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High-throughput imaging tools to phenotype traits in plants for stress environments

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Abstract
Despite the rapid development of plant genomic technologies, a lack of advancement in high-throughput image based plant phenotyping capabilities limits our ability to dissect the genetics of quantitative traits. Effective, high-throughput image based phenotyping platforms have recently been developed to solve this problem. In high-throughput phenotyping platforms, a variety of imaging methodologies are being used to collect data for quantitative studies of complex traits related to the growth, yield and adaptation to biotic or abiotic stress (drought, disease, insects, and salinity). These imaging techniques include visible (RGB) imaging, spectroscopy imaging (multispectral and hyperspectral remote sensing), thermal infrared imaging, fluorescence imaging. This paper presents a brief review on these imaging techniques and their applications in plant phenotyping. The features used to apply these imaging techniques to plant phenotyping for abiotic stresses are described and discussed in this review.

Keywords: High-throughput imaging tools, phenotype traits, plants, stress environments

Introduction
World agriculture is facing major challenges to ensure global food security, posed by several abiotic stresses. The major abiotic stresses (drought, high salinity, cold, and heat) negatively influence the survival, biomass production and yields of staple food crops hence, threaten the food security worldwide. Most prevailent abiotic stress that limits plant growth and productivity is dehydration stress which is common under drought, salinity and high temperature stresses. Earlier, there was a huge challenge in understanding the key molecular mechanisms for breeding tolerance in crop plants against these stresses due to their multigenic and quantitative nature (Collins *et al.,* 2008) [6]. In the last decade and half, revolution in the genomics and gene technology, have boosted the confidence of providing solutions for these challenges and has led to generation of huge repository of genomic information. To harness this information for crop improvement, novel approaches are required to identify quantitative phenotypes and to explain the genetic basis of agriculturally important traits in a high-throughput fashion. But, due to rapid development in genotyping and lagging behind of phenotyping, a genotype to phenotype gap has been created. To help bridge this gap, a comprehensive framework for high-throughput phenotyping is the need of the hour (Figure 1).
Advances in high throughput genotyping have offered fast and inexpensive genomic information and paved the way for the development of large mapping populations and diversity panels of thousands of recombinant inbred lines for phenotyping under various abiotic stresses. Although molecular breeding strategies have placed greater focus on selections based on genotypic information, they still require the following phenotypic data: (1) phenotypes are used for selection and to train a prediction model in genomic selection; (2) a single phenotyping cycle is used to identify markers for subsequent selection through generations within the maker-assisted recurrent selection; and (3) phenotyping is necessary to identify promising events in transgenic studies. Phenotyping advances are essential for capitalizing on developments in conventional, molecular, and transgenic breeding.

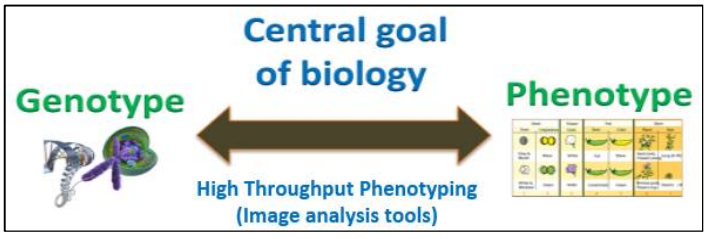

Fig 1: Bridging the gap between genotype and phenotype through high-throughput phenotyping

Responses of Crop Plants to various abiotic stresses

Drought

Crop production, worldwide is limited by drought more than by any other environmental stress (Cattivelli *et al*. 2008) [2]. This problem will further be exacerbated by climate change, which will result in increased crop demand for water.

Impact of drought stress on plant physiology

Gas Exchange

Under mild to moderate water deficits, one of the earliest plant responses is stomatal closure, concomitant with the reduced water potential and turgor associated with even a small decrease in relative water content (Lawlor and Tezara 2009, Chaves *et al*. 2003) [26]. Reduced stomatal conductance limits water loss and CO_2 diffusion, and hence photosynthetic assimilation. Ultimately, reduced photosynthetic assimilation rates result in reduced vegetative growth, and for many crop seven mild drought stress results in reduced yield.

