3 Traits Associated with Drought and High-Temperature Stress and Its Associated Mechanisms in Legumes

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Abstract

Developments in crop management practices and novel breeding methods are important to sustain crop productivity for the current and upcoming challenges caused by drought and high-temperature stresses because the occurrence of these stresses during crop growth stages is determinantal to crop yield. Direct selection for yield per se under any abiotic stress conditions is often ineffective because of the low heritability for yield. One of the ways to increase the selection efficiency for stress tolerance is to select for any secondary traits which are easy to measure, presenting high heritability, and correlate highly with grain yield under stress situations. In this chapter, the secondary traits like plant water status, green leaf area duration, limited transpiration, canopy temperature depression, root architecture, early morning flowering, membrane integrity, photochemical efficiency, stem carbohydrate mobilization, and yield-associated traits are discussed. The above plant traits can be quantified under both controlled and field environments. The possibility of converting these traits under controlled environments into a method of quantification at field scale depends on the advancements in allied sectors of sciences, like spectroscopy, remote sensing, aeronautics, and high-end computing facilities. The use of these traits as a selection tool in crop breeding will pave the way for the development of drought and high temperature stress-tolerant genotypes.

Keywords

Legumes · Drought · High-temperature stress · Phenotyping traits · Yield

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3.1 Introduction

Phenotype is defined as the observable effect of a genotype and its interaction with a given environment. Phenotyping is the application of protocols and methodologies to measure the traits specifically related to the structure of plant or function to facilitate the right selection in the breeding program or to complement genotypic data for the identification of associated genes. Under plant breeding, selection can be defined as the science of discriminating among the biological variants in a population to detect and pick the desirable recombinants. However, the identification of recombinants that leads to superior phenotype is challenging because of the interaction of genotype with the environment. The major challenge in phenotyping is that it involves a large workforce, is expensive, and is prone to error if the methodology was not meticulously followed. Ample literature is available on the mechanisms of tolerance to drought and HT. However, many of the traits that have been reported to be promising for stress tolerance are not feasible for screening genotypes in large-scale or high-throughput mode.

In this chapter, we consider the traits which can be recorded at the lab, controlled environment, and field level. More information about field-based high-throughput phenotyping systems can be found in the review article by Cobb et al. (2013). The innovative use of technology and cautious development of tools to automate the processes without sacrificing predictive power will be very critical in the phenotyping platforms. Standardized phenotyping structures are not feasible practically for all research-related questions, but with thorough consideration and defined objectives, several techniques can be harnessed to examine specific characters under high-throughput settings. Agronomically important traits that are observable at the canopy level can help in discriminating the genotypes based on their capability to capture and use the natural resources, and these traits serve as a proxy for important agronomic characters. If traits associated with stress tolerance are identified and validated in a wide range of crops, then methodologies could be developed to quantify those traits under high-throughput systems using technologies such as digital imagery, remote sensing, robotics, thermography, and farm machinery.

3.2 Traits Associated with Drought and High Temperature (HT) Stress Tolerance and Its Phenotyping Method

3.2.1 Green Leaf Area Duration

Genotypes exhibiting the extended green leaf duration area are referred to as stay-green phenotypes. In contrast to cosmetic stay green, functional stay-green leaves supply more photosynthates to developing grains and can thus significantly contribute to grain yield (Thomas and Howarth 2000). Stay-green genotypes in various cereals have been reported, and selection of stay green has been targeted to improve the crop yield under drought and HT stress. Stay green can be studied at basic cell level, leaf level, or whole-plant level. At the cell level, Western blot analysis of

proteins from leaves will indicate the integrity of chloroplast-associated proteins. In stay-green genotypes, delayed degradation of proteins is correlated with the phenomenon of delayed senescence onset (Borrell et al. 2001).

At leaf level, chlorophyll index measured through SPAD meter or chlorophyll meter is most frequently used to assess the greenness of the leaves. The chlorophyll index obtained from the SPAD meter is a point estimate (not cumulative), and it represents a section of the leaf and can be an indicator of the leaf senescence process. Studies have indicated that there is a strong positive relationship between chlorophyll index with actual leaf chlorophyll content (Hebbar et al. 2016). These phenotyping tools can allow the frequent measurement to assess the rate of leaf senescence, which is otherwise difficult if conventional spectrophotometric measurements are employed.

