Manual of Advance Training Course
On
Application of plant phenomics tools for assessing responses of crop plants to drought and high temperature
(February 15-28, 2018)






Organised by
ICAR - National Institute of Abiotic Stress Management
(Deemed to be University)
Malegaon, Baramati, Pune, Maharashtra – 413 115

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Chapter 1 : Plant Phenotyping: Needs and scope

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Introduction

The food production system in India has to feed about 1.6 billion of Indians by 2050. The magnitude of challenge in accomplishing this task attains enormous dimensions mainly in 3 aspects *viz.* limited and shrinking resources for agriculture, concerns about technological foot prints on human and environmental health and predicted amplification of factors contributing to unfavourable environments due to climate change. Limited resources mainly land and water continue to constrain the food production system as their sectors contributig to the national economy will draw their share during development while episodes of droughts can place tremendous pressure on water supply. Use of excess fertilizers and water have left their foot print on water and land quality which tend to become unsuitable for use unless treated by using modern technologies. Impact of climate change is being witnessed in different forms such as increase in frequency and intensity of drought, flood *etc.*

Limited scope for expanding the land has placed emphasis on enhanced productivity of agricultural sectors for food security. Those land which are not very much suitable with or without deficit rains cannot be left uncultivated as the food production from favourable land has reached its optimum. Further, the global food production system also may get constrained in supplementing the food supply for countries like India particularly when climate change events are bound to amplify adverse effects of natural disasters. Hence, there is a need for improved efficiency in input use, tolerance to abiotic stresses as well as high yield potential. Hence, there is an increase in expectations from possible intervention of modern sciences like biotechnology, molecular biology, nano-technology, information technology, computation and electro-mechanics and robotics, remote sensing *etc.* However, the success of these sciences in providing the viable solutions for crop management for farmers largely lies in deep insight into the features of the agro-ecologies as well as plant responses to the environment at the smallest scale with greatest intensity of applications. On the other hand improved genetics of crops facilitated by breeding and management techniques for different agro-ecologies is the key for enhancement and stabilization of crop production. As witnessed during the recent years, our knowledge about the genome of crop plants has enhanced remarkably by advances

in molecular biology and robust techniques to understand the genes and their locations in chromosome. However, this knowledge remains incomplete unless the plant responses to a set of environment and to exogenous treatments are related to genetic information about the crops generated so far. Hence there is an emphasis on plant characterization which is often referred as plant phenotyping.

The traditional phenotyping procedures- which deal with plant characteristics have not allowed a thorough functional analysis and have not led to a functional map between genotype and phenotype. This is often due to sufficient data on phenotype of plant to predict such relations with greater power. A focus on overcoming these shortcomings has led to an emerging and increasingly important branch of biological sciences termed "phenomics" (Furbank, 2009; Furbank and Tester, 2011). Phenomics is a technology that enables high-throughput phenotyping for crop improvement in response to present and future demographic and climate scenarios. Phenomics has been evolved as a novel area of biology and involves high-dimensional phenotypic data at multiple levels of organization for full characterization of the complete set of phenotypes of a genome. A plant phenotype consists of structural, physiological, and performance-related traits of a genotype in a given environment. Plant phenotypes are inherently complex because they result from the interaction of genotypes with a multitude of environmental factors. This interaction influences structural traits associated with developmental and growth of plants as well as physiological traits contributing plant functioning. Both the structural and physiological traits eventually determine plant performance in terms of biomass and yield. Plant responds to various components of its growing environment by adjusting its morphology, anatomy, phenology and cellular metabolism. Consequently growth, development and productivity of crop increase or decrease in favourable and unfavourable environments, respectively. Much of the achievements so far in improving the productivity of crops is attributed to empirical selection for yield and yield components. This was more apparent in favourable growth environment than in those affected by drought, high temperature, salinity etc. Hence, there is now an enhanced focus on traits associated with tolerance to these abiotic stresses. Further genes associated with such traits are the keys for further improvement as plant responses to stresses are manifestation of gene action in cellular and molecular mechanisms. Phenomics is emerging as a science that aims at non-destructive methods that allow screening of genotypes in a large scale and thereby complement genomic efforts to identify genes relevant from crop improvement both under favourable and unfavourable environments.

Why we need phenomics platform?

- The traditional approach with emphasis only on yield components for improvement of crop productivity is not as efficient as it was at the beginning of green revolution.
- Trait based selection is essential for each of the diverse agro-ecosystems vulnerable to climate change
- It is necessary to phenotype plant population that have been generated for genetic dissection of plant responses to stresses
- Multiple studies in phenomics highlight findings, such as relationships between traits and plant growth behaviour. In this way, the challenges of extracting multi-parametric phenotypic information along with the genetic variability can be adequately met.
- Large collection of germplasm of different crops needs to be phenotyped for identifying promising source of stress tolerance.

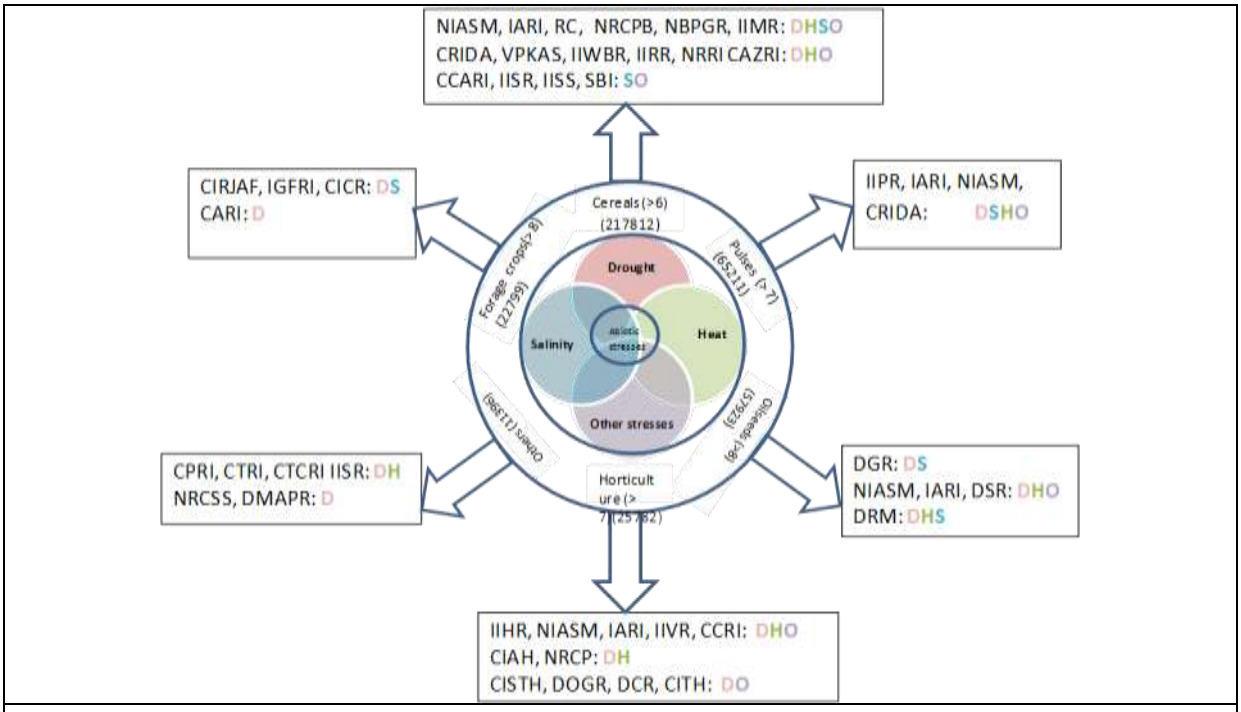

Fig. 1. Institutes involved in abiotic stress research

More than 40 institutes, more than 60 SAUs and private organisations are investigating abiotic stress tolerance in more than 56 crops (Fig. 1) and a total of 4.3 lakh accessions are in germplasm bank needs phenotyping services. In addition, with emphasis on common protocol for phenotyping in the context of global demand for phenotype database of different crops, high throughput and image based screening protocols are gaining immense importance.

In addition to the existing variation in germplasm, new genetic resources are being created regularly by those having expertise in plant genetics and breeding for targeting genes and traits intended for crop improvement. These newly created germplasm in the form of RILs, MAGIC population, association mapping population need to undergo process of phenotyping for targeted traits. Hence, plant phenomics is going to play a crucial role in years to come and has great prospects.

Chapter 2 : Non invasive tools to assess plant responses to abiotic stresses such as drought, heat and salinity

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Introduction

Drought has been a recurring feature of agriculture in India (Srinivasa Rao *et al.*, 2015) and it occurs over an extended period of time and space, making it unpredictable and the losses are not quantifiable easily. But the impact of drought on the techno-economic and socio-economic aspects of agricultural development and growth of the nation is severe and results in huge production and monetary losses. During the period 1900–2014, the number of occasions on which large Indian population got affected from drought was more than any other natural disaster. In the past, India experienced 24 large-scale droughts with increasing frequencies during the periods 1891–1920, 1965–90 and 1999–2012. Long-term rainfall data for India indicate that rainfed areas experience 3–4 drought years in every 10-year period. Of these, two to three are in moderate and one or two may be of severe intensity. Occurrence of drought is very frequent in the meteorological subdivisions like Maharashtra, West Rajasthan, Tamil Nadu, Jammu and Kashmir, and Telangana. The risk involved in successful cultivation of crops depends on the nature of drought (chronic and contingent), its duration, frequency and timing of occurrence within the season and the soil type.

There is increasing evidences that climate change related elements are contributing to accelerated resource degradation and the resultant abiotic stresses. The average increase in temperature in India during 1901 and 2005 has been 0.51^{o}C compared to 0.74^{o}C at global level. The increase was in the order of 0.03^{o}C per decade during 1901-1970 while it was around 0.22^{o}C per decade for the period from 1971 to 2004 indicating greater warming in the recent decades. Increase in the 21st century is projected to vary between 3 to 6^{o}C with southern regions registering 2-4^{o}C increase while the increase (>4^{o}C) would be more pronounced in the northern states and eastern peninsular region. The resultant heat stress would have serious impact on agriculture.

It is increasingly evident that the gains in agricultural output provided by the green revolution have reached their ceiling whereas the world population is

expected to reach nearly nine billion by 2050. The recent plateau in genetic gain in productivity of crop also indicates that possibly we are at attainable maximum productivity of crops with traditional method of crop improvement even with all the favourable factors for crop growth in place for high productivity zones. Therefore in addition to increasing the yield of crop plants in normal soils, there is an absolute need to enhance productivity and stability of crop yield in less productive lands, including salt affected lands. This is more relevant to highly populated countries like India where an estimated 6 to 7 million ha land is affected by salinity/alkalinity and about 2.0 million ha of salt affected land is being reclaimed. Further, it is being predicted that salt affected area is likely to increase to an extent of 16.2 million ha by 2050 mainly due to expansion in irrigated area, intensive use of natural resources responsible for second generation problems and also due to predicted climate change.

In this context, there is a need for concerted effort to improve tolerance to drought, high temperature and salinity are to be incorporated through genetic improvement. This needs suitable traits for introduction into the existing cultivars and we have to search for source of such traits and genetic variability existing for this trait. This is prerequisite for identification of genes associated with these traits that contribute to stress tolerance. Though this approach is not new, the advances in genomics have added new dimension to this approach for enhancing our capacity to develop new cultivars with stress tolerance. Much of these advances is apparent in enhanced capacity to understand genes in crop plants. However, the characterisation of plant responses to stresses can greatly complement genomic efforts.

Destructive phenotyping methods that include harvesting plant responses for assessment of water relations and other physiological responses to stresses limit our studies to very few plants and make this exercise cost and labour intensive. Hence, in the first generation of instrumentation for non invasive studies several equipments such as photosynthesis meters, stomatal conductance meter, SPAD meter, chlorophyll fluorescence meter, NDVI sensors emerged as handy tools for physiologists, breeders and agronomists for field studies. The current phenotyping platforms include a variety of imaging methodologies to obtain high-throughput non-destructive phenotype data for quantitative studies of complex traits, such as growth, tolerance, resistance, architecture, physiology, yield, and the basic measurement of individual quantitative parameters that form the basis for more complex traits (Chen *et al.*, 2014; Li *et al.*, 2014). Here, an attempt has been made to focus on non invasive methods which use images for assessing plant responses. These methods are based on images captured by background system that senses

different bands of wavelength in electromagnetic spectrum. They include visible, infrared, fluorescence, NIR/SWIR, hyper spectral/multispectral *etc*.

Imaging systems

Visible	: Colour, morphology, geometry
Infrared	: Canopy temperature
Fluorescence	: Efficiency of photosystem
NIR/SWIR	: Water content, thickness
Hyper spectral/ multispectral	: Spectral stress indices

Available Imaging Devices for High-Throughput Phenotyping

Visible Light Imaging

In plant science, visible light imaging has been broadly adopted due to its low cost and simplicity. Using this imaging system, with a similar wavelength (ranging from 400 to 700 nm) perception as the human eye, two-dimensional (2D) images can be used to analyze numerous phenotypic characteristics and to record the changes in plant's biomass (Golzarian *et al.*, 2011). To spread the spatial and volumetric information of phenotype images, three-dimensional (3D) imaging approaches have been developed, which could provide more accurate estimations of the morphological features (Clark *et al.*, 2011).

Therefore, during the integration of 2D and 3D image analysis, visible light imaging techniques are popular components for the integrated plant phenotyping platform (Yang *et al.*, 2013). It represents raw data of a phenotype image in spatial matrices based on the intensity values relating to photon fluxes (red~600 nm, green~550 nm, blue~450 nm) of the visible light spectral band. Although, it is the most trivial method in plant phenotyping, the drawback is that visible images only provide physiological information, and the common problem is created by the overlapping adjacent leaves and soil background during segmentation process (Li *et al.*, 2014).

Infrared Imaging

Infrared imaging technologies are used for screening objects of internal molecular movements which emit infrared radiation. Two popular infrared imaging devices- a near-infrared (NIR) and a far-infrared (Far-IR, also called IR thermal) - can be used to screen radiation images. Many studies have combined visible and NIR imaging to detect vegetative indices due to the fact that healthy plants reflect a large proportion of NIR light (800–1400 nm), whereas soil reflects little NIR light. Moreover, soil and

unhealthy plants reflect considerably more red light as compared with healthy plants.

The major advantage of visible light and NIR imaging are that they can assess plant health status response to different stress conditions. Visible and NIR digital imaging techniques are more suitable for screening multi-traits and nitrogen status under stress condition (Rajendran *et al.*, 2009). For drought resistance, IR thermal imaging can be used to visualize temperature differences. A thermal infrared imaging technique has been introduced in both, laboratories and fields, and can characterize mutant screens, drought tolerance, salinity tolerance, osmotic tolerance, tissue tolerance, and Na^+ exclusion. It can be used to compare chlorophyll pigments, leaf color and canopy temperature. Infrared imaging has improved drought resistance and/or salinity resistance research by quantifying the osmotic tolerance in response to drought or salinity stress.

The benefits of the infrared imaging technologies are that they provide spatial resolution and more precise measurement under changing environmental conditions, and in field trials a large number of plots can be imaged at the same time (Li *et al.*, 2014). One limitation of thermal imaging in the field is that it needs to include correction of soil background, wind impact and effects of transient cloudiness.

Fluorescence Imaging

Fluorescence imaging is used from laboratory to field. This imaging technique describes the information about the plant metabolic status that can be obtained by the artificial excitation of the plant photo systems and observation of the relevant responses (Li *et al.*, 2014). It is based on charge-couple device (CCD) cameras with sensitive fluorescence signals, where the signals occur by illuminating samples with visible or ultraviolet light. There are two types of fluorescence (red to far red region and the blue to green region) generated by the ultraviolet illumination ranging from 340 to 360 nm, and is expressed as a principle of underlying multi color fluorescence imaging. This technique offers the simultaneous capture of fluorescence emission, and provides a quick way to probe photosystem II status *in vivo* (Maxwell and Johnson, 2000).

There have been several uses of fluorescence imaging proposed for early detection of stress responses to biotic and abiotic factors before a decline in growth can be measured (Baker, 2008; Jansen *et al.*, 2009; Chen *et al.*, 2014b). To screen large mutant collections and to characterize mutants with different photosynthetic pigment composition, portable fluorometers, and fluorescence cameras are widely used. Furthermore, fluorescence imaging technique provides powerful diagnostic

tool to resolve the heterogeneity problem of leaf photosynthetic performance, and is used in many areas of plant physiology. Most of the fluorescence imaging applications is limited to the seedling level or the single leaves of model crop. However, it is necessary to develop more robust software and standard procedures for the fluorescence image phenotyping, processing, and data analysis.

Spectroscopy Imaging

The use of spectroscopy imaging is very promising for plant phenotyping. It measures the interaction of solar radiation with plants, and originated from remote sensing of vegetation research (Li *et al.*, 2014). Spectral measurements of the electromagnetic spectra can be obtained through multispectral or hyperspectral cameras that are capable of scanning wavebands of interest at high regulation. Multispectral and hyperspectral measurements of the absorption band in the infrared range are used to describe various water statuses that estimate the canopy water content. The best usable examples of spectral measurements is the derivation of a number of reflectance vegetation indices from simple differences between two wavelength reflectance values to normalized reflectance values. The reflected spectra carry the information about plant architecture and health condition, which can be used to evaluate growth characteristics.

Beyond visible and infrared imaging methods, hyperspectral imaging method can divide images into bands, thus providing a huge portion of the electromagnetic spectrum of the images. The high spectral resolution of hyperspectral technologies make it an essential method for detecting the severity of damage caused by insects. The application of spectroscopy imaging is well-suited for field phenotyping when combined with aerial platforms, but the cost of the spectral cameras and its related infrastructures are relatively expensive.

Structural Tomography and Other Imaging

In recent times, modern optical 3D structural tomography and functional imaging techniques have been developed and extended to improve living plant visualization. Functional imaging such as chlorophyll fluorescence imaging and PET (Positron emission tomograpy) are used for finding photosynthetic performance, stress, and focuses on physiological changes. The combination of structural tomography and functional imaging can screen more precise physiological activity of plant. Another novel imaging technique, MRI (magnetic resonance imaging) is used for imaging of internal physiological processes occurring *in vivo*. Screening the dynamic changes in plant functions and structures by the combining technique of MRI and PET provides a novel functional and structural imaging procedure.

The FRET (Förster resonance energy transfer) sensor is another of the non-invasive advanced imaging technologies for high-resolution measurement of small molecules in living tissue based on genetically encoded, ratiometric fluorescent sensors that bind to and report on levels of the target molecule. It is used for molecular phenotyping, and a single FRET sensor can lead to discoveries of multiple pathways and processes involved in the dynamics of the sensor target. The cellular/subcellular location of interest has to be properly characterized and expressed by a FRET sensor, and measurements can be easily acquired with high temporal and spatial resolution. As the application example, FRET has been used in plant tissue to study calcium and zinc dynamics with subcellular spatial and real-time temporal resolution, the characterization of sugar transport in roots of insect seedlings, the identification of novel sugar transporters. To address many basic questions of plant growth and development, FRET could be an outstanding technology for advanced phenotyping.

Each of these digital photonics-based systems acquires phenotype image data from plant laboratories, greenhouse or fields, and monitors these with special imaging sensor via a remote system. Table 1 illustrates a summary of optical photonics-based key techniques and applications in advanced phenotyping.

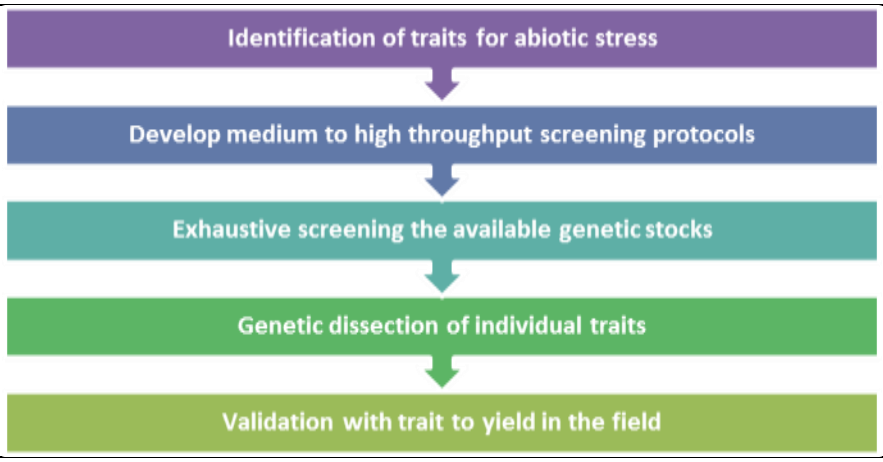


Fig. 1. Application of non-destructive phenotyping in genetic dissection of trait

Table 1. Plant Phenotyping Platforms

Name	URL	Description
PHENOPSIS	*http://bioweb.supagro.inra.fr/phenopsis*	Represents specific setups for automated phenotyping, allowing a culture of approximately 200–500 Arabidopsis plants in individual pots with automatic watering and imaging system.
WIWAM	*http://wiwam.be*	Like PHENOPSIS, WIWAM is an automated imaging platform simultaneously handling a large number of plants and measuring a variety of plant growth parameters with automatic watering and imaging system at regular time intervals (Skirycz *et al*., 2011).
PHENOSCOPE	*http://www.observatoirevegetal.inra.fr/observatoirevegetal_eng/Scientific-platforms/Phenoscope*	This automated phenotyping platform is an integrated device, allowing simultaneous culture of 735 individual Arabidopsis plants and high-throughput acquisition, storage and analysis of quality phenotypes (Tisne *et al*., 2013).
GROWSCREEN	*http://www.fz-juelich.de/ibg/ibg-2/EN/methods_jppc/GROWSCREEN*	This platform was developed to study plant leaf growth fluorescence and root architecture from seedling under control condition for visual phenotyping of large plant populations (Jansen *et al*., 2009).
TraitMill	*http://www.cropdesign.com*	High-throughput gene engineering platform developed by Crop Design. This is a highly versatile tool that enables large-scale transgenesis and automated high resolution phenotypic plant evolution.
PHENODYN	*http://bioweb.supagro.inra.fr/phenodyn*	This platform monitors plant growth and transpiration rate with stressful environmental condition.
Plant Scan	*http://www.csiro.au/Outcomes/FoodandAgriculture/HRPPC/PlatScan.aspx*	This is an automated high-resolution phenomic center which provides non-invasive analysis of plant structure, morphology and function by utilizing cutting edge information technology including high resolution cameras and 3D reconstruction software.
LemnaTec	*http://www.lemnatec.com*	Visualize and analysis 2D/3D non-destructive high-throughput imaging, monitor plant growth and behavior under entirely controlled conditions in a robotic greenhouse system.
QubitPhenomics	*http://qubitphenomics.com*	Integrated conveyor and robotic high-throughput plant imaging system for the laboratory, growth chamber and field phenotype screening and phenotyping.

HRPF	*N/A*	High-throughput rice phenotyping facility (HRPF) designed with two main sections: rice automatic phenotyping (RAP) and yield trait scorer (YTS). This high-throughput platform was developed for automatic screening of rice germplasm resources and populations throughout the growth period and after harvest.

Table 2. Imaging systems for plat phenotyping

Imaging system	Description	Phenotypic trait parameters	Application purpose
Visible light	The visible light imaging technique is camera sensitive and produces gray or color scale images.	Image-based projected biomass, dynamic growth, color, shape descriptors, root architecture, seed morphology, panicle traits, etc.	This imaging technique can be used to assess plant growth status, biomass accumulation, nutritional status, or health status (Golzarian *et al*., 2011).
Thermal infrared	Thermal infrared imaging sensor includes near-infrared, multispectral line scanning cameras. This imaging technique produces time series or single-time-point analysis based data.	Leaf area index, shoot or leaf temperature, surface temperature, insect infestation of grain, leaf and canopy water status, composition parameters for seeds, disease severity, etc.	This imaging technique used to characterize the plant temperature responses to the water status and transpiration rate and detect difference in stomatal conductance of the plant for adoption abiotic stress (Chen *et al*., 2014).
Fluorescence	Fluorescence imaging technique detects chlorophyll and other fluorophores signals using fluorescence cameras.	Photosynthetic performance, quantum yield, non-photochemical quenching, leaf disease severity assessments, leaf health status, etc.	It provides a fleet way to probe photosystem status in vivo, diagnosing early stress responses before decline growth, useful for disease detection in genetic disease resistance (Chen *et al*., 2014b), mapping QTLs for growth-related traits, characterizing mutants with numerous photosynthetic pigment compositions, etc.
Hyperspectral	This imaging technique use hyper spectral, thermal cameras produced continuous, or discrete spectra raw data.	Water content, leaf growth and health status, panicle health status, grain quality, pigment composition, etc.	This imaging technique used to measure spatiotemporal growth patterns during the experiment and provide insight into the diversity of growth dynamics (Chen *et al*., 2014b).
CT	It is based on X-ray digital radiography/computed tomography.	Grain quality, tiller, morphometric parameters, water content, flow velocity, etc.	This imaging is widely used to asses tissue density, measuring tiller numbers, grain quality, etc.

PET	Positron emission tomography.	Water transport, flow velocity, etc.	This is used to visualize distribution and transportation of radionuclide-labeled tracers involved in metabolism-related activities.
MRI	Magnetic resonance imaging.	Water content, morphometric parameters, etc.	The purpose of this imaging technique is to visualize metabolites, provides structural information, and monitor internal physiological processes occurring in vivo.

Table 3. Image analysis software

Name	URL	Description
ImageJ	http://imagej.nih.gov/ij	A popular, powerful, and extensible application used to process and measure alarge quantity of phenotypic traits captured by images.
IAP	http://iap.ipk-gatersleben.de	Large-scale plant phenotyping image analysis software for different species basedon real-time imaging data obtained from various spectra.
HTPheno	http://htpheno.ipk-gatersleben.de	A high-throughput (top and side view) plant phenotyping image analysis pipeline implemented as a plug-in for ImageJ.
Rosette tracker	http://telin.ugent.be/~jdvylder/RosetteTracker	Time-lapse visual, chlorophyll fluorescence, or thermal sequence of image analysis tool for quantification genotype effects of Arabidopsis thaliana, implemented as aplug-in for ImageJ.
PANorama	http://ricediversity.org	Flexible software which simultaneously measures multiple architectural andbranching phenotypes from images.
HPGA	https://www.msu.edu/~jincn/HPGA	A high-throughput phenotyping tool for plant growth modeling and functional analysis.
Phenophyte	https://vphenodbs.rnet.missouri.edu/PhenoPhyte/index.php	A web-based application which measures area-related phenotypic traits from imagery and multiple experimental setup.
SmartGrain	http://www.nias.affrc.go.jp/qtl/Smart Grain	Image analysis software for high-throughput phenotyping measurements of seed shape.
HYPOTrac	http://phytomorph.wisc.edu/HYPOTrace/download/index.htm	Automated hypocotyl growth and shape measuring software from grayscale images of Arabidopsis seedlings.
LAMINA	http://lamina.sourceforge.net	Automated leaves image analysis tool which measures a variety of characteristics related to leaf shape and size.

Leaf Analyzer	http://leafanalyser.openillusionist.org.uk/doku.php	An automated software for rapid and large-scale analyses of leaf shape variation.
Leaf Processor	http://gips.group.shef.ac.uk/resources.html	An application that semi-automatically stores a number of single-metric parameters and PCA analysis for leaf shape and size including contour bending energy.

Table 4. Traits for phenotyping

Organ	Phenotypic trait
Cell	Cell turgor, size, division
Tissue	Mesophyll conductance, PSII efficiency, stomatal conductance
Leaf	Photosynthesis rate, chlorophyll content, leaf shape, orientation, leaf expansion rate
Root	Length, architecture, biomass etc
Whole plant	Leaf no, yield component, water status
Canopy	Canopy temp, LAI, Biomass/area

Table 5. Types of traits for phenotyping

Physiological trait	Performance related trait	Structural trait
Canopy temperature	Biomass/ha	Leaf area index
Water content	Seed yield	Leaf number
Rate of photosynthesis		Leaf expansion rate
Mesophyll conductance		Number of layers
Cell turgor		Cell size

High throughput Plant phenotyping Initiatives

The throughput of a system is the amount of things it can do or deal with in a particular period of time. In plant phenotyping systems, throughput refers to the number of individual units at particular organizational levels within plants, or at the plant or canopy level, that can be analysed for a particular set of traits at a given time. Plant phenotyping has been a part of crop and variety selection since the time of human civilization. It has become common practice in plant breeding for selecting the best genotype after studying phenotypic expression in different environmental conditions

In recent years, there has been increased interest in development of high throughput phenotyping tools and techniques for screening of agronomic, physiological, and biochemical traits expressing especially under abiotic stress. These techniques have become much more advanced and have now entered the era of high-throughput field phenotyping. Several phenotyping platforms have been

developed around the world (table), which are fully automated facilities in greenhouses or growth chambers with robotics, precise environmental control, and remote sensing techniques to assess plant growth and performance. Several reports available on different aspects of phenotyping which is scattered among different source of information. Some of them are summarized in Table 1.

Table 6. List of works describing the use of automated high-throughput platforms to study plant responses to different stresses

Plant species	Type of stress	Type of automated analysis	Platform name/origin	Study/Reference
Tobacco	Biotic stress	Thermo imaging, TLCFIM	Self construction	Chaerle *et al*. 2006
Bean	Nutrient deficiency, biotic stress	RGB (top view), Thermo-imaging, TLCFIM	Self construction	Chaerle *et al*. 2007
Wheat	Salt stress	RGB (multiple views)	LemnaTec	Rajendran *et al*. 2009
Arabidopsis, Tobacco	Drought stress, chilling stress	RGB (top view), KCFIM	GROWSCREEN (self construction)	Jansen *et al*. 2009
Wheat, Barley	Salt stress	RGB (multiple views)	LemnaTec	Harris *et al*. 2010
Arabidopsis	Drought stress	RGB (top view)	WIWAM	Skirycz *et al*. 2011
Barley	Salt stress	RGB (multiple views)	LemnaTec	Golzarian *et al*. 2011
Soybean	Drought stress	RGB (two-views)	GlyPh (self construction)	Pereyra-Irujo *et al*. 2012
Arabidopsis	Drought stress	RGB (top view)	PHENOSCOPE	Tisné *et al*. 2013
Arabidopsis	Drought stress	RGB (top view)	PHENOPSIS	Bresson *et al*. 2013
Barley	Drought stress	RGB (multiple views), Thermo-imaging	Self construction, Semi automated	Cseri *et al*. 2013
Barley, (Wild species)	Drought stress	RGB (multiple views)	LemnaTec	Honsdorf *et al*. 2014
Grapevine	Drought stress	RGB (multiple views)	LemnaTec	Coupel-Ledru *et al*. 2014
Tomato	Drought stress	RGB (multiple views), hyperspectral NIR, SLCFIM	LemnaTec	Petrozza *et al*. 2014
Arabidopsis	Drought stress	RGB (top view), hyperspectral NIR	LemnaTec	Harshavardhan *et al*. 2014

Plant species	Type of stress	Type of automated analysis	Platform name/origin	Study/Reference
Barley	Drought stress	RGB (multiple-views), hyperspectral NIR,	LemnaTec	Chen *et al*. 2014
Wheat	Drought stress	RGB (multiple views), Thermo imaging	Self construction, semi-automated	Fehér-Juhász *et al*. 2014
Arabidopsis	Heat stress, drought stress	RGB (top view)	PHENOPSIS	Vasseur *et al*. 2014
Barley	Salt stress	RGB (multiple views)	LemnaTec	Schilling *et al*. 2014
Rice	Salt stress	RGB (multiple views) SLCFIM	LemnaTec	Hairmansis *et al*. 2014
Brachypodium	Nutrient deficiency	RGB (multiple views)	LemnaTec	Poiré *et al*. 2014
Arabidopsis	Drought stress	RGB (top view)	WIWAM	Clauw *et al*. 2015
Barley	Drought stress	RGB (multiple views)	LemnaTec	Neumann *et al*. 2015
Sorghum	Nutrient deficiency	RGB (multiple views), hyperspectral NIR	LemnaTec	Neilson *et al*. 2015
Pea, Field cultivars	Cold stress	RGB (multiple views), KCFIM	PlantScreen	Humplík *et al*. 2015

Phenomics facilities

India

- ICAR-NIASM, Baramati
- ICAR-IARI, New Delhi
- ICAR-IIHR, Bangalore
- ICAR-CRIDA, Hyderabad

China

- CAAS, Beijing
- Harbin
- Agripheno, Pudong, Shanghai

Australia

- Victoria laboratory, Horsham,
- ACPGF, Adelaide

Germany

- IPK Gatersleben
- Plant Science Research Centre, Julich

France

- INRA science and impact, Dijon
- INRA Montpellier

Netherland

- Keygene

Italy

- Metaponto (ITALY)

United Kingdom

- Rothamsted Research Station
- Aberystwyth University

Canada

- McGill Phenomics Platform, Canada

USA

- Donald Danforth Plant Science Center, St. Louis, Missouri, USA
- Arkansas State University, AR, USA

High throughput plant phenomics facility at NIASM

The installation of facility was completed on September 01-09-2015 by LemnaTec, GMBH, Germany under National Innovations for Climate Resilient Agriculture (NICRA). The facilities allow screening of 216 plants at a time and it is possible to screen thousands of lines for responses of plants to a particular phase of crop growth with staggered planting and growth initially under natural condition. The facility is equipped with cameras for acquiring images in visual, infrared and near infrared range for morpho-physiological traits, surface temperature and plant water relations respectively. It has programmable and automated irrigation and weighing system to create and monitor soil moisture stress. Automated temperature regulation can allows screening for high temperature tolerance. Robust software and hardware allow acquisition, storage and analysis of huge set of images. Research projects facilitated by this technology vary from large scale screening of early growth and tolerance to abiotic factors like soil moisture stress, salinity and nutrient imbalance. It has diverse application ranging from phenotyping for known traits to identification of novel or surrogate traits associated with stress tolerance. The facility has been provided with dedicated power supply and also the power backup for uninterrupted functioning.