Reactive Oxygen Species

Stomatal closure as a result of drought coincides with exposure to high photosynthetically active radiation. The rate of electron production exceeds the rate of electron use in the Calvin cycle, when leaves are subjected to excess incident radiation relative to the available intracellular CO_2. Reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), the hydroxyl radical ($HO^{\bullet}$), the superoxide anion ($O^{2\bullet-}$), and hydrogen peroxide (H_2O_2), are therefore produced, particularly in the chloroplasts, which are both the main producers as well as targets of ROS (Sofo *et al*.2005). These Reactive Oxygen Species react with proteins and lipids, causing damage to cellular structures and metabolism, particularly those associated with photosynthesis (Lawlor and Tezara 2009) [26]. This situation will ultimately damage the photosynthetic apparatus, unless either photo protective mechanisms are available to down-regulate photosynthesis, or the decline in CO_2 assimilation coincides with an increase in the strength of another sink for the absorbed radiation. This photoprotective mechanism varies across the genotypes. Hence, phenotyping large number of germplasm for this photoprotective trait will give us best material for breeding varieties tolerant to drought stress.

Osmotic Adjustment

Osmotic adjustment is the lowering of osmotic potential due to the net accumulation of solutes in response to water deficits (Zhang *et al*. 1999) [44]. Osmotic adjustment is often induced during drought (Chaves *et al*. 2009) [4], with solutes accumulating, resulting in the maintenance of a higher turgor potential at a given leaf water potential (Zhang *et al*. 1999) [44]. Different types of compatible solutes can be responsible e.g. various sugars, organic acids, amino acids, sugar alcohols, andions. Concentrations of soluble sugars (sucrose, glucose, and fructose) are altered by drought – ingeneral concentrations increase (Chaves and Oliveira 2004) [5] – although under severe dehydration they may decrease (Pinheiro *et al*. 2001) [33]. Soluble sugars act as signalling molecules under stress (Chaves and Oliveira 2004) [5] , interact with hormones, and modify the expression of genes involved in photosynthetic metabolism – generally resulting in a reduction in source activity such as photo assimilate export and an increase in sink activity such as production of lipids and proteins (Chaves *et al*. 2009) [4] .

Salinity

Due to poor quality irrigation water, inadequate drainage, salt water flooding of coastal land, and salt accumulation in dry areas (Kijne 2006) [24], salinity is impacting more agricultural lands worldwide. Salinity is a soil condition characterized by a high concentration of soluble salts, mostly chloride and sulfates of sodium in the soil. Soils are classified as saline when the electrical conductivity (EC) is 4 dS/m more, which is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of approximately 0.2 MPa (USDA-ARS 2008) [41]. Soil salinity creates both osmotic and ionic stresses in plants. Presence of salts in the soil solution reduces the ability of the plant to take up water, and this leads to reduction in the growth rate, referred as the osmotic or water-deficit effect of salinity. If an excessive amount of salt enters the plant in the transpiration stream, it causes injury to cells in the transpiring leaves, resulting in further reduction in plant growth. This is called the salt-specific or ionic effect of salinity (Greenway and Munns 1980) [15]. Both chloride and sodic salts cause damage to the root system of crops. The chloride-triggered injury is identifiable by the extensive leaf blade scorching symptoms whereas the accumulation of sodic salts results in leaf mottling and leaf necrosis.

High Temperature stress

High temperature stress is a serious threat to plants because the stress causes membrane integrity loss, production of ROS, aggregation and inactivation of proteins, and metabolic and cellular disequilibria, ultimately leading to cell death (Los and Murata 2000; Iba 2002) [29, 20]. Photochemical reactions in thylakoid lamellae in the chloroplast stroma are thought to be the primary sites of injury during heat stress (Wise *et al*. 2004) [43]; thus, one critical aspect of heat tolerance in plants is the continual maintenance of photosynthesis.

Approaches for developing stress tolerant crop plants

The success of the crop-breeding program largely depends on the availability of natural variation among the germplasm resources. Large number of cultivated and wild germplasm in major crops, preserved in the International and National Agricultural research institutes, provide unique resources for systematic screening for discovery of novel variability to improve adaptation of crop plants in environments affected by various abiotic stresses. Accurate Phenotyping procedures are critical and need of the hour for identifying useful germplasm for crop improvement program as well as for deciphering the genetic basis of the mechanisms associated with abiotic stress tolerance.