At the plant level, the expression of a stay-green trait can be evaluated using a stay-green score, or measuring canopy reflectance through a green seeker, and a spectroradiometer. Spectral reflectance from canopies over the visible and near-infrared (NIR) regions is mainly influenced by the canopy structure, leaf pigmentation, protein contents, and leaf water (Homolova et al. 2013). A healthy green canopy absorbs most of the red spectrum and reflects most of the NIR spectrum because chlorophyll molecule absorbs blue and red color and mesophyll region reflects the near-infrared spectrum. The normalized difference vegetation index (NDVI) is widely used to quantify the leaf senescence process or greenness of the canopy. The NDVI can be obtained at ground level, and from high, low, and satellite altitudes. The portable NDVI sensor, namely green seeker, provides rapid information on leaf area index and green area index. $\mathrm{NDVI} = (R_{\mathrm{NIR}} - R_{\mathrm{Red}})/(R_{\mathrm{NIR}} + R_{\mathrm{Red}})$, where R_{NIR} is the reflectance at the NIR region, and R_{Red} is the reflectance at the red region. The majority of the portable NDVI sensors have their own sources of light, which allows the measurements to be made at any time of the day and any light condition. For NDVI sensor without a light source, measurements can be made on a bright sunny day with negligible wind, because slight wind or breeze can significantly alter the plant canopy structure. The plant surface should be devoid of dew, irrigation, and rain. Measurements can be made at any of the developmental stages or regular intervals from the emergence to maturity stage depending on the objective of the study. If the objective of the experiment is to compare the genotypes, measurements during heading and anthesis can be avoided because differences in phenology will confound the result. To identify the abiotic stress-tolerant genotypes, measurement during and after the stress (recovery) is generally recommended to discriminate between susceptible and tolerant genotypes. To track the rate of leaf senescence, NDVI measurements from the anthesis period to physiological maturity are generally followed. The genotype that maintains greenness, canopy green area, and duration are mostly associated with a higher yield.

Green leaf area duration in terms of NDVI can also be assessed using unmanned aerial vehicles or systems (UAV or UAS). Aerial vehicle systems have the capacity of acquiring images with temporal and spatial resolutions. When compared with other remote sensing platforms such as manned aircraft and satellites, UAVs can be deployed effortlessly and even have lower operational costs. Regardless of the class

of UAV used, a range of customizable cameras and sensors can be integrated for agricultural studies. The images should be collected around solar noon under clear-sky conditions. Flight path, speed, and sensor parameters like aperture, exposure time, frame rate, and sensitivity are important so that there will be adequate overlap between images for mosaicking. After acquiring the images, preprocessing operations like mosaicking and radiometric calibration should be adopted for better results. After preprocessing, the UAV imagery has to be converted to reflectance data for the extraction of different vegetation indices.

3.2.2 Plant Water Status

Plant water status can be quantified through either psychrometric methods or pressure chamber methods, but both these methods are time consuming, very laborious, and less suited for plant breeding or screening genotypes for stress tolerance (Jones 2007). It is observed that relative water content (RWC) is often a good surrogate for pressure potential, and predawn water potential is a surrogate for soil water potential (Jones 2007). RWC evaluates the existing water content of the sampled leaf tissue relative to the maximal water content it can hold at complete turgidity. A major disadvantage of the RWC technique is the considerable time lag between obtaining and sampling the result. Further, the four weighing operations are required, which is time consuming. To overcome this disadvantage, relative tissue weight (a ratio between tissue fresh weight to tissue turgid weight) is used because relative tissue weight is linearly related to RWC (Smart and Bingham 1974).

Loss of water through the transpiration process changes the pattern of canopy reflectance, which indicates a reduction in the absorption of light by leaf due to both the radiative properties of water and drought-related changes in the leaf morphological properties, and leaf physiological status (Ollinger 2011). The extent of the increase in canopy reflectance under drought conditions is related to the duration of stress as well as the response of genotype. Penuelas and Filella (1998) have used canopy reflectance at specific wavelength bands in the visible and NIR region for estimating the plant water status. Hyperspectral active or passive sensors provide measurements of wavelengths in the visible (~400–700 nm) ranges and NIR (~700–2500 nm) ranges, from which different indices are calculated. The important NIR-based index is the water index, WI = R_{970}/R_{900}, which is used to quantify relative leaf water content (Peñuelas et al. 1993). Based on the WI, Babar et al. (2006) and Prasad et al. (2007) have developed normalized water indices NWI-1 = $([R_{970} - R_{900}]/[R_{970} + R_{900}])$, NWI-2 = $([R_{970} - R_{850}]/[R_{970} + R_{850}])$, NWI-3 = $([R_{970} - R_{880}]/[R_{970} + R_{880}])$, and NWI-4 = $([R_{970} - R_{920}]/[R_{970} + R_{920}])$ to screen spring wheat genotypes for drought tolerance. These five indices are now widely used as a selection tool for grain yield under drought stress in wheat (Prasad et al. 2007). Apart from the above, the available water absorption bands at 1450, 1900, and 2100 nm, overtones at 750 and 1250 nm, and numerous spectral vegetation indices for drought have been established for the recovery of crops' status under drought stress (Claudio et al. 2006; Cohen 1991; Hunt Jr et al. 1987).