Objectives

- To develop plant phenomics protocols for characterization of responses of crops to abiotic stresses mainly drought, high temperature and salinity
- To identify alternative traits to accelerate characterisation of plants responses to complex and difficult to measure traits associated with stress tolerance
- To identify promising genotypes that have attributes contributing to stress tolerance
- To identify traits and genes associated with tolerance to drought, high temperature and salinity
- To complement efforts of plant breeder and molecular biologists involved in investigation of genes associated with tolerance to abiotic stresses in field and horticultural crops
- To develop plant phenome database by employing common methods and comparable procedures
- To facilitate development low cost indigenous plant phenotyping tools for controlled and field experiments by validation of results in HTP phenotyping

Fig. 2. LemnaTec Scanalyzer process and function

Optimisation of field phenotyping protocols

Optimisation of phenomics protocol

For efficient phenotyping image based protocols needs to be optimised for each crop. Hence several experiments were carried out with different crops such as mungbean,

chickpea, soybean, maize and wheat. Methods have been standardized to predict biomass of mungbean based on area sensed by visible camera as derived from specifically designed image analysis configuration. Methods are being optimised for other crops.

Extraction of images from original images

Fig. 3. Extraction of images from original images

Visible area as surrogate parameter

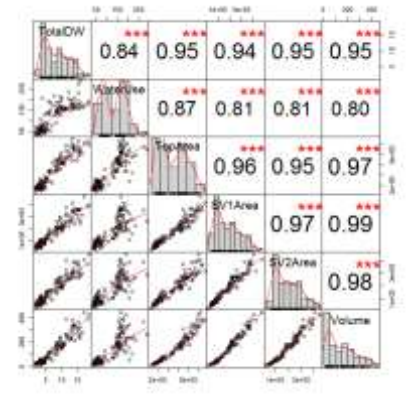


Fig. 4. Visible area derived from 24 Genotypes grown with or without water stress in three replications (144 pots). Area and volume derived from images could explain the variation in water use and biomass (Total DW) in mungbean. This optimised method can now be used to assess mungbean genotypes for their capacity to utilise soil moisture effectively during the growth period.

Field Phenomics initiatives

Optimisation of thermal imaging

The development of new germplasm with higher tolerance towards water stress is a main objective for many breeding programmes. However, breeding for drought tolerance is a complex task because of the absence of precise screening methods. To hand pick superior alleles, it is essential to evaluate large numbers of genetic

resources under actual field conditions. Genotypic variability in terms of performance under water conditions may be the result of differences in water uptake from the soil at dissimilar rates (Berger *et al.* 2010). The commonly used methods for phenotyping genotypic performance to drought are laborious and destructive (Roy *et al.* 2011). Water stress induces stomatal closure in plants to prevent transpiration and thus loss of water. This leads to an increased canopy temperature. Canopy or leaf temperature, as measured using thermography (thermal infrared sensing or imaging), provides a powerful monitoring tool for a broad range of plant stresses that affect any aspect of plant water relations specifically stomatal conductance, because a major determinant of leaf temperature is the rate of evaporation or transpiration from the leaf. Thermography is a non-invasive imaging method that gives access to temperature by use of the blackbody law. In the domain of plants, the measurement of the temperature is an important physical parameter. Additionally, apparent temperature provided by thermography is also indirectly related to other functional or structural parameters, like leaf orientation, heat capacity, surface properties, infrared (IR) absorption, and transpiration rate (Kana and Vass, 2008; Fiorani *et al.*, 2012). Thermography has therefore been widely tested and shown useful on plants at various observation scales from canopy down to single leaf and in various biological contexts, including, for instance, evaluation of stomatal aperture (Leinonen *et al.*, 2006), plant water content (Wang *et al.* 2010a, 2010b), plant freezing (Wisniewski *et al.*, 2008), leaf water loss (Raina *et al* 2016) and the development of pathogens (Chaerle and der Staeten, 2001; Chaerle *et al.*, 2007; Belin *et al.*, 2013). Both infrared thermometry and infrared thermography have been widely used in field studies for both irrigation scheduling and for genetic screening particularly based on stomatal response to drought and salinity stress and to select for stomatal mutants especially that involving altered abscisic acid (ABA) metabolism affecting stomatal closure.

Although infrared thermometry can be used as a cheaper alternative for screening for drought tolerance the much greater time and labour requirement means that it is nothing like as well suited for high-throughput systems as is thermography, especially when automated image analysis procedures are used.

Prototype of tools for image based phenotyping

Taking into consideration the need to accelerate phenotyping in field efforts have been made to develop phenotyping tools. A hand operated track mounted trolley was designed for imaging purpose which hosts a camera and a Lap Top PC. The system acquires images of each plot in the experimental field after recognising the barcode. Images are stored with plot name. Tools have also been developed to rapidly analyse these images. Promising results have been obtained with image

acquisition and analysis tool. This field based, semi-automated platforms potentially allow high-throughput phenotyping at a low cost.

Phenotyping for canopy temperature

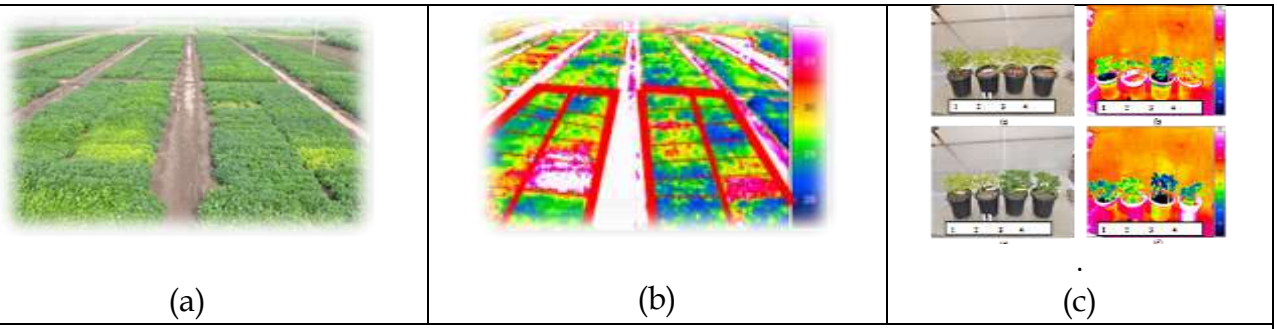
Identification of superior genotypes

VC-6173-C, a mung bean cultivar, could keep its canopy cooler than local variety of mung bean even under drought condition. This genotype was able to maintain a high stomatal conductance under drought, which helps plant keeping its canopy cooler.

The variation for canopy temperature among low Leaf water loss (LWL) and high LWL mungbean genotypes under drought conditions was monitored in field. A cooler canopy was revealed in high LWL genotype and higher canopy temperature observed in low LWL genotype. This can be explained by a sharp reduction in stomatal conductance of this genotype when compared to high LWL genotype.

Genotypes were identified which have higher yield with higher minimum canopy temperature and those with cooler canopy temperature as compared to locally adapted cultivars. Higher yield in some genotypes despite its higher CT throughout its growth period as compared to other genotypes could be partially attributed to high net assimilation rate and quantum yield, as indicated by chlorophyll fluorescence parameters.

CTD measured at the reproductive stage explained a major proportion of the variation in grain yield both under sufficient and deficit soil moisture conditions in soybean. Simple methods were developed to process the thermal image.



(a) (b) (c)

Fig. 5. Soybean field as viewed through visible imaging (a) Infrared imaging system (b) and pot culture (c)

Phenotyping for photosynthetic efficiency under stress

All plant material that contains chlorophyll pigments will emit red fluorescence upon illumination. This chlorophyll fluorescence has an enormous potential as a non-destructive probe to investigate the physiology and structure of the photosynthetic apparatus. Chlorophyll fluorescence is one of the most popular techniques in plant physiology because of the ease with which the user can gain detailed information on the state of photosystem II (PSII) at a relatively low cost. Chlorophyll fluorescence imaging provides information on photosynthetic performance without Destruction or contact with the living plant. The chlorophyll fluorescence involves an emission of red light from chlorophyll a pigments that can be used to assess photosynthetic functions, thereby allowing for plant health monitoring (Maxwell and Johnson, 2000; Takayama and Nishina, 2009). The imaging technique of chlorophyll fluorescence has been used to evaluate The heterogeneous distribution of photosynthetic activities over a leaf surface, and thus to detect photosynthetic dysfunctions caused by biotic and abiotic stress factors and recently, the objects of chlorophyll fluorescence imaging have been scaled up to the levels of a whole plant. The recently increased interest in the use of chlorophyll fluorescence techniques has been mainly due to research in crop improvement and in particular for the screening of desirable plant traits and linking genomic information with phenological responses (Furbank et al., 2009).

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Chapter 3 : Water stress – Watering and precision stress management with Lemna Tec technology

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Introduction

In general it can be stated that in all cases where plants are not watered to full saturation, but where specific, accurate soil humidity levels are to be maintained for each single plant, automated watering is almost the only way to perform such tests when larger numbers of plants are involved.

For this purpose LemnaTec has developed specific water management hard- and software tools particularly useful in connection with high-throughput conveyor systems, but also conveniently used as stand-alone modules.

The following text provides a short description of the hardware and different watering modes which can be employed.

Watering hardware

Each plant is identified every time before individual watering, either by barcode or preferably by using RFID-chips. The plants are weighed before watering and the results are written to the central database, which controls watering as well as imaging. Depending on the watering mode programmed beforehand, the plants will then be watered individually, using a high-precision hose pump, which is able to deliver pure water as well as nutrient solutions or saline water.

Fig. 1. Watering and watering station. Plants are lifted pneumatically from the belt to obtain precise weights.

In all cases plant weights will be stored in the database each time a plant passes the scales and is weighed, either to document the evaporation process or to check the plausibility of watering volumes. The whole process is generally designed to take not more than 30 seconds, but longer times may be needed for high-precision watering combined with high individual volumes.

Where to water – top or bottom?

LemnaTec adapts watering to the specific needs of the plants, which may even change in the course of one growth period. Watering from top and bottom both have specific features which might be either advantageous or disadvantageous, depending on and the aim of the experiment.

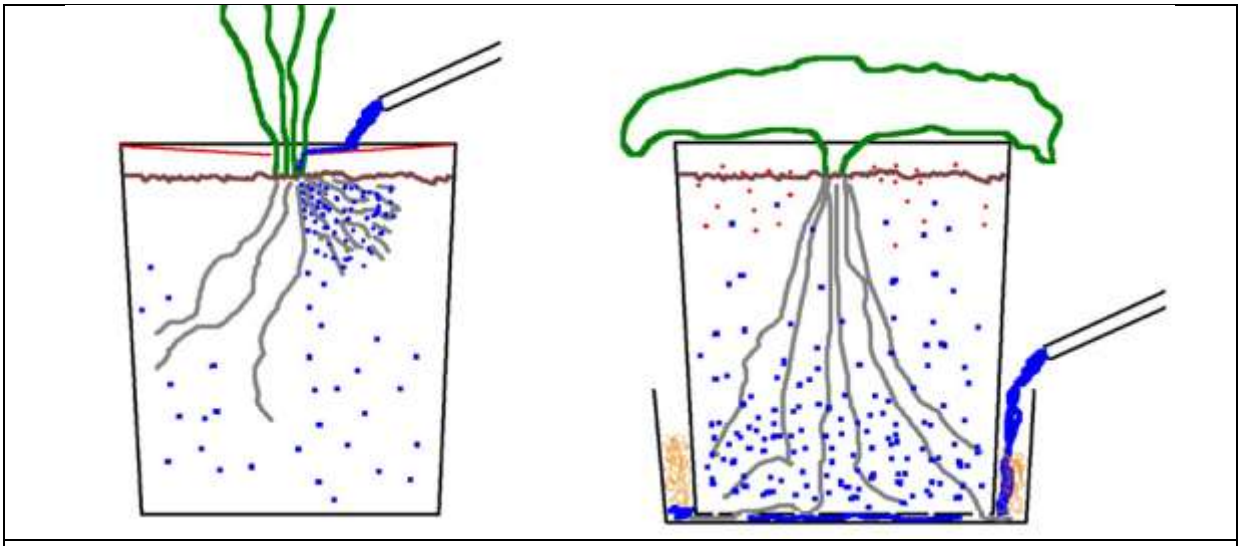

Fig. 2. Schematic sketch showing different features of top and bottom watering

Watering from top

Top watering can be done with pots without drainage holes – where excess watering is methodically avoided by target watering – as well as with open pots. Almost any amount of water can be added to plants of any size as even small plants will be able to get water close to the soil surface. Nevertheless, plants covering the whole pot diameter (e. g. salad) or those having very sensitive lower leaves will interfere with the watering nozzle, even if this is made of highly flexible rubber. For drought stress experiments where low amounts of water are to be added repeatedly, roots may unnaturally accumulate close to the surface in the high-moisture area. On the other hand, almost any soil can be used, as water transport can follow gravity and does not depend on soil capillarity.

Watering from bottom

For the plant to take up water from the bottom, pots with drainage holes and soil with a sufficient capillarity are essential. Nevertheless, there always remains the risk that small amounts of added water may not disperse sufficiently. This could be a problem for small plants with a small root area. Soils with a gradient to higher humidity in lower soil layers can simulate longer drought periods and also humidity fed by ground water. Researchers may check if a more " natural" humidity gradient like this may specifically stimulate root growth or lead to more realistic root phenotypes. Due to evaluation of the top soil, salt accumulation in top layers can be the result of intentional simulation of saline conditions or just a negative side effect. There is a certain risk that plant roots may leave the pot through the drainage holes and take water directly from the waterphase in the saucer.

Degrees of automatisation

Plants are either put on the scales manually (stand-alone system) or transported there via conveyor belt systems, if desired in combination with imaging or comprehensive greenhouse management systems. Minimum options, including just a conveyor belt and a pump and weight system, are available as well.

It is particularly important for fully automated systems – in order to determine several important factors with presumably high biological relevance – that watering plans should include much more than only watering modes, for example:

1. The watering frequency for every day – once, several times, invariably when arriving at any watering station.
2. The time-frame in which watering may take place. While for some plants it may be preferable to water them late in the evening to minimise water stress at night, for others late watering may induce the risk of humidity-related diseases.

The days on which watering can take place

1. An alternative protocol about what is going to happen if a predefined water application could not take place (repeat, repeat partially, just carry on).
2. A minimum amount of water applied to guarantee for example the flooding of the whole soil surface in order to provide an even water distribution. This value may also be used to imply a defined drought stress repeatedly inflicted on the plants.)

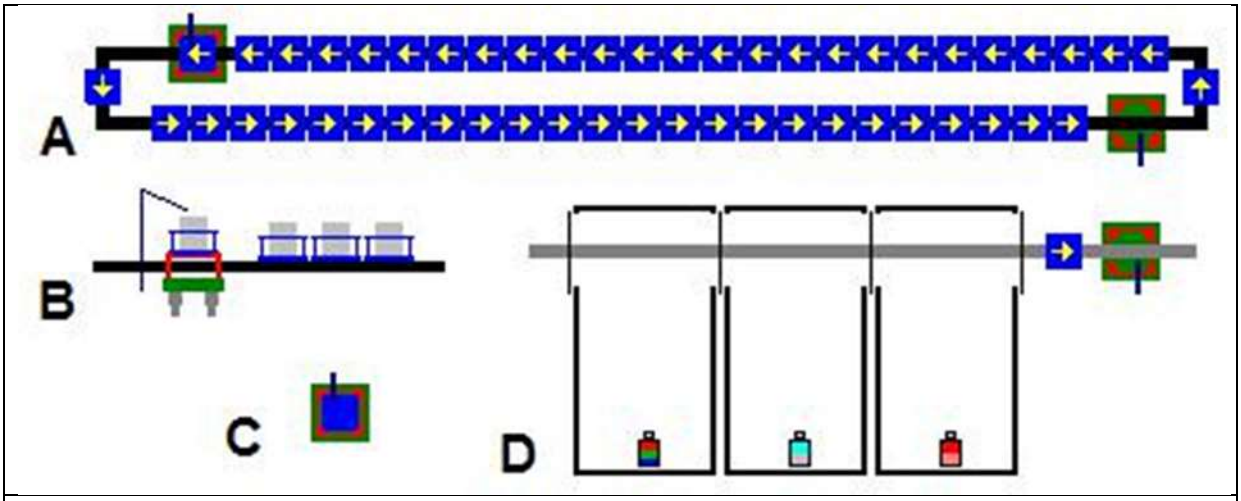

Fig. 3. Options for watering systems:
A: A pump and watering station as part of a greenhouse management system, which may be stand-alone and simple as the one shown here or part of a large multi-line system;
B: Schematic system from the side with scales (green) and pneumatic units (grey), lifting the pallet from the belt for weighing;
C: Stand-alone unit without conveyor belt, linked to a computer system;
D: A watering unit may be directly linked with imaging units, to water plants after imaging.

3. Other triggers that induce watering, e. g. those based on results from former imaging (e. g. the symptom of leaf rolling ...)

These factors determine when watering is actually performed, even if plants arrive at the watering stations more frequently during the day due to imaging or greenhouse rotation. The following part first describes the basic watering modes and subsequently specific watering protocols which might be created based on these modes.

Mode 1 – dose watering:

1. An individually definable amount of solution is delivered to each plant without taking any weights into consideration. Application of this mode is mostly:
2. Delivery of nutrient solutions or salt solutions, as an addition of fertilisers or stressors does generally not relate to actual water loss, but a defined dosage per plant is needed.
3. Exchange of water or salt solutions in hydroponic or soil substrates with excess watering to ensure full exchange of enriched solutions.

Mode 2 – target watering, standard method:

1. For each plant a target weight is defined up to which it is subsequently watered when it arrives at the watering station and the watering plan actually allows watering (watering frequency may be restricted, hourly values limited or repetitions scheduled).
2. The target weight might include not only the pot and solid dry weight (which should remain constant for all pots within a test series wherever possible), but also for example estimates derived from imaging of shoot and root biomass growing over time. Target weights may be changed at every point in time.

The application of simple target watering mainly achieves:

1. To keep the humidity in relatively large and well-watered pots on a near to constant level,
2. To generate well-defined drying down schemes,
3. To maintain salinity of pots on a relatively constant level by replacing evaporated water,
4. To perform surplus watering with water or saline solutions while keeping the surplus to a minimum, thus avoiding extensive leaching of soil material.

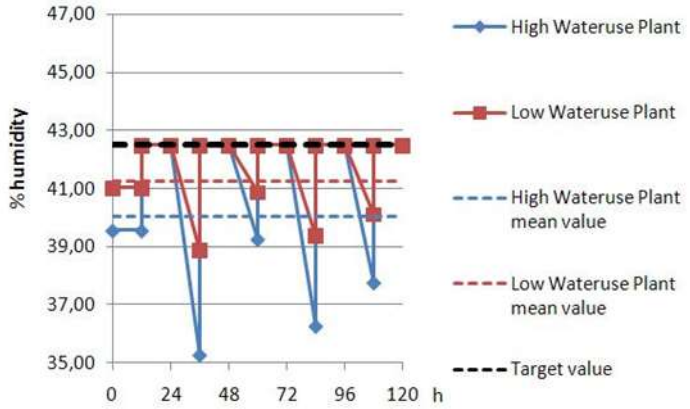

Fig. 4. Watering to a predefined target weight corresponding to a defined percentage of soil humidity will bring the soil humidity for all plants to the same value only once a day. Plants that need much water will evaporate more, and thus the average value during the day will be significantly lower than that for plants needing less.

Mode 3 – dynamic/adaptive target watering:

While simple target watering creates similar soil humidity values for all plants only immediately after watering, dynamic watering adds a definable offset (in % of water

loss under the target weight, which may be limited to an absolute value) to provide plants consuming large amounts of water with more water, thus compensating for their faster loss. As a result not the maximum, but the average soil humidity level is kept near to constant, independent of the individual water consumption of each plant caused by size or changing climatic situations.

The most important application of this mode is with cases where the soil water capacity is not too large in relation to the daily evaporation volume of the plant, due to small pots or low water availability in drought stress experiments (where low soil water humidity is to be maintained).

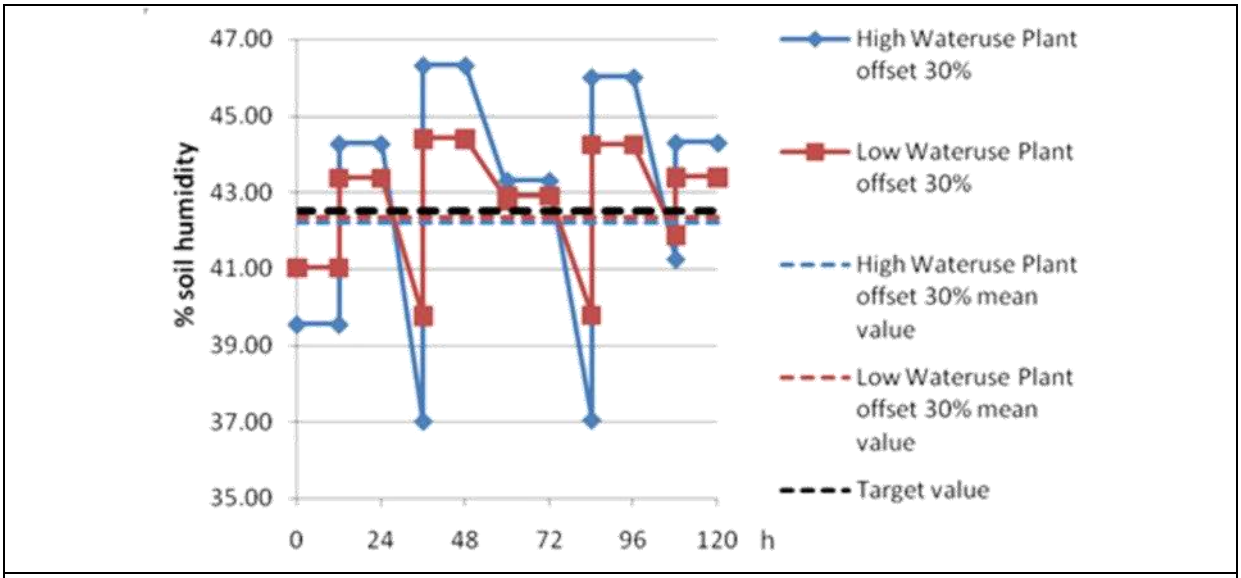

Fig. 5. Dynamic watering takes the higher consumption of water by some plants into account by adding a percentage offset to the water consumed relative to the target value. In most cases a 30 % offset is sufficient to keep the average humidity of plants with different water demand nearly constant over time. Values depend on absolute water consumption, water holding capacities and pot sizes.

Examples for applications

Defined dry-down slope for plants of different sizes over defined time ranges

While drying down over a longer period is quite normal in natural climates, simulating a similar stress pattern in the greenhouse is a complex process. Due to the low amount of soil available for each plant, the drying process needs finally to be simulated by a sequence of incrementally reduced target weights. Such watering can be easily programmed by setting the target weight lower with every watering. It should be considered that in the end the plant with the least consumption defines how pots can dry down, if the process should be as fast as possible (see Fig. 5). Such drought schemes with plateau phases on different dryness levels might be much

more useful and stable to measure a genetic, physiological or proteomic response to specific stress conditions.

The slope is chosen to be only slightly less shallow than the water reduction of a plant with less demand for water. But on day 8 less water is evaporated than the expected 5% per day. This form of programmed drying provides the best possible comparison between plants as the stress is nearly identical. By using dynamic watering (see above), the average soil humidity could be held even more constant than in the example shown here for a predefined target weight.

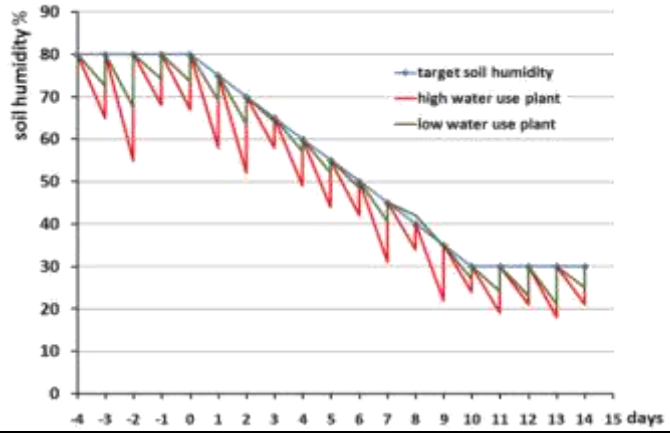

Fig. 6. Fast, but consistent drying down of all plants from 80 to 30% soil humidity.

Alternating drought cycles between lower and upper target values

By setting a rather high minimum watering volume, plants only get watered if adding the minimum volume or more water does not surpass the target value again. Thus plants will dry down as fast as they can, all facing nearly the same maximum stress. And, nevertheless, all will definitely survive independent of the actual water consumption rate of each individual plant.

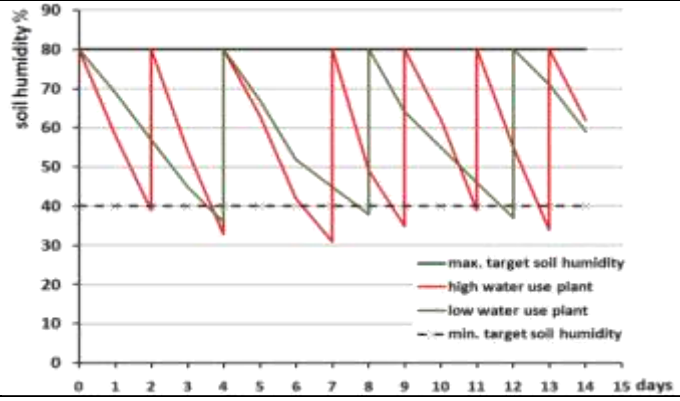

Fig. 7. In this example plants are only watered when the humidity level has fallen below 40 % of soil humidity. Different plants reach this level at different time intervals due to different evaporation rates. While the plant with the high water

demand is watered 6 times within 14 days, the plant with the low demand falls below the 40 % level only 3 times.

Monitoring plant reaction to different kinds of drought stress

The comprehensive monitoring of plant reactions to different drought stresses becomes both measurable in the amount of water the plant needs at different soil humidity levels and in the visible plant phenotype at any of the monitored wavelength ranges.

By integrating enough weighing stations, the decrease of weight can be followed up more thoroughly than with weighing only at the watering stations. Based on water consumption and results from image-based leaf area estimations, water consumption efficiencies can be calculated and normalised.

The core of the LemnaTec Scanalyzer 3-D technology, the imaging units, can provide a wide range of parameters representing different plant reactions. For details on the reactions described in the following survey, please consult the respective LemnaTec papers.

Visible imaging

1. Plants change leaf orientation when heavily stressed by drought
2. Plants may start leaf rolling under stress conditions
3. Longer high-stress conditions reduce growth rates of the plants and can alter leave colour.

NIR-imaging

Leaf water potential is reduced under drought stress. NIR-imaging of the water band (1450-1550 nm) shows strongly reduced absorption for plants loosing water due to drought stress.

Fluorescence imaging

The intensity of chlorophyll fluorescence will change if plants have to stop assimilation and need to eliminate photonic energy by other means than chemical reactions.

IR-imaging

To minimise water loss, plants minimise evaporation. As a consequence, leaf temperature measured by infrared imaging rises when the evaporative cooling is stopped.

The examples show that the LemnaTec detection systems provide a wide range of sensors and quantitative measurements to investigate how plants react on drought stress at different time scales.

Conclusion

The automated plant watering employed by the LemnaTec systems provides a unique and very efficient way to mimic stress patterns similar to those in the field, homogeneously for all plants of a batch, while at the same time using all advantages of the controlled greenhouse environment, including application of different, defined stressor schemes to different plant groups growing side by side. Particularly for drought and salinity tests with large numbers of plants, an automated water management system based on individualised plant water supply is essential. It can impose specific stress on the samples and keep factors as constant as possible within compared replicates or groups, even if these differ in size or daily water consumption. The graphs show that every drought is unlike any other drought, and different watering regimes − which can all be implemented in programmed watering − create rather different stress patterns. The scientist is then required to select what he or she needs for the specific environmental conditions to be simulated.

At the same time and in full correlation to the quantitative measurement of water consumption and water status in the soil, the imaging with a range of different sensor systems allows the scientist to see which strategies the analysed plants use to cope with the precisely defined stress situations. Such quantitative measurements as a key to dynamic plant phenotyping can be used in gene identification (e. g. QTL-analysis), selection for further breeding or stability and performance testing of newly developed lines.

(Source: LemnaTec guide)

Chapter 4: LemnaControl Planning watering jobs

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Developing a watering plan

The watering plan wizard helps you to develop a customised watering scheme.

- LemnaControl >> Configurations >> Watering plan >> Change selection

A watering scheme is essentially a table listing:

- the time points when to water plants
- which plants should be watered
- the amount of water a plant should receive

The first step is to select a set of samples for which a single watering plan is going to be applied homogenously

- ... >> Change selection
- ... >> Attribute >> contains: wheat
- ... >> CarID >> corresponds to: 1

In this example, we first make a selection of samples by picking only those whose attribute contains the substring "wheat". We thereby capture samples with attribute labels "WheatB07-X5", and "WheatB07-X2".

The second filter demonstrates a more stringent sub-selection; here only the CarID that matches the value "1" is retained.

Notice: the selection of the target sample set for a specific watering plan is done incrementally.

- ... >> **Selection of a pump configuration**

Most of the systems have only one pump installed. However, you can install more pumps in order to supply the plant with different nutrition resources. For example, pump 1 provides buffer solution A, and pump 2 supplies buffer solution B. You may also define different pump configurations, e.g. a slow pump configuration to avoid

forming ditches in the soil, and a fast pump configuration to swamp a tray carrying a pot quickly (cf. section Calibrating a pump).

... >> Watering period

Specify the time span of the watering scheme. You may define two different watering schemes in non-overlapping time span to address different growth stages.

- **... >> Watering time window**

- ... >> Watering frequency: [daily|several times a day NUMBER times|less than once a day NUMBER days]

In case you are using small pots, you can meet the daily water consumption of a plant by a frequent and low amount of water supply. You can water your plants once a day (option daily), more than once a day (option several times a day), or once a day after a day interval (option less than once a day).

- ... >> Type of watering: [absolute volume | target weight]

There are two types of watering. You can water your plants either with a fixed amount of x ml of water, i.e. an absolute volume, or you can apply as much water until a desired weight is arrived (target weight). In both cases the type of watering, i.e. absolute volume or targe tweight, can be applied uniformly for each watering time point.

You can also specify different watering values for each time points. Simply use the option "manual", then enter or modify vlaues in the watering scheme table.

- ... >> OK

- ... >> Create another watering plan?

You can specify another watering plan using the same sample selection. For example you may want to define three different watering plans.

Importing a watering plan

The watering plan developed with the watering wizard ultimately creates a table, where each row represent the watering of a single plant at one time point. This table could have been generated externally, e.g. using R, and then saved as a flat file (.csv or .txt). To import the flat file into LemnaControl, the table must meet certain tabular format requirements:

- The number and order of columns are fixed
- Columns are separated by semicolon or tab
- Each line defines a new watering job
- Column entries are (in sequential order)

sample ID (alphanumeric)

watering date time (YYYY-MM-DD HH:MM:SS)

watering time window: starting hour (integer from 0 to 24)

watering time window: ending hour (integer from 0 to 24)

quantity (integer; ml for absolute volume; g for target weight)

watering type: Absolute, TargetWeight, or TargetWeightOffset

Dynamic Offset (integer; only in conjunction with TargetWeightOffset; otherwise use 0)

failure behaviour: Add, AddWithLimit, Skip

Failure Behaviour Limit (integer; only in conjunction with AddWithLimit; other 0)

pump configuration IDs (comma separated list of pump config database IDs; IDs can be determined from pump config dialogue)

To import the table use

LemnaControl >> Import >> Watering jobs >> Wizard

Here is an example table (with tabulator as column separator).

Notice: Double-check that the table has been successfully imported to the database by consulting the watering job monitor.

Chapter 5: Image analysis

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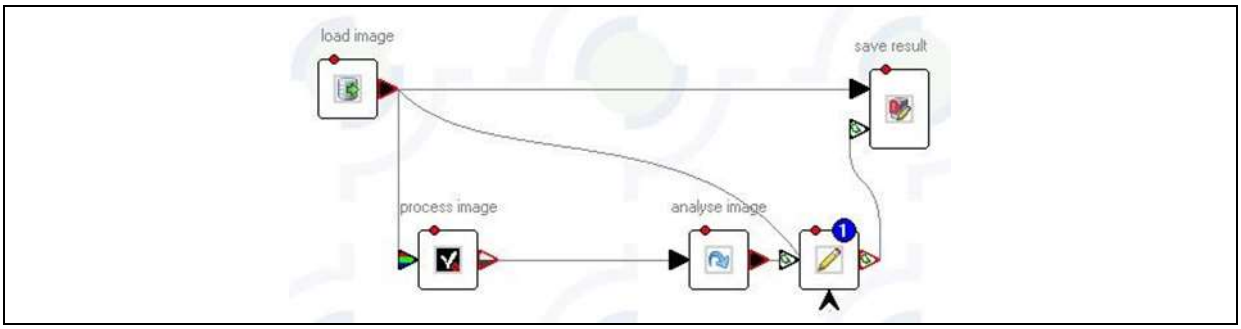
Manually Editing Image Objects

This lesson will show you how to build a user-interface for your LemnaGrid app. You will learn how to interact with the user-interface to create, remove, or refine image mask from the image processing output.

We will use the "app-1" app you made in lesson 1. To get started, open the app-1.iac in LemnaGrid and add the Object user manipulation device (device group: User interaction). Edit the workflow to match the one below:



With this pipeline we get a user-interface to refine the recognition of a coloured object from a white background.

