Manual of ICAR Sponsored Short Term Training Course On Phenomics: Perspectives for Application in Improvement of Abiotic Stress Tolerance in Crop Plants

(July 20-29, 2017)





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

Manual of ICAR Sponsored Short Term Training Course On Phenomics: Perspectives for Application in Improvement of Abiotic Stress Tolerance in Crop Plants

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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.

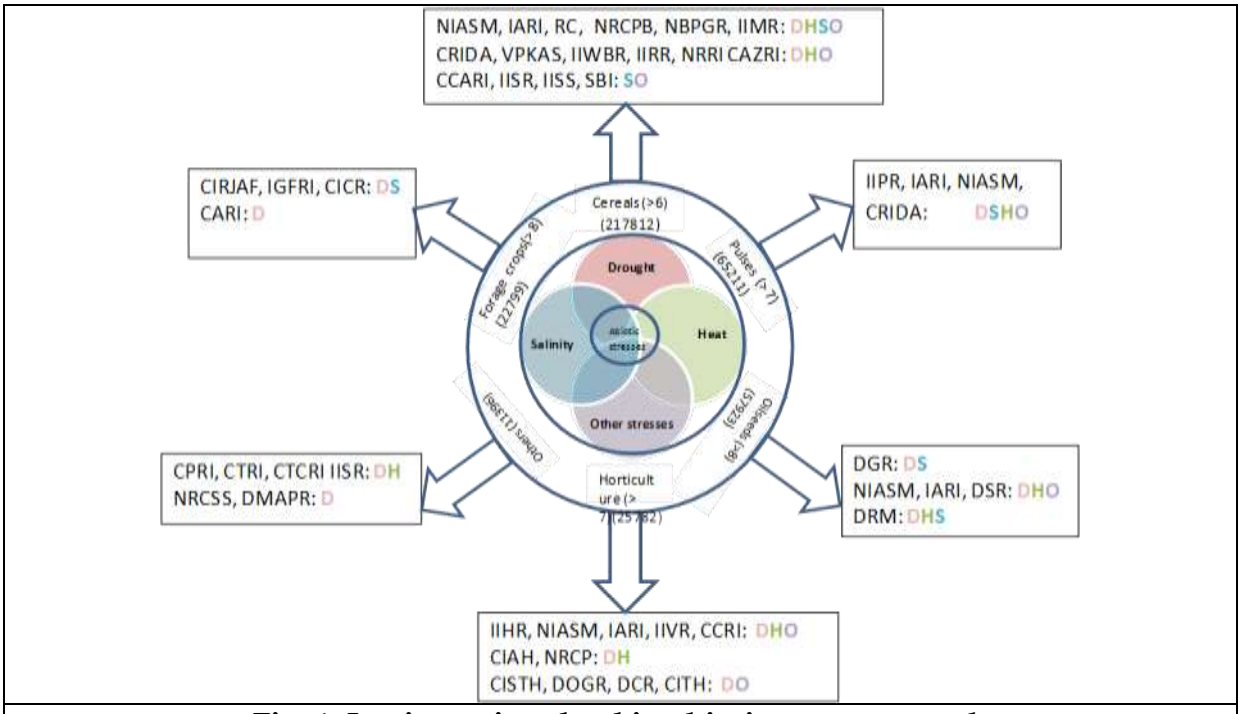

Fig. 1. Institutes involved in abiotic stress research

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.

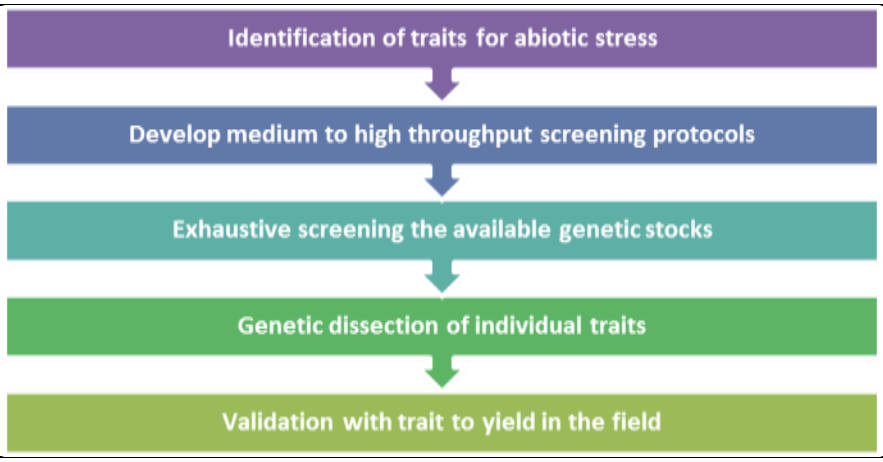


Fig. 1. Application of non-destructive phenotyping in genetic dissection of trait