Plant Phenotyping

Plant phenotyping is the comprehensive assessment of complex plant traits such as growth, development, tolerance, resistance, architecture, physiology, ecology, yield, and the basic measurement of individual quantitative parameters that form the basis for more complex traits. The plant phenotype includes these complex traits, and examples of their direct measurement parameters are the root morphology, biomass, leaf characteristics, fruit characteristics, yield-related traits, photosynthetic efficiency, and biotic and abiotic stress response. Given the rapid development of high-throughput genotype screening in plant breeding and genomics for related growth, yield and tolerance to different biotic and abiotic stresses, there is a call for more effective and reliable phenotyping data to support modern genetic crop improvement. To accomplish this goal, phenotyping enlists

expertise from the biological sciences, computer science, mathematics and engineering. In recent years, high throughput integrative phenotyping platforms have been deployed in growth chambers or greenhouses. These platforms use robotics, precise environmental control and imaging technologies (hardware and software) to assess plant growth and performance (Figure 2). High-throughput integrative phenotyping facilities provide an opportunity to combine various methods of automated, simultaneous, non-destructive analyses of plant growth, morphology and physiology, providing a complex picture of the plant growth and vigour in one run, and repeatedly during the plant's life-span. Particular methods used in integrative plant phenotyping are often not new and usually represent those which have already been used for a number of years in basic research, e.g. non-invasive methods that employ visible or fluorescence imaging. High-throughput then allows analysis of the plants on a large scale. This enables users to apply statistics to discover subtle but significant differences between the studied genotypes and treatment variants.

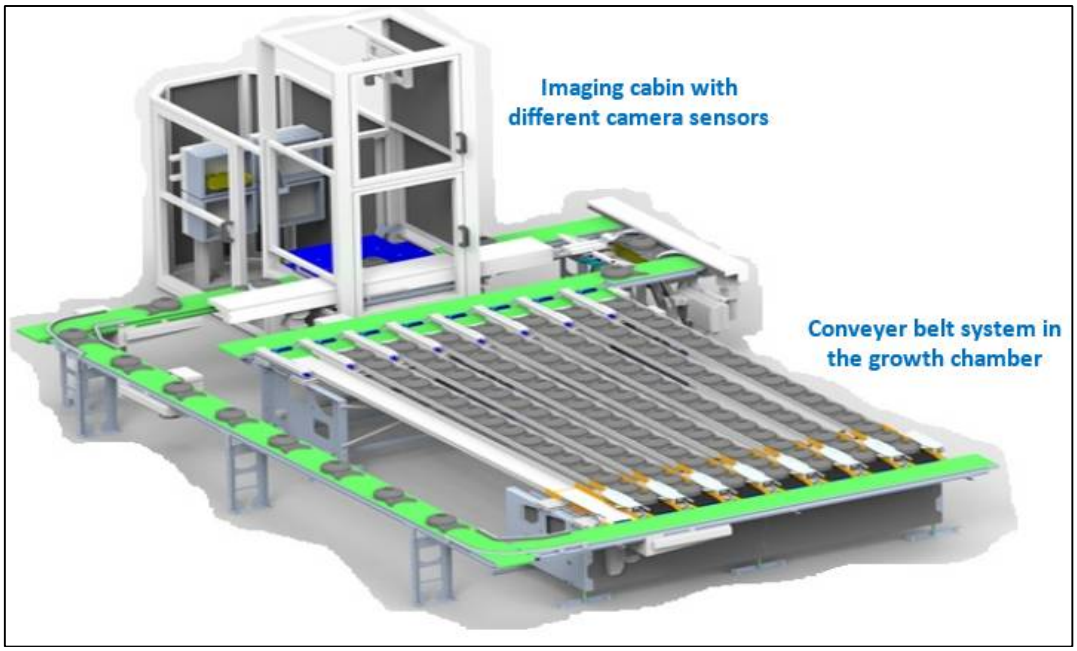

Fig 2: A High throughput Phenotyping Facility with imaging cabin and growth chamber with a conveyer belt system.

Non-destructive analysis of growth and physiology of plants

Various non-invasive sensors used in high through put phenotyping platforms are visible red-green-blue (RGB) imaging, chlorophyll fluorescence imaging (CFIM), thermo-imaging, and hyperspectral imaging.