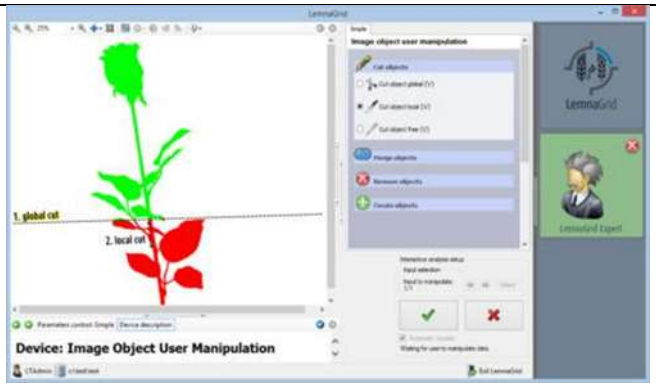
Note: you need to enable the interactive mode of Object user manipulation by right-click the device and select 'add to interactive analysis' and accept the default settings. The label in the context menue then changes to 'Remove from interactive analysis'

Cut objects

Object user manipulation offers three methods to split an object into two parts. The easiest way to partition an object is by using the method 'cut object freely'. Trace with the cursor the interface of two target objects, e.g. the boundary between the red flower and the green stem. Other methods are:

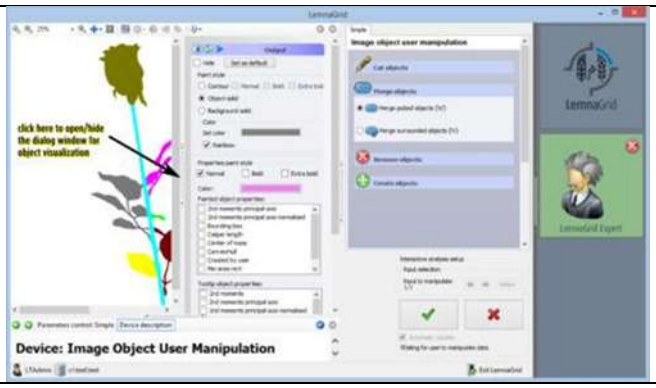
cut object globally

cut object locally



Merge objects

Image segmentation can occasionally return fragmented objects where solid ones were expected. By using '*merge picked objects*' and left-click on objects to be merged, two objects can be grouped together. Alternatively, multiple objects can be summarised directly with 'merge surrounded objects'. Left-click and drag a polygon line around objects to be merged.



Remove objects

If your object detection algorithm returns false positives, then you can use this option to reduce the list of objects. To remove an object from the list:

- Remove picked object
- Remove surrounded object
- Remove object within area

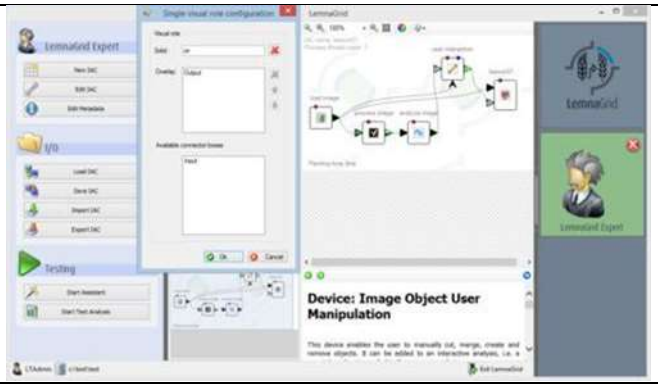
For example, you can delete a particular object by picking (remove picked object). To undo accidentally deleted objects press CTRL+Z.

Note: If you visualise objects as solid and with rainbow colour, then you can better see individual objects.

Create objects

Your algorithm to detect objects may not be sensitive enough. As a result part-ofs or whole objects may not be captured. You can add an object to your image object list by drawing a contour. For example, 'create objects >> free-form ' allows to freely outline the shape of an object. Left-click to add points. Right-click to finish selection. Other options (polygon, free circle, circle) enables the creation of geometric shapes as objects.

To better evaluate the image processing result using user interaction, it is helpful to overlay the image object list with the original image. Right-click on the Object user manipulation device and select 'Add visualization box'. Set 'Name = ori', then drag 'ori' from the 'Available connector boxes' to 'Solid' text field. Then drag 'Output' to Overlay and confirm the changes (Figure 3). You get an additional input arrow at the bottom of the device. Connect the output from DB Data Reader to the new arrow.



Save your LemnaGrid app as app-2 to the database.

Running a LemnaGrid app with user-interface

Method 1

- LemnaBase >> Image Analysis >> Start Interactive Analysis

Similar to a batch processing you need to provide the following information:

- IAC = your LemnaGrid app
- snapshots = set of snapshot images to be processed
- label for reanalysis = a unique identifier of your job, for example use PROJECT_NAME + DATETIME

Method 2

Run a batch image processing as explained in lesson 1 using app 2. Evaluate the result in SnapShot viewer and select 'Enqueue for post-processing' if the result needs to be revised .

Then

- LemnaBase >> Image Analysis >> Post Processing >> Post Process All

Note: you may need to update the post-processing job table with the 'Refresh Job Queue' button.
You now can refine the image processing result within the interactive session.
Note: after post-processing the result from the first analysis will be overwritten.

Develop your image segmentation

This lesson will show you how to explore and combine different image segmentation approaches. What is image segmentation? A procedure to partition an image into regions or categories, which correspond to different objects (e.g. leaves) or parts of objects (e.g. leaflets). Image segmentation returns a simplified representation of an image.

Several concepts in image segmentation are implemented in LemnaGrid. Often there are more than one solution to do the job. In other cases it is the combination of different methods that returns accurate results.

Thresholding

Thresholding is a simple and widely used method of segmentation based on grayscale images. On the basis of a threshold value (t), each pixel value is scrutinised and classified into foreground (white) or background (black) pixel. The resulting binary image has binary large objects (blobs, aka image regions) with pixel value either 0 to t, or in the range t+1 to 255.

Convert a colour image into an intensity (greyscale) image with HSI to grey converter (device group: Color to grey conversion). Look up the definition of Hue Saturation Intensity colorspace. Add Threshold (device group: Threshold) to your pipeline, double click on the device to define a threshold value and to obtain a binary image. Adjust your workflow to match the following one:

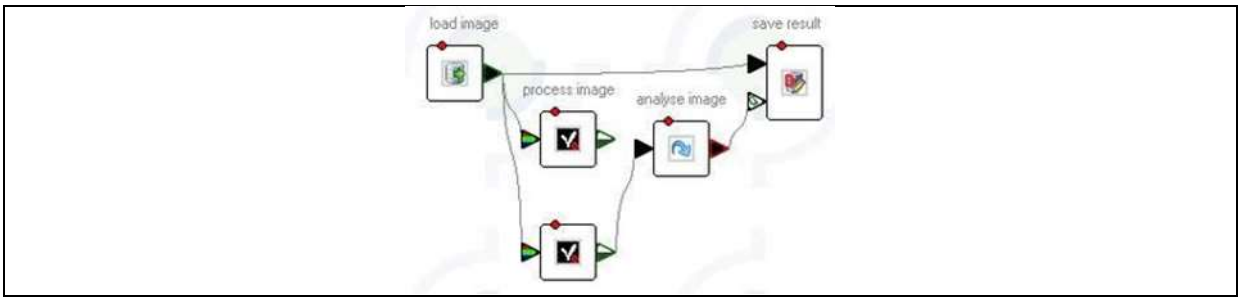
Note: two-sided (bandpass) thresholding is also possible classify pixel value within a range. Check the advanced settings in the Threshold device.

Multivariate classifier

Multivariate classifiers use information in the Red, Green, and Blue (R/G/B) channel to categorize pixels into fore- and background. Two classifiers are available in LemnaGrid in the device group Color Picker: Fore/Background separation, and NN fore/background separation.

Fore/Background separation

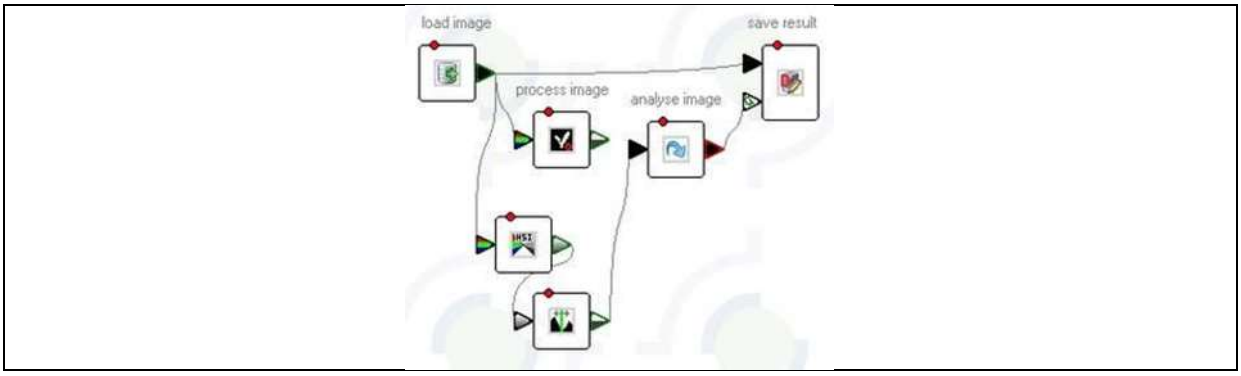
Given a palette of RGB colors and a 'Radius of a box', each pixel's RGB value is analysed. Use Fore/Background separation on the original image to create this following workflow:



- To create a colour palette for foreground pixel classification:
- Right-click on 'Foreground'
- 'Remove all colors'
- 'New color', 'Define Custom Colors'
- Specify three different R/G/B values to match the object
- Now move the mouse to any target pixel on the input image
- Mouse-click to pick a representative color
- Try adjusting the 'Radius' value and see how it affects the result

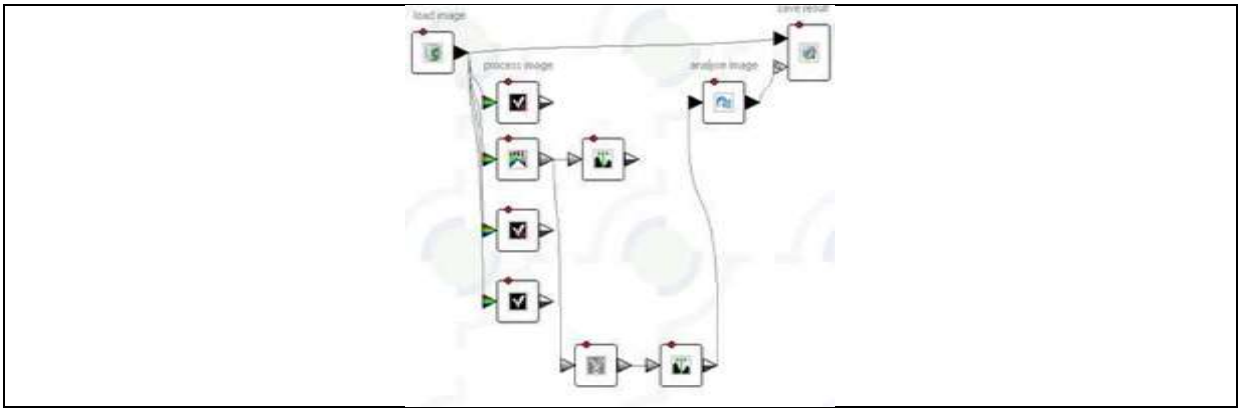
NN fore/background separation

fore/background separation utilises a colour palette for the foreground and a colour palette for the background to classify pixels. Colours are classified as fore- or background based on the Nearest Neighbourhood algorithm. Replace the Fore/background separation with NN fore/background separation. Edit both colour palettes with corresponding representative colours.



Edge-based segmentation

Boundaries or edges of an object are often associated with sharp intensity contrast at the region boundaries. Edge detection is a simple and basic method used to segment simple images with few features, e.g. a grayscale image. Several well-described edge detection methods are available in LemnaGrid, including Laplace filter, Prewitt filter, Roberts filter, Scharr filter, and Sobel filter. Create another path in your workflow to implement an edge-based segmentation:

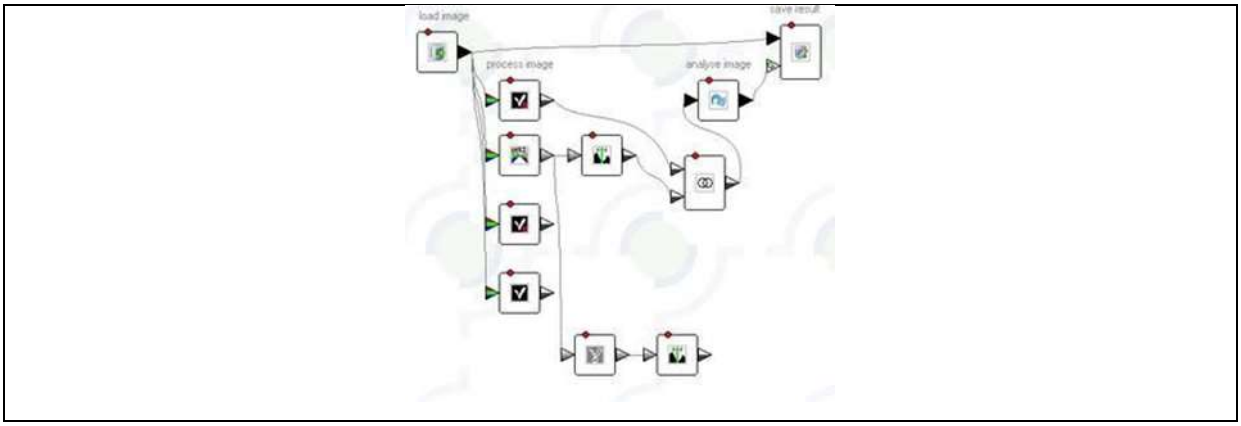


The result of Sobel filter (device group: Filter >> Edge) is a grayscale image where the pixel values indicate the degree of edges. The edges identified by edge detection are often disconnected. To segment an object from an image however, one needs closed region boundaries. Use Threshold to find an appropriate value to segment the image.

Set operations

Our workflow is now extended with various image segmentation approaches. We can combine the output of each to create a new image mask. Use Logical operations (device group:

Logical operations) as shown below:



Logical operations has three setting options:

- AND := returns intersecting pixel set
- OR := returns union pixel set
- XOR := returns disjoint pixel set

Try the different settings and check their results.

Note: you can cascade Logical operations to include more input images in your set operations. Or you can increase the number of inputs of this device by right-click, and 'Change input box number'.

Lemnagrid Use

It is easy to test different image segmentation approaches in your LemnaGrid app.

- LemnaGrid provides the building blocks (devices) to implement different image segmentation strategies.
- LemnaGrid Designer allows you to build parallel and open ended paths (approaches) in your workflow.
- Each path can be connected to combine results.

Remove noise

Binary images may contain numerous imperfections (small pixel artefacts) or wrongly picked foreground pixels coming from defect camera pixels, dirt or other disturbances. Hence binary regions obtained by thresholding are often distorted by noise and texture. This lesson will teach you how to use morphological operations to improve your binary image.

Fill areas

FILL AREAS (device group: Filters >> Morphological operations) inverts the colour of blobs in a binary image of a defined size.

Two main options are available:

- Fill areas up to size X (in pixels), i.e. pixels of a blob with an area of <= X are inverted (black to white, or white to black)
- Fill only controls which blobs are to be inverted: foreground, background or both

Erosion

EROSION (device group: Filters >> Morphological operations) removes small blobs from a binary image while reducing the size of objects at the same time. By subtracting the eroded image from the original image, you can obtain the contour of your object. Apply EROSION on your binary image output and check results with different erosion steps.

Dilation

DILATION (device group: Filters >> Morphological operations) enlarges objects with a given number of dilating steps. Use DILATION on your binary image. See what happens by increasing the number from 1 to 8.

Note/Tip: small step dilation are used to smooth fringes of an objects. The larger the dilation step is the more pixilated becomes an object.

Note: Morphological operations may be combined in a series. For the operations erosion and dilation, you can use Multi step morphological (device group: Filters >> Morphological operations) to define a sequence of erosion/dilation, and the degree of each operation.

Median filter

MEDIAN FILTER is used to smooth images, especially those with a lot of pixel noise. Try using the filter on a colour, grayscale, or binary image.

Tip: try using the Median filter with asymmetric filter mask sizes. You can remove horizontal or vertical thin lines, for example, with 9x3 or 3x9.

Describe shapes

Time to give your LemnaGrid app an "analytical" quality. This lesson will show you how to describe object form and shape in your image with a set of geometric parameters. To get started, load app-1.iac from lesson 1 in LemnaGrid.

Universal converter

The Universal converter converts binary large objects (blobs) to image objects. For each image object a set of shape descriptors (morphometric parameters) are calculated on the fly.

Here is a brief list of the computed parameters:

Category	Parameters
Area & Perimeter	Area, convex hull area, perimeter (boundary point count), shape factor, compactness
Enclosure & Orientation	Width, height, eccentricity
Centroids & Radii	Centre of mass, caliper length, second moments, second moment ratio
Other parameters	Object count, sub-object count

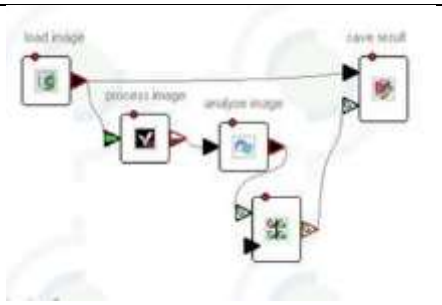
Note: a detailed list is available in the LemnaGrid Workshop article: "Application of the Universal converter" and "Morphometric parameters automatically determined for each image object".

Image object composition

Image object composition groups a set of spatially disconnected objects. This device helps to sum-up all objects within a region of interest to one single object. For example, grass

leaves of one plant are mostly detected as individual objects due to the torsion of the leaves although they belong to one plant (object). Using Image object composition all leaves canbe merged to yield into a single plant.

Edit your pipeline to match the one below



In the default setting all image objects are grouped into a single object with Image object composition.

Note: check the optional input of Image object composition by mouse over the indented black triangle.

Chapter 6: Color Analysis using LemnaGrid: Phenotyping leaf senescence process

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Background

Change in leaf color is an indicator of stress experience by plants or developmental changes. These two events can be separated when a plant under stress is compared with other which is grown under normal condition. The stress induced senescence of leaves can be determined by monitoring the leaf color or loss in leaf greenness. There are several methods to estimate the greenness of leaves and they include spectrophotometric measurement of chlorophyll or non destructive measurements with chlorophyll SPAD. Several image based methods have evolved to assess the same based on color pixels.

In this paper we present a work flow to quantify color changes on leaf surfaces, typically denoted as lesions, using LemnaTec imaging technology and software. As examples, we looked at three fungal diseases on sugar beet (*Beta vulgaris* L.) leaves.

Plant material

Plant leaves varying in their color (greenness) can be collected from field or controlled environments. Leaf discs of 4 cm diameter to be cut from these leaves and images to be taken in the Scanalyzer HTS with back light illumination for transmission measurements. Standardized light conditions, ideally diffuse light, are important to ensure good image quality for unsupervised automated analysis. In the following section there is a protocol to detect and characterize green, yellow and brown regions in leaf tissues based on color classification using the LemnaGrid software.

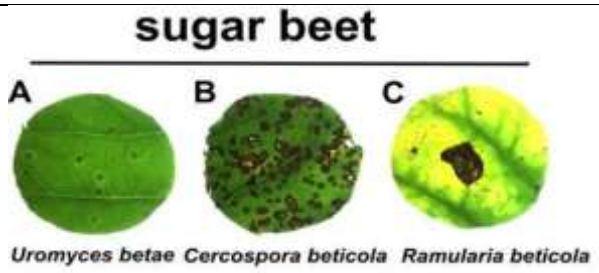

Fig. 1. Sugar beet leaves measured with Scanalyer HTS.

Approach

LemnaGrid operates in a sequential fashion: reading an input image from the database, applying image processing operations, and writing analysis output back to the database. The image processing workflow implemented in LemnaGrid is is shown in Figure 2. The principal steps are:

Load images from database. Subsequent demosaicing is the process to reconstruct a full colour image from the spatially under-sampled colour channels from the colour filter array (image sensor).

The GREEN Channel in the RGB image is used to discriminate the leaf from the white background and to separate leaves into green, yellow and brown tissue. As result one obtains and image mask (binary image).

Brown spots are detected using two filters:

(i) Adaptive region of interest (ROI) threshold and (ii) Colour-based classification. The adaptive ROI filter computes differences of each pixel value with respect to its local neighbourhood. This method is used to detect small spots. Large spots are detected using the colour-based classification, where a set of manually predefined signature spot colours is used. Both results are combined to a binary image.

Post-processing, transformation of interconnected pixels to objects, assignment of colours and shape parameters to each object.

The remaining, not classified green/yellow pixels are filtered.

The global threshold for the GREEN pixel value is used to separate between green yellow.

Saving data to database.

Note that the analysis parameters were once determined for sugar beet leaves. Thereafter all images were analysed using the same set of parameters, a prerequisite for automated and unsupervised image processing.

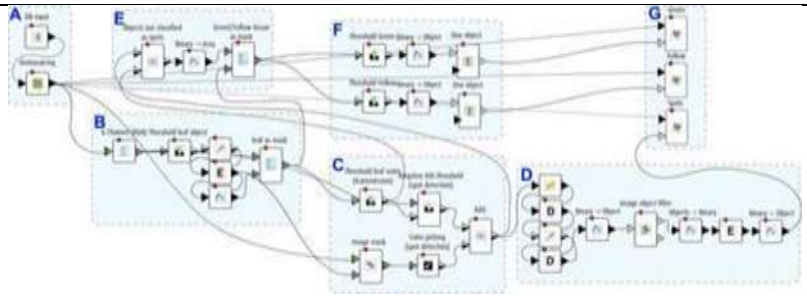

Fig. 2. Implementation of the image processing in LemnaGrid software.
The Grid can be downloaded using the LemnaShare (previously LemnaNet) program. Icons represent devices and do specific tasks. The dataflow is from left Database Input (A) to right Database writing (G). Blue boxes highlighted with capital letters represent modules identical to Fig. 2 and are described in the text.

Results

Using the proposed image processing approach with LemnaGrid we can detect colour changes in leaf images (Fig. 3).

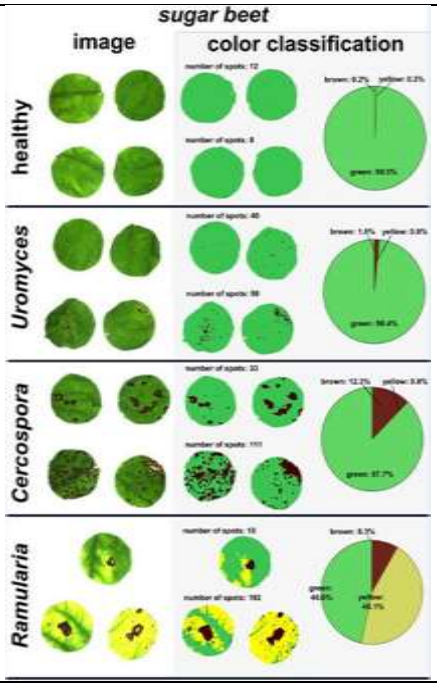

Fig. 3. Left: Sugar beet leaf discs with yellow/brown disease symptoms caused by *Uromyces betae*, *Cercospora beticola* or *Ramularia beticola*.
Middle: Leaf discs with colour classification into green, yellow and brown areas using the introduced image processing approach. The number of detected spots per image is given.
Right: Pie chart summarizing the average colour distribution over all sampled leaf discs. 8 healthy leaf discs, 6 with *Uromyces*, 10 with *Cercospora*, and 3 with *Ramularia* were analysed.

Conclusion

A workflow is needed to quantify symptoms on leaves caused by stresses. The analysis is robust. Repeating this analysis with a larger dataset can allow establishing a footprint to identify stress symptoms based colour changes in leaves caused by various biotic and abiotic stresses.

Chapter 7: Near Infrared Imaging

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Near infrared (NIR) or short wave IR-imaging (SW-IR) can be used, for example, to get detailed information on the watering status of plant leaves and their reaction to limited water availability or external drought (e. g. during growth or storage periods). The following image shows how various lettuce cultivars dry down over time, changing the NIR-absorption of the leaves in the NIR-absorption band between 1450 nm and 1600 nm.

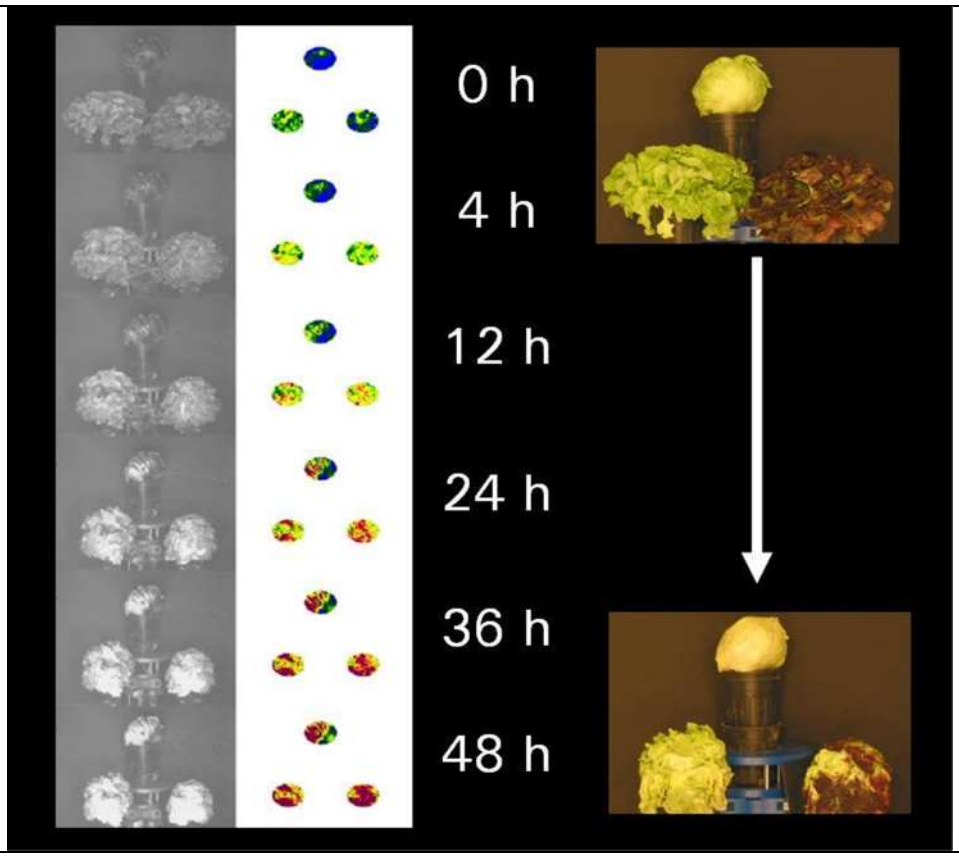

Fig. 1. An iceberg lettuce (top row) and two oak leaf lettuces (green and red, bottom row) dry down in warm ambient conditions.
NIR-imaging shows a strong increase in reflectance as the water in the leaves is extremely reduced. Blue false colours represent high water content, while red colours symbolise low water content (high reflectance).

This test of various lettuce reactions to the same environmental conditions is just one application example of NIR-imaging; in this case it is used to quantify the water dynamics in plants.

Similar tests can of course be carried out with various plants, even in pots, to assess their reaction to water stress under drought conditions.

The diagram below shows the quantitative data, expressed as false colour classes, of several absorption ranges.

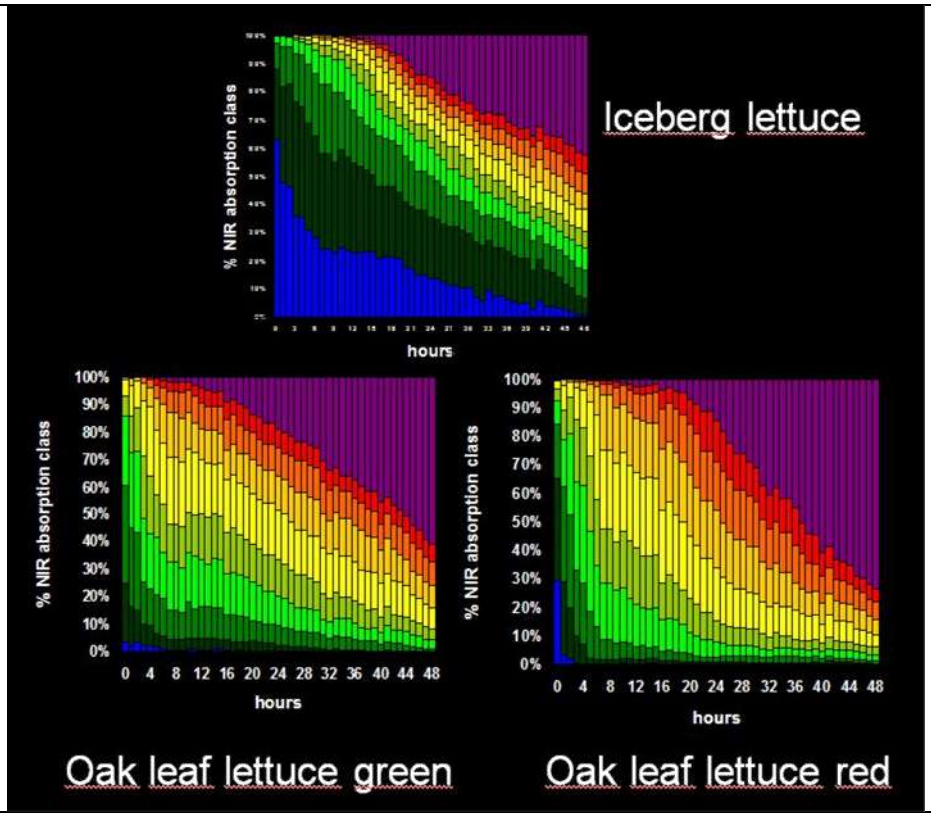

Fig. 2. The drying dynamics of the outer leaves of 3 lettuce cultivars
While keeping the whole head together, only the surface layer (which finally defines the purchase decision of the customer in the shop) is measured. The less blue and the more green/yellow/red/violet colour classes appear the dryer is the lettuce.

Figure 2 shows how non-destructive imaging generates high-content data on water loss dynamics. While the iceberg lettuce contains the highest amount of water (largely blue) and dries out most slowly, the red oak leaf lettuce dries down the most overall during the measurement period. Nevertheless the large number of intermediate values shows that this drought process proceeds more slowly in the first few hours, particularly in comparison with the green oak leaf lettuce. Such profiles emphasise the immense power of imaging to provide different phenotypic patterns.

The sensitivity of the technology is clearly revealed by the fact that within the first few hours significant changes towards drying down are already depicted as major shifts between the colour classes.

These values might be correlated to e. g. sensory data such as crunchiness and good mouth feel, or to agriculturally important traits such as efficient water usage or drought stress tolerance.

HighContent Screening -Cereal NIR-Phenotyping

This wheat test with its fast reaction to drought is just one application example of NIR-imaging; in this case it is used to quantify the water dynamics in plants.

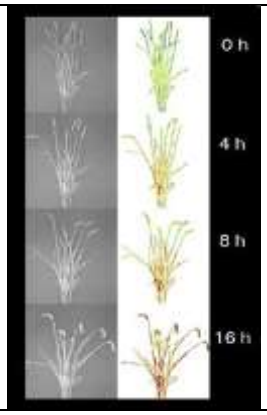

Figure 3: A bunch of wheat dries down in warm ambient conditions
NIR-imaging shows a strong increase in reflectance as the water in the leaves is extremely reduced. Blue/green false colours represent high water content, while yellow/red colours symbolise low water content (high reflectance).

The diagram below shows the quantitative data, expressed as false colour classes, of several absorption ranges.

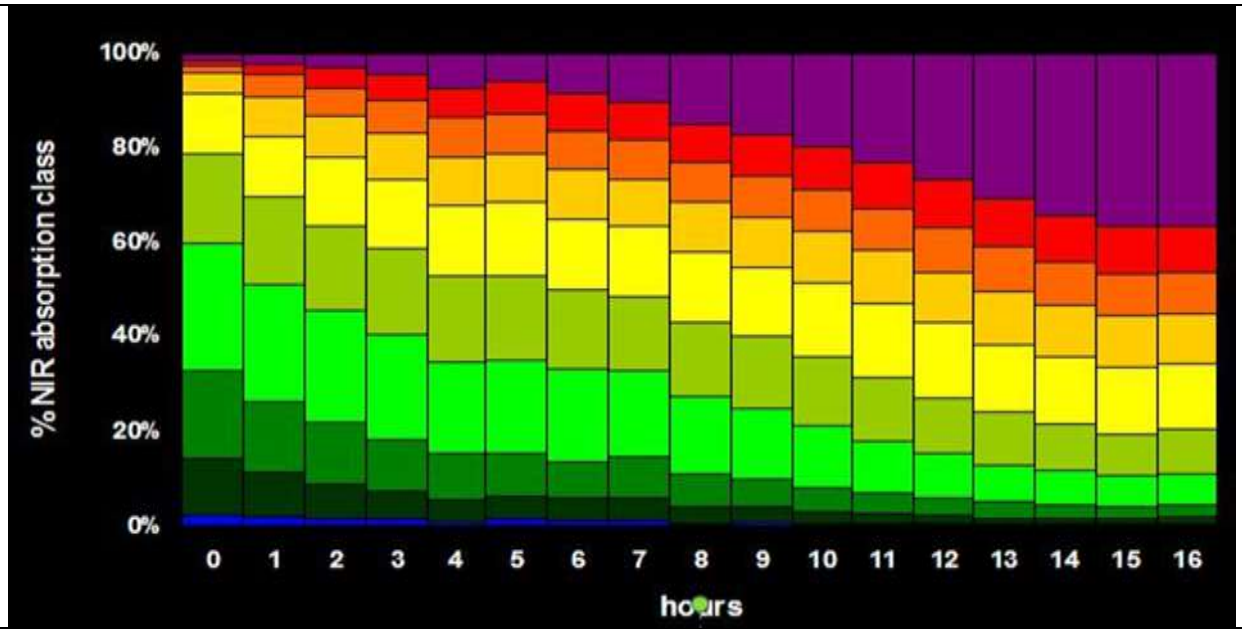

Fig. 4. The drying dynamics of a wheat plant over a 12 hour period.
Blue/green false colours represent high water content, while yellow/red/violet colours symbolise low water content (high reflection).

Figure 4 shows how non-destructive imaging generates high-content data on water loss dynamics. The sensitivity of the technology is clearly revealed by the fact that within the first few hours significant changes towards drying down are already depicted as major shifts between the colour classes.

Conclusion

The LemnaTec Scanalyzer is a comprehensive phenotyping platform highly suitable to quantify morphological traits e. g. of cereals like wheat – and in fact any other plant over the whole length of an entire life cycle.

The example above provides only a first impression of the unlimited capabilities of the system concerning the quantitative characterisation of water dynamics in vegetables and other plants. All results based on biologically relevant parameters that are generated in this way will be reproducible.