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

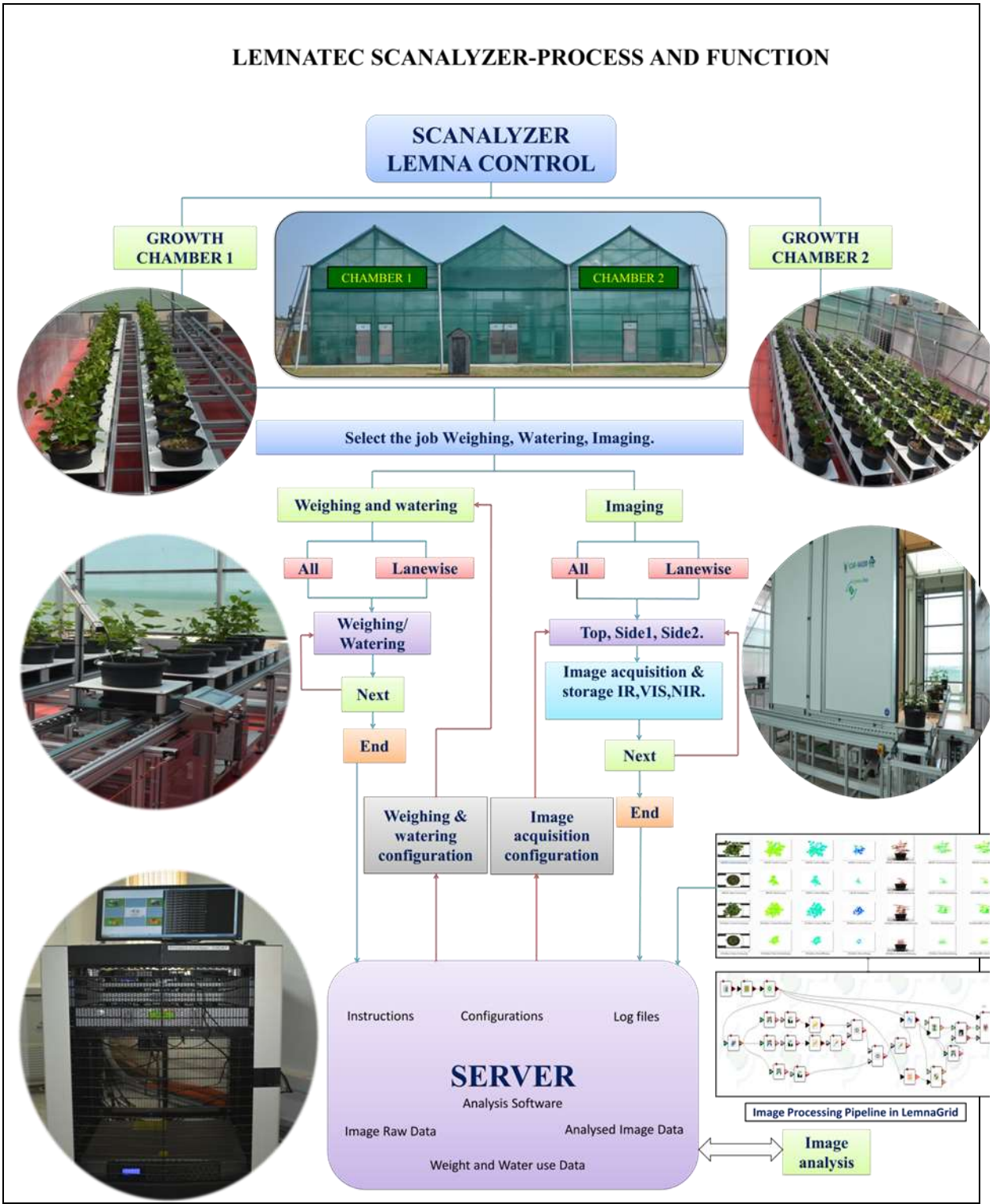


Fig. 2. LemnaTec Scanalyzer process and function

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

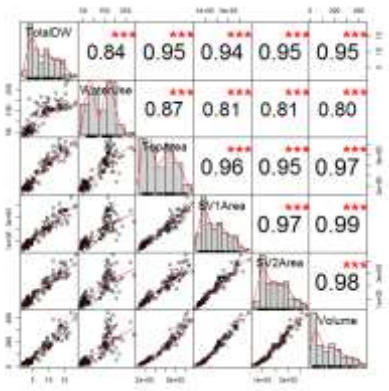


Fig. 4. Visible area derived from 24 Genotypes grown with or without water stress in three replications (144 pots). Area and volume derived from images could explain the variation in water use and biomass (Total DW) in mungbean. This optimised method can now be used to assess mungbean genotypes for their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

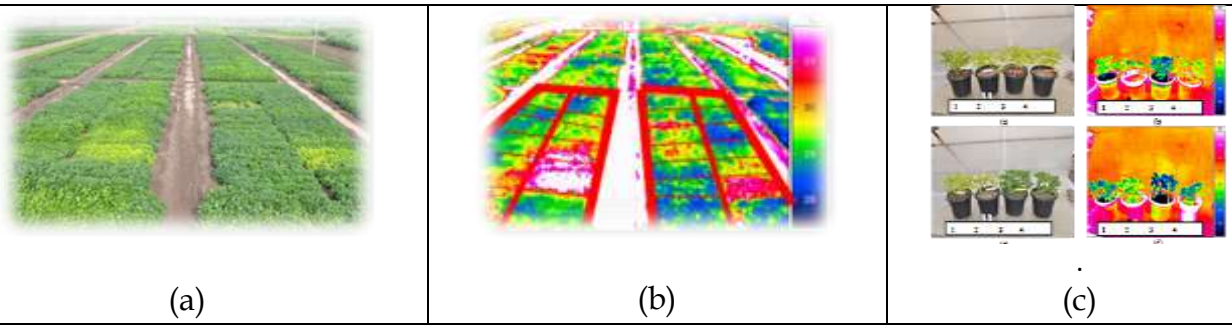
Identification of superior genotypes

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.