3.2.3 Canopy Temperature Depression

Among the various traits that are associated with plant water status, the easiest method is a measurement of canopy temperature depression (CTD), which shows good correlations with parameters associated with plant water relation parameters (Mahan et al. 2012). Canopy temperature depression is expressed as the difference between the air and canopy temperature ($CTD = T_{air} - T_{canopy}$). Under high-drought conditions and solar radiation, stomatal conductance gets decreased because the soil moisture is inadequate to meet the evapotranspiration demands, resulting in an increase in canopy temperature. Canopy architecture influences the canopy temperature through mutual shading or leaf angle.

Canopy temperature depression is a negative or positive value based on the air temperature and canopy temperature. The CTD is influenced by both environmental and biological factors (Bahar et al. 2008). However, studies revealed a significant correlation between CTD and leaf water potential (Cohen et al. 2005), stomatal conductance (Rebetzke et al. 2012), and grain yield (Balota et al. 2007; Reynolds et al. 1994) under drought-stress conditions; therefore, it is used as a criterion for drought tolerance selection. Genotypes with cooler canopies than other genotypes in the same environment indicate the drought tolerance ability. Higher transpiration indicates cooler canopy and higher conductance of stomata, favoring net photosynthesis, and a lower canopy temperature in crops under drought indicates a relatively higher capacity for consuming soil moisture or for upholding a healthier plant water status. However, the suitability of CTD as an indicator of yield must be evaluated for the individual environment and in particular plant species (Blum et al. 1989) as the genotypes which keep their canopy cooler by deeper root system in the medium to deep soils may not perform better when grown on shallow soils.

Apart from this, CTD has remained a good estimate for screening genotypes to HT stress tolerance; measurement by CTD using infrared thermometer has some common genetic base under both drought and HT stress (Pinto et al. 2010). Under HT stress, due to the high vapor pressure deficit (VPD), the plants that are well watered raise their transpiration rate to cool the canopy through the evaporative cooling process. The cool canopies are associated with an increased rate of stomatal conductance and root lengths. Increased root length can explore the deeper layer of soil, and in a situation with higher VPD, it can extract more moisture, and it will be used to cool the canopy through open stomata. These genotypes are usually referred to as HT escaper. If subsoil moisture is not available for enhanced transpiration, the stomata close, leading to yield penalty. Real HT-tolerant genotypes give high yield under HT stress and also have inherent high leaf temperatures. Mutava et al. (2011) have studied sorghum [*Sorghum bicolor* (L.) Moench.] genotypes exhibiting higher leaf temperature and higher yield in the sorghum diversity panel, which can be used for improving drought and HT stress tolerance in sorghum.

Infrared thermal imaging is a remote sensing technique commonly used for quantifying canopy temperature (Jones and Schofield 2008). In earlier days, thermocouples and mercury thermometers were commonly used to measure leaf temperature. It was cumbersome and does not represent the canopy temperature