Visible RGB imaging of plant shoots

Apart from the importance of root-growth analysis, a key descriptive parameter in plant physiology is the growth of plant shoots. Although there are numerous secondary traits describing the morphology of shoots in particular species and their developmental stages, the primary and universal trait is biomass formation. Shoot biomass is defined as the total mass of all the aboveground plant parts at a given point in a plant's life (Roberts *et al.,* 1993) [34]. This trait can be easily assessed by a simple weighing of the fresh (FW) and dry (DW) masses. However, this involves the destruction of the measured plant thus only allowing end-point analyses. Similarly, leaf area and consequently the plant growth rate are usually determined by manual measurements of the dimensions of plant leaves (Rouphael *et al.,* 2010, Cemek *et al.,* 2011, Misle *et al.,* 2013) [35, 3, 31]. Such measurements are highly time consuming and thus cannot be used for large scale experiments. For this reason, plant phenotyping facilities prefer to evaluate the growth rate using imaging methods which employ digital cameras with subsequent software image analysis. This enables a faster and more precise determination of the leaf area (Green *et al.,* 2012, Zhang *et al.,* 2012, Tessmer *et al.,* 2013) [14, 45, 40] and other parameters called projected area (Figure 2), or hull area in the case of monocots (Furbank *et al.,* 2011, Honsdorf *et al.,* 2014) [9, 17]. In general, non-invasive techniques of shoot growth determination have proven very reliable and high correlations between the digital area and the shoot fresh or dry weights, respectively, were reported in Arabidopsis, tobacco (Walter *et al.,* 2007) [42], cereals (Golzarian *et al.,* 2011, Fehér-Juhász *et al.,* 2014) [12, 8] and pea (Humplík *et al.,* 2015) [19].

Chlorophyll fluorescence imaging (CFIM)

One of the chlorophyll (Chl) fluorescence methods is chlorophyll fluorescence induction (CFIN), i.e., the measurement of the Chl fluorescence signal during illumination of the sample following prior dark adaptation. Since the first paper on CFIN by Kautsky and Hirsch (1931) [23], CFIN has been one of the most common methods used in photosynthesis and plant physiology research: it is inexpensive, non-destructive, and above all, provides a great deal of information about the photosynthetic function of the sample (reviewed, e.g., by Lazár D. 1999, Lazár D. 2006) [27, 28].

Use of pulse amplitude modulation (PAM) techniques for the measurement of CFIN together with the application of the saturation pulse (SP) method enables the separation of photochemical and non-photochemical events occurring in the sample (Schreiber *et al.,* 1986) [38]. Chl fluorescence is excited and measured with the help of weak measuring flashes, whereas photosynthesis is maintained by actinic illumination

and saturation of photosynthesis is achieved by the SPs. Since Chls absorb in blue (Chl a at 436 nm and Chl b at 470 nm, respectively) and red (at about 650 nm for Figure 3 The illustrative figure presenting outcome of simultaneous analysis of control and salt-stressed Arabidopsis plants, using RGB, hyper spectral and Chl fluorescence imaging. The 18 DAG old soil-grown Arabidospis plants were treated with 250 mM NaCl (salt-stressed) and water (control) and after 48 hours were analysed by different sensors for comparison in: morphology (top-view RGB imaging can be used for computation of rosette area or shape parameters), spatial distribution of vegetation index reflecting changes in the chlorophyll content (NDVI) provided by VIS/NIR hyperspectral camera, and the changes in maximal quantum yield of PSII photochemistry for a dark-adapted state (ΦPo, also referred as FV/FM) reflecting the photosynthetic activity of the plants obtained from KCFIM. Humplík *et al*. Plant Methods (2015) 11:29 Page 4 of 10 both Chls a and b) regions of visible spectrum, the measuring and actinic light is the light with one of the above wavelengths, usually 650-nm. The saturation pulse is usually generated by white light. On the other hand, Chl fluorescence emission spectrum at room temperature shows two peaks centred at about 680 and 735 nm. To avoid a possible overlap of the 650-nm excitation light with Chl fluorescence emission, the Chl fluorescence signal is detected at wavelengths longer than 700 nm.

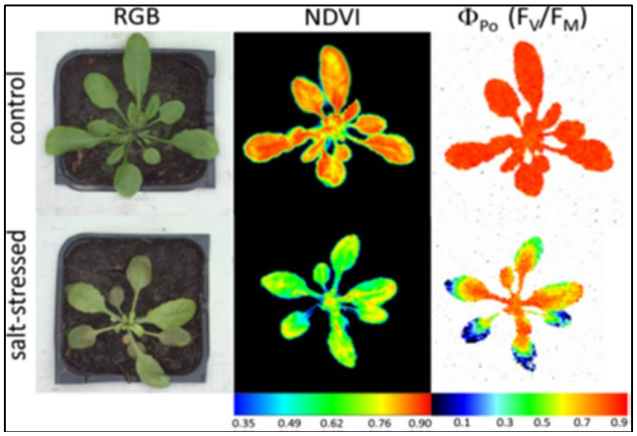

Fig 3: The illustrative figure presenting outcome of simultaneous analysis of control and salt-stressed Arabidopsis plants, using RGB, hyper spectral and Chl fluorescence imaging (Adopted from Humplík *et al.,* 2015) [19].