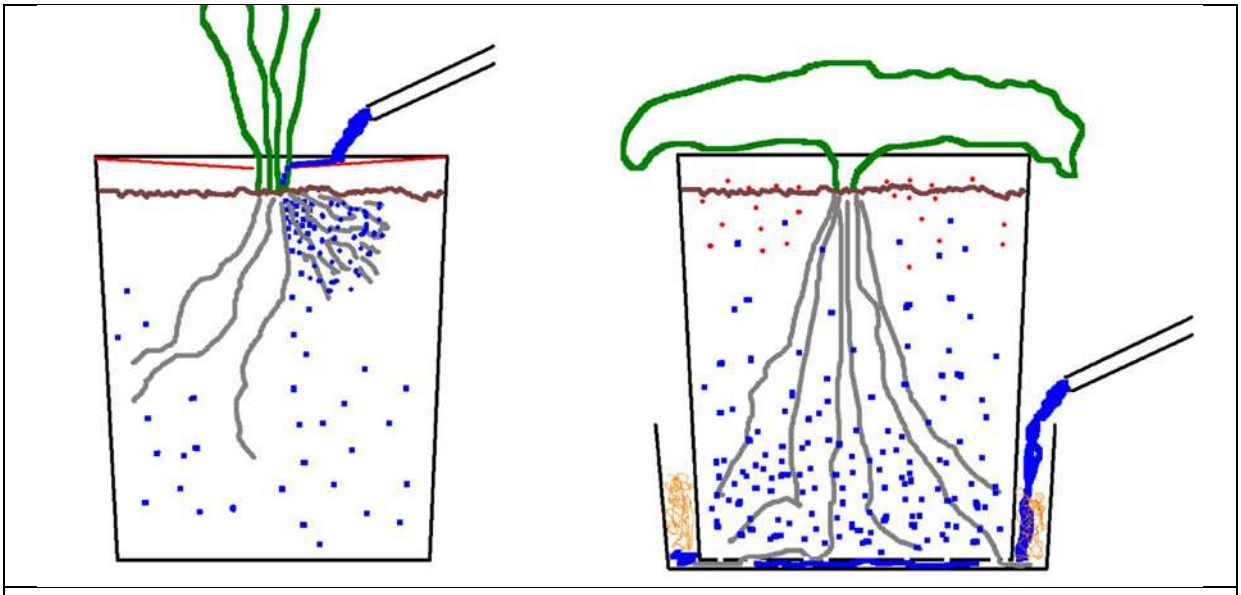

Fig. 2. Schematic sketch showing different features of top and bottom watering

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.)

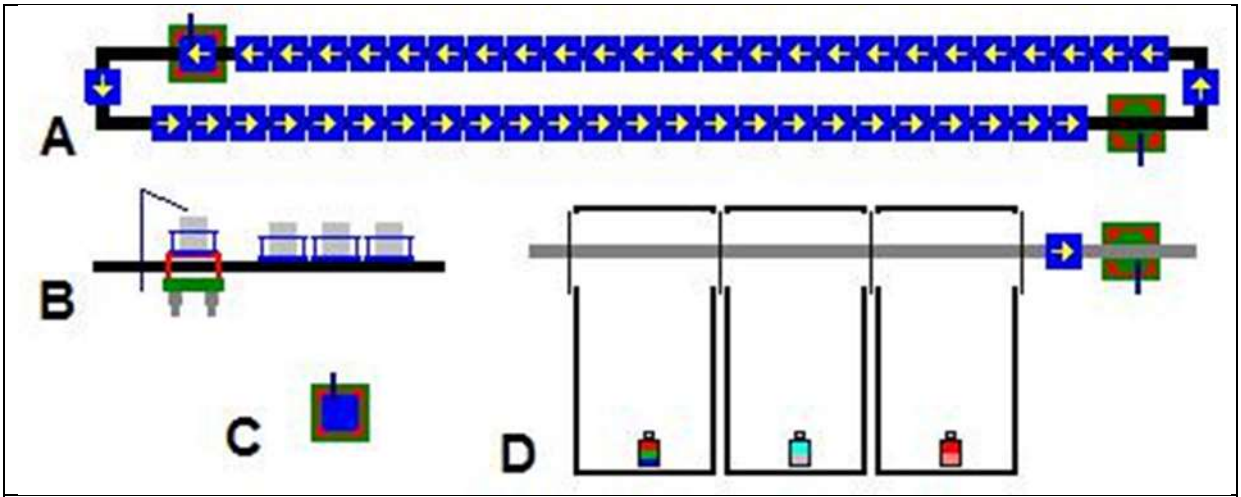

Fig. 3. Options for watering systems:
A: A pump and watering station as part of a greenhouse management system, which may be stand-alone and simple as the one shown here or part of a large multi-line system;
B: Schematic system from the side with scales (green) and pneumatic units (grey), lifting the 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.

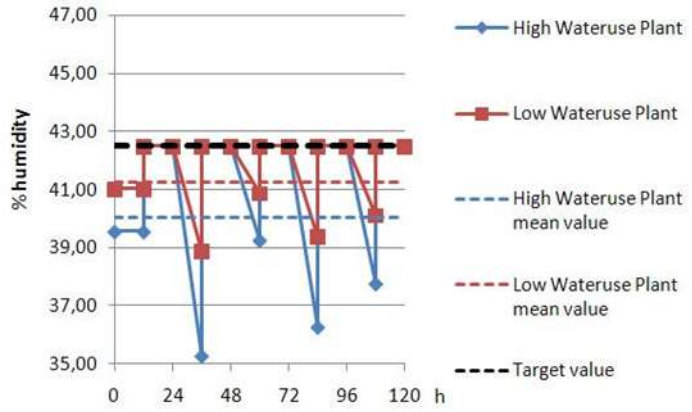

Fig. 4. Watering to a predefined target weight corresponding to a defined percentage of soil humidity will bring the soil humidity for all plants to the same value only once a day. Plants that need much water will evaporate more, and thus the average value during the day will be significantly lower than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