Moreover, the LemnaTec systems can be customised for various applications, depending on individual research requirements.

Chapter 8: Canopy temperature/IR thermography as a trait for phenotyping for drought and heat tolerance

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The surface temperature of the canopy is related to the amount of transpiration resulting in evaporative cooling. IR based thermometer/ camera allows canopy temperature (CT) to be directly and easily measured remotely and without interfering with the crop. It is well documented that CT is correlated with many physiological factors like plant water status, stomatal conductance, transpiration rate, crop yield etc. CT used routinely, particularly for stress diagnostic and breeding selection of stress adapted genotypes: (i) under drought conditions it is related to the capacity to extract water from deeper soil profiles and/or agronomic water use efficiency (WUE); (ii) under irrigated conditions it may indicate photosynthetic capacity, sink strength and/or vascular capacity –depending on the genetic background, environment and developmental stage; and (iii) under heat stress conditions is related to vascular capacity, cooling mechanism and heat adaptation.

CT is an integrative measurement (i.e., scoring the entire canopy of many plants within a plot), and so has advantages over other methods used for stress detection, such as stomatal conductance and water potential, because it integrates a larger area of plant/ crop measurement, is non-destructive, does not interfere with stomata (which are sensitive), and is faster and not laborious. However, trait expression shows interaction with both developmental phase and time of day (e.g., pre-heading and/or morning readings are usually lower due to lower incident solar radiation and air temperature), which can be used to relate different canopy traits and stress tolerances.

Infra-red thermometry or IR thermography measures temperature of the target by measuring the radiant thermal energy emitted by the target. Infrared is a type of electromagnetic radiation, which is emitted, to greater or lesser degree, by all objects that have temperature. IR spectral region of 8 to 13 µm is typically used for thermal remote sensing.

Factors influencing the canopy temperature

- **Stomatal features:** shape, size and structure of stomata.

- **Leaf Characters:** Number and orientation of leaves, presence of cuticle and waxiness on leaf lamina and stem
- **Plant water status:** Water content of plant/leaf in relation to that required for optimal growth.
- **Crop Yield:** high photosynthesis
- Emissivity of objects.
- **Time of day:** Measurements typically from 11:00h to 14:00h, Avoid cloudy and rainy days.
- **Environment condition:**Measurements must be in clear sky and there is little or no wind. The plant surfaces are dry and not wet from dew, irrigation or rain.
- **Plant phenological stage:** Stage should be objective based and interval should be roughly 5-7 days between each measurement– to give a reasonably heritable estimate of trait expression.

Always take measurements of the part of the plot which is most exposed to the sun, and ensure to avoid the shadow of the operator and/or shadows from the neighbouring plots.

Image capturing process in Variocam HR inspect 575

- Press the button AL. The thermographic system automatically focuses and the temperature scaling of the false-colour image is automatically optimised according to the current scene. Or Adjust the view mannualy to focus the object.
- In the live mode, joystick movements ↑, ↓ change the selected temperature level and joystick movements ←, → change the selected temperature range.
- Pressing the Enter button switches between the live mode and the focus mode.
- In the focus mode, joystick movements ↑, ↓ focus over larger or shorter distances to the object.
- For Storage of the thermal image press the S button. The live image freezes, i.e. camera goes into stop mode. Pressing the S button again saves the thermal image on the SD card. Pressung the C button interrupts the saving process. The camera will return to the live mode after the saving process.
- For switch off press button CL, the dialogue for switching off is selected and confirmed by pressing Enter.

Image processing

- From menu "File", select submenu "Open file" and open the desired thermograms (*.irb files).
- Select the desired colour palette via the "Scale", which is located on the right-hand of the thermogram.
- Via menu "View", you can display further image elements, measurement data, annotations and parameters in addition to the thermogram.
- By pressing the right mouse key on the colour scale, the dialogue "Level/Range", where the temperature level and range can be adjusted as desired by moving the scroll bar. The adjustment is also adopted for subsequent thermal images.
- With the help of the respective functional buttons on the symbol bar, points of measurement, areas, etc. as well as the display of temperature maximum and minimum can be activated.
- For inserting the analysed thermal images into the reports, select the menu "Report". Alternatively the images can be stored in common image formats suc as .jpg, .bmp etc

Prototype of tools for image based phenotyping

Taking into consideration the need to accelerate phenotyping in field efforts have been made to develop phenotyping tools. A hand operated track mounted trolley was designed for imaging purpose which hosts a camera and a Lap Top PC. The system acquires images of each plot in the experimental field after recognising the barcode. Images are stored with plot name. Tools have also been developed to rapidly analyse these images. Promising results have been obtained with image acquisition and analysis tool. This field based, semi-automated platforms potentially allow high-throughput phenotyping at a low cost.

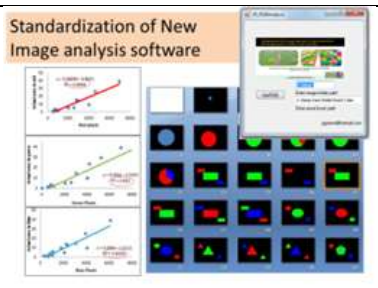

Fig. 1. Prototypes to screen genotypes in field (a) pot culture (b) tools for rapid analysis of images (c)

IR thermometer Vs Thermal camera

- A IR thermometer gives number whereas, thermal imaging cameras generate an image.
- A IR thermometer reads the temperature of one single spot whereas, a thermal imaging camera gives you temperature readings for each pixel of the entire thermal image.
- Because of advanced optics, thermal imaging cameras can also resolve temperatures from a longer distance. This allows you to quickly inspect large areas and hance, simultaneous response recording for large set of genotypes.

Limitations of thermal imaging

- Thermal cameras are more expensive and It is greatly Influenced by being around any object and environment hence, necessitating the use of reference.
- Imaging sensor calibration and atmospheric correction are often required for high efficiency.

Chapter 9: Partial root zone drying: Deficit irrigation strategy for drought stress management in horticultural crops

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Introduction

Drought is one of the most common environmental stresses that may limit agricultural production worldwide. However, in many countries as a consequence of global climate changes and environmental pollution, water use for agriculture is reduced. Water is also becoming scarce not only in arid and drought prone areas but also in regions where rainfall is abundant In recent years, water-saving irrigations techniques used are pressurized irrigation system i.e drip and sprinkler irrigation system. These systems improve the water productivity (WP) and quality of produce in horticulture crops as well as in cereal crops.

Strategies to improve water productivity under water scarcity

1. Cultivation of plants with high water-use efficiency or plants with greater drought tolerance
2. Investment in water-efficient technologies for growing plants as in deficit irrigation techniques

Deficit irrigation (DI)

Deficit irrigation is an optimization strategy in which irrigation is applied during drought-sensitive growth stages of a crop. The correct application of DI requires thorough understanding of the yield response to water (crop sensitivity to drought stress). In regions where water resources are restrictive it can be more profitable for a farmer to maximize crop water productivity instead of maximizing the harvest per unit land. The saved water can be used for other purposes or to irrigate extra units of land.

Advantages

- Maximizes the water productivity
- Allows economic planning and stable income due to a stabilization of the harvest in comparison with rainfed cultivation

- Decreases the risk of certain diseases linked to high humidity (e.g. fungi) in comparison with full irrigation
- Reduces nutrient loss by leaching of the root zone, which results in better groundwater quality and lower fertilizers needs as for cultivation under full irrigation
- Controls of vegetative growth and canopy density (reduce pruning in grapevine)
- Improvement of irrigation water use efficiency and saving water for irrigation
- Increases in nutrient use efficiency (especially N)
- Improvement of fruit or yield quality (potato, grape, tomato, pepper, apple, maize)

Constraints

- Exact knowledge of the crop response to water stress is important.
- There should be sufficient flexibility in access to water during periods of high demand (drought sensitive stages of a crop).
- A minimum quantity of water should be guaranteed for the crop, below which DI has no significant beneficial effect.
- An individual farmer should consider the benefit for the total water users community (extra land can be irrigated with the saved water), when he faces a below-maximum yield
- Because irrigation is applied more efficiently, the risk for soil salinzation is higher under DI as compared to full irrigation.

In recent years, the two main approaches for developing practical solutions to manipulate vegetative and reproductive growth used. That has been: Regulated deficit irrigation (RDI) and Partial rootzone drying (PRD). However, these developments have been possible only as a consequence of better understanding of physiological responses to water deficit and the widespread use of drip and other forms of micro-irrigation that enable the precise control of water application rate and timing. RDI and PRD have become established water management techniques. Therefore, great emphasis is placed in the area of crop physiology and crop management with the aim to make plants more efficient in water use through RDI and PRD irrigation practice under dry conditions.

Partial root-zone drying irrigation (PRD)

Partial root-zone drying (PRD) is a modified form of deficit irrigation (DI) (English *et al*., 1990), which involves irrigating only one part of the root zone in each irrigation event, leaving another part to dry to certain soil water content before rewetting by shifting irrigation to the dry side; therefore, PRD is a novel irrigation strategy since half of the roots is placed in drying soil and the other half is growing in irrigated soil (Ahmadi *et al*., 2010a). Schematic diagram of FI, DI and PRD are shown in Figure 1 (after Davies and Hartung, 2004). Partial root zone drying imposes a soil deficit within alternating sides of a root zone, but plants so managed should remain turgid.

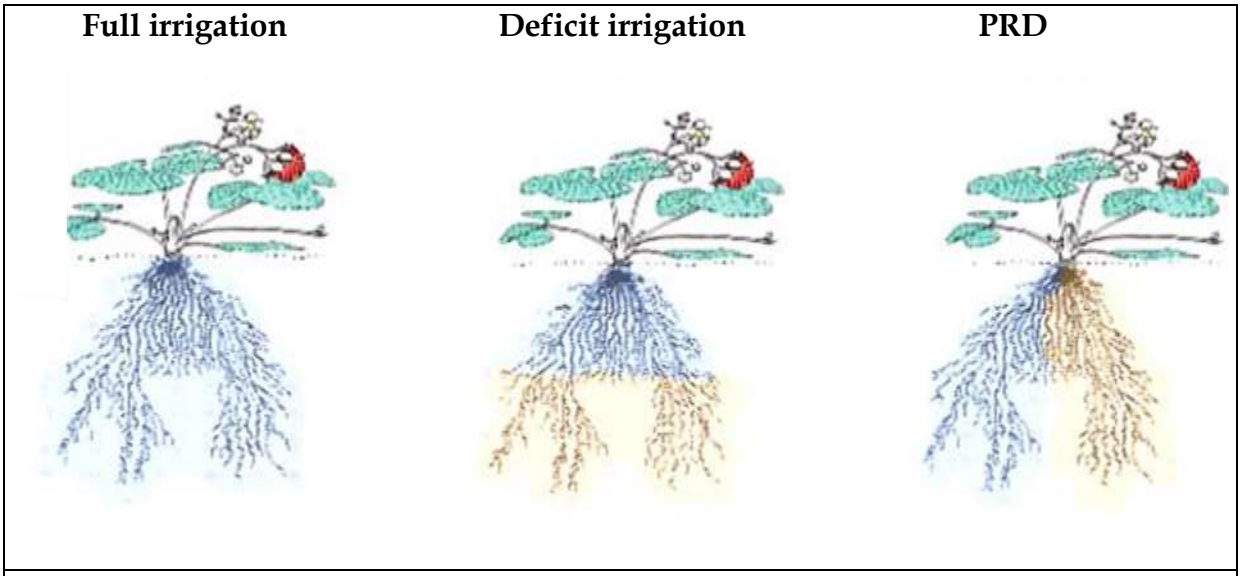


Fig. 1. Schematic of the irrigation pattern in FI, DI, and PRD (After Davies and Hartung, 2004)

Principle of PRD

When a part of the root zone dries out, ABA produced in the roots in drying soils and is transported by water flow in xylem to the shoot for regulating the shoot physiology. The increase in abscisic acid in the xylem flow roots to leaves triggers the closure of stomata as response to water stress and reduced shoot growth and transpiration. After 10–15 days, the wet and the dry root zone are inverted. However, due to alternating wet and dry zones, roots have continuous access to water. Thus, the plant continues to grow and flowering and fruit development will not affect. Alternating the wet and dry zones of the roots means that repeated surges of ABA are delivered to the shoots, maintaining conditions of reduced shoot growth and reduced transpiration, but with no significant effects on flowering and fruit development (Fig 2)

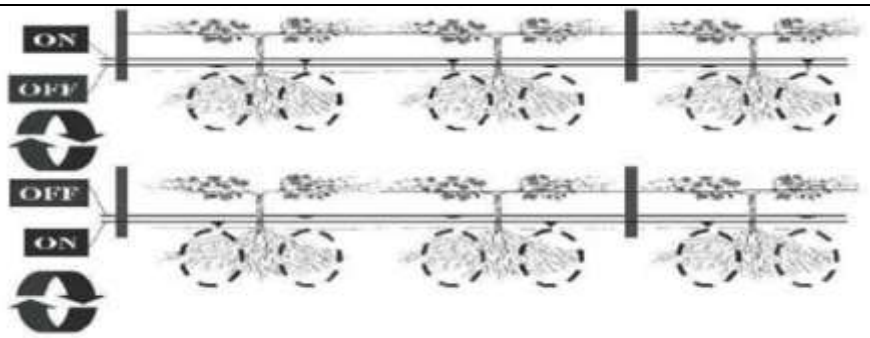


Fig. 2. Partial root zone drying using two above-ground drip lines in a vineyard

Wetting and drying each side of roots are dependent on crops, growing stage, evaporative demands, soil texture and soil water balance (Saeed *et al*., 2008). Yet there is little understanding on mechanism of PRD effects on crop growth, therefore, no definite solid procedure exist on determining the optimum timing of irrigation for each side. ABA is a plant hormone that is produced in the roots in drying soils and is transported by water flow in xylem to the shoot for regulating the shoot physiology (Kang and Zhang, 2004). Therefore, in PRD roots sense the soil drying and induce ABA that reduce leaf expansion and stomatal conductance and simultaneously the roots in wet soil absorb sufficient water to maintain a high water status in shoot (Zegbe *et al*., 2006; Liu *et al*., 2006a; Ahmadi *et al*., 2010a).

Partial root-zone drying irrigation (PRD): Theory

The effect of water stress on plants at physiological, biochemical and molecular levels and a crop that is imposed to PRD as a water-saving irrigation may show diverse responses to water stress in terms of these three responses levels according to the severity and timing of the water stress (Fig 3).

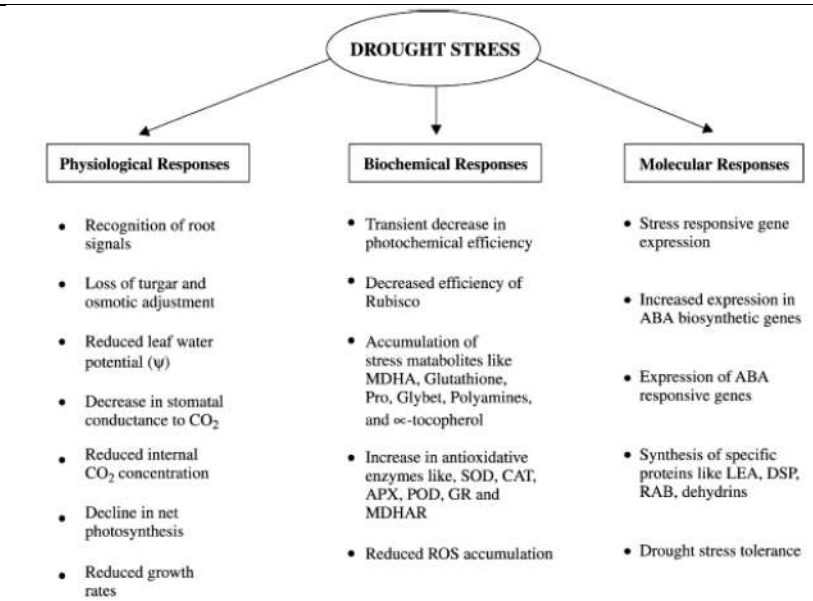


Fig. 3. Physiological and molecular bases of drought stress tolerance (After Shao *et al.*, 2008)

Chemical and hydraulic signaling in PRD

The conventional view of drought is that soil drying induces restriction of water supply and these results in a sequential reduction of tissue water content, growth and stomatal conductance. This kind of reaction requires that the plants have some mechanism for sensing the availability of water in the soil and regulating stomatal conductance and leaf growth accordingly. Such control has been termed non-hydraulic or chemical signaling.

Chemical signals can be negative or positive messages. Negative messages are supplied by turgid roots and promote stomatal opening and shoot growth. Positive messages, whose production increases as the soil dries, may be an inhibitor such as abscisic acid (ABA). Changes in mineral composition and pH of xylem sap may provide additional signals. Hydraulic signaling, which represents transmission of reduced soil water availability *via* changes in the xylem sap tension.

Roots in drying soil produce more ABA than under normal conditions and it is moved as an anti-stress root chemical signal to shoot through transpiration stream and limits the stomatal conductance. At mild water stress, ABA as a major chemical signal (CS) acts earlier than the change in plant water status i.e hydraulic signal, HS. However, under severe water stress, both CS and HS may be involved in regulating plant physiological processes. At severe water stress, the leaf water potential in mesophyll cells decreases and stomata will close to a greater extent that inhibits the photosynthetic rate (Taiz and Zeiger, 2006). In some plants, CS and HS occur independent of each other, while in others they take place dependently. A balance between CS and HS occur in PRD. In PRD, roots on the irrigated side absorb enough water to maintain high shoot water potential, and the roots on the non-irrigated side produce ABA for possible reduction in stomatal conductance. This mechanism optimizes water use and increase water productivity.

Agricultural benefit of root-to-shoot chemical signaling

PRD reduced vine vigour, canopy density and increased the quality, yield of fruit and improved water-use efficiency (Loveys *et al.*, 2000). It also resulted in leaf expansion rate in wheat (Ali *et al.*, 1998), maize (Bahrun *et al.*, 2002), soybean (Liu *et al.*, 2005a), potato (Liu *et al.*, 2006c), and tomato (Topcu *et al.*, 2007). Excessive plant vigour is a major problem for many fruit crops, since the use of assimilates in leaf

growth restricts fruit set and development. Alternating wet and dry zones of the root system are essential to trigger the continuous root-to-shoot signal. This is necessary because the root system is not able to maintain root ABA production for long periods (Loveys *et al*., 2000). The frequency of the switch is determined according to soil type and other factors such as rainfall and temperature. In most of the published data the PRD cycle includes 10 to 15 days (Davies *et al*., 2000; Stoll *et al*., 2000).

Gas exchange in PRD

Water is lost as transpiration and CO_2 is absorbed for photosynthesis through stomata. Therefore, any variation in stomata opening affects stomatal conductance (gs) and photosynthesis rate (An). The An is not as responsive to mild water stress as leaf expansion. This is because An is much less sensitive to a decrease in turgor pressure compared with leaf expansion (Taiz and Zeiger, 2006). However, severe water stress usually influences both An and gs. Reduced stomatal conductance in early stages of water stress inhibits transpiration rate more than it reduces the intercellular CO_2 concentration that is the driving factor for photosynthesis. In other words, due to non-linear relationship between An and gs, and a lower sensitivity of An than gs to water stress, water productivity increases at mild water stress (Davies *et al*., 2002; Liu *et al*., 2005c; Liu *et al*., 2006a).

Root development and water uptake

Root development and distribution are affected by spatial and temporal soil water distribution (Wang *et al*., 2006). Further, they affect water and nutrient uptake from the soil to maintain the physiological activities of the above-ground part of the crop. Mild water stress in soil leads to preferential root growth into the moist soil zone and water uptake through root system expansion and increasing root length density (RLD, cm root per cm3 soil) (Benjamin and Nielsen, 2006; Songsri *et al*., 2008). Earlier studies indicated that PRD enhanced the extension and inhibition of primary and secondary roots (Kang *et al*., 2000b), increased root growth (Dry *et al*., 2000) and root mass (Kang *et al*., 2000a; Mingo *et al*., 2004), improve ABA-induced root hydraulic conductivity (Glinka, 1980; Taiz and Zeiger, 2006; Thompson *et al*., 2007), and increased the nutrient uptake (Wang *et al*., 2009).

Plant water uptake rate is enhanced after re-watering in water stress condition compared to full irrigation. This is obtained due to improvement of hydraulic conductivity of root systems that is subjected to water stress (Kang and Zhang, 2004). The root system can partially compensate for the increasing limited

water availability on the non-irrigated side of PRD due to an increase in root hydraulic conductivity.

Practical application of RDI and PRD: Irrigation management strategies

Before making irrigation plan it is important to know the characteristics of soil in the field including:

- Number and thickness of layers (identifying impermeable layers in the soil that may cause drainage and surface run-off problems)
- Soil texture
- Soil structure
- Field water capacity, wilting point
- Rate of infiltration
- Rooting depth of plants that will be growing
- Soil chemical analyses to identify possible chemical/nutrient problems (e.g. acidity, salinity, nutrient deficiency).

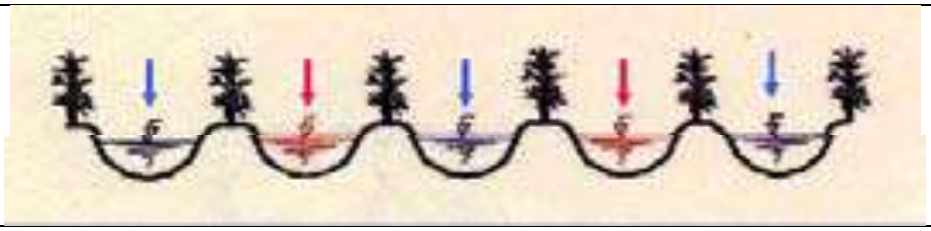
Irrigation methods for applying RDI and PRD

PRD and RDI could be applied in the field by different irrigation methods including:

- Furrow irrigation
- Drip irrigation

Furrow irrigation system

PRD System should be applied as the two rows configurations and the both furrows should be irrigated alternately. After the switching period, wetted furrow started to dry out and dry furrow will be irrigated.



RDI System should be applied at the same time in all rows, but with 50-70% water amount needed for full treatment

In drip surface or subsurface for PRD irrigation two irrigation lines should be set up and operated separately with the distance between emitters of 60cm (for potato). This way lateral of one emitter will irrigate one part of the root system and emitters of other lateral will irrigate other half of root system. In FI and RDI irrigation one lateral is used for irrigation with the distance of 30cm between emitters. Irrigation in FI and RDI should cover a total root area.

Difference between RDI and PRD

RDI	PRD
Site must be responsive to irrigation	
Can be used with furrow irrigation	Drip irrigation preferred, alternate row furrow possible
Water must be available on demand	
Control of fruit size	No/ negligible effect on size
Vegetative growth control	Vegetative growth control
Potential for yield loss	No loss of yield
Positive effects on fruit quality	Possible improvement in quality
Marginal water savings	Significant water savings
No irrigation hardware modification	Significant changes required. Can be retrofitted.
Soil water monitoring recommended	
High-level management skills required	

(Ref: Kriedemann and Goodwin, 2003)

Precaution to be taken while implementing PRD

- Best PRD responses occur in soils with high values of readily available water (RAW). Shallow soils with low RAW can allow relatively small volumes of applied water to deplete rapidly. To some extent this can be overcome by more frequent irrigation.
- Use of PRD in soils with poor infiltration characteristics may also cause problems if sufficient water cannot be supplied through what is effectively 50% of the normal soil surface area.

- The amount and timing of irrigation applied to the 'wet' side should be sufficient to prevent the development of significant water deficits (soil moisture tension should remain higher than 50 kPa).
- If soil moisture monitoring is available, the irrigated side of the plant should be switched when water extraction from the "dry" side becomes negligible. In sandy soils and under hot dry conditions this may be only a few days. In soils with a higher water retention characteristic and under less stressful conditions, the cycle time may become several weeks.
- Use of PRD should not result in significant reduction in midday leaf water potential when compared with standard irrigation practice.
- When PRD is being implemented in an existing orchard, total soil area wetted by the irrigation system (wet plus dry sides) should not vary significantly from that wetted by the original irrigation system. For example, conversion from flood to drip may wet only a small fraction of the available roots. The PRD irrigation system should aim to wet about half the roots at any one time.
- Correctly implemented PRD should not result in major effects on fruit quality. With Navel oranges, PRD using very low water application rates saw a reduction in fruit size in heavily cropped trees but this problem was not evident at higher water inputs. A reduction in water input, applied by flood or by drip, may result in a small but significant reduction in the percentage of juice and an increase in acid. There should be no effect on sugars and sugar/acid ratios may change accordingly.
- Response to PRD varies between species. It is still not known how some plants will respond.

Conclusion

Partial root zone drying is a very useful and significant step in improving the water use efficiency, increasing productivity, and improving quality of produce of perennial horticultural crops. While there is some risk of water stress to the plant, but with careful soil water monitoring these risks can be minimized. The cost of implementing PRD varies depending on the irrigation system employed. The additional outlay of installing PRD, is economical where the cost of irrigation water is high and as water becomes an increasingly valuable and scarce resource.

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Chapter 10: Modeling the Nitrogen stress for variable rate N application in rice and wheat crops

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Introduction

Nitrogen, phosphorous and potassium are regarded as key nutrients among all the nutrients added to the soil for enhancing crop yields. Nitrogen (N) plays a key role in the plant life cycle and affects crop yields significantly. It plays many roles in plants and is a component of chlorophyll, which is necessary for photosynthesis. Nitrogen is typically taken up in larger amounts than other nutrients and is the most common, and most important, limiting nutrient for non-legume agricultural crops. Not only does N nutrition affect yield, but it also affects the quality (protein or sugar content) of crops such as grain and sugar beets, for example. Plants absorb nitrogen as a mineral nutrient mainly from soil, and it can be may come in the form of ammonium ($NH4^+$) and nitrate ($NO3^-$). However, soil N supply is often limited, which forces farmers to increase the amount of N fertilizers in order to achieve better crop yield. However, farmers may provoke nitrogen over-fertilization, which hinders optimum plant productivity, as plants are not able to absorb the excess of N-fertilizer. This entails unnecessary expenditure on the part of farmers. Nitrate leaching, soil denitrification, and volatization are the main processes for N-fertilizer excess loss, contributing to environmental pollution. Nitrate leaching contaminates groundwater and other bodies of water, which may contribute to eutrophization. In addition, volatized N contributes to global warming by releasing nitrous oxides (i.e., NO, N_2O), which are considered greenhouse gases. Most of the crop plants generally require nitrogen throughout their growth period. Irrespective of the crop, all plants tend to grow at a slow pace in the beginning, rapidly in the "grand growth period" (the period at which elongation of cells, tissues and formation of organs take place) and again slow during maturity. Accordingly, nitrogen is also taken up by the plants in keeping with the pace of plant growth. Therefore use of nitrogenous fertilizers should be so timed as to ensure its supply to the plant throughout its growth period especially during grand growth period. Nitrogenous fertilizers are very soluble in water, therefore liable to be leached. As such it is necessary to apply nitrogenous fertilizers in split doses of two-four, depending on the type of soil and the duration of the crop. When the fertilizer is applied at sowing time, it is called basal dressing; and the dose applied in standing crop is called top dressing. Plants require

phosphorus mainly during the early root development and early growth period. Besides, almost all phosphatic fertilizers release phosphorus very slowly to the plant growth unlike nitrogenous fertilizers. They are, therefore, applied only at the time of sowing i.e. basal dressing. Having discussed the importance of split application of nitrogen as compared to single application of phosphorous and potash, the ways to optimize the nitrogen application holds relevance.

Review: Variable rate N fertilization and N Management

Nitrogen fertilization rate is the most important N management decision regarding potential to achieve optimum crop yield, influence nitrate loss to water systems, and return maximum economic profitability. The first step to do this would be to know the status of Nitrogen in growing medium (for basal dose application) or the plant (for top dressing of Nitrogen). Soil nutrient testing is a management tool that can help accurately determine the available nutrient status of soils and guide the efficient use of fertilizers. Having done the Soil nutrient analysis the deficient nutrients are addressed by applying corrective fertilizer dose. However this does not ensure that the Nitrogen demand of the crop will be satisfied by soil supply because of numerous channels of N loss and therefore supplying N in synchronous with the crop N demand remains the only way to increase nitrogen use efficiency. The crop N demand is reflected by the canopy NDVI values as established by several research done across globe. The invention of analog-based, pulse-modulated, two-band, active lighting sensors (Beck and Vyse, 1995) and the equivalent digitally based sensor (Stone *et al*., 2003, 2005) have contributed to the potential use of these technologies for variable-rate application of N fertilizers. One of the more common reflectance indices used in agriculture is the normalized difference vegetation index (NDVI). The index is computed as (NIR – Red)/(NIR + Red), where NIR is the fraction of emitted near-infrared radiation returned from the sensed area (reflectance) and Red is the fraction of emitted red radiation returned from the sensed area (reflectance). Work by Filella and Penuelas (1994) and Liu *et al*. (2004a) noted that red edge reflectance can be indicative of plant chlorophyll content and biomass. Kanke *et al*. (2011) reported that NDVI better detected differences in plant growth, especially at early growth stages, than red edge reflectance. Spectral measurements of plants correlated with numerous physiological and morphological factors affecting growth and yield. Because of the difficulty in accounting for all confounding factors, models for computing N fertilizer rates are generally empirical and plant species specific and do not account for environmental factors, particularly rainfall, and their interactions with plant growth factors.Biggs *et al*. (2002) proposed a reference strip, where fertilizer is applied at a sufficient rate such that crop yield

reaches a response plateau, that would subsequently be used to manage N fertilization. He patented a concept to measure reflectance with an optical sensor of the strip and the adjacent field rate and calculated the N application rate based on the ratio of the two readings (Biggs *et al*., 2002). The sensors were mounted on a center pivot irrigation system and paired measurements were made on-the-go.

Researchers use linear or exponential models to describe the relationship between vegetative indices and plant yield. Linear relationships have been identified between yield and NDVI for corn (Diker *et al*., 2004), wheat (Nidumolu *et al*., 2008; Liu *et al*., 2004b), tomato (*Solanum lycopersicum* L.) (Bala *et al*., 2007), cotton lint (*Gossypium hirsutum* L.) (Plant *et al*., 2000), and barley (*Hordeum vulgare* L.) (Kancheva *et al*., 2007). Multiple linear regression was used for winter wheat (Salazar *et al*., 2006; Kumar *et al*., 1999). Exponential relationships were used for NDVI and yield in cotton lint (Plant *et al*., 2000), winter wheat (Enclona *et al*., 2004; Raun *et al*., 2005), spinach (*Spinaciaoleracea* L.) (Jones *et al*., 2007), canola (*Brassicanapus* L. var. *napus*) (Osborne, 2007), and corn (Raun *et al*., 2005). One model incorporated additional variables to account for other confounding factors such as the date of planting (Kumar *et al*., 1999). Raun *et al*. (2005) recognized that N algorithms should account for the independence of the crop response to additional N and potential maximum yield. As such, they must be measured individually. Because N is highly mobile (Khosla and Alley, 1999), the maximum potential crop yield is temporally and spatially (Girma *et al*., 2007) variable, and the amount N available from soil nitrification or denitrification varies greatly from year to year (Johnson and Raun, 2003). Furthermore, there is a strong agronomic basis for the argument that N algorithms must account for these factors by year and location. Any algorithm that combines the two without considering their independence will result in flawed recommendations (Raun *et al*., 2011).

Algorithms using other strategies, such as the sufficiency concept for recommending fertilizer N (Varvel *et al*., 2007), do not account for the temporal variability of these factors. An example of the sufficiency approach is work done by Varvel *et al*. (2007), which used normalized chlorophyll meter readings and relative or normalized yields to calculate N application rates. The use of a sufficiency index approach is appropriate for soil nutrients that are immobile, but models based on data averaged across years disregard the variability of yield responsiveness to N applied preplant and the yield response to unlimited N, both bound by the environment. As a result, the final N rate recommended is fixed to a sufficiency percentage determined from historical data and not tied to the yield level that would be achievable that year. Furthermore, the potential yield achievable is fundamental to calculating the total N demand for cereal crops in any crop year.

Lukina *et al*. (2001) proposed that the midseason N fertilizer required to maximize the grain yield for a specific season could be used to calculate the midseason N application rate. They proposed the following to predict the N application rate: $[(YP_{max} - YP_0)GN]/0.70$, where YP_{max} is the maximum potential yield, YP_0 is the potential yield with no additional fertilizer, GN is the predicted amount of total N in the grain, and 0.70 is the expected efficiency of the N fertilizer under ideal conditions. This method of determining in-season fertilizer need was shown to decrease large-area N rates while increasing wheat grain yields when each 1-m^2 area was sensed and fertilized independently. Later research by Raun *et al*. (2005) suggested that midseason N fertilizer rates be based on predicted yield potential and a response index. Their work showed that they could increase the N use efficiency by >15% in winter wheat, compared with conventional methods, at a 0.4-m^2 resolution.Ferguson *et al*. (2002) suggested that improved recommendation algorithms may often need to be combined with methods such as remote sensing to detect the crop N status at early, critical growth stages followed by carefully timed, spatially adjusted supplemental fertilization to achieve optimum N use efficiency. Later work by Noh *et al*. (2005) confirmed that it was technically feasible to design a machinery-mounted multispectral imaging sensor to reliably and accurately detect crop N stress.

Zillmann *et al*. (2006) indicated that sensor-based measurements can be used efficiently for variable N application in cereal crops when N is the main growth-limiting factor. They further cautioned that the causes of variability must be adequately understood before sensor-based, variable-rate fertilization can be properly used to optimize N side dressing in cereals.Ortiz-Monasterio and Raun (2007) showed that using a combination of an N-rich strip, together with the use of a Greenseeker sensor and an algorithm to interpret the results from the sensor, allowed farmers to obtain significant savings in N use and thus farm profits.