because it is a point measurement. Hence, principles of thermometry and thermal imaging have been translated into the most commonly used remote sensing tools such as infrared thermometer (IRT) and thermal imaging cameras for assessing the canopy temperature of crop plants. Infrared thermometry can report the subtle differences in canopy temperature in fields and controlled conditions (Winterhalter et al. 2011). Thermal imaging has mostly preferred to quantify plant water relations because of the fast data collection and nondestructive nature, and it can also include a huge number of individual plants in a single image for calculating the temperature measurements. For higher accuracy, such measurements should be preferred when the canopy covers the soil, which otherwise contributes to background noise. These techniques were successful in differentiating drought tolerance among genotypes when used for assessing the irrigated crops on windless and cloudless days with high VPD. One of the most important issues while applying thermal imaging is to filter out the background soil from the image and get more precise canopy measurements. Also, the use of thermal imaging is influenced by air temperature, solar radiation, humidity, and wind speed, which keep one fluctuating under natural conditions. Repeated measurements can take these influences into account when assessing the genotypes for tolerance to high temperatures or drought. Hence, recently Internet of Things (IoT) is being used to replace handheld IRTs and wired IRTs for monitoring canopy temperature in wireless mode. The sensors are installed at the center position of each plot/genotype where there are maximum ground cover, uniform growth, and 0.15 m above the plant canopy height. A base station unit will be established at the edge or corner of the field, which collects the data transmitted by the sensors. Every sensor collects data from a circular field of view (60°) with a 0.15 m diameter every minute, and this is auto-averaged to every 15 min and is reported wirelessly to the base station. The canopy temperature data collected in the base station will be transmitted to a computer system for archiving process and subsequent analysis. Such automation in monitoring and assessing the high-temperature responses of crop genotypes can accelerate selection processes aiming at climate-resilient cultivars.

3.2.4 Limited Transpiration

The limited transpiration trait is usually referred to as a slow-wilting trait. Considerable intraspecific variations in the stomatal response to a change in vapor pressure deficit (VPD) have been reported in soybean (Fletcher et al. 2007), peanut (*Arachis hypogea* L., Devi et al. 2010), sorghum (Gholipoor et al. 2010), pearl millet [*Pennisetum glaucum* (L.) R. Br., Kholova et al. 2010], and chickpea (*Cicer arietinum* L., Zaman-Allah et al. 2011). Measurement of restricted transpiration is a semi-high-throughput phenotypic technique because it is not quick, but this trait can be measured simultaneously in a large number of samples. Restricted transpiration under high VPD by partial closure of the stomata may be associated with the decreased hydraulic conductance of leaf and root in plants, which limits the flow of water from roots to leaf (Sadok and Sinclair 2010). It has been assumed that the hydraulic conductivity connected with limited transpiration trait is related to the

transmembrane transport of water via aquaporins. Therefore, application of aquaporin inhibitors, namely cycloheximide, mercury, and silver ions, has the potential to evaluate the expression of restricted transpiration trait.

An indirect approach for the identification of limited transpiration traits in a set of genotypes is through looking at a delay in canopy wilting under water-limiting field conditions. However, it cannot be definite because delayed wilting could be associated with other reasons too. An effective method to evaluate delayed wilting is to measure the canopy temperature under irrigated field conditions. Genotypes with higher canopy temperature and high VPD under well-watered conditions could indicate partial closure of stomata and be associated with limited transpiration rate. However, the environmental conditions should be unique because the difference in leaf temperature can be from several possibilities like temperature, light, relative humidity, and nutrition (Sinclair et al. 2017).

In another case, the expression of restricted transpiration can be quantified by measuring the transpiration rate and weight of the pot at different VPD in the intact plants as detailed by Riar et al. (2015), and here more care should be taken to reduce the evaporation from the soil. An alternate method is to study the stomatal conductance of plants under field conditions during the natural daily variations in VPD (Shekoofa et al. 2014). However, it is limited by the weather conditions when the measurements were made and also the number of lines that are repeatedly measured throughout a day for stomatal conductance. The LeasyScan platform allows quick measurement of plant leaf area and pot weight, and by using this platform, pot weight can be measured every hour to arrive at the transpiration rate. Using 3D laser scanning, the leaf area can be estimated. By using leaf area and transpiration rate, the limited transpiration trait can be phenotyped in a large number of genotypes. Evidence of concept in observing the limited transpiration has been confirmed in corn (*Zea mays* L.), pearl millet, cowpea (*Vigna unguiculata* L.), sorghum, and peanut (Sinclair et al. 2017).

3.2.5 Root Architecture

Huge phenotypic plasticity of root characters in response to soil physical and chemical conditions was observed, and lack of cost-effective and high-throughput screening techniques makes root studies highly challenging. Root architecture denotes the spatial arrangement of root systems, which determines the plant anchorage, ability of roots to absorb nutrients and water, and intra- and interplant competition. The rooting system of the plant responds to environmental stimuli through appropriate adaptive changes in morphological, structural, and physiological processes, which is referred to as root plasticity, and thus exploiting this through breeding by integrating the physiological phenes and root architectural traits will guide in breeding the genotypes for drought tolerance (Kashiwagi et al. 2006; Lynch 2011; Osmont et al. 2007).