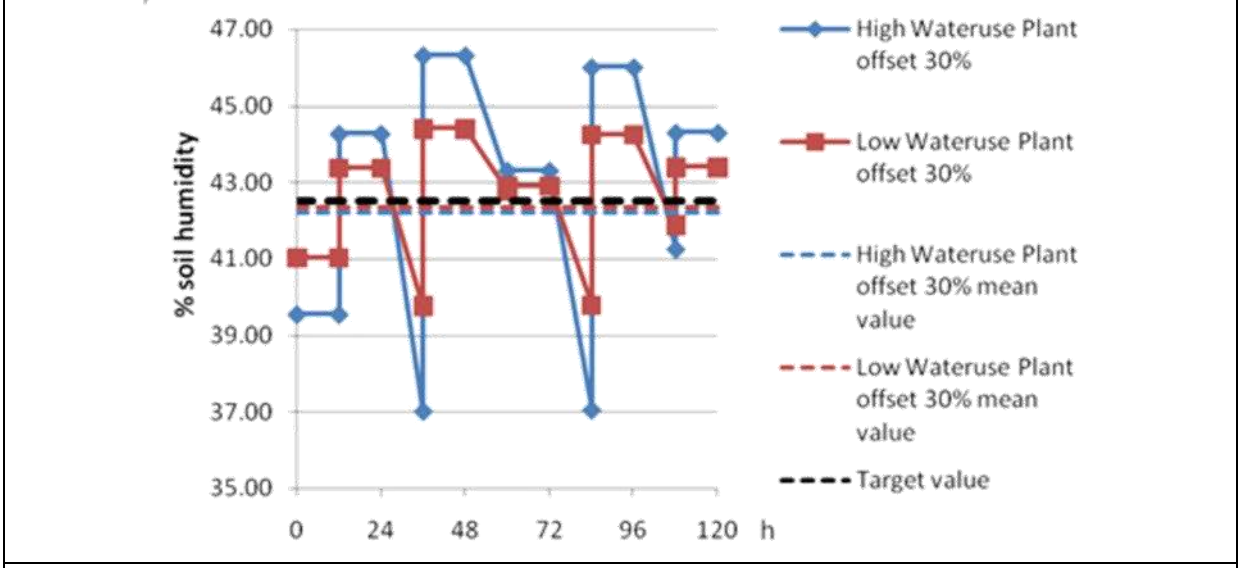

Fig. 5. Dynamic watering takes the higher consumption of water by some plants into account by adding a percentage offset to the water consumed relative to the target value. In most cases a 30 % offset is sufficient to keep the average humidity of plants with different water demand nearly constant over 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.

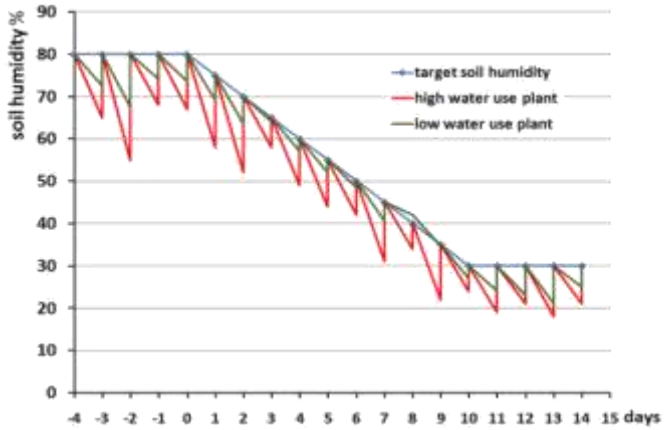

Fig. 6. Fast, but consistent drying down of all plants from 80 to 30% soil humidity.

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.

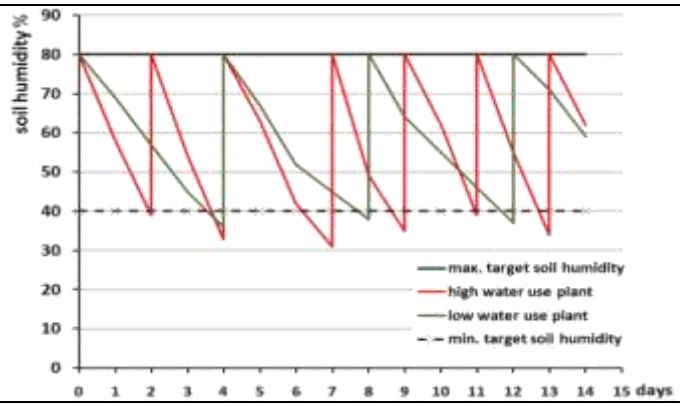

Fig. 7. In this example plants are only watered when the humidity level has fallen below 40 % of soil humidity. Different plants reach this level at different time intervals due to different evaporation rates. While the plant with the high water demand is watered 6 times within 14 days, the plant with 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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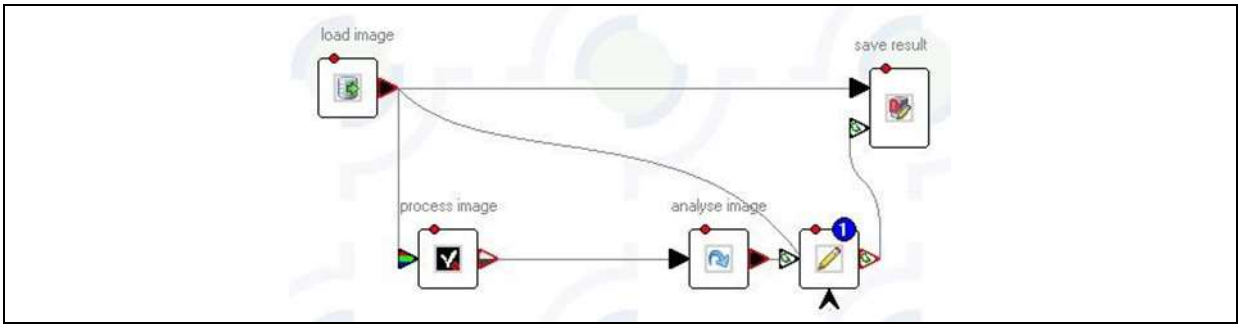
Manually Editing Image Objects

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.