In the images, different colours are used to show different fluorescence intensities according to a chosen false colour scale (as mentioned above, fluorescence emission is always above 700 nm, red light). An additional advantage of the CFIM is that it provides a huge amount of data which can be thoroughly analysed and used for early detection of plant stress as shown, e.g., by Lazár *et al*. 2006 [28].

Thermo imaging

Plants maintain their canopy cool by the process of transpiration and when the stomata are closed, plant canopy temperature increases. Based on this principle, thermal imaging was used for the first time to detect the changes in the temperature of sunflower leaves caused by water deficiency (Hashimoto *et al.,* 1984) [16]. The ability to maintain cooler canopy under abiotic stresses like drought, salinity and high temperature varies from genotype to genotype, hence canopy temperature difference (CTD) has been used as a trait to screen large number of germplasm for various abiotic stress tolerance. In addition to transpiration, stomata also drive water vapour, both parameters being typically determined by leaf gas exchange measurements. However, leaf gasometry involves contact with leaves which often interferes with their function. Further, leaf gasometry is time-consuming, limited by sample size and/or large number of samples required. In addition to heat emission, plants can lose heat by conduction and convection, which in fact represent mechanisms of a non-photochemical quenching of excited states. For this reason, it is not unexpected that an increased thermal signal correlates with an increase in non-photochemical quenching (Kaňa and Vass 2008) [22]. Given the foregoing, thermo imaging is a very suitable method for plant phenotyping (Fehér-Juhász *et al.,* 2014, Siddiqui *et al.,* 2014) [8]. Like Chlorophyll fluorescence imaging, it uses cameras to measure spatial heterogeneity of heat emissions, usually from leaves; the heat is electromagnetic radiation in the infrared region, usually between 8–13µm. Generally, thermal imaging has been successfully used in a wide range of conditions and with diverse plant species. The technique can be applied to different scales, e.g., from single seedlings/leaves through whole trees or field crops to regions. However, researchers have to keep in mind that environmental variability, e.g., in light intensity, temperature, relative humidity, wind speed, etc. affects the accuracy of thermal imaging measurements and therefore the measurements and their interpretations must be done with care. Although thermal imaging sensors have been integrated into the in-house phenotyping platforms with controlled-environment (see section The use of phenotyping methods to study plant stress responses) the majority of studies have been performed so far in field conditions (Jones *et al.,* 2009, Grant *et al.,* 2012, Costa *et al.,* 2012) [21, 13, 7].

All aspects of thermal imaging used for the exploration of plant-environment interactions, as well as an overview of the application of thermo imaging in field phenotyping, were recently reviewed (Costa *et al*, 2013).

Hyperspectral imaging (VIS-NIR, SWIR)

The absorption of light by endogenous plant compounds is used for calculations of many indices which reflect the composition and function of a plant. Such indices are, for example, the normalized difference vegetation index (NDVI) (Rouse *et al.,* 1974) [36], an estimator of the Chl content, and the photochemical reflectance index (PRI) (Gamon *et al.,* 1992) [10], an estimator of the photosynthetic efficiency. The absorption of a compound (e.g., water) at a given wavelength (Carter G A, 1991) [1] can also be used for direct estimation of the compound contents in the plant. For practical reasons, measurement of absorbance is replaced here by measurements of reflectance. Depending on the measured wavelengths of reflected signal, various detectors are used, usually VIS-NIR (visible-near infrared region (400–750) - (750–1400 nm)) and SWIR (short wavelength infrared region; 1400–3000 nm). Measurements of the reflectance signal in VIS-NIR and SWIR regions originate from methods of remote sensing (Huber *et al.,* 2014, Lamb *et al.,* 2014 and Saberioon *et al.,* 2014) [18, 25, 37]. However, due to the high value of the information they carry, they are very suitable methods for plant phenotyping (Garriga *et al.,* 2014, Mahajan *et al.,* 2014, Petach *et al.,* 2014) [11, 30, 32]. The reflectance signal can be detected at selected wavelengths or separated spectral bands (so-called multispectral detection).The whole spectral region can also be measured even for each pixel when cameras are

applied and the hyperspectral imaging is carried out (Figure 2). Whereas the hyperspectral imaging in the VIS-NIR spectral region is used for evaluation of several indices as mentioned above, the SWIR spectral region is mainly used for the estimation of the plant's water content. Several aspects of plant reflectance were recently reviewed (Ollinger S V, 2014).

Summary

Advanced integrated phenotyping technologies that combine molecular techniques and non-invasive sensors contribute to the momentous progression of high-throughput plant development research especially under stressed environments. This advanced research enables observation of high throughput phenotypic traits and how these traits change depending on environment and genotype.

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