Measurement of root system architecture is hindered not only by various complexities (physical, chemical, biological) of soil medium, but also by lack of

comprehensive information about the root system architecture and life span of the root system of a plant (Ahmadi et al. 2011; McCully 1995). A lot of phenotyping and sampling methods for roots in the field have been suggested, namely monoliths, soil profiling, rhizotron, nail plates, trenching, probes, shovelomics, visualization, and digitalization of roots in the field (Costa et al. 2014; Pierret et al. 2003; Trachsel et al. 2011; Wu et al. 2015), to obtain information on root length, dry matter, surface area, dry weight, diameter, diameter class, and structure.

Spatially dispersed monolith sampling can be used for assessing crop root system architecture under the field. However, commonly used auger core sampling might suffer large errors when illustrating spatial distributions of roots. Shovelomics is an alternate high-throughput method for phenotyping root system architecture in the field, which provides a rapid sampling and quantification of rooting depth but not fine details of the root system. The heterogeneity of soil structure and composition can cause a confounding effect on the root system architecture within the same field. Several software packages (RootScan, RootNav, DART, GiARoots, RootSystemAnalyzer, RootReader, IJ Rhizo, RootReader3D, and RooTrak) were developed for extracting quantitative data and imaging roots from the captured root images (Lobet and Draye 2013).

Direct measurements of root traits under field conditions can be made by removing the soil, which can cause the death of the plant, the loss of root material, and the loss of geometric information. Earlier, digging of a trench close to growing plants to visualize the whole root was employed. Even though the trench method permits for a precise in situ observation of roots grown in the field, it is very slow and laborious. Apart from trench methods, researchers are using excavation techniques like soil coring to study the root architecture. Soil coring is done by introducing a metal cylinder down into the soil to obtain a soil core. The soil core is usually divided into segments with the same length, and each segment is washed over a screen to collect all the roots. The collected roots are scanned on a flatbed scanner for measuring subsequent length. The roots can also be dried, weighed, and counted. The soil coring method is used to estimate the rooting depth and root length density. With the development of the tractor hydraulic system, now the soil coring method is well automated.

Another method of excavation technique practiced is root crown phenotyping or shovelomics because the root crown is considered as the backbone of the root system. Using a regular shovel, the root crown which is the upper part of the root structure attached to the shoot is excavated, washed, and analyzed for root architecture. This procedure provides information on root placement in the soil and the number of roots, and their lengths and angles. Shovelomics is also a destructive method.

Field rhizotrons are considered as an enhanced version of trenches; in this method, a trench is dug, a glass window is positioned tightly over the vertical cut plane, and a roof is installed over the pit. A customized camera is inserted into the tube to image the soil with the roots around the tube. Through this method, root initiation, growth, and turnover of individual roots over a period can be assessed. A major limitation of this technique is low throughput (numbers of samples are small

per unit time) and is highly influenced by soil properties. To reduce the environmental variability and increase the throughput, researchers are using rhizobox, which works similarly to rhizotron. In most cases, the rhizoboxes are maintained at an angle that forces the root to grow along with the glass so that it can be monitored frequently. However, in this method, it will be difficult to distinguish between thin roots and soil. Now researchers are using clear media such as agar (in Petri dishes) to grow roots, making the roots visible, and the images of the Petri dishes with roots can be analyzed for obtaining information on root angle.

On the other hand, X-ray computed tomography and magnetic resonance imaging are the two technologies that can be used for imaging the root systems over some time without destroying the plant. With X-ray computed tomography, both roots and soil are imaged, and custom-made software tools are required to segment the roots. On the contrary, magnetic resonance imaging can be adapted to image only roots in such a way as to avoid segmentation. However, these two techniques are low throughput and expensive. In summary, though there are many ways to grow and observe roots, appropriate methods should be selected based on the experimental question and ease of use.