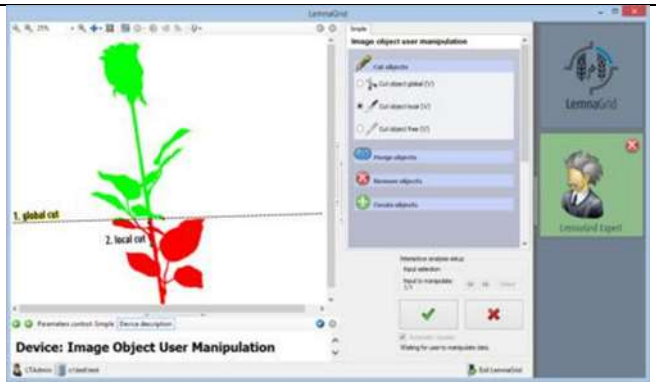
Note: you need to enable the interactive mode of Object user manipulation by right-click the device and select 'add to interactive analysis' and accept the default settings. The label in the context menue then changes to 'Remove from interactive analysis'

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:

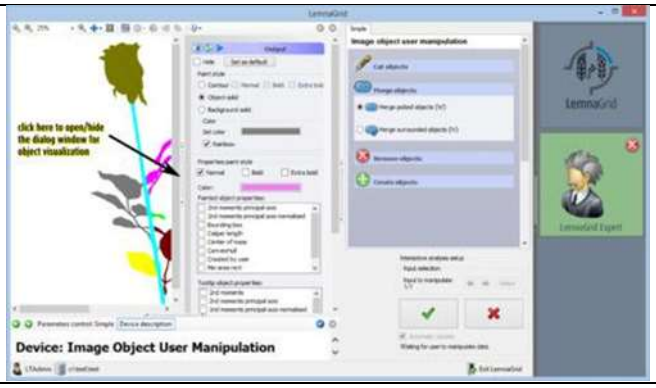
cut object globally

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

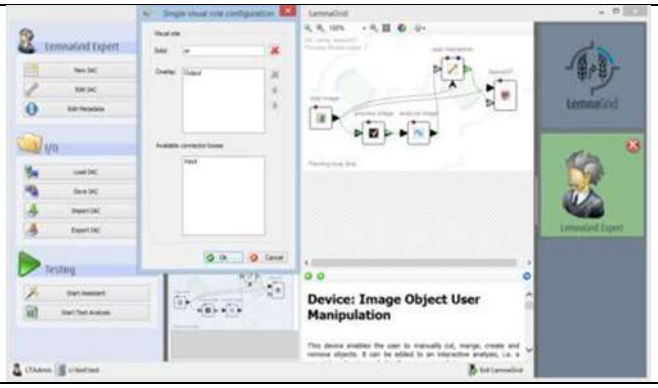
For example, you can delete a particular object by picking (remove picked object). To undo accidentally deleted objects press CTRL+Z.

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:

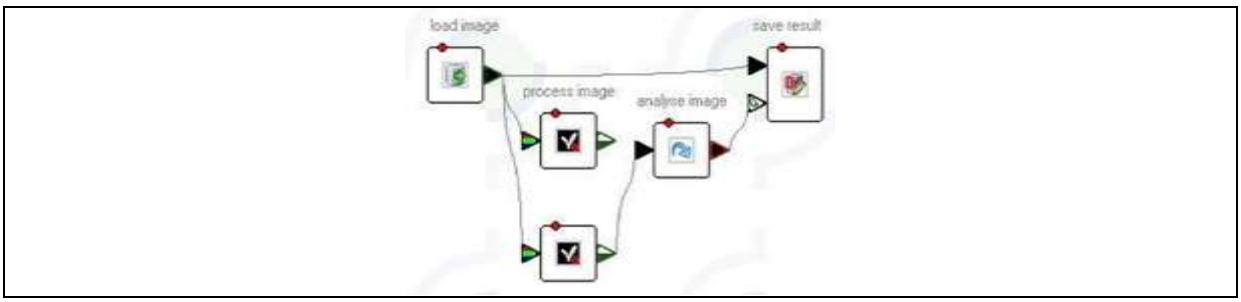
Note: two-sided (bandpass) thresholding is also possible classify pixel value within a range. Check the advanced settings in the Threshold device.

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

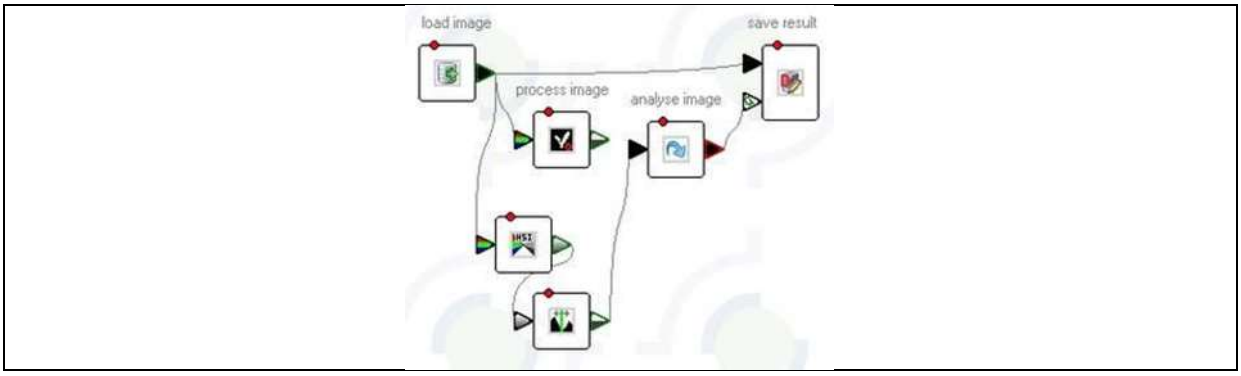
Given a palette of RGB colors and a 'Radius of a box', each pixel's RGB value is analysed. Use Fore/Background separation on the original image to create this following workflow:



