for the interpretation and comparison of drought studies. Site characterisation is essential to select sites within minimum soil variability. This information can also be used to delineate experiments within a field to further reduce unwanted experimental error. Many new high throughput phenotyping tools for abiotic stress are now being used in field studies for abiotic stress. These new tools are unlikely to provide greater selection gains in yield than direct selection for grain yield itself. However, combined with molecular information they will help further elucidate the genetic and physiological basis of abiotic stress tolerance. Together the integration of knowledge from agronomy, physiology, breeding and molecular biology will facilitate yield increases in farmers’ fields.

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