Modeling variable rate N fertilization

Several trials on Greenseeker based N Management have been done in Indo Gangatic plains by PAU, Ludiana, DWFSR, Modipuram and few at CIAE, Bhopal for rice and wheat crops. These were found suitable for generating site specific N fertilization recommendations (Bijay-Singh *et al.,* 2010) based on green seeker readings. The methodology for modeling the N dose estimation (Nitrogen fertilizer optimization algorithm) (NFOA) adopted from William Raun *et al.,* 2005 of Oklahoma State University, USA, has following steps

Development of INSEY-GY relationships : first and subsequent years

1. Measuring NDVI using greenseeker sensor
2. Estimating Yield Potential/ In Season Estimate of Yield:(INSEY)
3. INSEY= NDVI/days from planting to sensing, days
4. Generating the Yield Prediction Equation

Quantifying fertilizer N requirement : second year onwards

5. Establish pre-plant N Rich Strip (NRS)
6. Determine Response Index (RI)= NDVINRS/NDVITest plot
7. Predict potential yield (YP0) with no added fertilizer N from the equation for grain yield and in season estimates of grain yield (INSEY) - -YP0 =a*(INSEY)b or exponential function using nitrogen rich strip (NRS) NDVI readings and RI
8. Predicting the Potential Response YPN to Applied N
9. YPN =(YP0*RI)
10. Computing Grain N Uptake at YP0 and YPN
11. Generating a Fertilizer N Rate Recommendation
12. Fertilizer N Requirement = (grain N uptake YPN – grain N uptake YP0/(0.5 to 0.7)
13. Computing the Final Fertilizer dose based on percentage of N in the fertilizer (eg. Urea has 46% N)

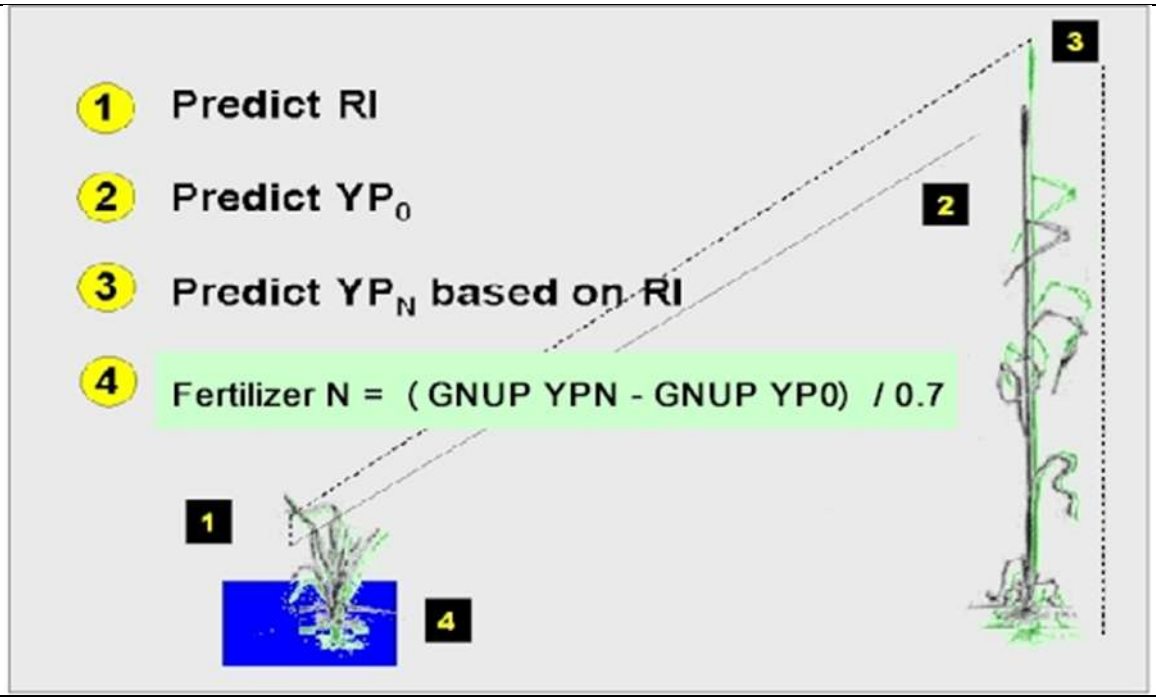

Fig. 1. Nitrogen fertilization algorithm Pictorial representation

(A Hypothetical Example)

Steps

Lay out an experiment with reps and N level as shown above Table 1.

1. Take NDVI observations at different dates after emergence (DAE), from each subplot with different Nitrogen application rates, at some regular interval

Note: the emergence datecorrectly or else use date of planting/ sowing date. Else use the planting date as reference.

2. Count the Number of vegetation period from the emergence date or planting date, taking into account only the vegetation period with the Growing Degree Days (GDD) higher > 0 4GDD = [(Tmin + Tmax)/2] – 4.4°C >0

Table 1 Layout of the experiment for calibration of Optical sensors for N response

No	N0	N30	N60	N90	N120	N150	N180
R1							
R2							
R3							
R4							

Table 2 NDVI Measurements: Replication-I

NDVI Readings Days after first emergence*	N0	N30	N60	N90	N120	N150	N180
15							
30							
45							
60							
75							
90							
105							
120							

*Exclude the non-vegetation period (snow period) when GDD< 0 or count the period as vegetation period when GDD>0 as per equation below:

GDD = (Tmin + Tmax)/2 – 4.4°C>0)

Where, Tmin, T max are minimum and maximum air temperature expressed in ^{0}C.

1. Fill the Table 2. (above) from actual field data on NDVI Measurements

2. Calculation of Response Index (RI) using equation:

RI = (NDVINRS/NDVIi=0; n and d=0, n)

Where NDVINRS refers to NDVI of the N- Rich strip or plot where N is maximum and there is no N deficiency (hidden or otherwise. NDVIi=0; n and d=o, n refers to NDVI of each N treatment and Replication on different dates from initial date of emergence.

Table 3 Response Index calculations:

RI at days after emergence	Replication -I							R-II	RIII
	N0	N30	N60	N90	N120	N150	N180		
15									
30									
45									
60									
75									
90									
105									
120									
Yield Mg/ha									

Fill the above table with the calculated data and if possible plot all the data points on a graph and show the average trend line.

3. Calculation of INSEY (In Season Estimated Yield) using following equation:

INSEY= NDVIi=0;n; D=0,n / DAS Where DAS or DAE = Days after sowing or emergence as the case may be

Table 4 INSEY data calculations:

Note: If there is a non-vegetation period of (say of 40 days where GDD <0) discount this 40 days period from the total days from emergence to till time of taking the specific reading.

Nitrogen level	Days after sowing								Crop yield, Mg/ha
	15	30	45	60	75	90	105	120	
N0									
N30									
N60									
N90									
N120									
N150									
N180									

Collect crop yield data from all the N level plots and treatment replications.

Table 5 Crop yield data (Rep 1-Rep 4)

Crop yield, Mg /ha	N0	N30	N60	N90	N120	N150	N180
R1							
R2							
R3							
R4							
Average							

4. Establish equation describing Yield as function of the INSEY:
Plot all the INSEY at different dates against averaged crop yield data for different N levels on a graph describing yield as function of INSEY.

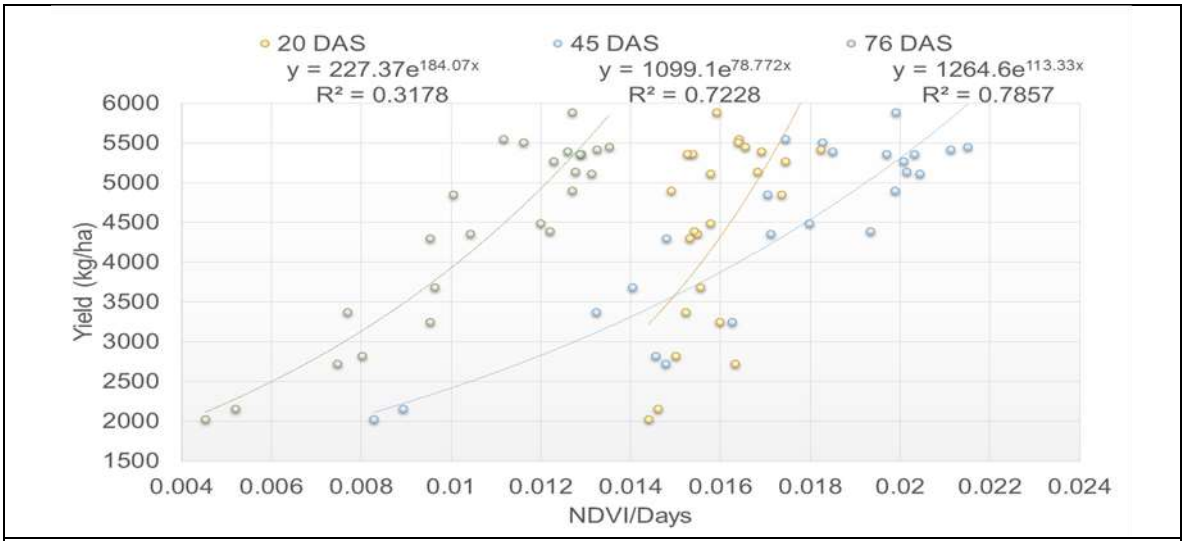

Fig. 2. Yield prediction equations

Methods for prediction of Maximum crop yield based NDVI data

1. Sense the N Rich Strip (NRS) or plot where N is maximum and there is no N deficiency
2. Sense a strip parallel to the NRS (Farmer Practice or FP)
3. Determine how many days from planting to sensing (days, GDD>0)
4. Compute INSEY (NDVI/days from planting to sensing where GDD>0)
5. Predicted yield YP0 = Predicted or potential yield based on growing conditions up to the time of sensing, that can be achieved with no additional (topdress) N fertilization (units: Mg/ha). For this purpose equation should be developed YP0= Function (INSEY)
6. YPN = Predicted or potential yield that can be achieved with additional (topdress) N fertilization based on the in-season response index (RINDVI) (units: t/ha) = (YP0)*(RINDVI)

Generating a Fertilizer N Rate Recommendation

1. RINDVI= NDVI from plots receiving adequate but not excessive preplant N, divided by NDVI from plots where no preplant N was applied
2. Computing Grain N Uptake at YP0 and YPN: The predicted amount of N that will be removed in the grain at harvest (using our equation generated from 1E) is computed as follows:

Grain N uptake, YP0 = Grain Yield (YP0) * expected % N in the Grain or Forage
GNUP_YP0 = YP0*0.0239 GNUP_YPN = YPN*0.0239

Where 0.0239 represents (0.0239 kg N uptake / kg grain 0r 2.39% N in the grain for winter wheat grown

For example, if YP0=3000 kg/ha, and desired yield is YPN=6000 kg/ha than
GNUP_YP0 = YP0*0.0239=71.7 kg/ha GNUP_YPN = YPN*0.0239 =143.4 kg/ha.
N= GNUP_YPN- GNUP_YP0=143.4-71.7=71.7 kg/ha

1. Computing the Final Fertilizer N Rate: The fertilizer N rate to be applied is computed by subtracting the predicted amount of N to be removed in the grain at YP0 from the predicted amount of N to be removed in the grain at YPN, divided by Nitrogen use efficiency. This value can range anywhere from 50% to 70%.
2. By dividing N to NUE or 71.7/0.6=113.6 kg/ha we get amount of fertilizer rate should be added into the soil in order to achieve potential crop yield of 6000 kg/ha.

Case Study

Based on the above mentioned methodology Yield prediction equations were developed based on three years data at ICAR-CIAE, Bhopal for wheat and rice crops. The Nitrogen fertilizer optimization algorithm (NFOA) given in hypothetical example was used to calculate the N top-dress dose in wheat and paddy crop. Two software (multilingual android app (Fig. 3) and a web based app (Fig. 4)) were developed using these Yield prediction equation and NFOA for estimating nitrogen fertilizer requirement of the target crop based on NDVI values measured using greenseeker sensor.

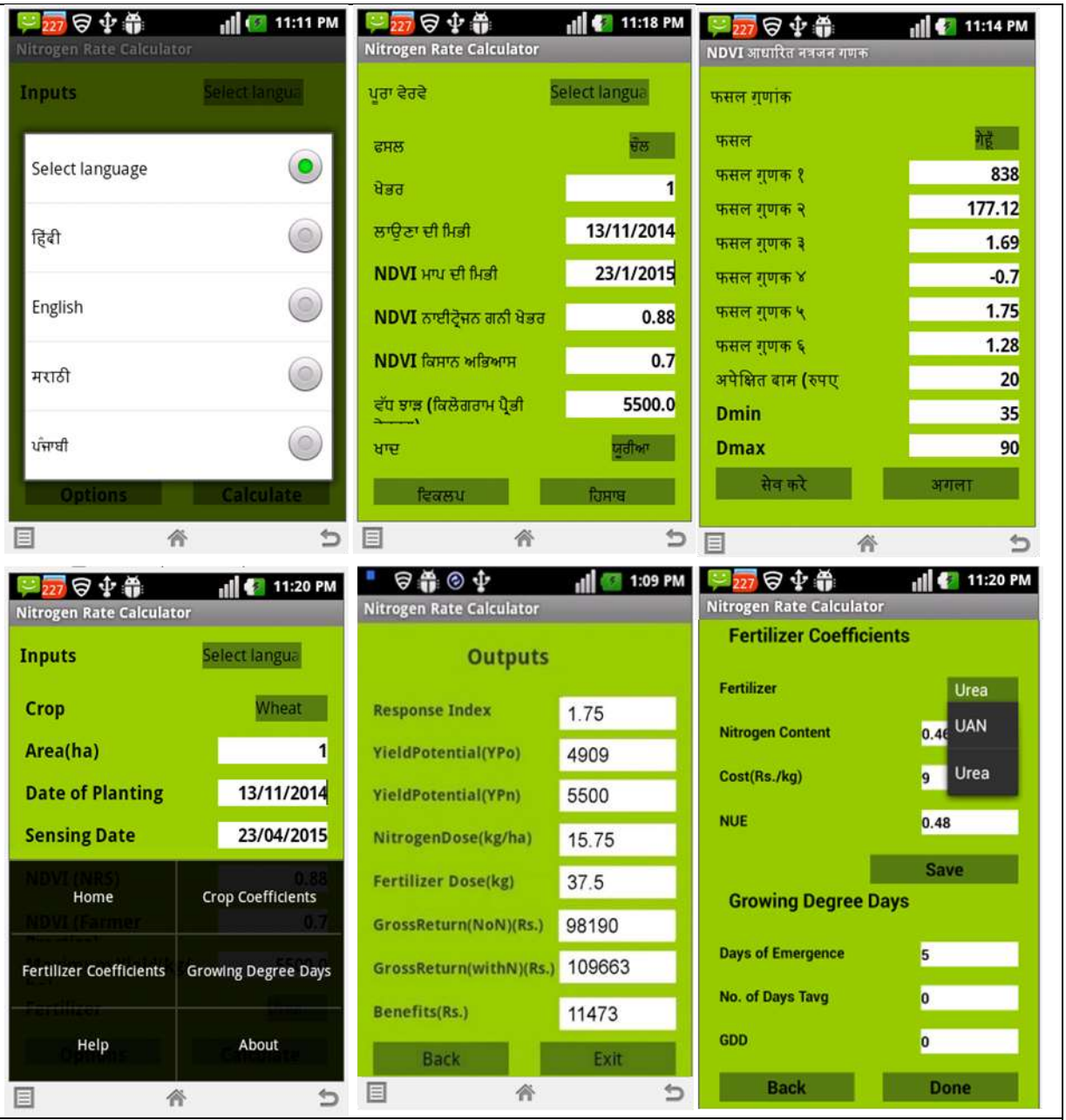

Fig. 3. Screen shots of the Multilingual Nitrogen fertilizer dose Calculator (Android App ver.1.02)

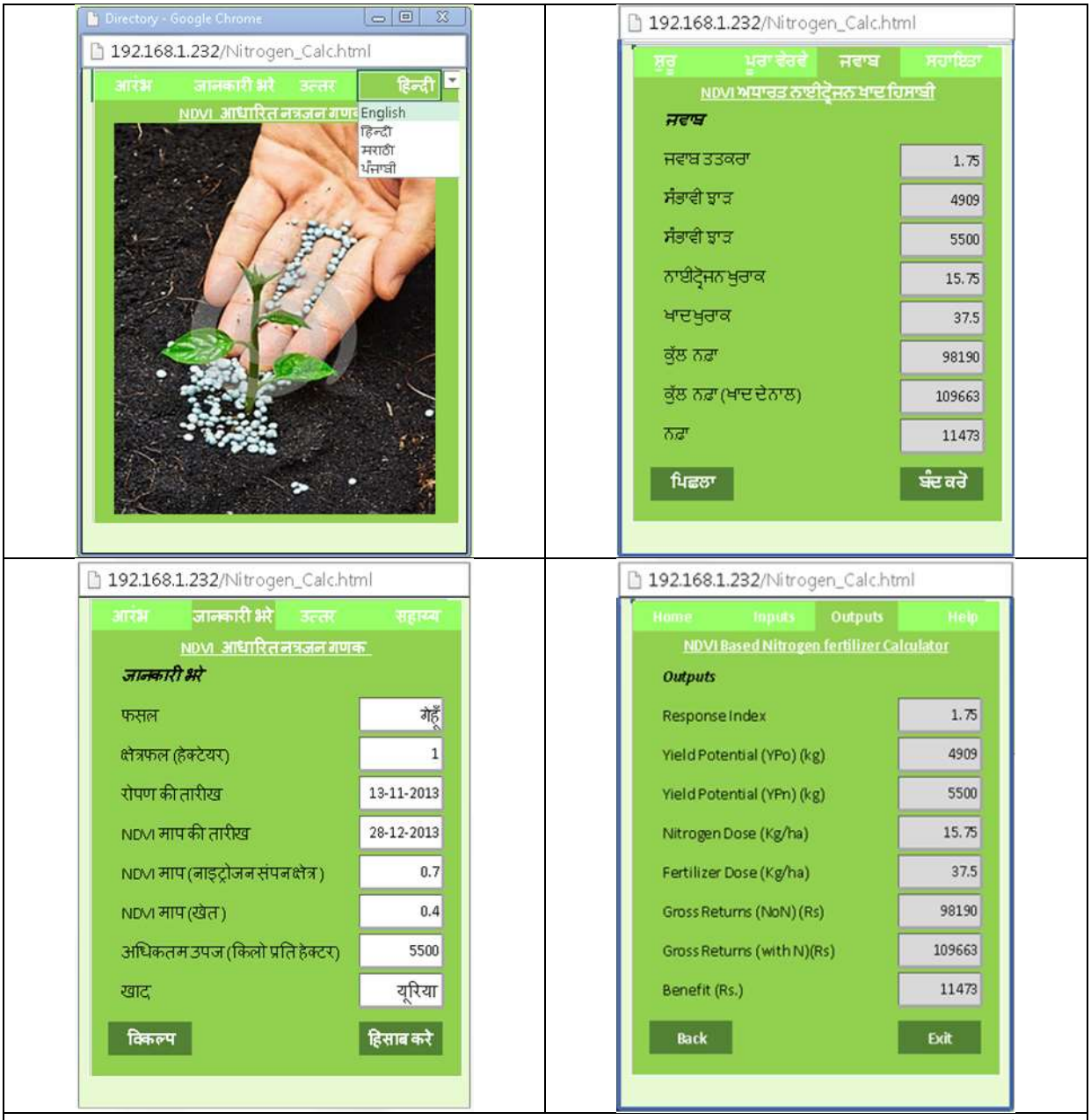

Fig. 4. Screen shots of the Multilingual Nitrogen fertilizer dose Calculator (Web App)

Validation studies were carried out to test the yield response to the Nitrogen dose recommendation generated using app. Randomized block design was used for validation of the yield prediction equation developed based on earlier field experiments by applying the NDVI based nitrogen fertilizer recommendation (generated using android app) during 3rd irrigation (Nr3I), 4th irrigation (Nr4I) and during both 3rd and 4th irrigation (Nr3I+4I). Farmers practice (FP) (120 kg/ha N) and plot with no Nitrogen (N0) were also laid for comparison Crop and soil samples required for calculation of yield and other agronomic parameters were collected for

rice and wheat crop. The results obtained showed Highest Partial factor productivity (PFP) and Agronomic use efficiency of Nitrogen in Nr3I treatment for both paddy and wheat crop. However Highest yield was observed for Nr(3I+4I) treatment in both paddy and wheat crop.(Fig. 5a &b). The potential of saving in greenhouse gas emissions/ha due to reduced urea use is summarized in Table 6.

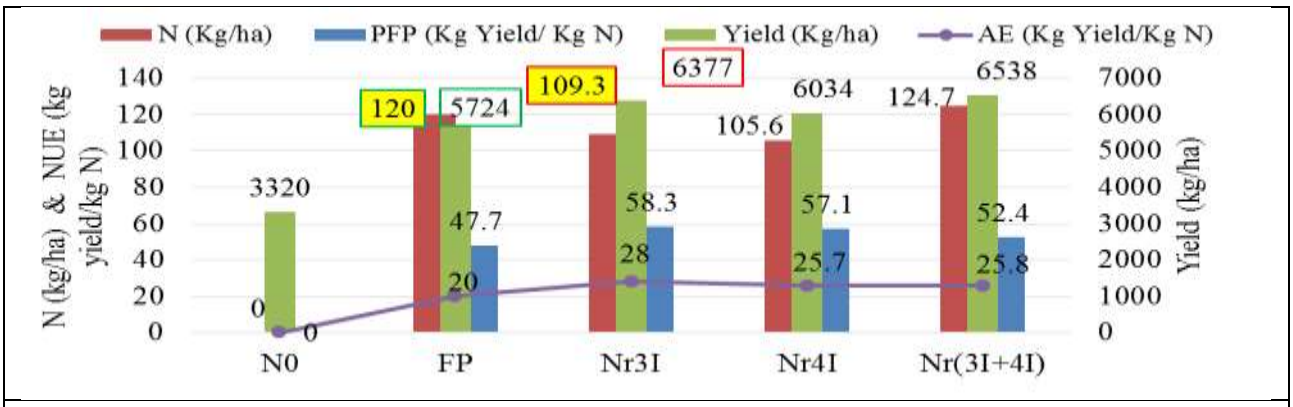

Fig. 5a: Yield and N use efficiencies for Paddy crop using NDVI based recommendation

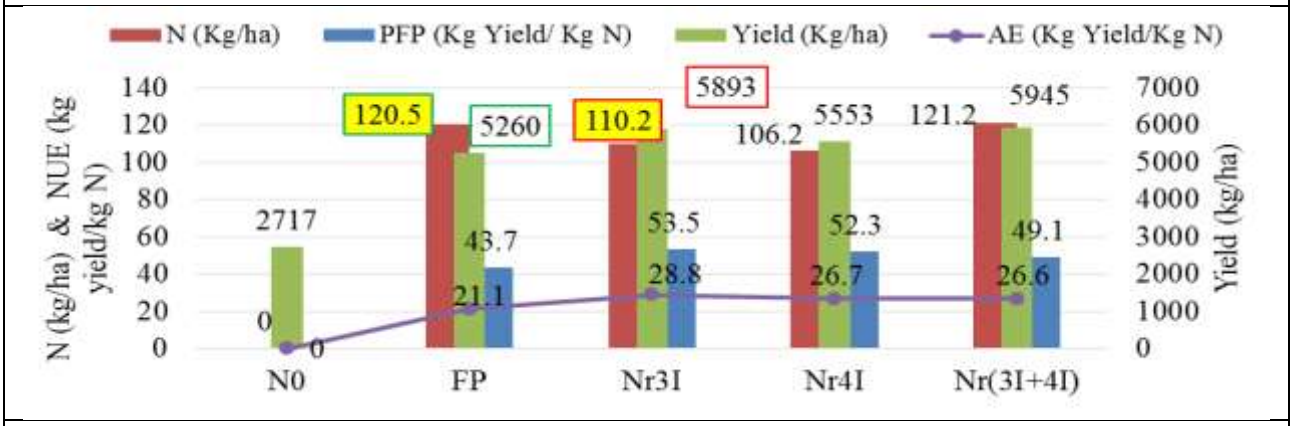

Fig. 5b: Yield and N use efficiencies for Wheat crop using NDVI based recommendation

Table 6: Potential of saving in greenhouse gas emissions/ha due to reduced urea use

Saving in emissions/ha due to reduced urea use	Paddy			Wheat		
	FP	Nr3I	Nr(3I+4I)	FP	Nr3I	Nr(3I+4I)
Average Yield (kg/ha)	5724	6377	6538	5260	5893	5945
N Dose (kg/ha)	120	109.3	124.7	120	110.2	121.2
Urea Dose (kg/ha)	260.87	237.61	271.09	260.87	239.57	263.48
Difference	0	23.26		0	21.30	
Emission coeff. for Urea (kg CE/kg Urea)	0.42					
Reduced CE emission (kg CE/ha)	-	9.77	-	-	8.95	-
CE fertilizer émission (kg CE/1000 kg grain)	19.14	15.65	17.41	20.83	17.07	18.61

Reduced CE fertilizer emission (kg CE/1000 kg grain)	0	3.49	1.73	0	3.76	2.22

Conclusions

The indication of Nitrogen stress affecting crop yield can be modelled using NFOA algorithm and other similar approaches. Most of the approaches established till date use spectral reflectance based devices that measure nitrogen stress with indices similar to NDVI. These approaches can be used for variable rate top dress nitrogen dose recommendation and application in rice and wheat crops for higher yields, reduced fertilizer consumption and overall lower carbon emissions.

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Chapter 11: Soil Plant- Interaction in Tropical Horticulture and implication of phenotyping

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Introduction

In India, presently around 9.2 m ha and 6.9 m ha area are under the cultivation of vegetables and fruits, respectively. Further increase in production is possible through bringing higher production potential crops under large area and converting waste lands, estimated to be around 11 m ha into productive lands. Farmers, scientific communities and policy makers have always been concerned about adverse impacts of abiotic stresses on agriculture and over exploitation of natural resources. About 42% (6 m ha) of degraded land in India mainly suffers with hard pan and having shallow soil depth. Resultant edaphic and drought stresses in these lands reduce the longevity and potential yields of orchards especially due to high vulnerability to droughts. Soil erosion, land degradation and multiple nutrient deficiencies are also very common features on these basaltic soils. Moreover, the impact of climate change on land degradation has drawn worldwide attention wherein the importance of geological formation has been taken as an important stress parameter to define the quantum of degradations. As proportions of productive lands are gradually declining with anthropogenic activities, it is axiomatic that the food security for ever increasing population would have to be met through adaptation and mitigation strategies for harsh agro-ecosystems in order to sustain productivity of horticultural crops. The negative impacts of shallowness in terms of low water retention, hard rocks and murrum etc. are the major constraints for establishment of orchards in shallow basaltic soils of Maharashtra. The poor and depleted soil fertility remains a primary constraint to agricultural productivity in most of tropical and sub-tropical regions. The elevated temperatures, changing precipitation patterns and extreme weather events also greatly affected on agriculture production (IPCC, 2007). The average rate of crop production increase by only 1.3% per year, but it cannot keep pace with population growth. By connecting the genotype to the phenotype, high yielding, stress-tolerant plants can be selected far more rapidly and efficiently than is currently possible. However, the lack of access to phenotyping capabilities limits our ability to dissect the genetics of quantitative traits related to growth, yield and adaptation to stress. Now days, plant

phenotyping greatly helps the genetic analysis of abiotic stress tolerance to further elucidate the stress tolerance mechanisms. However, conventional methods of plant phenotyping are laborious and destructive as compared to the recently developed high-throughput, non-destructive imaging technologies (Roy *et al*. 2011; Yang *et al*. 2013). The recent phenotyping techniques, being non-destructive, enable acquiring quantitative data on plant growth, health, and water use under abiotic stress by taking multiple images of the same plant at different time points and at different wavelengths (Morison *et al*. 2008 and Jones *et al.* 2009). Therefore, these technologies are being routinely applied to quantify traits related to stress tolerance in a number of crop plants (Berger *et al*. 2010 and White *et al*. 2012).

The nature of soil, shaped by its chemical, physical and biological properties, plays a key role in determining the growth, productivity and reproductive success of individual plants, the relative performance of coexisting plant species, and ultimately the production and productivity. Plants can influence soil properties through inputs of chemical compounds and organic matter, by impacting upon hydrological processes and surface soil temperatures, as well as by providing habitats and/or resources for microscopic and macroscopic organisms (van Dam 2009; Bardgett & Wardle 2010). Plant influences on biotic and abiotic soil properties may alter the soil's ability to support these same individuals, other individuals of the same species or other plant species. Changes to soil properties that are caused by plants, which in turn influence the performance of plants are termed 'plant–soil feedbacks' (Bever, Westover & Antonovics 1997; Wardle 2002; Ehrenfeld, Ravit & Elgersma 2005; Kulmatiski & Kardol 2008).

Gaining a greater understanding of plant–soil feedbacks and underlying mechanisms is improving our ability to predict consequences of these interactions for plant community composition and productivity under a variety of conditions. Future research will enable better prediction and mitigation of the consequences of human-induced global changes, improve efforts of restoration and conservation and promote sustainable provision of ecosystem services in a rapidly changing world.

While there has been a rapid increase in understanding the biological, chemical and physical mechanisms and their interdependencies underlying plant–soil feedback interactions, further progress is to be expected from applying new experimental techniques and technologies, linking empirical studies to modelling and field-based studies that can include plant–soil feedback interactions on longer time scales that also include long-term processes such as litter decomposition and mineralization.

Root systems play an essential role in ensuring plant productivity. Experiments conducted in controlled environments and simulation models suggest that root geometry and responses of root architecture to environmental factors should be studied as a priority. However, compared with aboveground plant organs, roots are not easily accessible by non-invasive analyses and field research is still based almost completely on manual, destructive methods. Contributing to reducing the gap between laboratory and field experiments, there is need of a novel phenotyping system like GROWSCREEN-Rhizo, which is capable of automatically imaging roots and shoots of plants grown in soil-filled rhizotrons/fields. These findings have good potential to characterise root geometry and temporal growth responses with relatively high spatial accuracy and resolution for in-situ studies of Orchard fruit crops. It will allow the design of high-throughput screening methodologies simulating environmental scenarios that are relevant in the field and will support breeding efforts and improved management practices towards better resource use efficiency and stability of crop yields.

Phenotyping under controlled condition

Although filed phenotyping is the best option to select genotypes of our interest in the target environment for yield and its component, the phenotyping in controlled environment facilities is advantageous for imposing abiotic stresses uniformly, which is not possible in field conditions. The studies on influence of abiotic stress factors like excess or limited moisture stress, high temperature and salinity are conducted under controlled conditions. The controlled condition under which the plants are grown should be relevant to the conditions prevailing in the field (Izanloo *et al.* 2008). Evaluation under controlled conditions is advantageous in terms of collecting data at a particular stage when genotypes being tested differ in durations to attain certain phenological stage. Growing plants in pots allows for strict control of water stress imposed on test genotypes and the homogeneity of stress severity; such control is seldom achieved under field conditions, particularly when genotypes under test differ in phenology and biomass.

Phenotyping under field condition

Ultimately, evaluation of crop plants for yield performance under particular abiotic stress needs to be done under field conditions. Field phenotyping helps to identify tolerance traits in the ultimate target environment and helps in evaluating many genotypes at a time. Unlike controlled growth condition, in field evaluations, there are certain factors which impact the quality of the phenotypic data to be collected (Tuberosa, 2011) listed the following factors to be evaluated carefully to ensure the

collection of meaningful phenotypic data in field experiments under water limiting conditions. The factors are the experimental design, heterogeneity of experimental condition between and within experimental unit, size of the experimental unit and number of replicates, number of sampled plants with in each experimental unit and genotype-by-environment-by-management interaction. Though the field evaluations are conducted on the ultimate target environmental conditions or crop management during the experimentation might influence the plant's phenotype. Thus the variability caused by these factors must be kept to the minimum so as to collect quality phenotyping information. In field evaluation, techniques like measuring canopy spectral reflectance (Gutierrez *et al.* 2010) and screening under high temperature stress (Hazra *et al.* 2009) and drought stress (Ashraf *et al.* 2005) are employed. The phenotyping methodologies like line source irrigation, withholding irrigation to impose water stress (Rao and Bhatt, 1992), imposition of salinity stress and conducting evaluation trails during high-temperature periods in the hotspot areas are a few techniques that are followed under field condition.

Phenotyping sites for different abiotic stress

Drought stress:

- 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.
- 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.
- 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.

Nutrient stress:

- Remove depleted nutrient from the soil.
- 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.

Saline stress:

- Automatic saline solution circulatory system
- Perforated pots in saline water tanks
- At booting stage transfer of plants to saline condition to checked flag leaf condition
- Supported hydroponics system used for imposing a controlled and homogeneous salt-stressed

Thermal stress:

- Off season planting/ staggered planting
- Maintained heat stress condition in phytotron facility

Different imaging techniques in plant phenotyping used to detect abiotic stresses

Imaging Techniques	Sensor	Resolution	Phenotype Parameters	Examples	Environment conditions
Fluorescence imaging	Fluorescence cameras	Whole shoot or leaf tissue, time series	Photosynthetic status (variable fluorescence), quantum yield, non-photochemical quenching, leaf health status, shoot architecture	Wheat (Bürling *et al*., 2010), Tomato (Mishra *et al*., 2012)	Controlled environment, field
Thermal imaging	Near-infrared cameras	Pixel-based map of Surface temperature in the infrared region	Canopy or leaf temperature	Wheat (Manickavasagan *et al*., 2008)	Controlled environment, field
Visible light imaging	Visible spectral range	whole organs or organ parts, time series	Projected area, Growth dynamics, Shoot biomass, Yield traits, Panicle traits, Root architecture, Imbibition and germination rates, Early embryonic axis growth, Height, Size morphology, Flowering time	*Arabidopsis thaliana* (Joosen *et al*., 2012), Rice (Clark *et al*., 2011)	Controlled environment, field
Hyperspect	Near-infrared	Crop	Leaf and canopy	Wheat	Controlled

ral imaging	instruments, spectrometers ,hyper spectral camera	vegetation cycles, indoor time series experiments	water status; Leaf and canopy health status; panicle health status; leaf growth; Coverage density	(Moshou *et al*., 2005)	environment; Field

Near infrared imaging	Near-infrared cameras, multispectral line scanning cameras, active thermography	Continuous or discrete spectra for each pixel in the near-infrared region	water content parameters for seeds, leaf area index	Soybean (Bolon *et al*., 2011)	Controlled environment

Conclusion

The quick development of germplasm and their 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. Plant phenomics is a simply plant physiology in 'new clothes', but it promises to bring physiology up to speed with genomics by introducing the incredible recent advances made in computing, robotics and image analysis to the wider field of plant biology. Phenomics provides the opportunity to study previously unexplored areas of plant science, and it provides the opportunity to bring together genetics and physiology to reveal the molecular genetic basis of a wide range of previously intractable plant processes. The future challenges of characterizing crop plant for desirable traits require the advances we have seen in information technology, and there is a need to build on these advances for global food security. The better knowledge of the physiological, biochemical, molecular and genetic basis of the mechanisms promoting tolerance to abiotic stress will enhance the capacity to improve crop yield under hostile environments.