- 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

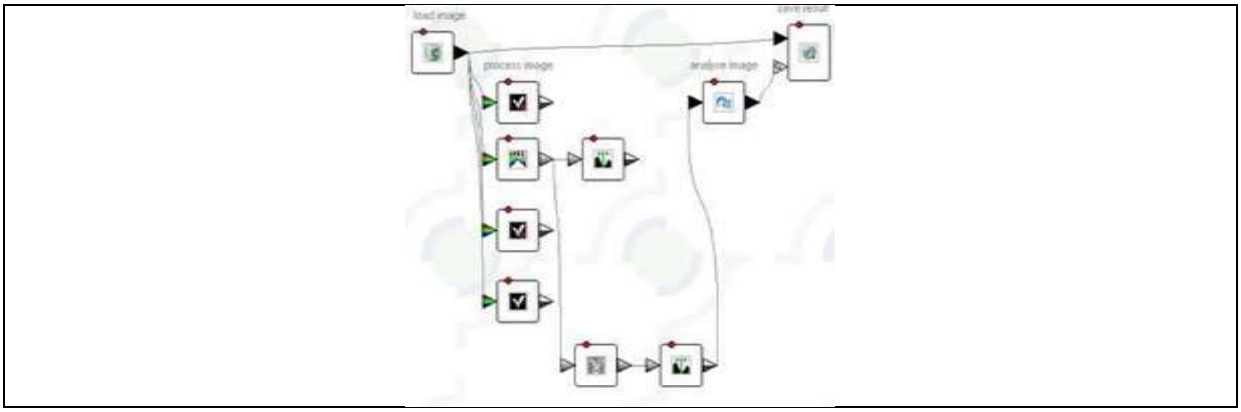
NN fore/background separation

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:

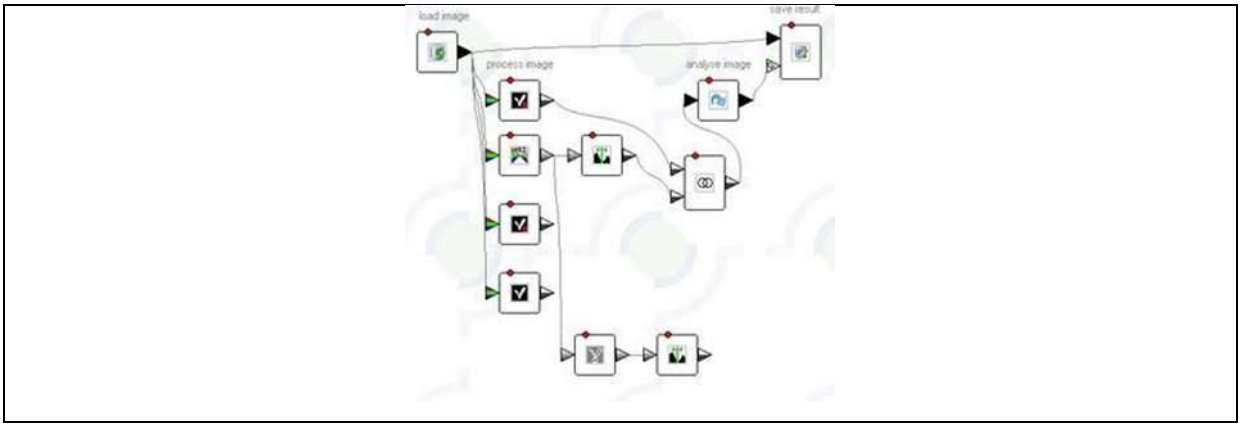


The result of Sobel filter (device group: Filter >> Edge) is a grayscale image where the pixel values indicate the degree of edges. The edges identified by edge detection are often disconnected. To segment an object from an image however, one needs closed region boundaries. Use Threshold to find an appropriate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

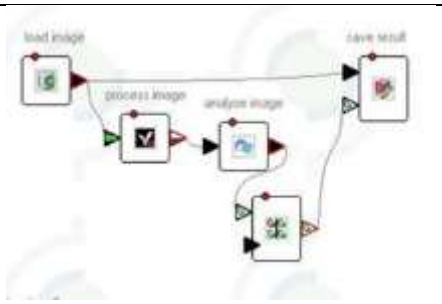
Note: a detailed list is available in the LemnaGrid Workshop article: "Application of the Universal converter" and "Morphometric parameters automatically determined for each image object".

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.

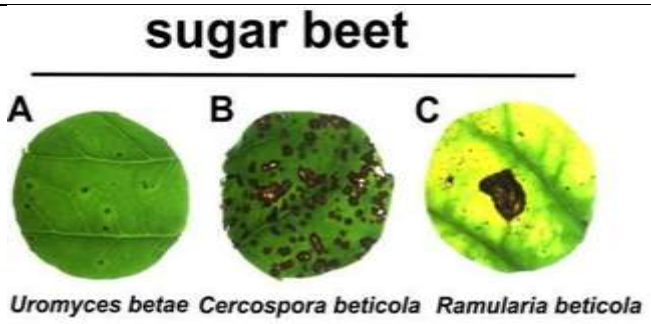

Fig. 1. Sugar beet leaves measured with Scanalyer HTS.

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.