3.2.6 Membrane Stability

Maintaining cell membrane stability is one of the adaptation mechanisms under abiotic stress. Cell membrane stability can be assessed directly through electron microscopy and indirectly by lipid peroxidation, electrolyte leakage, and chlorophyll *a* fluorescence. The level of lipid peroxidation detected as malondialdehyde (MDA) is an indicator of free radical damage to the cell membranes because lipid peroxidation alters the physiological functions of cell membranes. The traditional technique to detect MDA content in plants is the thiobarbituric acid-reactive substance (TBARS) test using the spectrophotometry technique. The TBARS such as aldehydes and malondialdehyde react with thiobarbituric acid at low pH and form [TBA]-MDA adduct, which is a pink chromogen having a maximum absorbance at 532 nm; the formed adduct is quantified through a spectrophotometer. The TBARS test is a standard test and is sensitive for microsomal and liposomal membrane lipid peroxidation tests. It rarely measures the free MDA content of the lipid system. TBA reactivity depends on the lipid content of the sample (Bhattacharjee 2014).

Also, high-performance liquid chromatography (HPLC) was used to determine MDA in plants (Davey et al. 2005). However, the HPLC method requires lots of time, chemical, and complex sample preparation (utmost care has to be undertaken to ensure the loss of oxidized material and artificial peroxidation). Kong et al. (2016) have assessed the feasibility of hyperspectral imaging with 400–1000 nm to detect MDA content in crops after herbicide application. The result indicated that the extreme learning machine model achieved the optimal prediction performance with 23 wavelengths selected by competitive adaptive reweighted sampling.

Assessment of damage to the thylakoid membrane under stress is a reliable measure of a plant's susceptibility to HT stress (Ristic et al. 2008). Impairment in

the thylakoid membrane can be estimated by determining chlorophyll *a* fluorescence trait and measuring the ratio of constant fluorescence (*O*) and the peak of variable fluorescence (*P*) (Ristic et al. 2008). An increase in the *O*/*P* ratio represents the damage in thylakoid membranes; the higher the increase, the greater the damage. Larcher (1995) has observed a good correlation between chlorophyll fluorescence and electrolyte leakage (an indicator of membrane damage).

With the recent developments in tracer techniques, fluorescent dyes and nucleic acid stain (Sytox green) were used as molecular probes to track the membrane damage. After the brief incubation period with the stain, the nucleic acids of dead cells will fluoresce bright green. This property makes the stain Sytox green a simple, quantitative single-step dead cell (compromised membrane) indicator for use with fluorescence microscopes (Prasad and Djanaguiraman 2011).

3.2.7 Photochemical Efficiency

Chlorophyll fluorescence displays the fate of excitation energy in the photosynthetic apparatus that has been used as an early, in vivo indicator of stress (Yamada et al. 1996). In most of the studies, dark-adapted chlorophyll *a* fluorescence parameters are used to understand the reactions of plants to environmental cues. However, it is challenging under field conditions, due to time constraints (dark-adaptation time) to perform the dark-adapted test if the study involves many genotypes or treatments. Dark-adaptation times vary with a crop from 10 to 60 min, and some researchers use pre-dawn values for the basal fluorescence (F_o). The F_o measurement and its light-adapted equivalent F_o' are fundamental to the analysis of fluorescence. F_o' is measured immediately after switching off the actinic light, but accurate measurement of F_o' is difficult. Many fluorometers have the ability to apply a weak far-red light to measure both F_o and F_o'. Application of saturating pulse to a dark-adapted leaf triggers a maximum value of fluorescence by closing the reaction centers. At this time, in a non-stressed healthy leaf, there is no non-photochemical quenching (NPQ) since the leaf is dark-adapted leading to a maximum value of fluorescence (F_m). The F_v/F_m ratio is an indicator of the maximum quantum yield of PSII photochemistry. The value of F_v/F_m ratio in an unstressed leaf will be $\geq$0.80, and the presence of stress will decrease this ratio through photoinhibition or inactivation of PSII (Long et al. 1994). Thus, measuring the F_v/F_m ratio after an appropriate dark adaptation is the most commonly used technique to quantify stress in leaves. To attain this precisely in a light-adapted leaf, we need to make sure that PSII is fully oxidized, and this can be succeeded using a pulse of far-red light. Precise measurements of basal fluorescence in the field are challenging due to the relaxation kinetics of the chlorophyll molecule under the dark-adapted state. When a leaf is dark-adapted, the movement of electrons in the thylakoid should stop almost immediately. However, NPQ "relaxes" more leisurely because the protective NPQ processes remain active. Therefore, to obtain the true maximum value of fluorescence, we must allow the leaf to remain in the dark for a span of time ample for these processes to complete (i.e., NPQ to become zero) (Murchie and Lawson 2013).