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Chapter 12: Chlorophyll Fluorescence measurements and use in plant phenotyping

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Inroduction

Chlorophyll fluorescence is one of the most highly informative, rapid and non-destructive diagnostic methods for the detection and quantification of damage in the photosynthetic apparatus caused by environmental stress. No investigation into the photosynthetic performance of plants under field conditions seems complete without some fluorescence data. Light energy absorbed by chlorophyll molecules in a leaf can undergo one of three fates: it can be used to drive photosynthesis (photo-chemistry), excess energy can be dissipated as heat or it can be re-emitted as light—chlorophyll fluorescence. These three processes occur in competition, such that any increase in the efficiency of one will result in a decrease in the yield of the other two. Hence, by measuring the yield of chlorophyll fluorescence, information about changes in the efficiency of photochemistry and heat dissipation can be gained. Fluorescence occurs mostly from chlorophyll a of PSII in the red region of the spectrum (685nm) and therefore it is emitted as red light. More than 90% of absorbed light is utilized by photosynthesis. Only about 1 to 2% light is utilized by the fluorescence process (Maxwell and Johnson, 2000). The spectrum of fluorescence is different to that of absorbed light, with the peak of fluorescence emission being of longer wavelength than that of absorption. Therefore, fluorescence yield can be quantified by exposing a leaf to light of defined wave-length and measuring the amount of light re-emitted at longer wavelengths. Several research documents are available in support of chlorophyll a fluorescence to assess PSII status under light (Luttge 2000), cold (Koscielniak and Biesaga-Koscielniak 1999), heat (Srivastava and Strasser 1997; Bukhov and Carpentier 2000), and water stress (Georgieva *et al*. 2005, 2007; Goltsev *et al*. 2012).

Fluorescence parameters

Chlorophyll fluorescence can be used as a non-intrusive method of monitoring photosynthetic events. There have been very many fluorescence parameters defined in the literature. The aim is to provide information on the parameters that can be

usefully used in crop improvement programmes to identify differences in plant performance non-destructively and rapidly. Consequently, the focus will be on the fluorescence parameters associated with the induction of fluorescence on exposure of dark-adapted leaves to light and the operation of photosynthesis under growth and other light conditions. It gives a good measure of the photochemistry and electron transport rate and can be related to photosynthetic efficiency. For example, environmental stresses affect the PSII efficiency and there is decrease in Fv/FM. The fluorescence parameters (as given in Table1 and references) are very useful in monitoring and judging the physiological state of the plants under environmental stresses such as water deficit, temperature, nutrient deficiency, polluting agents, attack by pathogens.

There are two types of chlorophyll fluorescence meters – time resolving (Continuous Light) fluorimeters and pulse modulated fluorimeters (Hall et. al. 1993). Time resolving fluorimeters give the fluorescence parameters (F and Fo) for dark adapted leaves and only record Kautsky curves. However, the fluorescence measurements of the light adapted leaves require pulse modulated fluorimeters. Commercially available fluorimeters as well as integrated photosynthesis systems with fluorimeter are available for monitoring the fluorescence parameters. The fluorimeters models are PEA (Hansatech), PAM-2000 (Walz) etc. The models of integrated photosynthesis systems with fluorimeter include LI-6400 (LICor), CIRAS-2 (PP-Systems), HCM-1000 (Walz) , LCpro+ (ADC).

Table1. Fluorescence parameters and their physiological relevance (Baker, 2008)

Parameter(s)/ Definition	Physiological relevance
F= Fluorescence emission from dark adapted leaf F′=Fluorescence emission from light adapted leaf	Provides little information on photosynthetic performance because these parameters are influenced by many factors. F′ is sometimes referred to as Fs′ when at steady state
Fo=Minimal fluorescence from dark adapted leaf Fo′=Minimal fluorescence from light adapted leaf	Level of fluorescence when QA is maximally oxidized (PSII centers open)
Fm=Maximal fluorescence from dark adapted leaf Fm′=Minimal fluorescence from light adapted leaf	Level of fluorescence when QA is maximally reduced (PS II centers closed)

Fv=Variable fluorescence from dark-adapted leaves Fv′=Variable fluorescence from light adapted leaves	Demonstrates the ability of PS II to perform photochemistry (QA reduction)
Fq′= Difference in fluorescence between Fm′ and F′	Photochemical quenching of fluorescence by open PS II centers.
Fv/Fm = Maximum quantum efficiency of PSII photochemistry	Maximum efficiency at which light absorbed by PSII is used for reduction of QA.
Fq′/Fm′=PS II operating efficiency	Estimates the efficiency at which light absorbed by PS II is used for QA reduction. At a given photosynthetically active photon flux density (PPFD) this parameter provides an estimate of the quantum yield of linear electron flux through PS II. This parameter has previously been termed $\Delta F/Fm'$ and φ PS II in the literature.
Fv′/Fm′=PS II maximum efficiency	Provides an estimate of the maximum efficiency of PS II photochemistry at a given PPFD, which is the PS II operating efficiency if all the PS II centers were 'open' (QA oxidized).
Fq′/Fv′=PS II efficiency factor	Relates the PS II maximum efficiency to the PS II operating efficiency. Nonlinearly related to the proportion of PSII centers that are 'open' (QA oxidized). Mathematically identical to the coefficient of photochemical quenching, *qP*.
NPQ=Non photochemical quenching	The non photochemical quenching from Fm to Fm′. Monitors the apparent rate constant for heat loss from PS II. Calculated from (Fm/Fm′)–1.
qE=Energy-dependent quenching	Associated with light-induced proton transport into the thylakoid lumen. Regulates the rate of excitation of PS II reaction centers.

qL=Fraction of PS II centers that are 'open'	Estimates the fraction of 'open' PSII centers (with QA oxidized) on the basis of a lake model for the PSII photosynthetic apparatus. Given by (Fq'/Fv')(Fo'/F')
φF=Quantum yield of fluorescence	Number of fluorescent events for each photon absorbed

Setting of instrument before start of experiment

1. Focus & Zoom:Place a testing plant under the FC. The testing plant should be similar to the plants that will be used in. Increase El. Shutter and Sensitivity to see a nice image. Change False- color scale (right mouse click on the scale right to the image) to Black & White. Zoom and Focus the objective to see sharp image. FOCUS and ZOOM should be kept during the whole experiment otherwise the level of absolute fluorescence signal could be changed. Then arrange the plant to final position, leaves of interest perpendicular to camera.
2. Camera settings: Let measuring flashes switched on and adjust El. Shutter and Sensitivity in LIVE WINDOW. Change False- color scale to Extended spectrum or Extended spectrum 3_0_3 (the most sensitive color scales for human eye). Keep El. Shutter as low as possible (low resolution CCD between 0-1, high resolution CCD between 1-2), otherwise measuring pulses would be too strong causing actinic effect. Adjust Sensitivity by trucking the bar to get a signal in the range of 200-500 digital units (dark blue or blue color).
3. Light settings – ACTINIC LIGHT: Choose intensity of Actinic light (Act1 or Act2): (a) either desired absolute light intensity can be chosen with respect to cultivation conditions, or (b) it can be adjusted according to the fluorescence transient. (a) Place a light meter under FC to the position and distance
4. Protocol: Click on the magic hat pictogram in top panel – Protocol & Menu Wizard and choose measuring protocol. There are some predefined protocols on the left side. User alone can define own protocols by using Wizards on the right side. The predefined protocol Fv/Fm Page 2 is a simple protocol determining Fo, Fm and Fv/Fm. This can be used to check if Saturating pulse is strong enough before running any other more complicated protocol.
5. Importing settings: Click button Use in the bottom of LIVE WINDOW to import camera and light settings to the protocol.
6. Measure: Click red flash icon, Start Experiment, in the top panel to run the measurement.

Methods to achieve dark adaptation

Sr. No	Method	Limitations
1	A leaf can be put into a leaf clip shielding it from ambient light.	If the ambient light intensity is high, and the leaf is not entirely flat, there is a chance that some stray light reaches the shielded area
2	Detached leaves can be kept for a while between wet filter paper	Consequences for the physiological state of the leaf
3	Measurement in dim light under lab conditions	Leaves can still absorb and use most of the green light for photosynthesis
4	Measurements directly in the field at nigh	Measurements differ from measurements following a relatively short dark-adaptation

W**hat can go wrong during a fluorescence measurement on leaves?**

- Unopened or partially opened leaf clips
- Clip may shift in smooth leaves while attaching measuring head.
- Stray light may enter the leaf clip if leaf is not flat

Chlorophyll fluorescence: NIASM initiative

Photosynthetic system (PSII) of spikes of T. durum is more tolerant than that of *T. aestivum.*

Chlorophyll fluorescence based photosynthetic efficiency was measured in spikes of two T. aestivum and three T. durum wheat cultivars which were developed in central zone of India. It was observed that the T. durum wheat had high photosynthetic efficiency than T. aestivum as indicated by chlorophyll fluorescence parameter (Fv/Fm) at similar phenological stage. In addition, the rate of decline in photosynthetic efficiency with increase in desiccation was high in T. aestivum than in T. durum. Similar trend was observed in each of the spikelets except terminal ones. Durum wheat had relatively less moisture than the aestivum throughout the measurements suggesting that better photosynthetic efficiency in the former than in the later was intrinsic. The results also indicated that chlorophyll fluorescence of spikes could be employed for phenotyping responses of wheat germplasm for drought tolerance.

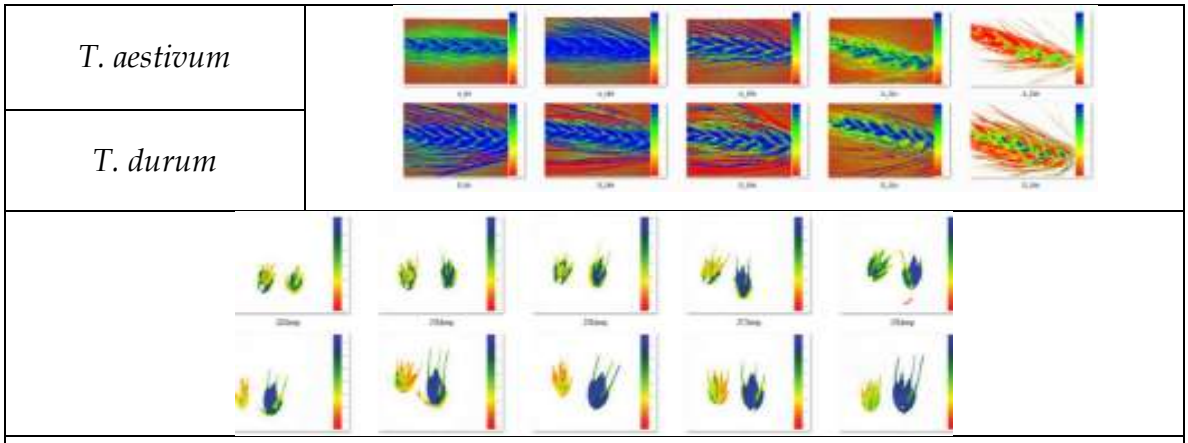

Fig. 1. Chlorophyll fluorescence imaging to assess desiccation tolerance

Photosynthetic system (PSII) of excised leaf of high and low LWL mungbean genotypes.

Variation in chlorophyll fluorescence in excised leaves of high and low LWL genotypes were studied. The initial fluorescence was identical (0.8) in both the high and low LWL genotypes. However, high LWL genotype recorded a sharp reduction in photosynthetic quantum yield within a period of 7 h of excision while low LWL genotype was able to maintain its fluorescence 48% higher than the high LWL genotype. The decrease in the chlorophyll fluorescence of high LWL genotype is further evident by a faster disappearance of the blue colour pixels from its leaf image while low LWL genotype reveals preponderance of blue colour pixels even up to 7 h post excisions

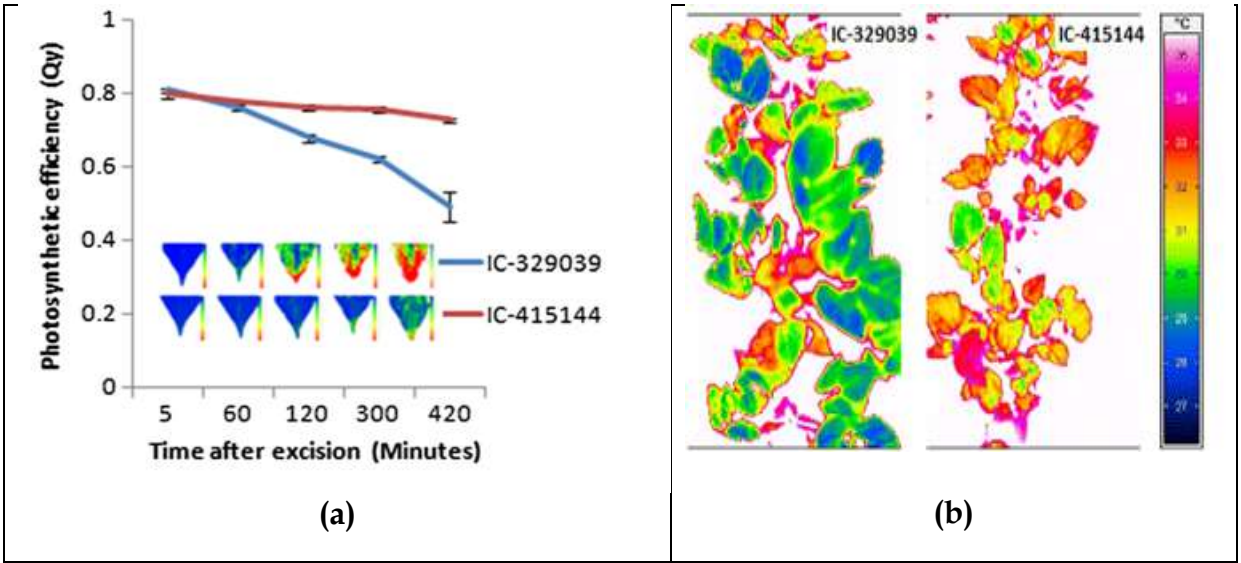

Fig. 2. Fluorescence images depicting change in chlorophyll fluorescence of excised mungbean leaves of high and low LWL genotypes over a period of indicated time points (a) Thermal images of the high and low LWL genotypes exposed to drought

(b)

Photosynthetic system (PSII) sensitivity of dragon fruit to temperature was less than that of other fruit crops.

We used Chlorophyll fluorescence technique to identify fruit crops (pomegranate, sapota, sweet orange, grape, karonda, acid lime and mango) tolerant to desiccation. Photosynthetic efficiency in terms of Fv/Fm was measured in five leaves of each of the above fruit crops. Results revealed that photosystem of pomegranate were less sensitive to desiccation when compared with the same in other crops under this experiment. High sensitivity to desiccation was conspicuous in Mango as revealed by rapid decline in Fv/Fm values which indicate sensitivity of plants to stress. The rate of decrease in quantum efficiency with moisture stress was in the order of karonda < acidlime < sweetorange < grape < sapota < mango indicating that karonda was more tolerant than others.

Photosystem of pomegranate were less sensitive while sensitivity to desiccation was conspicuous in Mango

We employed Chlorophyll fluorescence imaging to study sensitivity of 11 different fruit crops viz; acid lime, karonda, sweet orange, grape, jamun, pomegranate, sapota, mango, guava, custard apple and dragon fruit to temperature. Chlorophyll fluorescence imaging technique was preferred for phenotyping for photosynthetic efficiency of plants based on photosystem performance. We conducted experiments with leaves of different fruit crops mentioned above clearly revealed that chlorophyll fluorescence ratio (Fv/Fm) decreases as temperature increased but with varying degree of sensitivity among the fruit crops. Studies revealed that sensitivity of photosystem of dragon fruit to temperature was less than that of other fruit crops. The rate of decrease in quantum efficiency with rise in temperature was in the order of dragon fruit< acid lime < karonda < sweet orange <grape < jamun < pomegranate < sapota < mango < guava < custard apple indicating that acid lime was more tolerant to temperature than others.

Chapter 13: Hyper-spectral remote sensing for phenotyping

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Introduction

The term hyperspectral is used to define spectra consisting of large number of narrow, contiguously spaced spectral bands. Hyperspectral remote sensing, also known as imaging spectroscopy is a relatively new technique used by researchers and scientists to detect terrestrial vegetation, minerals and land use/land cover mapping. Technological advancements have enabled imaging spectroscopy to be extended beyond laboratory settings to satellites so that its applications can be focused over a global extent. Within the electromagnetic spectrum, it is well known that not all spectral bands are available for remote sensing purposes. The technique of hyperspectral remote sensing combines imaging and spectroscopy within a single system thereby resulting in large data sets that require sophisticated processing methods. Generally, hyperspectral data sets will be composed of about 100 to 200 spectral bands possessing relatively narrow bandwidths unlike the multispectral data sets which possess just 5-10 bands of relatively larger bandwidths.

It is evident that since in hyperspectral imaging, the data is collected in a large number of contiguous (spectrally without any gap in the region of interest) spectral bands, much finer spectral content in the target can be resolved, as compared to that obtained from a multispectral imager (Fig.1).

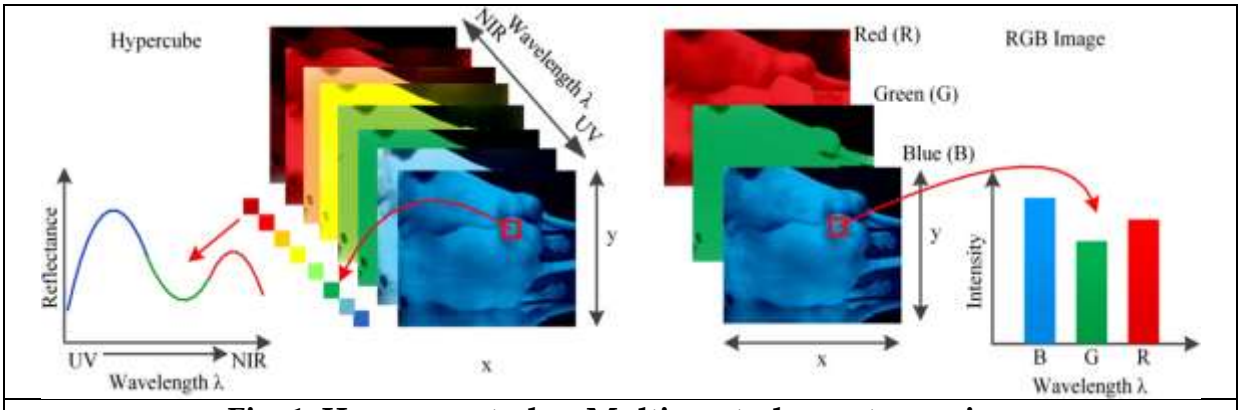

Fig. 1. Hyper-spectral vs Multispectral remote sensing

A hyperspectral imager measures the energy collected from an object in a two dimensional spatial domain and the spectral information acquired along the third direction (Fig.2).

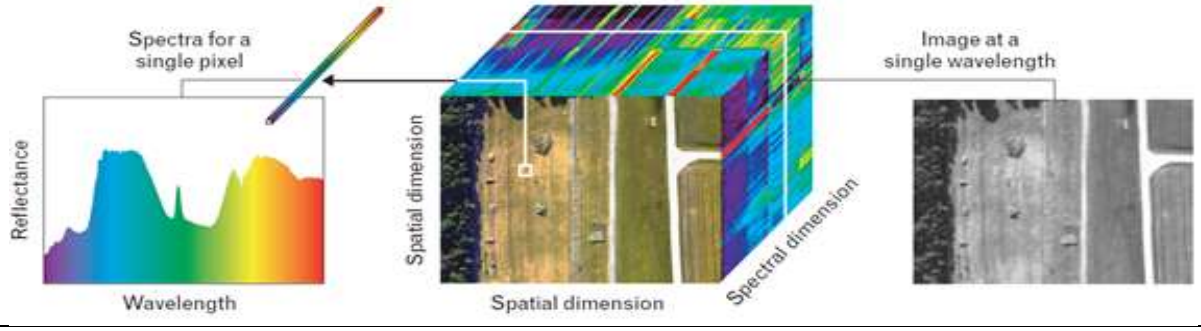

Fig. 2. Three dimensions of hyperspectral cube

The resulting 3D data set is often referred to as subject cube or data cube. The fig.3 demonstrates this concept appropriately.

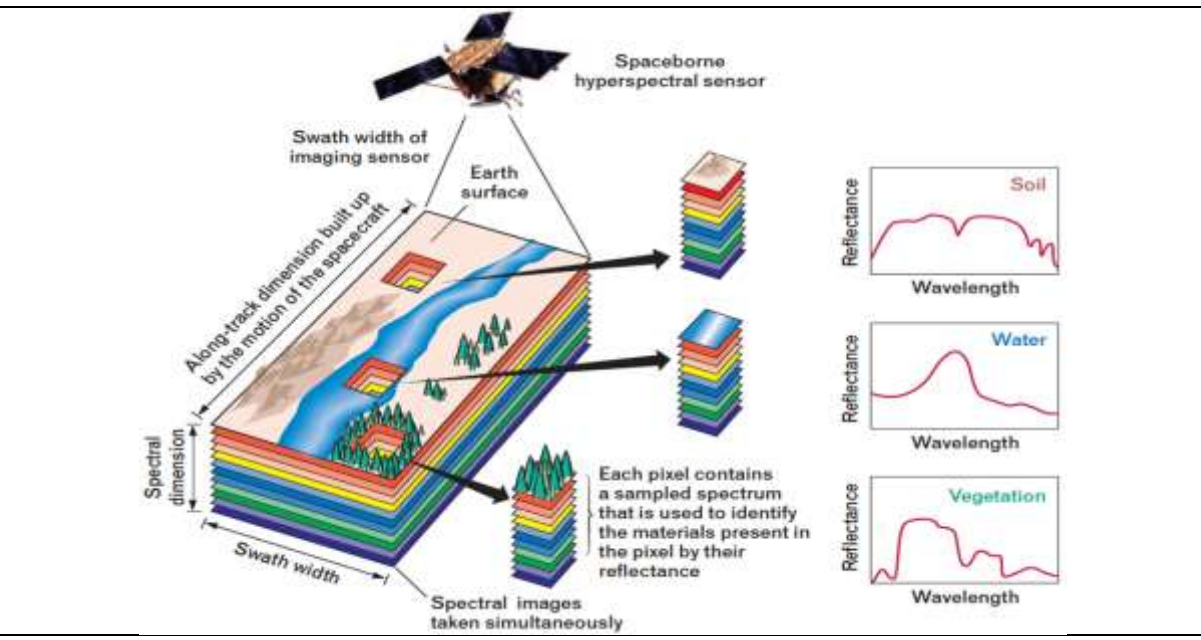

Fig. 3. Concept of data cube generated by a spaceborne hyperspectral imager

Multi/Hyper-spectral remote sensing for vegetation

Imaging plants is more than just 'taking pictures'. The application of imaging spectroscopy to plant phenotyping came from research on the remote sensing of vegetation. In the visible spectrum (400–700 nm), reflectance by single leaves or canopies is particularly low. This low reflectance is explained by the absorption by leaf pigments, primarily chlorophyll, with a characteristic peak of reflectance in the

green region of approximately 550 nm. With the transition from the visible to near infrared (NIR) wavelengths, there is a sharp increase in reflectance, or the so-called 'red edge'. In the NIR (700–1200 nm), a large proportion of incident radiation is reflected by leaves from scattering within the leaf mesophyll. With increasing wavelengths of up to 2500 nm, the reflectance decreases gradually because of increased absorption by the water present in the leaves.

Healthy plants interact (absorb, reflect, emit, transmit and fluoresce) with electromagnetic radiation in a manner different from that of infected/stressed plant interactions and the wavelength of the incident radiation which, thus forms the signature of that object. This finding is primarily explained by the fact that plants have different optical properties. Imaging techniques are very helpful for detecting these properties, especially for those that cannot be seen by the naked eye. The general shape of reflectance and transmittance curves for green leaves is similar for all species. It is controlled by absorption features of specific molecules and the cellular structure of the leaf tissue. Because of the strong absorption by photoactive pigments (chlorophylls, anthocyanins, and carotenoids) at visible wavelengths, the canopy has low reflectance. In the near-infrared wavebands, the canopy has high reflectance because of multiple scattering at the air-cell interfaces in the internal leaf tissue. In wide wavebands of shortwave infrared, healthy leaves have low reflectance because of absorption by water, proteins and other carbon constituents. Because of their high water content (emissivity between 0.97 and 0.99), healthy leaves emit radiation in the thermal infrared band (≈10 μm) according to their temperature. The leaves appear green because the green light band (550 nm) is reflected relatively efficiently when compared with the blue, yellow and red bands, which are absorbed by photoactive pigments. At approximately 670 nm, reflectance changes cause the red edge to shift to shorter wavelengths (the sharp transition from low visible reflectance to high NIR reflectance).

Leaves represent the main surfaces of plant canopies where energy and gas are exchanged. In the visible region (400-700 nm), absorption by leaf pigment, namely chlorophyll a and b, carotenoids, xanthophylls, and polyphenols leads to low reflectance. Chlorophyll a and b have typical absorption bands in the blue region at around 430/450nm and in the red region at around 660/640 nm. Thus a weak reflectance peak at around 550 nm (green region) is induced, thereby giving green colour to plant. The spectra in Near Infrared (NIR) region (700/1300 nm) mainly arise for the internal structure of the leaf. Around 40-50% of energy is reflected with in this range while <5% is absorbed. The third important region is Short Wave Infrared (SWIR) region (1300-2500 nm). This region is characterized by water content of the leaf. Hence this region has three strong water absorption channels (1400 nm, 1900 nm, 2700 nm) and secondary features at 960, 1120, 1540,

1670, and 2200 nm. Therefore, with decreasing water content of the leaf, the reflection in this region increases shown in (Fig.4).

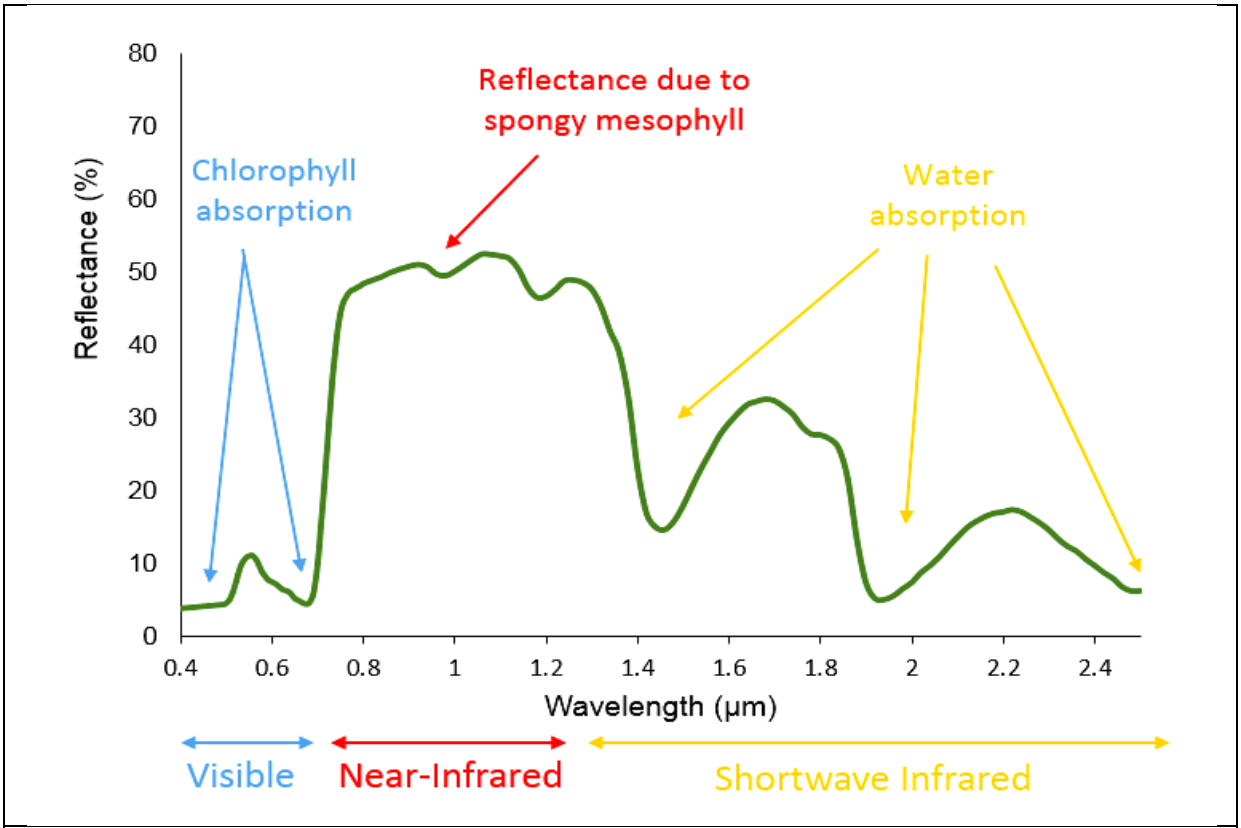

Fig. 4. Spectral bands response in vegetation

Stress Detection

Increased reflectance in the green and red region is the most important leaf reflectance responses to plant stress. It may be noted that green reflectance changes as soon as plant faces stress, however, in NIR region changes in reflectance is observed only when stress goes beyond a certain level. The water stress also gets clearly detected in red and NIR portions of the spectrum. Example of the spectral change due to stress is shown in (Fig.5).

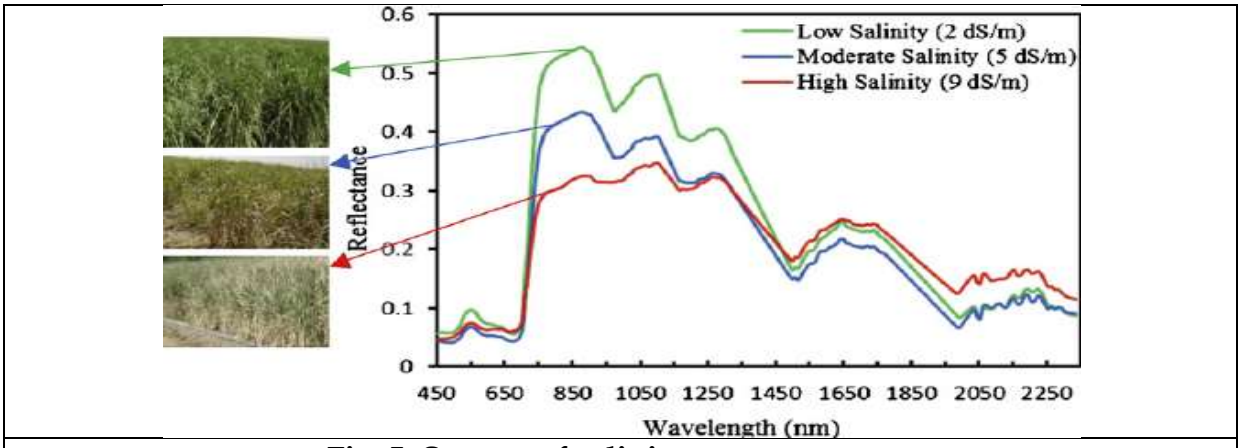

Fig. 5. Spectra of salinity stress response

However, The manifestations of water and nutrient stress in these plants expressed as changes in leaf area index and leaf chlorophyll content could be correlated well through power functions using the amplitude as well as wavelength of the red edge peak (703 nm) and the area of the red edge peak (between 680 and 780 nm). They also found that the differences in the parameter, amplitude of the red edge peak was discernable only when the water stress was well developed. On the other hand, indices such as SRPI, PRI, mSR_{705} and mND_{705} and NPCI were found more useful ($R^2 > 0.80$) for determining plant nitrogen status.

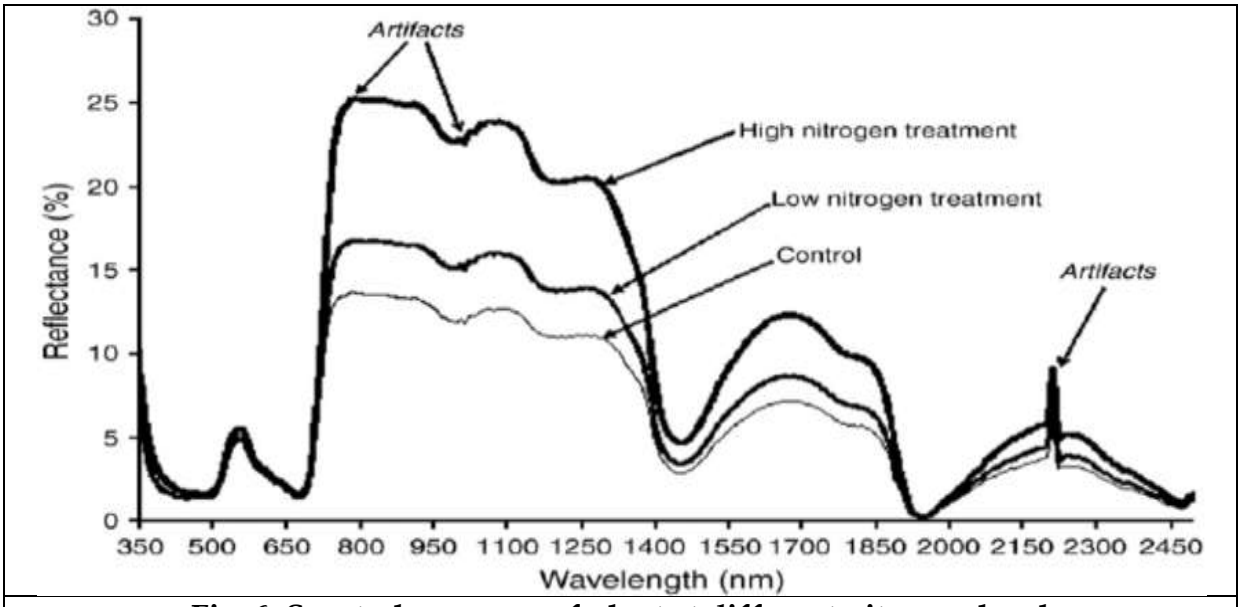

Fig. 6. Spectral response of plant at different nitrogen level

For a typical crop canopy, reflectance is low between the 480 and 680 nm region due to the strong absorption by chlorophylls and other pigments, but is high in the NIR region due to the microcellular structures in leaf material and canopy structures (Thomas and Oerther, 1972). The real-time target information can be effectively

extracted and utilized by analyzing canopy spectral characteristics and developing key spectral vegetation indices. The main task of agronomic remote sensing is to determine the sensitive bands 9single of a combination in the form of indices) of spectrum reflection and their derived parameters characterizing vegetation canopies for indicating growth status and then to determine the quantitative relationships between spectral properties and agronomic parameters.