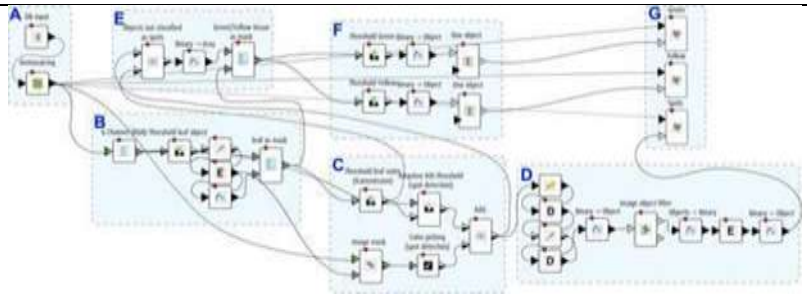

Fig. 2. Implementation of the image processing in LemnaGrid software.
The Grid can be downloaded using the LemnaShare (previously LemnaNet) program. Icons represent devices and do specific tasks. The dataflow is from left Database Input (A) to right Database writing (G). Blue boxes highlighted with capital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

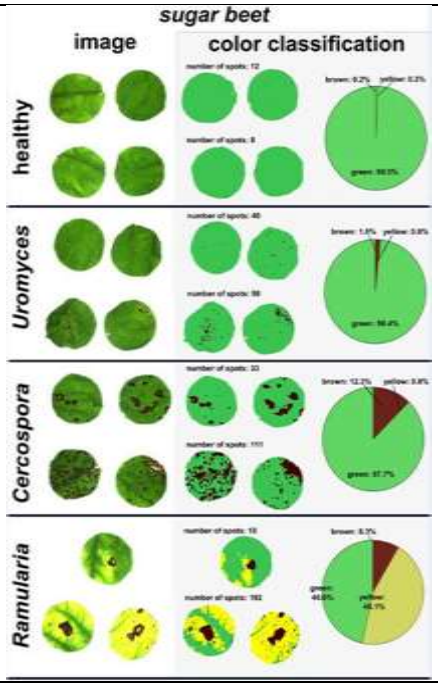

Fig. 3. Left: Sugar beet leaf discs with yellow/brown disease symptoms caused by *Uromyces betae*, *Cercospora beticola* or *Ramularia beticola*.
Middle: Leaf discs with colour classification into green, yellow and brown areas using the introduced image processing approach. The number of detected spots 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.

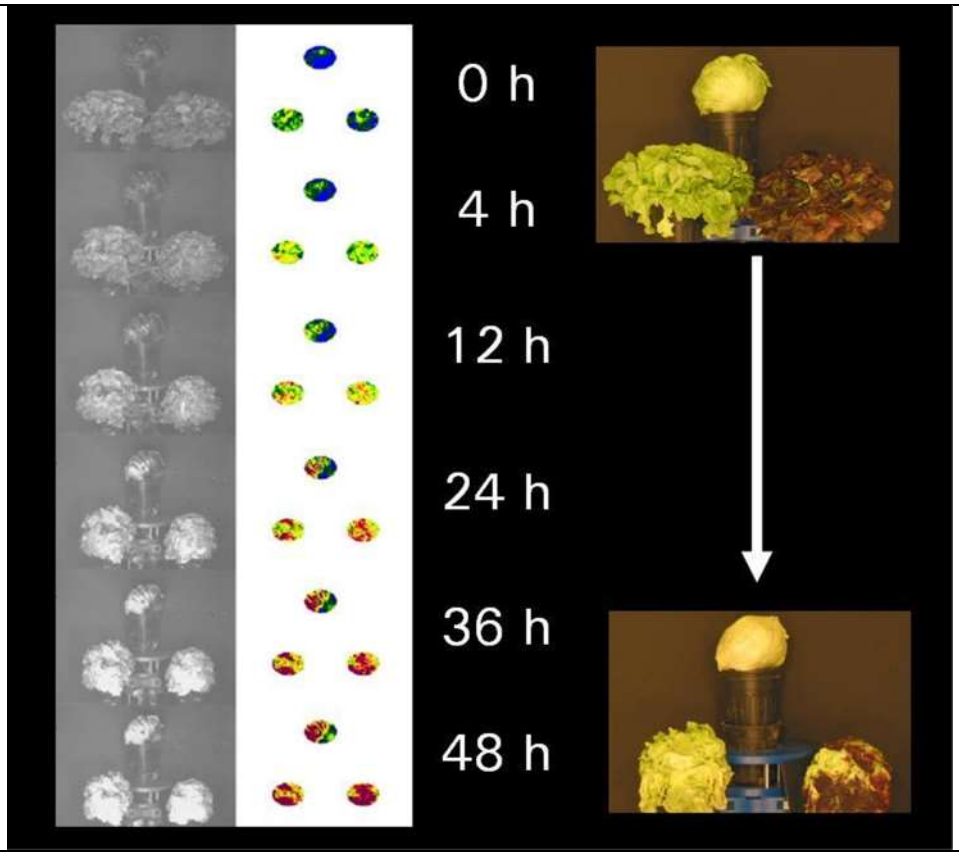

Fig. 1. An iceberg lettuce (top row) and two oak leaf lettuces (green and red, bottom row) dry down in warm ambient conditions.
NIR-imaging shows a strong increase in reflectance as the water in the leaves is extremely reduced. Blue false colours represent high water content, while red colours symbolise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