Due to the ease of measurement of the maximum efficiency of PSII photochemistry in the light, this is widely used as an indicator of the operating efficiency of PSII in the light. However, care should be taken in this measurement because PSI may contribute to fluorescence when measurements are made above 700 nm and the existence of "multiple turnovers" of PSII during the saturating pulse. From the value of F_v/F_m ratio, the rate of electron transport can be calculated using the photosynthetic active radiation (PAR) value and a fraction of light intercepted by PSII and PSI. It is difficult to measure the latter, and an assumption of equal absorption is made. Although these standard values are expected to be constants, they will differ between the leaves having different optical properties or the same leaf suffering from stress treatments. For instance, relating the electron transport rate (ETR) values between control (fully hydrated leaf) with a drought-stressed leaf (low turgor value) is not appropriate.

Similarly, leaf samples with different pigment contents or photosystem stoichiometry will vary for a light interception, causing inaccuracies in ETR calculation (Walters 2005). The measurement of F_o' can be open to error if the far-red light applied does not sufficiently oxidize Q_A and if the relaxation of NPQ causes F_o' to rise quickly after the actinic light has been switched off. Thus, care should be taken during measuring the PSII quantum yield to assess the impact of stress on the plants. Now, imaging chlorophyll fluorescence as a diagnostic tool is becoming increasingly popular for screening germplasm. Chlorophyll fluorescence imaging has been combined into many phenotyping platforms for high-throughput analysis. Imaging provides additional evidence on the spatial and temporal heterogeneity of measured parameters. Now, chlorophyll fluorescence imaging is integrated with infrared gas exchange techniques, thermography, and hyperspectral imaging to explore and integrate various traits to understand the stress response (Bauriegel et al. 2011).

3.2.8 Yield-Forming Traits

Grain yield is the final product of many processes, and in cereals, for example, it is primarily determined by yield-associated traits like the number of spikes/panicles per plant, number of grains per spike/panicle, and individual grain weight. Seed numbers are a function of seed set, and the decrease in seed set percentage under stress is linked with early or delayed flowering, asynchrony of male and female reproductive development, and impairments in parental tissues, in male and female gametes (Zinn et al. 2010). Estimation of seed set percent is a more accurate estimation of the response of gametes to HT stress. However, data from marked floret instead of whole spike/panicle will provide a good estimate because within a spike each floret will have a different developmental stage or day of anthesis (Aiqing et al. 2018). During estimating seed set percentage, researchers may consider ill-filled seeds/grains as a seed, because the formation of seed indicates the function of gametes. However, it will be wise to discard the ill-filled grains during calculating the seed set percentage because it will overestimate the seed yield potential of the genotype. A strong positive correlation between seed set and pollen availability, and seed set and seed

yield, was established (Prasad et al. 2017, 2019). Limited gamete functions may be considered the most important factor for seed set under drought and HT stress environments. Gamete functions depend on its viability, which can be evaluated by viability assays like staining and in vitro and in vivo germination.

The choice of pollen viability method depends on the crop or species. Viability has been defined as having the ability to dwell, nurture, germinate, or develop, and the loss of viability is a constant variable. Thus, the viability of pollen grains has been used to define the ability of pollen grains to germinate on the stigmatic surface, germinating in vitro, picking up certain stains, and effective seed set following pollination. Viable pollen grains cannot germinate under in vitro or in vivo conditions if the circumstances are not favorable (environment and pistil response). Therefore, assessment of pollen viability based on seed set indicates the response of both male and female gametes. Methods of determining pollen viability are enormous, and the method of determining pollen fertility (ability to set the seed) is through quantifying seed set percentage.

All the methods of assessing pollen viability depend on factors like cytoplasm content, enzyme activity, plasmalemma integrity, and environmental conditions. However, none of the methods can be able to confirm that the pollen is inviable and unable to fertilize and set seed. Therefore, it gives a likelihood estimate. The approaches used to evaluate the pollen viability are measuring the respiration rate (very rarely used), staining techniques (vital stain to indicate membrane integrity, presence of the cytoplasm, respiration rate, enzyme activity, and starch content), in vitro germination, and capacity to set seeds. In vitro pollen germination method is rapid, fully quantitative, and reasonably simple, and it is highly correlated with seed set percent in many species (Prasad et al. 2019). The results depend on the time of pollen collection, the composition of the medium, the temperature of the growth medium, and the duration of the test. Low germination under in vitro conditions indicates that the pollen is still fertile and able to set seed. The in vivo method is more valid than the in vitro method. However, it must be accompanied by a stigma receptivity test. The major drawbacks are that vital stains tend to stain old and dead pollen, overestimation of viability, pollen with cytoplasm or starch is not necessarily fully fertile, and immature or aborted pollen grains also pick the stains (Dafni and Firmage 2000). The important elements that showed up during assessing pollen viability are (1) information about the test environment, (2) freshly collected pollen preferred for the assay, (3) testing in parallel the dead pollen as a negative control, (4) testing hydrated vs. dehydrated pollen to understand the effect of moisture level, and (5) running several tests simultaneously to understand which method is best for the test species.