Hyperspectral Imaging Techniques for Plant Phenotyping

Plant phenotyping is intended to measure complex traits related to growth, yield and adaptation to stress with a certain accuracy and precision at different scales of organization, from organs to canopies. Rapid development of high-throughput genotype screening in plant breeding and genomics for related growth, yield and tolerance to different biotic and abiotic stresses, has necessitated a call for more effective and reliable phenotyping data to support modern genetic crop improvement. Quantitative measurement strongly benefits from novel imaging technologies but needs standardized experimental protocols, including imaging sensor calibration and a precise definition of raw data processing routines, as part of the best practices for plant phenotyping.

Plant phenotyping based on spectral reflection information relies on the properties of the light emerging from the canopy after multiple interactions (such as reflections, transmissions, and absorptions) with the tissues of the plant. The canopy spectral signature from this diffusely reflected radiation is described by the ratio of the intensity of reflected light to that of the illuminated light for each wavelength in visible (400–750 nm), near-infrared (750–1200 nm) and shortwave infrared (1200–2400 nm) spectral regions.

In plant phenotyping, spectral reflectance indices are used for fast, non-destructive measurements of green biomass, canopy chlorophyll content, leaf and canopy senescence (or if they stay green) and plant water status. The derivation of a number of reflectance vegetation indices, from simple differences between two wavelength reflectance values to normalized reflectance values, is often used. Several indices have been introduced in both field research and breeding programs for large-scale phenotyping and dynamic estimations of the biomass, greenness, nitrogen content pigment composition, photosynthetic status, and water content. Multispectral and hyperspectral measurements are widely used to estimate the canopy water content as an indicator of water status, which uses the absorption bands in the infrared range to describe various water indices. Moreover, the use of high resolution spectroscopy and wavelet analysis can also provide high sensitivity to the canopy water content. The high spectral resolution hyperspectral

measurement makes it a promising method for assessing rice leaf growth, for determining the condition of rice panicles; near infrared reflectance spectroscopy as a high-throughput screening tool for pest and disease resistance in a sugarcane breeding program; Two hundred and twenty-two wheat genotypes were scanned over the 1100–2300 nm wavelength range by a fiber-optic probe; vegetation indices for the precision phenotyping of quantitative stripe rust reactions etc. Investigators are also looking into the possibility of using specific bands in the NIR to the mid-infrared region to estimate tissue water content noninvasively and to design screening protocols for genotypic differential responses to drought. In further extending the number of measured wavelengths, imaging spectroscopy opens new possibilities for extracting spectral features related to plant health and disease status.

Results interpretation requires the integration of experimental metadata within data schemas for the measured phenotype, genomic data and environmental data (Physiological: Leaf Nitrogen Content, Leaf Chlorophyll content, Net Photosynthesis, Transpiration rate, Stomatal Conductance, Equivalent Water Thickness, Proline concentration, Glycine betaine, SOD activity, Sucrose content, Commercial cane sugar; Soil: Moisture (dynamics) Initial available N, P, K, S, Zn, Fe, Mn and organic; carbon, pH, EC, Soil type and texture etc.). Some of the hyper-spectral bands identified for trait identification are given in Table 1. Modern hyperspectral imaging techniques have high resolution and allow for the visualization of multi-dimensional and multi-parameter data. Imaging techniques are used to quantify complex traits under related growth, yield and applications to stress for plant phenotyping in controlled environmental systems (in growth chambers or in the greenhouse) or in the field. As the plants traits and image interpretation are complex issues, the use of satellite imaging technique especially hyper-spectral to monitor plant growth and dynamic responses under stress in real time are a real challenge and needs to be taken care with greater caution.

Table 1: Hyper-spectral bands responsible for identifying plant characters

Hyperion band no.	Region of electromagnetic spectrum	Central wavelength (nm)	Frequency of occurrence	Agricultural importance (according to Thenkabail *et al.*[35])
9	Visible	436.99	2	Blue absorption peak; sensitive to senescing, chlorophyll *a*
25		599.80	2	Absorption pre-maxima; sensitive to biomass, soil background
26		609.97	2	
27		620.15	2	
29		640.50	2	
30		650.67	2	
32		671.02	2	Absorption maxima; maximum chlorophyll absorption, greatest soil crop contrast
33		681.20	2	
39	Red edge	742.25	2	Red edge region, sensitive to vegetation stress and dynamics
40		752.43	2	
42	NIR	772.78	4	Early NIR; more sensitive to changes in chlorophyll content than a broad NIR band
43		782.95	2	
44		793.13	2	
45		803.30	2	Centre of NIR shoulder; strong correlation with total chlorophyll
50		854.18	2	
52		874.53	3	Correlation with biomass, LAI
86	Moisture sensitive	1003.30	2	Rapid reflectance rising spectra after moisture absorption; sensitive to plant moisture status, biomass and LAI
87	NIR (MSNIR)	1013.30	2	
88		1023.40	2	
89		1033.50	2	
90		1043.59	2	
91		1053.69	2	
92		1063.79	2	Post-reflectance peak in NIR; sensitive to biomass and LAI
94		1083.99	2	
159	Early MIR (EMIR)	1739.69	2	Reflectance post-peak in EMIR; sensitive to biomass, cellulose and lignin
185	Far MIR (FMIR)	2002.06	2	Moisture absorption trough in FMIR; sensitive to plant moisture

Chapter 14: Membrane stability index (MSI) and Relative water content (RWC) – phenotypic indicators of plant tolerance to abiotic stresses

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Introduction

Abiotic stresses, including water deficit, salinity and extreme temperatures, are the most important to be considered in the selection of new genotypes because it affects crop production. Among these stresses, drought is one of the most adverse factors of plant growth and productivity. During water deficit, many physiological and biochemical processes are disturbed. Understanding the multiple mechanisms by which plants respond to water stress is a challenge to enhancing crop drought tolerance. Plant response to drought can be studied by identification of traits that are related to drought tolerance at the physiological, cellular, biochemical and molecular levels. Genotypes possessing the ability to maintain cell membrane integrity and high relative water content traits throughout grain filling are potential candidates to assure yield in semi-arid regions.

Membrane Stability Index (MSI) as a drought tolerance test

Many studies point to cell membrane as an initial site of stress injury, the function and structure of plant cell membranes is drastically damaged by environmental stress. Thus, evaluation of cellular membrane integrity as a measure of environmental stress tolerance appears to be relevant criterion (Sullivan, 1972). Most commonly, changes in the electrical impedence and electrolyte leakage have been measured to detect stress injury of cell membrane. Leakage will vary in relation to the membranes' abilities to take up and retain solutes and, therefore, will reflect stress induced changes in both membrane potentials and membrane permeability. Sullivan and Ross (1979) have conducted many experiments concerned with the relationship between electrolyte leakage following a desiccation treatment and the general ability of the plants to tolerate stress, and they found that membrane integrity and stability to the stress as evaluated by electrolyte leakage correlate well with tolerance of other plant process to the stress. To date, this method has been successfully used to measure membrane integrity in various crop plants subjected to a variety of environmental stress (Blum *et al*., 2001, Talaat and Shawky, 2014; Furlan

et al. 2017). Electrolyte leakage measured was markedly influenced by age, sampling part and season, degree of stress hardening, and plant species. Therefore, these factors should be taken into consideration at the measurement.

Estimation of MSI

The general protocol involves the application of stress to the leaf after it has been subjected to hardening, followed by the measurement of electrolyte leakage using the electro conductometric method (Blum and Ebercon, 1981).

1. 100 mg of clean leaf discs or pieces of leaf tissue cut with scissors or even whole small leaves are detached and placed in standard glass vials containing double distilled water (DDW) in two sets (The total area of leaf material per vial is about 15 to 25 cm^2. The exact area is not important and it does not have to be the same for all vials. In the case of screening, at least 10 vials (samples) are prepared for each genotype. All the tubes should be closed with caps or aluminium foils).

2. Heat one set of the sample at 40 °C for 30 min in a water bath and measure the electrical conductivity (C_1) on conductivity meter after cooling to room temperature (the bottom portion containing leaf samples will be completely below the water surface level during heating in water bath).

3. Heat another set at 100 °C on a boiling water bath for 10 min and measure its conductivity on the conductivity meter after cooling to room temperature (C_2).

4. Calculate MSI using the formula

$$\mathbf{MSI\ (\%) = (1-C1/C2)\times 100}$$

Relative water content (RWC) as a drought tolerance test

Leaf relative water content (RWC) is an important indicator of water status in plants (Sinclair and Ludlow 1985); it reflects the balance between water supply to the leaf tissue and transpiration rate (Lugojan and Ciulca 2011). RWC was used successfully to identify drought resistance in several crops like soybean (Mutava *et al*., 2015), tomato (Zhu *et al*., 2014), maize (Efeoğlu *et al*., 2009) and common bean (Rosales-Serna *et al*., 2004). The cultivars having high RWC, are more resistant against drought stress (Schonfeld *et al*., 1988). Trials can be rapidly screened for genotypes which maintain high leaf RWC values during water deficit stress, and vice-versa. Generally, it seems that osmoregulation is one of the main mechanisms preserving turgor pressure in most plant species against water loss from plant, so it

causes plant to continue water absorption and retain metabolic activities. Leaf RWC is easily and simply measured, without the need for expensive specialized instruments.

Estimation of RWC

The method has long been in use, even before its re-examination (Barrs and Weatherley, 1962), when it was termed 'relative turgidity'. It gained increasing appreciation with time and experience. The method is simple and this is one more advantage. It estimates the current water content of the sampled leaf tissue relative the maximal water content it can hold at full turgidity. Normal values of RWC range between 98% in fully turgid transpiring leaves to about 30-40% in severely desiccated and dying leaves, depending on plant species. In most crop species the typical leaf RWC at around initial wilting is about 60% to 70%, with exceptions.

General precautions to be followed for sampling

- All components of leaf water relations change during the day as irradiance and temperatures change. For no more than two hours at and after solar noon, the change is very small. This is the time "window" for leaf sampling, unless a daily curve of RWC is of interest.
- Avoid the plant samples which are wet from dew, irrigation or rain.
- Take 4 to 6 samples (replications) from a single treatment or genotype. Each sample represents a different plant, if possible.
- Take top-most fully expanded leaves, unless the interest is in profiling leaves on the plant.
- In large broad-leaves (sunflower, cotton, etc) leaf discs should cut from the leaves, to obtain a total of about 5-10 cm^2/sample. Sample size does not have to be the same for all samples.
- Avoid large veins.
- Leaf discs should be large enough (around 1.5 cm in diameter) so as to reduce the area of cut leaf surface/sample.
- Various leaf disc cutters were designed by laboratories and might be available commercially. Alternatively a sharp cork borer may be used, cutting the leaf over a piece of dense rubber or a large rubber stopper.
- In smaller composite leaves (groundnuts, alfalfa, clover, chickpeas) several leaflets make up a fast and convenient sample. In cereals, a sample may constitute of a mid-leaf section of about 5-10 cm^2 cut with scissors. With larger leaves such as maize or sorghum a section measuring, say, about

1x7 cm can be cut with scissors from the area between the mid-vein and the edge.

- Samples should be immediately placed in a picnic cooler (around 10 ^{0}C-15 ^{0}C) but not frozen on ice. Samples should reach the lab as soon as possible. This is why leaf sampling should be done quickly and it is important to enlist as much help as possible for the job.
- With good and careful work the method should normally result in about 2% to 3% of RWC being a statistically significant difference between treatments.

Procedure

1. Weigh the leaf discs immediately after collection and record the fresh weight (FW).
2. Place the samples in labelled petri plates containing distilled water. Saturate the leaf samples for three hours.
3. Take the leaf samples out of the plate, and quickly and carefully blot dry with filter/tissue paper.
4. Weigh the leaf samples to get turgid weights (TW).
5. Dry the samples in an oven at 80 °C for 24 hours or until constant mass and reweigh the samples (dry weight, DW).
6. The RWC can be calculated as follows:

$$\mathbf{RWC = (FW - DW)/(TW - DW) \times 100}.$$

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Chapter 15: Measurement of stomatal density and size in crop plant

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Introduction

Stomata are minute pores on the leaf surface which are surrounded by pairs of adjacent guard cells that tightly regulate the pore aperture. These microscopic pores regulate the vital processes like leaf temperature, water evaporation and gas exchange by changing the osmotic pressure in the guard cells. Stomata are distributed in both the lower surfaces (abaxial) and upper surfaces (adaxial) of the leaves. Usually the lower surface of a dicot leaf has a greater number of stomata while in a monocot leaf they are more or less equal on both surfaces. In most of the floating plants, stomata are found only on the upper epidermis. Number of stomata per unit area of the leaf is known as stomatal density and often ranges from 20 to 1190 mm^{-2} (Willmer and Fricker 1996). Stomatal density and distribution of the stomata plays an important role in determining the rate of gas exchange and water loss from a leaf. Stomata take part in photosynthesis reactions by providing CO_2 through it as well as involves in the transpiration of water which is essential for nutrient uptake from soil to the plant body. Conversely, excess water loss from plants under drought stress is disadvantageous and might exert damaging effects resulting in plant death. Stomatal aperture is tightly regulated by divergent exogenous stimuli, such as light, drought stress, pathogens, temperature and others. These stimuli are sensed and signaled to the guard cells via endogenous signaling molecules including phytohormones, hydrogen peroxide (H_2O_2) and Ca^{2+} ions. Abscisic acid (ABA) is among the major players in terms of stress related stomatal closure. Other phytohormones, such as ethylene, jasmonates and salicylic acid, also function in the regulation of stomatal aperture.

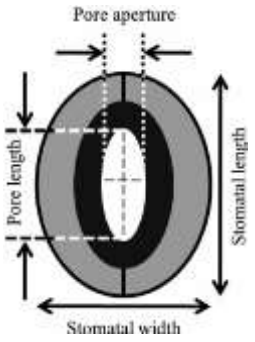
Information on variation of stomatal density and distribution is of significant importance for plant breeder as this character can affect CO_2 diffusion into the carboxylation sites and also the amount of energy used in transpiration (Jarvis and McNaughton 1986). The balance between carbon gain through photosynthesis and water loss through transpiration may also affect instantaneous Water Use Efficiency (WUE) (Borthakur et al. 2017). In the context of climate smart agriculture, information on these aspects may play a crucial role in developing climate efficient clones.

Two different methods are describing here for measuring size and density of stomata. Both required a stereo microscope with attached camera and image analysis software.

1. Using isolated epidermal layer of leaf

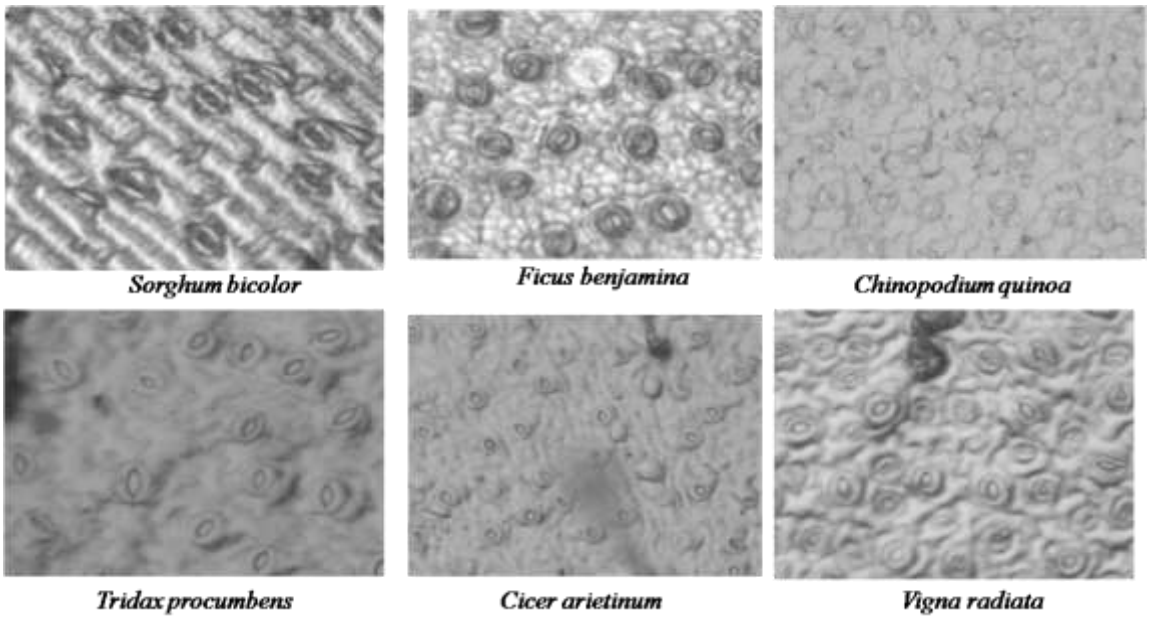
The distribution of stomata on the upper and lower surfaces of the leaf can be studied by removing the peels of the leaf from the adaxial or abaxial surfaces and observing the same under a microscope. This assay can also be used to identify the substances stimulating stomatal closure in various crops (Shen et al. 2015).

1. Collect the fresh fully opened leaves
2. Gently peel the epidermis using sharp pointed forceps
3. Float the peels with cuticle side in contact with the solution [MES buffer (10 mM MES, 50 mM KCl, 100 µM $CaCl_2$, pH 6.15)] in a Petri dish.
4. Incubate the peels under bright light for 1 hour to promote stomatal opening
5. Observe under microscope for opened stomata by either transferring peels to a microscopic slide or directly in the Petri dish.
6. Capture the images.
7. Calculate stomatal density by counting number of stomata in a specified unit area using the image analysis software and can be expressed as number of stomata per mm^2.
8. Use *calibrate scale* in the image analysis software for measuring the width and length of the stomata and stomatal pores directly from the saved image as shown in figure (Figure courtesy- Savvides et al., 2011)



9. Stomatal size and stomatal pore size will be obtained by multiplying length and width respectively (Franks and Beerling, 2009). In the case of closed stomata, pore size represents the length between the junctions of the guard cells at each end of the stoma, and may indicate the maximum potential opening of the stomatal pore, but not the aperture of opening that actually occurs (Malone et al., 1993; Maherali et al., 2002).

Note: Stomatal closure assay can be studied by keeping the peels with opened stomata in MES buffer with 10 µM ABA. If the intention is to determine only stomatal density, then skip 3-4 steps; isolated peel can be mounted on a slide and can be observed on microscope)



Distribution of stomata among various plants

2. Leaf impression method (Borthakur et al. 2017)

- Clean the abaxial epidermis of the leaf using a degreased cotton ball
- Spread a thin layer of clear nail varnish in the mid-area between the central vein and the leaf edge
- Leave it to dry (for approximately 20 min).
- Peel off thin film (approximately 5 mm×15 mm) from the leaf surface
- Mount it on a glass slide and cover with a cover slip
- View under microscope with a camera and computer attachment
- Stomatal density and size can be calculated as mentioned in the above procedure.

Note: This method provide only the information about current status of stomata

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Chapter 16: Advances in in-situ Root Phenotyping

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The studies on crop root system are often neglected and thus still remain as an underexplored target for improvements of crop yields and productivity. A promising finding for crop root systems is that increased yield and productivity is attainable with improved root system architecture traits, being explained as an optimized spatial configuration of root structures in the soil. Roots are often much more variable than shoots, and are affected by variations of climate, soil conditions, tillage practices, plant varieties, soil nutrient, other crop and soil management practices and water availability throughout the season. Thus, adoptions of appropriate crop management practices diminish the likelihood of root limitations. Understanding of temporal and spatial root architectural development is very important for crop yield maximization particularly under extreme environments. However, much emphasis has not been given on the improvement of crop root systems as compared to above ground plant characteristics. This discrepancy might be ascribed to their hidden nature at below ground and of variable nature, both of which enormously complicate observation and conducting the research trials and not easily instrumented or observed due to relatively high cost and technical difficulties in sampling, data collection and analysis.

The variation in root system morphology can lead to difference in structural and mechanical strength of root system, which determines the plant susceptibility to lodging. Physiological characteristics also differ between cultivars and can be important in determine the outcome of processes such as nutrient acquisition. The rate of uptake of nutrients per unit root length depends on the nutrient availability but also varied considerably between cultivars (Uren and Reisenaur, 1988; Meier and Leuschner, 2008;). The number and length of root hairs in the field depends upon soil and management conditions. These root hairs are responsible for the uptake of lesser mobile nutrients such as phosphorus (Itoh and Barber, 1983). The potential uptake of water and nutrients is mainly governed by the root length and surface area of the plant (Judd et al., 2015;-Sathiyavani et al., 2017). Root diameter plays an important role in root development and function (Wu et al., 2016) and it is also considered one of the most important parameters in rhizosphere modelling studies. Thus, in future

research for improving the crop productivity, emphasis should be given to selection of those kinds of traits that optimize acquisition of resources such as water and mineral nutrients particularly under extreme environmental conditions. Therefore, understanding the below ground root architectural developments particularly under abiotically stressed environments holds potential for the exploitation and desirable manipulation of root characteristics to enhance resource-use efficiency and crop productivity. Hence, advance techniques and tools are required to record the complex and vital process of root architectural development for a desired period or at a particular critical crop growth stages.

Techniques for root phenotyping:

There are many techniques for root phenotyping depending on the extent and nature of root phenotypes. The conventional and many contemporary techniques for root system architectural studies involve careful excavation and recording (Schuurman et al., 1971; Bohm, 1979; Barnett et al., 1983; Gregory, et al. 2009; Page`s et al., 2010; Trachsel et al., 2011), but are laborious, time consuming and destructive in nature. The advanced high end sophisticated techniques that are automatic, non-invasive in nature and likely involve some kind of spectroscopy or imaging (Zhu et al., 2011; Judd, et al., 2015). These techniques have their own merits and demerits, and some may be surrogates that measure properties (Barison & Uphoff. 2011) that simply correlate with root properties. Other methods like portable capacitance meter (McBride et al., 2008), optical imaging (French et al., 2009; Iyer-Pascuzzi et al., 2010; Vegapareddy et al., 2010) and three dimensional imaging, X-ray computed tomography and nuclear magnetic resonance imaging (Garbout et al., 2013; Metzner et al., 2015; Tracy et al., 2015; Daly et al., 2015; van Dusschoten et al., 2016) are also used for various type root studies but these techniques are more expensive and not much popular. The most common methods/ techniques used for root studies with their advantages and disadvantages as reported by Judd, et al., 2015 are given in Table 1.

In-situ root Phenotyping:

Non-destructive methods such as using rhizotrons, mini-rhizotrons, and radioactive tracers are mainly used for continuous observation of root extension or to investigate the distribution of live roots throughout the crop growing season.

The most common techniques being used for in-situ root phenotyping of crop plants are discussed here:

Table 1. Overview of most frequently used methods to measure or to analyze root systems, and selected studies reporting or using them. (Adapted from Reubens *et al*., 2007).

Method	Information Type	Destructive to roots?	Advantages (+)/Disadvantages (−)
Field methods			
Photographs or drawings	Qualitative analysis, 2D root morphology	No	(+) Copy of the exact root structure visible, easy and rapid (photographs) (−) tedious (drawings), blurry (photographs), no statistical inference or quantitative information, only qualitative commentaries, 2D only, problems with root overlap
Trench/window	2D spatial root distribution	Yes/No	(+) easy to record root data, repeated measurements on specific roots (−) static, limited 2D area, roots and structure could be destroyed by digging process, aberrant root growth along installed window
Pinboards/monoliths	Length, weight, diameter, distribution pattern	Yes	(+) view some natural arrangement of roots (−) requires some skill, labor-intensive, large losses of fine roots
Auger/core	Length, weight, diameter, distribution pattern	No	(+) easy (−) requires large number of samples, labor-intensive, sampling depth limited, time-consuming processing in lab
Rhizotron/minirhizotron/ mesorhizotron	Dynamic 2D information on root morphology, growth and turnover	No	(+) repeated measurements on specific roots (−) expensive, possibly labor intensive (construction and analyzing data), aberrant root growth along window
Above-ground rhizotrons	Dynamic 2D information on root morphology, growth and turnover	No	(+) repeated measurements on specific roots (−) aberrant root growth along window
Container methods			

Root washing	Root dry weight, shoot:root ratio, diameter, distribution pattern	Yes	(+) whole root system visible (−) large losses of fine roots, loss of natural positions/architecture, time-consuming, tedious
Root rating	Root density, appearance, branching and distribution pattern	No	(+) easy, rapid (−) subjective measurement, qualitative, human error
Transparent containers/substrates	Root density, appearance, branchingand distribution pattern	No	(+) whole root system visible, 3D, more natural architecture (−) different environment compared to soils and soilless substrates
Horhizotron™	Length, weight, diameter, distribution pattern	No	(+) repeated measurements on specific roots, lightweight materials used (−) only for large plant use—starting with 3.78–11.35 L root balls, materials not permanent/fixed, easily breakable, aberrant root growth along window
Mini-Horhizotron, rhizometer, hydraulic conductance flow meter	Root density, appearance, branching and distribution pattern	No	(+) repeated measurements on specific roots, lightweight materials used, materials permanent, hard to break (−) only for small plant use—seeds/plugs/liners, aberrant root growth along window
Digital imaging			
Image Analyzing Computer	Branching and distribution pattern	Yes	(+) less time-consuming, less subjective (human) (−) harvested roots, only photographing small sections of roots at a time, problems with root overlap
WinRHIZO, RootReader	Root density, angles, appearance, branching and distribution pattern, root length, root surface area	Yes/No	(+) easy, rapid, less subjective (human), greater range of measurements (−) may only work on washed roots (destructive), problems with root overlap
NMR and X-ray CT	Root length, growth, volume repartition	No	(+) report image of whole root system (−) far from being practical, roots grown in small containers only

Rhizotron/ soil columns/ pipes systems/ perspex or glass/ observation chambers rhizolaboratory method:

The rhizotron can be defined as a facility or building designed underground for viewing and measuring plant roots and underground structures through transparent surfaces that may be in contact with the natural soil (Klepper and Kaspar, 1994). It is a tool for making non-destructive, repeated measurements of root systems at a large field-scale (Judd et al., 2015). Rhizotrons are one of the earliest non-destructive techniques for observing root growth in soil. The aim of these methods is to increase the understanding of:

- The interaction between growth and functioning of plant part above and below the ground and the relevant soil processes;
- The relationship between unfavourable growth conditions in soil and atmosphere and dry matter utilization and partitioning and thus good for crop growth model studies;
- Root growth and performance in situation where biotic factors and abiotic factors may interfere

Design and equipment:

Rhizolabs contains soil container lowered into the soil (125x125x200 cm depth). Plants are grown in and around these containers to initiate a crop situation as closely as possible. The containers are closed system suitable for water balance studies.

The undisturbed soil profile and water table can be installed specific requirement. The sensors can be accommodated at different soil profile and the following observations can be made:

- *Root development pattern:* The rooting pattern can be studied and can be recorded with an endoscope or a mini video camera placed in horizontal root observation tubes at depth intervals of 10-15 cm. Sensors and cameras can be installed to measure soil conditions and record time-lapse photography. Roots growing along the transparent wall can be traced as the roots grow, to provide information on speed of root growth and root density (Glinski et al., 1993).

- *Soil water content and water tension:* About 48 tensiometer or 160 capacitive water sensor monitor can be fitted in and temp. and soil conductivity are recorded. Since each container has separate irrigation system, the water level and water extraction per layer and the corresponding rooting pattern can be assessed.
- *Soil temperature, composition of soil solution and soil atmosphere:* Thermocouple and other sensors in the profile provides 200 measuring point for soil temperature. For soil solution sampling, the ceramic section cups and microporus tubes are fitted in the profiles and small gas exchange cells are used for soil atmosphere. These measurements help us to collect information on local nutrients concentrations and soil respiration.
- *Crop evapotranspiration, Ps rate and soil gas exchange:* There are transparent crops enclosure that can be used as per our desire for any length of the time. Air temperature and CO_2 concentration can be controlled and evapotranspiration, photosynthesis and respiration can be measured from CO_2 and H_2O concentration difference between entry point and exit hole.

For data acquisition and data base management a small computer is attached with the rhizotron that collect and records the data and computed.

The major advantages of using rhizotron facilities are the taking of successive measurements on the same individual root and continuous monitoring in change of root growth while keeping the controlled environmental condition (Huck and Taylor, 1982). However, the biggest disadvantage of the rhizotron is its expense of construction and operation (Klepper and Kaspar, 1994). Other disadvantage includes the finite number of repetitions, the immobility of the structure and changing of the soil environment when the rhizotron is installed (Huck and Taylor, 1982). Also, the viewing surface of the rhizotron may not be representative of the roots in the bulk soil at depth and after research is conducted, the soil might need to be replaced, in which case the replacement soil may have altered soil biology as compared to the native profile (Klepper and Kaspar, 1994).

In-situ root scanner/ mini-rhizotron technique:

The technique called the mini-rhizotron is used to monitor the rate of root growth under different environmental conditions. It is a well-established technique in long-term root dynamics studies (Johnson and Mayer 1998, Satomura et al. 2001; Roberti et al., 2014). In this technique, a video camera /scanner enable to capture non-destructive, high resolution, digital images to monitor in-situ root growth and development. The growth and behavior of roots can be monitored for an entire crop growing season through successive measurements on the same site and same depth. The in-situ root images are analyzed with an available root analysis software package and data thus collected from the mini-rhizotrons can be used to draw a dynamic picture of root system in terms of root count, length, diameter, surface area and volume. It is a non-destructive but expensive method for root studies. It has the advantages of least soil disturbance and suitable for long-term monitoring of root dynamics. However, this system does not represent the whole root systems because of limited assessable area with a single tube. There is the possibility of missing root turnover because of the long gap between observations and the amount of production and dead root between captures is not observed, so it can be underestimated (Rygiewicz et al., 1997). Therefore, gap between the measurements should be minimized.

Even then, this is most employed technique for in-situ root dynamic studies in India. This technique is discussed in details in the chapter "*In-situ Root Phenotyping through*

Mini-rhizotron Technique ".

Choudhary et al., 2016 have used minirhizotron technique for periodic monitoring of the rooting patterns of ratoon sugarcane. The standard access tubes of 1.8 m length were installed in the field and in-situ root images representing 0.2 m soil depth were captured using a Root Scanner CI-600. The images were analyzed with a CI-690 RootSnap! Software. They reported that root dynamics of sugarcane was significantly affected due to drought and the impact of drought could be mitigated through conservation agriculture and fertilizer management practices. Similarly, minirhizotron technique was also used to monitor the root growth of feba bean (*Vicia faba* L.) throughout the crop growing season under different tillage treatments in vertisol (Romero et al., 2011). This technique was

also used for measuring the root dynamics in other crops like European beech (*Fagus sylvatica* L.) (Meier and Leuschner, 2008), wheat (Romero et al., 2010), chickpea (Romero et al., 2012), soybean (Torrion et al., 2012), apple orchard (Zanotelli et al., 2012). The minirhizotron technique also being used to estimate production, biomass and turnover of ectomycorrhizal mycelium (Wallander et al., 2013).

Wide-view optical scanner method:

The wide-view optical scanner has been developed by Dannoura et al., 2008. Like minirhizotron, this technique is suitable for continuous monitoring of root growth and performing detailed studies of individual roots. The scanner system facilitates the analysis of image data and allows continuous monitoring by automating the capture and by fixing the position of the optical scanner. In comparison to mini-rhizotrons, the viewing area of root windows is usually much larger and not curved. However they can cause a bigger disruption to the soil and installation could be complicated.

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Chapter 17: In-situ Root Phenotyping through Mini-rhizotron Technique

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In research, much emphasis has not been given on the improvement of crop root systems as compared to above ground plant characteristics. This discrepancy might be ascribed to their hidden nature at below ground and of variable nature, both of which enormously complicate observation and conducting the research trials and not easily instrumented or observed due to relatively high cost and technical difficulties in sampling, data collection and analysis. Hence, advance techniques and tools are required to record the complex and vital process of root architectural development for a desired period or at a particular critical crop growth stages. The growth and behavior of roots can be monitored for an entire crop growing season through successive measurements on the same site and same depth by mini-rhizotron technique.

Equipment and experimental set-up for in-situ root studies

Image acquisition software:

The system (CI-600 and CI-601 Root scanner) for automating image capture for mini-rhizotrons has recently been developed by CID, Inc, WA, USA (Fig. 1). With the introduction of the CI-601, image acquisition can be performed automatically and remotely.

The CI-600 features:

- Very portable and quick operation
- Linear scanning with no distortion
- High-resolution images up to 23.5 million pixels
- 360-degree scans (21.59 x 19.56 cm)
- 100, 300, and 600 DPI scanning resolutions

- Included tablet computer to power scanner, operate control software, and save images
- USB interface for laptop computer image storage
- Ability to observe root growth and behavior over multiple growing seasons

Fig. 1. CI-600 scanner for in-situ root image recording.

Mini-Rhizotrons/ Rhizo-tubes: The clear acrylic tubes having 6.4 cm inner diameter and 7.0 cm outer diameter and 105 cm standard length are used for installation in the field (Fig. 2 & 3). However, tubes with greater length (180 cm) are also can be utilized.

Fig. 2. Mini-rhizotron for in-situ root image acquisition

Experimental set-up:

The series of holes of around 7.5 cm diameter and 45^0 angles from the soil surface are dugged in the field by using a motorized root auger and transparent acrylic tubes are inserted into the holes (Fig. 3). A wooden platform for guiding the motorized root auger can be used while making the holes at 45^0 angles (Fig. 4). The best centre for single site auguring is about 1/3 of the distance from the plant base to mid-way between the two rows. The above ground portion of tubes should either be painted black or covered with a black cotton cloth to prohibit entry of light in the tube. The installation of tubes should be done prior to sowing of the crop.

Fig. 3. Motorized root auger for digging the hole in the soil. and transparent acrylic tubes are inserted into the holes

Fig. 4. Wooden platform for guiding the motorized root auger.

In-situ root image acquisition using the CI-600:

The CI-600 root scanner connected with a laptop computer through USB as shown in Fig. 5 is inserted into the tubes to monitor root growth at a desired depth. When the plant begins to build a network of roots, images of the structure and behavior of the roots can be recorded. The CI-600 scanner head rotates within the tube to scan roots and it provides nearly 360^0 high-resolution images of soil and roots of 21.59 x 19.56 cm size. The connected images of different depths can be captured by moving the camera along the tube and these images can be saved in computer for further analysis.

Fig. 5. In-situ root image acquisition by using CI-600 root scanner.

Image analysis system:

Image analyses systems provide an opportunity to facilitate analyzing procedure. They offer a rapid assessment of root characteristics like length and surface area, diameter and tips, root branching patterns etc. The image analysis software "CI-690 RootSnap!" has been developed by CID, Inc, WA, USA. It allows users to measure root growth and turnover dynamics, disease, and behavior over time by analyzing scanned images collected with the CI-600. The analysed images are stored in rsp formats and the software supports exporting data to Microsoft Excel for further statistical analysis.

Features of CI-690 RootSnap!:

- Monitor and analyse root development, architecture, and morphology.
- Map roots in a fraction of the time
- Multi-touch Interface, optimised for touch-screen
- Integrated image enhancement feature
- Automated "Snap to Root" functionality
- Comprehensive data analysis package
- Measures root length, area, volume, and diameter
- Time-series root analysis feature
- Intuitive and efficient user interface

RootSnap! is a faster and more reliable method for analyzing root images. It includes a revolutionary user interface that employs a combination of advanced image analysis and

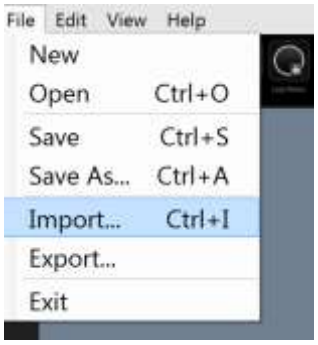
a multi-touch LCD screen, which allows users to quickly and easily trace roots using their fingers.

Analysis of in-situ root images with CI-690 RootSnap! Software:

After the RootSnap! software is installed on a touchscreen computer, the application can be accessed from the Start Menu or by double-clicking on the RootSnap! icon on the desktop.

To begin using RootSnap!, an image of a root needs to be imported into the program or an already saved project or session can be opened. The Menu Bar displays File, Edit, View, and Help and many other features.

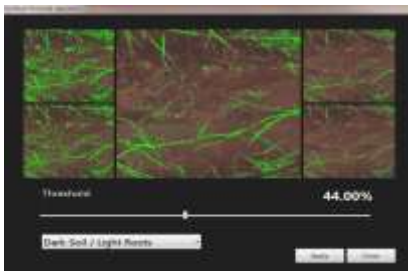
Import an Image:



- To import an image, select <File> <Import > or press Control + I
- Browse the computer or enter the file name of the image you wish to import.

Estimate Root Percent:

After an image is imported and the actual size is correct, the image's root/soil separation threshold should be set. Click the Estimate Root Percent icon on the toolbar. Adjust the threshold level and select the best image. The best image has neon green overlaying only the roots and none of the soil. This tool estimates how much of the root system is shown in the image. After the threshold is set and you click "Apply", the estimated root percent statistic will appear in the Image Details Panel.

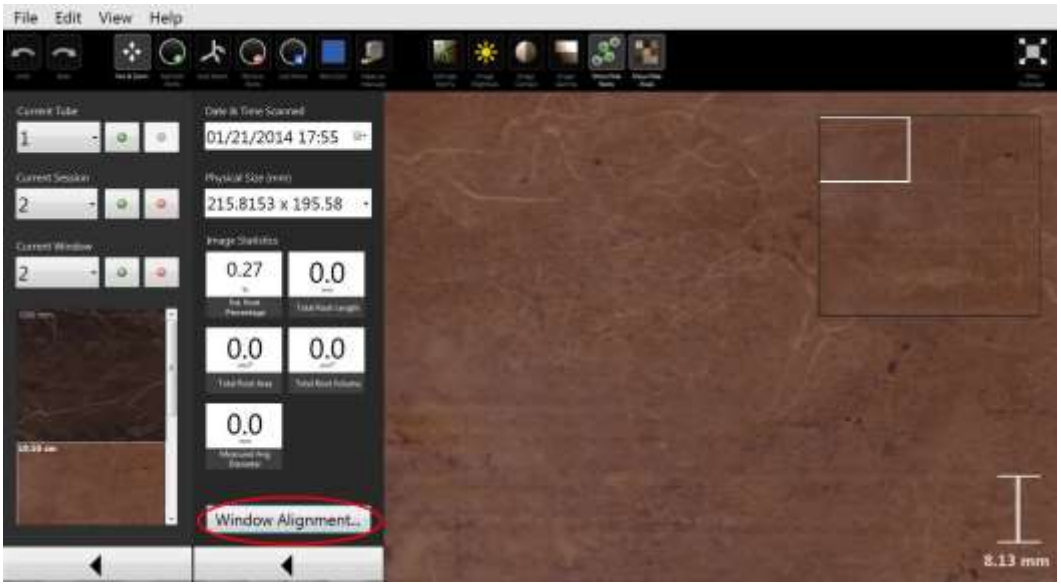


Window Alignment:

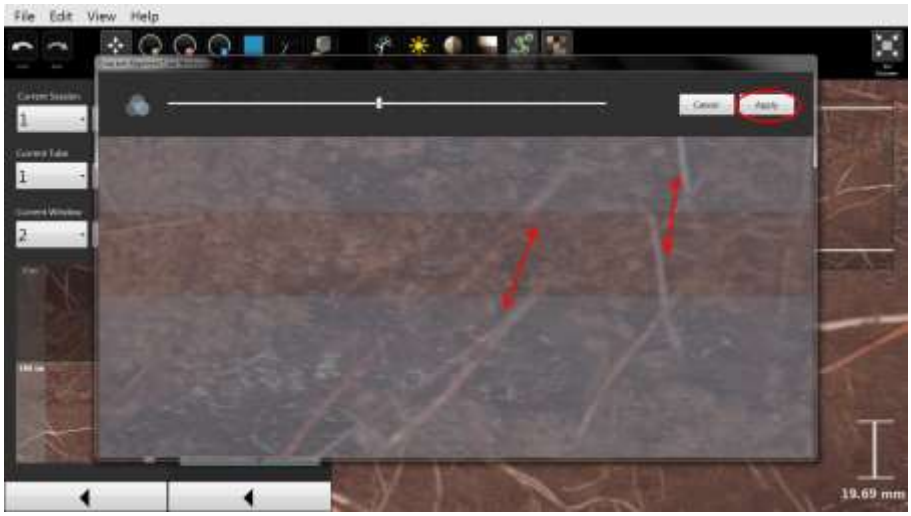
Multiple images may be lined up and have the overlap removed using the Window Alignment tool on the Image Details panel. It is easy to accurately eliminate the overlap and make sure that all scanned roots are fully included in the analysis.

To align window images:

- Load the images into RootSnap!.
- Starting with the second window from the top (Window 2), the Window
- Alignment button is at the bottom of the Image Details Panel. If you cannot see the Window Alignment button, check that RootSnap! is in full-screen mode (show/exit full screen button on right of tool bar) and check that Window 2 or lower is selected.
- To see the Image Details Panel, select View<Panels<Image Details from the top menu bar.



- Pressing the Window Alignment button will initiate a pop-up window.
- Align the images from Window 1 and Window 2 using the mouse or fingers.
- The images will become transparent where overlapping. This is in order to help accurately line up the images and roots.
- Use the mouse or fingers to fine-tune the exact overlap in the transparent "ghost effect" area. Align the images by overlaying the roots.
- Click <Apply> at the top of the screen when images are aligned or click <Cancel> to exit the crop and align feature.

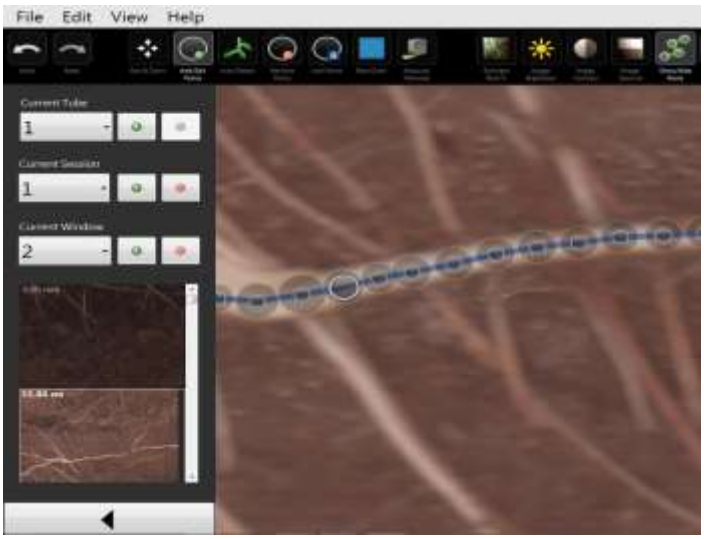


Beginning to Map Roots:

Select a window image to start mapping. Follow the steps as given below for mapping the roots:

1. Import image.
2. Rotate/flip the image.
3. Verify correct physical size in Image Details Panel.
4. Window alignment (if lower in tube then Window 1).
5. Adjust image Brightness, Contrast and Gamma
6. Estimate root percent.
7. Zoom in on root to map until it is at least as wide as your finger.

8. Place the first point in the center of the root. The first point is critical; it must be on the root!
9. Detect growth.
10. Check automatically mapped points for accuracy and diameter.
11. Move/place points past color change to keep automatically detecting growth.
12. Detect growth again.
13. Start mapping branches:
    a. Map a few points and detect growth.
    b. Dock branch to parent root.
14. Detect growth.
    a. Move points to end of branches.
15. Continue for rest of root system.



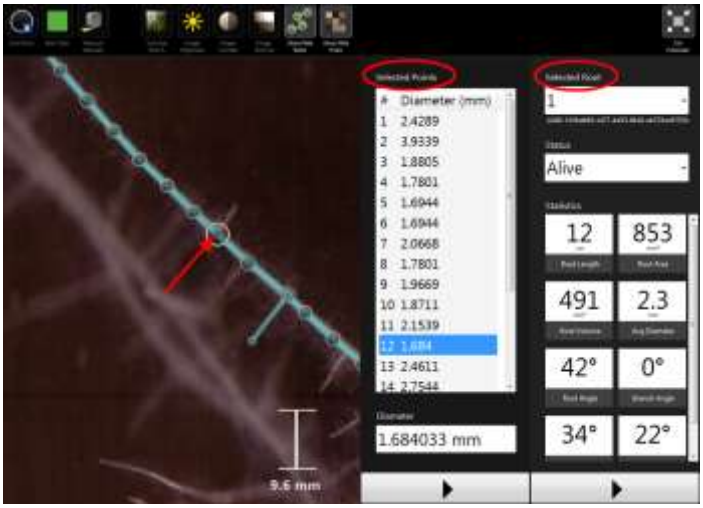
Tool bar options:

1. Undo & Redo
2. Pan and Zoom
3. Add/ Edit Points
4. Range
5. Snap to Root:

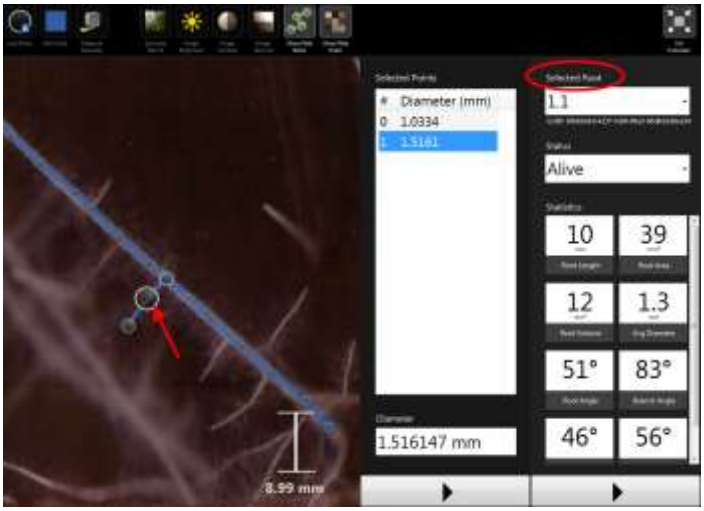
When this feature enabled, a root drawing that has just been traced will automatically "snap" to the center of the actual root. The center of the root is determined by the matching the identical color as the last root point, within the range of the tool, for the next root point.

6. Auto Detect: The Auto Detect tool will automatically find roots using the Snap To Root application of RootSnap!

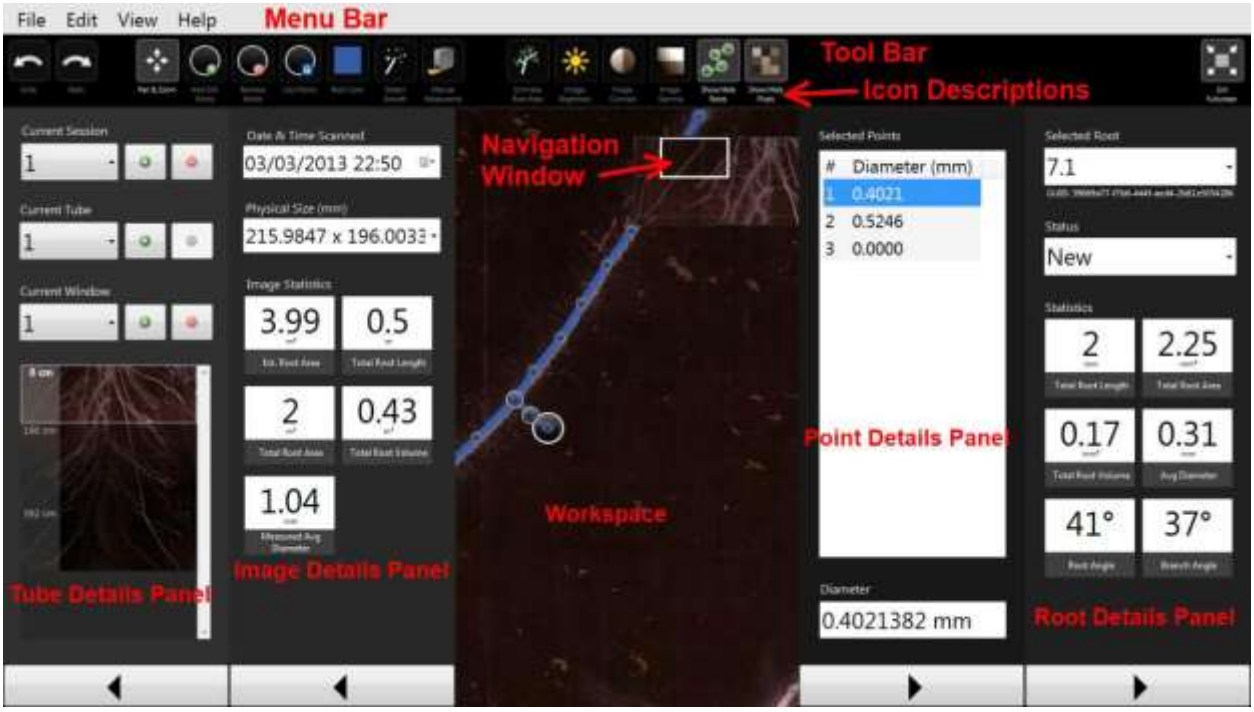
View data about the parent root in the Root Details and the Point Details panel: <View><Panels><Point Details> or <Root Details>. Use the Add/Edit Points tool to select a point on the parent root.



View data about the branch by selecting a branch point: A branch will have a current root with a decimal place. To change the name of the root, click the box and type a new name. The branch angle will also appear for points along a branch segment. The branch angle will be 0^{o} for a point on a parent root.



Edit Menu:	**View Menu:**	**Help Menu:**
1. Undo & Redo 2. Delete current root 3. Lock current root 4. Migrate roots 5. Tube angle 6. Default root status	1. Navigation window 2. Zoom 3. Panels 4. Layers 5. Toolbar 6. Icon descriptions 7. Large icons	



Exporting Data:

Data from RootSnap! projects, tubes, windows or sessions can be exported to be opened as a spreadsheet. Exported data is saved as .csv (comma separated value) files which can be opened using Microsoft Excel or similar programs and saved. Root data is displayed at the top of the file including the root id, length, average diameter, area, volume, mean angle, branch count, branch ids and point count. Option is also available to save the root image while exporting the data.

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Chapter 18: Improvement of water stress tolerance in crop plants through genetic engineering

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Introduction

Abiotic stress, mainly water stress, reduces average yields for most crop plants by more than 60% (Rodríguez *et al*. 2005). Water stress or water deficit is one of the major environmental factors which prevents plants from realizing their actual genetic potential and negatively impact on crop yield (Araus *et al.* 2002, Morison *et al*. 2008, Salekdeh *et al*. 2009). Adverse environmental factor, mainly limited soil moisture conditions, induce series of morphological, physiological, biochemical and molecular changes (Wang *et al.* 2001). To counter this adverse environmental factor, plants try to avoid the water stress condition through various ways. They try to escape the season of drought by early flowering. They may decrease the leaf area (LA), increase the efficiency of roots to uptake more water or may decrease activity of stomata. Slowing growth, osmotic adjustments and synthesis of antioxidants are some other mechanisms adapted by plants to combat adverse environmental conditions. These adaptations help plants to adapt to water stress conditions. Some plants are able to adapt to limited soil moisture by shortening their growth cycle or they avoid water stress by augmenting root growth to increase their water uptake (Molnar *et al*. 2004)). Unfortunately, the mechanisms by which crops maintain yield under water stresse are poorly understood since water stress can occur at different stages of the plant's development, with different effects on plant function, and thus requires distinct mechanisms for tolerance. In addition, a variety of abiotic stresses commonly occur during drought, such as high temperatures, high concentrations of salt and other toxic solutes and low availabilities of nutrients (Fleury *et al*. 2010, Salekdeh *et al*. 2009, Mittler 2006), and these vary by location and time.

Signaling pathways are induced in response to environmental stress and recent molecular and genetic studies have revealed that these pathways involve many components. The multiplicity of information embedded in abiotic stress signals underlies one aspect of the complexity of stress signaling (Chinnusamy *et al.* 2004). Nevertheless, most studies on water stress signaling have focused on primarily salt stress because plant responses to salt and drought are closely related and the mechanisms overlap (Zhu 2002). Responses to stress are not linear pathways, but are complicated integrated circuits involving multiple pathways and specific cellular compartments, tissues, and the interaction of additional cofactors and/or signaling molecules to coordinate a specified response to a given stimulus. Plants respond to these stresses at molecular and cellular levels as well as physiological level. Expression of a variety of genes has been demonstrated to be induced by these stresses. The products of these genes are thought to function not only in stress tolerance but also in the regulation of gene expression and signal transduction in stress response (Yamaguchi-Shinozaki *et al*. 2002, Shinozaki *et al*. 2003).

In addition, the sensitivity of many crops to a particular abiotic stress varies depending on their developmental stage. For example, rice is sensitive to salt stress at the young seedling stage, but much less at the reproductive stage (Flowers and Yeo. 1981, Lutts *et al.* 1995). It is suggested that stress tolerance mechanisms in a plant are controlled by a variety of genes, which are expressed at different times during the life of the plant (Witcombe *et al*. 2008, Fleury *et al.* 2010). Plant adaptations to most abiotic stresses involve a range of traits which combine to contribute plant tolerance. Individual genes have been reported to improve the stress tolerance in some crops, for instance, the transcription factor ZmNF-YB2 has been reported to improve drought tolerance in maize (Nelson *et al*. 2007). While in majority of cases, it is not a simple matter of identifying the single gene that will provide resistance to a particular abiotic stress.

This review describes recent advances in understanding mechanisms underlying plants response to water stress conditions. The main mechanisms such as signal transduction pathways, regulation of gene expression, ion transport, and detoxification mechanisms are also described. Emphasis has been given to transgenic plants that were developed for water

stress tolerance, based on different mechanisms associated with plants response to water stress.

Improvement of water stress tolerance in crop plants through genetic engineering

Many plants have multiple physiological, biochemical and molecular mechanisms that enable them to tolerate water stress conditions. Understanding the mechanisms by which plants perceive and transduce the stress signals to initiate adaptive responses and their engineering using molecular biology and genomic approaches are essential for improving water stress tolerance in crop plants. Several efforts in this direction have been carried out in many laboratories targeting manipulation of genes belonging to diverse categories. Genetic engineering strategies rely on the transfer of one or several genes that are either involved in signaling and regulatory pathways, or that encode enzymes present in pathways leading to the synthesis of functional and structural protectants, or that encode stress tolerance-conferring proteins. Attempts have been made to confer water stress tolerance in crop plants through biotechnological approaches and water stress tolerant varieties of crops such as wheat, soybean and rice have been developed.

Genes associated with plant's stress surveillance

Sensors initiate a signalling cascade to transmit the signal and activate nuclear transcription factors to induce the expression of specific sets of genes. Genes involved in stress signal sensing and a cascade of stress-signaling in model plant such as *Arabidopsis thaliana* has been of recent research interest (Winicov and Bastola 1997; Shinozaki and Yamaguchi-Shinozaki 1999). Components of the signal transduction pathway may also be shared by various stress factors such as drought, salt and cold (Shinozaki and Yamaguchi-Shinozaki 1999). Although there are multiple pathways of signal transduction systems operating at the cellular level for gene regulation, ABA is known component acting in one of the signal transduction pathways, while others act independently of ABA. The early response genes have been known to encode transcription factors that activate downstream delayed

response genes (Zhu 2002). Although, specific branches and components exist (Lee *et al*. 2001), the signaling pathways for salt, drought, and cold stresses all interact with ABA, and even converge at multiple steps (Xiong *et al*. 1999). Abiotic stress signaling in plants involves receptor-coupled phospho-relay, phosphoionositol-induced Ca^{2+} changes, Mitogen Activated Protein Kinase (*MAPK*) cascade, and transcriptional activation of stress responsive genes (Xiong and Zhu 2001). A number of signaling components are associated with the plant response to high temperature, freezing, drought and anaerobic stresses (Grover *et al*. 2001). One of the merits for the manipulation of signaling factors is that they can control a broad range of downstream events that can result in superior tolerance for multiple aspects (Umezawa *et al*. 2006). Alteration of these signal transduction components is an approach to reduce the sensitivity of cells to stress conditions, or such that a low level of constitutive expression of stress genes is induced (Grover *et al*., 1999). Over-expression of functionally conserved At-DBF2 (homolog of yeast DBF2 kinase) showed striking multiple stress tolerance in Arabidopsis plants (Lee *et al*. 1999). Transgenic tobacco plants produced by altering stress signaling through functional reconstitution of activated yeast calcineurin not only opened-up new routes for study of stress signaling, but also for engineering transgenic crops with enhanced water stress tolerance (Grover *et al*. 1999). Overexpression of an osmotic-stress-activated protein kinase, *SRK2C* resulted in a higher drought tolerance in *A. thaliana*, which coincided with the upregulation of stress-responsive genes (Umezawa *et al*. 2004). Similarly, a truncated tobacco Mitogen-activated Protein Kinase Kinase Kinase (*MAPKKK*), *NPK1*, activated an oxidative signal cascade resulting in cold, heat, salinity and drought tolerance in transgenic plants (Kovtun *et al*. 2000, Shou *et al*. 2004). However, suppression of signaling factors could also effectively enhance tolerance to abiotic stress (Wang *et al*. 2005).

Water stress induced genes

The complex plant response to abiotic stress involves many genes and biochemical-molecular mechanisms. Various genes respond to drought-stress in various species, and functions of their gene products have been predicted from sequence homology with known proteins. Many water stress inducible genes are also induced by salt stress and

low temperature, which suggests the existence of similar mechanisms of stress responses. Genes induced during water stress conditions are thought to function not only in protecting cells from water deficit by the production of important metabolic proteins but also in the regulation of genes for signal transduction in the drought stress response (Yamaguchi-Shinozaki *et al*. 2002, Shinozaki *et al*. 2003). Stress inducible genes have been used to improve the stress tolerance of plants by gene transfer. It is important to analyze the functions of stress-inducible genes not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation. Hundreds of genes are thought to be involved in abiotic stress responses.

Genes involved in transcriptional regulation

Transcription factors (TFs) are small molecules that attach to specific sites on a DNA molecule in order to activate or deactivate the expression of certain genes. A single gene encoding a specific stress protein does not always result in sufficient expression to produce useful tolerance, because multiple and complex pathways are involved in controlling plant drought responses (Bohnert *et al*. 1995) and because modification of a single enzyme in a biochemical pathway is usually contrasted by a tendency of plant cells to restore homeostasis. Targeting multiple steps in a pathway may often modify metabolite fluxes in a more predictable manner. Another promising approach is therefore to engineer the overexpression of genes encoding stress inducible transcription factors. Transcription factors typically regulate several genes and are likely to be used extensively in the next generation of genetically modified crops (Yamaguchi-Shinozaki and Shinozaki 1994, Chinnusamy *et al* 2005). Numerous transcriptional regulators are known to be involved in plant responses to water stress (Yamaguchi-Shinozaki and Shinozaki 2002); most fall into one of the large transcription factor families (*AP2/ERF*, *bZIP*, *NAC*, *MYB*, *MYC*, *Cys2His2 zincfinger*, *NFY* and *WRKY*); and some cis-elements, bound by these transcription factors, have been identified. For example abscisic acid-responsive elements (ABRE) (Mundy *et al*. 1990) are 50 upstream regions of abscisic acid-responsive genes that are bound by AREB/ABF transcription factors belonging to the basic leucine zipper family. These mediate at least one

of the abscisic acid-dependent pathways involved in responses to drought stress. Another cis-element is the dehydration responsive element/C-repeat (DRE/CRT) which is involved in one of the abscisic acid-independent pathways (Yamaguchi-Shinozaki and Shinozaki 1994).

Various DRE/CRT-binding proteins, coding for ERF/AP2 transcription factors, are induced by desiccation, salt treatment, and cold in some plant species. The first examples of transcription factor engineering to improve abiotic stress tolerance were overexpression of the *ERF/ AP2* factors *CBF1, DREB1A* and *CBF4*. Overexpression of these factors resulted in cold, drought and salt tolerance in Arabidopsis (Jaglo-Ottosen *et al*. 1998, Kasuga *et al*. 1999) and it was later shown the similar tolerance could be induced in many crop plants by overexpression of these factors (Pellegrineschi *et al*. 2004). Numerous transgenic Arabidopsis varieties with improved drought tolerance due to overexpression of various stress-regulated transcription factors have been reported, but similar results have also been obtained in crop plants. Typically a gene coding for a transcription factor in Arabidopsis is isolated, characterized and shown to improve drought response when overexpressed. The gene is then transferred to a crop plant where it often confers the same drought-tolerant phenotype. The *HRD* gene, coding for an *AP2/ERF*-like transcription factor) exemplifies this approach. Arabidopsis plants with a gain-of-function mutation in the *HRD* gene (hrd-D mutants) are drought resistant, salt-tolerant, and overexpress abiotic stress marker genes. Overexpression of the same gene in rice significantly improves water use efficiency both under well-watered conditions (50–100% increase) and under drought (50% increase). These plants also show enhanced photosynthetic assimilation and reduced transpiration). *HRD* gene overexpression conserves drought tolerance in both dicots and monocots. Various regulatory genes involved in drought tolerance are depicted in Table 1.

Genes for osmotic regulation and ionic balance

Early attempts to develop transgenic plants resistant to water stress focused on single action genes responsible for the modification of a single metabolite or protein that would confer increased tolerance to water stress. Recent reviews document progress in this area. Osmoregulation is one of the most effective ways evolved by stress-tolerant plants to combat abiotic stress, but most crop plants lack the ability to synthesize the osmoprotectants naturally

produced by stress-tolerant plants. Therefore genes concerned with the synthesis of osmoprotectants have been incorporated into transgenic plants to confer stress-tolerance. Overproduction of compatible solute osmoprotectants such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines), and sugars and sugar alcohols (e.g. mannitol, trehalose and galactinol) has been achieved in various target plants. Glycine betaine in particular has been extensively studied as a compatible solute, both by genetically engineering its biosynthesis in agriculturally important species and by its exogenous application (Chen and Murata 2008).When maize plants were transformed with the *betA* gene from Escherichia coli that encodes choline dehydrogenase, they accumulated glycinebetaine in tissues and were more tolerant to drought stress than wild-type plants at different developmental stages. Most importantly their grain yield was 10–23% higher than that of wild-type plants after three weeks of drought stress (Quan *et al*. 2004). In some cases the accumulation of compatible solutes also protects plants against damage by reactive oxygen species (ROS) (Bohnert and Shen 1999); in other cases the solutes have chaperone-like activities that protect other proteins maintaining their structure and function (Diamant *et al*. 2001, McNell *et al*. 1999]. Genes coding for heat-shock proteins, molecular chaperones and LEA proteins (reviewed in have been extensively used to improve drought responses in plants. These authors demonstrated that constitutive expression of two cold shock proteins – CspA from *E. coli* and *CspB* from *Bacillus subtilus* (both RNA chaperones) – conferred abiotic stress tolerance to transgenic Arabidopsis, rice, and maize. They obtained a greater than 20% increase in maize grain yield under water-limiting conditions in field trials, without observing pleiotropic effects on plant development. The improvement in drought response was observed in the late vegetative/flowering period as well as the grain-fill period: during these periods, three consecutive days of wilting can reduce grain yield by 30–50%. Stress tolerance conferred by manipulation of cold shock proteins is not only novel, but also appears as a highly promising approach to improving plant productivity in suboptimal growth conditions. Genes encoding enzymes that synthesize osmotic and other protectants are listed in Table 2.

Table 1: Various regulatory genes involved in drought tolerance

Gene	Gene action	Species	Phenotype	References
ABF3	Transcription factor	Rice	Drought resistance	Oh *et al*., 2005
Alx8	High APX2 and ABA	*Arabidopsis*	Drought resistance	Rossel *et al*., 2006
AREB1	ABRE-dependent ABA signaling	*Arabidopsis*	Drought resistance	Fujita *et al*., 2006
CAbZIP1	Plant development (dwarf phenotype)	*Arabidopsis*	Disease, drought and salt tolerance	Lee *et al*., 2006
CAP2	Transcription factor	Tobacco	Drought and salt tolerance	Shukla *et al*., 2006
DREB	Transcription factor	*Arabidopsis*	Increased tolerance to cold, drought and salinity	Kasuga *et al*., 1999
DREB1 or osDREB1	Transcription factor	Rice	Drought, salt and cold tolerance with reduced growth under non-stress	Ito *et al.,* 2006
DREB1A	Transcription factor	Tobacco	Drought and cold tolerance	Kasuga *et al*., 2004
DREB2A	Transcription factor	*Arabidopsis*	Drought resistance	Sakuma *et al*., 2006
FAD3 and FAD8	Increased fatty acid desaturation	Tobacco	Drought resistance	Meng *et al*., 2005
OsDREB1A	Transcription factor	*Arabidopsis*	Drought, salt, freezing tolerance	Dubouzet *et al*., 2003
OsMYB3R-2	MYB homeodomain, and zinc finger proteins	*Arabidopsis*	Drought, salt, freezing tolerance	Dai *et al*., 2007
TaPP2Ac-1	catalytic subunit (c) of protein phosphatase 2A	Tobacco	Drought resistance; maintain RWC and membrane stability	Xu *et al*., 2007
ZmDREB2A	Encodes HSP & LEA proteins	*Arabidopsis*	Drought and heat tolerance	Qin *et al*., 2007

Table 2: Genes encoding enzymes that synthesize osmotic and other protectants

Gene	Gene action	Species	Phenotype	Reference
Abc	Arg decarboxylase	Rice	Reduced chlorophyll loss under drought stress	Capell *et al*., 1998
Abc	Polyamine synthesis	Rice	Drought resistance	Capell *et al*., 2004
AtTPS1	Trehalose-6-phosphate synthase	Tobacco	Drought resistance; sustained photosyntehsis	Almeida *et al*., 2007
BADH -1	Betaine aldehyde dehydrogenase	Tobacco	Heat tolerance in photosynthesis	Xinghong *et al*., 2005
betA	Choline dehydrogenase (glycinebetaine synthesis)	Maize	Drought resistance at seedling stage and high yield after drought	Ruidang *et al*., 2004
Mt1D	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	Wheat	Drought and salinity tolerance of calli and plants	Abebe *et al*., 2003
Osm1…Osm4	Osmotin protein accumulation	Tobacco	Drought and salt tolerance in plant water status and proline accumulation	Barthakur *et al*., 2001
otsA	Trehalose-6-phosphate synthase (trehalose synthesis)	Tobacco	photosynthetic activity under drought. Increased carbohydrate accumulation.	Pilon-smits *et al*., 1995
otsB	Trehalose-6-phosphate synthase (trehalose synthesis)	Tobacco	photosynthetic activity under drought. Increased carbohydrate accumulation.	Pilon-smits *et al*., 1998
P5CS	Pyrroline carboxylate synthase (proline synthesis)	*Petunia*	Drought resistance and high proline	Yamada *et al*., 2005

P5CS	Pyrroline carboxylate synthase (proline synthesis)	Rice	Increased biomass production under drought and salinity stress	Zhu *et al*., 1998
P5CS	Pyrroline carboxylate synthase (proline synthesis) (tomato	Sugarcane	Drought resistance via antioxidant role of proline	Molinari *et al*., 2007
SMADC	S-adenosylmethioninedecarboxylase (polyamine synthesis)	Tobacco	drought, salinity, Verticillium and Fusarium wilts resistance	Waie and Rajam, 2003
SST/FFT	Fructan accumulation	Potato	Reduced proline accumulation at low water status	Knipp and Honermeier, 2006
TPS1	Trehalose synthesis	Tomato	Drought, salt and oxidative stress tolerance	Cortina and Culianez-Macia, 2005
TPS1 and *TPS2*	Trehalose synthesis	Tobacco	Maintenance of water status under drought stress	Karim *et al*., 2007

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