Cardinal temperatures (T_{min}, T_{opt}, and T_{max}) for pollen grain germination are used to screen germplasm for HT stress tolerance, and this proved to be a good screening tool. Results from in vitro studies on peanut, sorghum, pearl millet, rice, and coconut (*Cocos nucifera* L.) presented that genotypes varied in response to temperature for cardinal temperatures, and the differences in cardinal temperatures were mainly responsible for the tolerance/susceptibility level of genotypes to HT stress (Djanaguiraman et al. 2014, 2018; Hebbar et al. 2018). The genotypes having higher

ceiling temperature (T_{max}) for pollen germination values tend to be HT tolerant in most cases. However, research also indicated that there is no relationship between cardinal temperature and tolerance/susceptibility to HT stress because the cultivars which had a higher optimum temperature (T_{opt}) for pollen germination did not always have a T_{max} or vice versa (Kakani et al. 2002).

To evaluate the effect of HT on seed abortion (postfertilization stage), the number of seeds per pod and number of locules are used. The weight of individual grain is a product of the rate and duration of grain filling. Grain-filling duration can be expressed as the time between anthesis and physiological maturity; beyond this point, there will be no significant increase in grain dry matter. The average for grain-filling rate was calculated from the ratio of maximum grain weight to grain-filling duration, which was estimated from quadratic or cubic polynomial curves. Linear regression has been employed to find out the grain-filling rate, and the intersection of two regression lines has been used to attain the grain-filling duration. Among genotypes, genetic variability exists for grain-filling rate and duration and can be exploited for developing high-yielding cultivars.

3.3 Conclusion

Selection for grain yield per se under drought and HT stress is limited by the low heritability of grain yield under stressful conditions. This situation requires the identification of highly reliable secondary traits that are closely related to grain yield under stress and having high heritability. The traits like plant water status, canopy temperature depression, green leaf area duration, root architecture, membrane stability, gamete viability, and stem reserves are key traits among those listed in this chapter that are highly associated with stress tolerance. The relative importance of each trait under drought and HT stress was provided in Table 3.1. Regarding

Table 3.1 A subjective classification of the relative value of different trait measures for stress tolerance

	Stress	
Traits	Drought	High temperature
Green leaf area duration	+++	++
Plant water status	+++	+
Canopy temperature depression	++	+
Limited transpiration	++	+
Root architecture	+++	++
Membrane stability	+	+++
Photochemical efficiency	++	++
Early-morning flowering	−	+
Stem reserve mobilization	+++	+++
Yield-forming traits	++++	++++

More +s indicates greater value, while (−) indicates limited value

quantification of the above traits, they are semi- to high throughput in nature. The advent of high-throughput genotyping technologies urges us to gather high-quality phenotypic data for marker-based selection in crop breeding. However, a collection of phenotypic data is laborious and highly influenced by the genotype, environment, and management. Therefore, the development of the high-throughput phenotypic platform is critical for accelerating the breeding programs. Field-based high throughput increases the accuracy of the estimation and reduces the time, leading to the selection of genotype and the identification of genetic loci with high precision. Despite major improvements in phenotyping, there are still large shortcomings, namely quality of data collection, management of digital data, image resolution, and accurate analysis. Robust computational skills will be needed to handle the phenotypic data collected from the phenotypic platform. Care should be taken to phenotype at the target environment with standardized protocols. The field phenotyping process must go hand in hand with the methodologies to characterize and control the field variations, and user-friendly data management. Imaging and spectroscopy techniques can provide nondestructive measures of traits like chlorophyll fluorescence and green leaf area duration, and this technique can be extended to quantify other traits through surrogate parameters. All these advances in phenotyping are likely to accelerate genomics application for enhancing crop productivity under drought and HT environments.

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