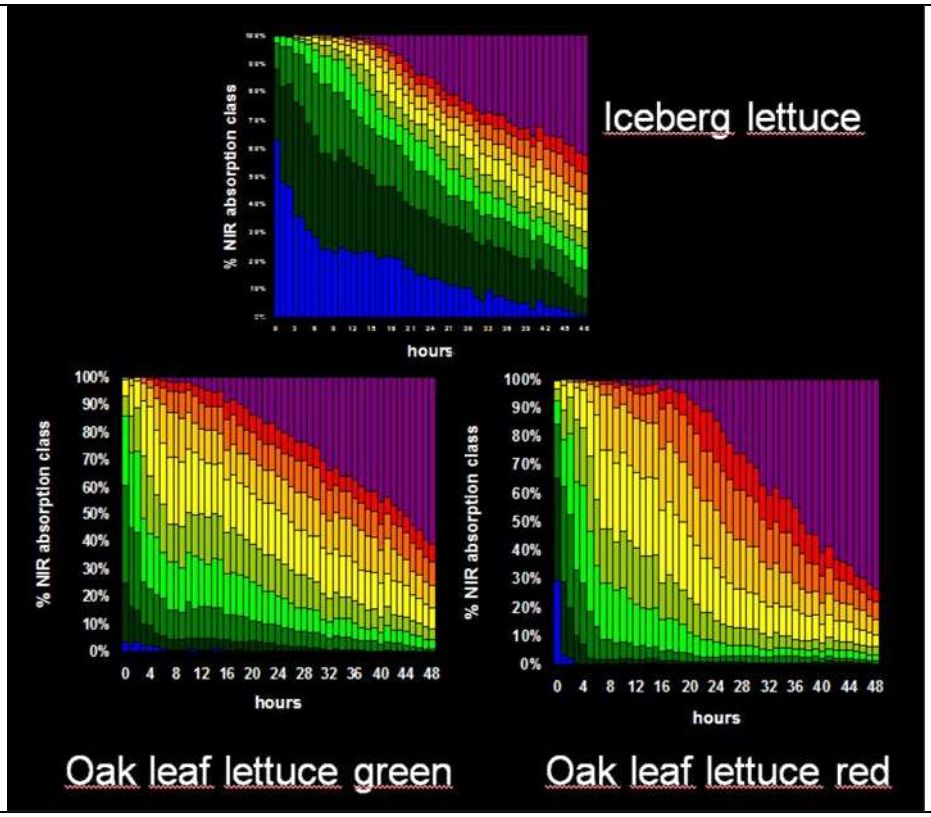

Fig. 2. The drying dynamics of the outer leaves of 3 lettuce cultivars
While keeping the whole head together, only the surface layer (which finally defines the purchase decision of the customer in the shop) is measured. The less blue and the more green/yellow/red/violet colour classes appear the dryer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

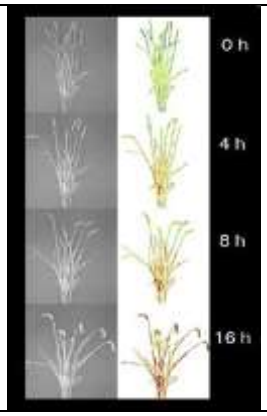

Figure 3: A bunch of wheat dries down in warm ambient conditions
NIR-imaging shows a strong increase in reflectance as the water in the leaves is extremely reduced. Blue/green false colours represent high water content, while yellow/red colours symbolise low water content (high reflectance).

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

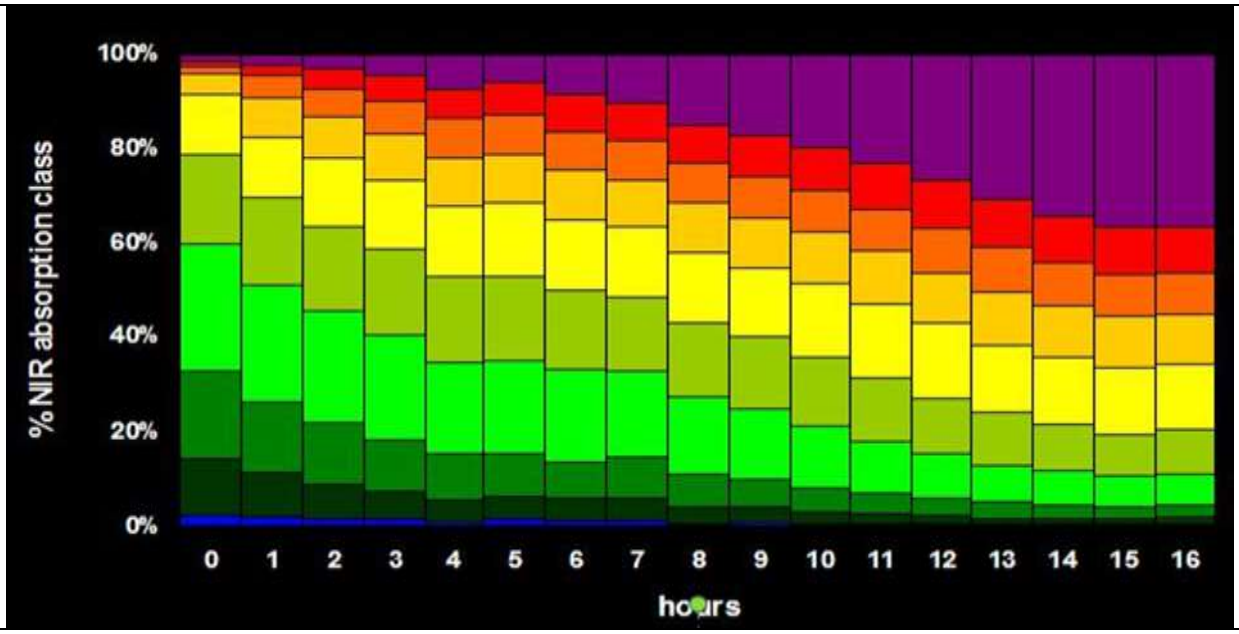

Fig. 4. The drying dynamics of a wheat plant over a 12 hour period.
Blue/green false colours represent high water content, while yellow/red/violet colours symbolise low water content (high reflection).

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.

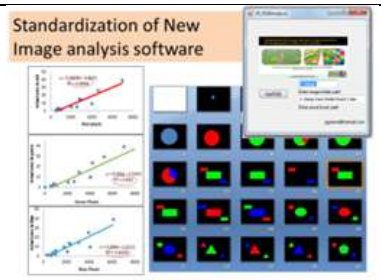

Fig. 1. Prototypes to screen genotypes in field (a) pot culture (b) tools for rapid analysis of images (c)

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.

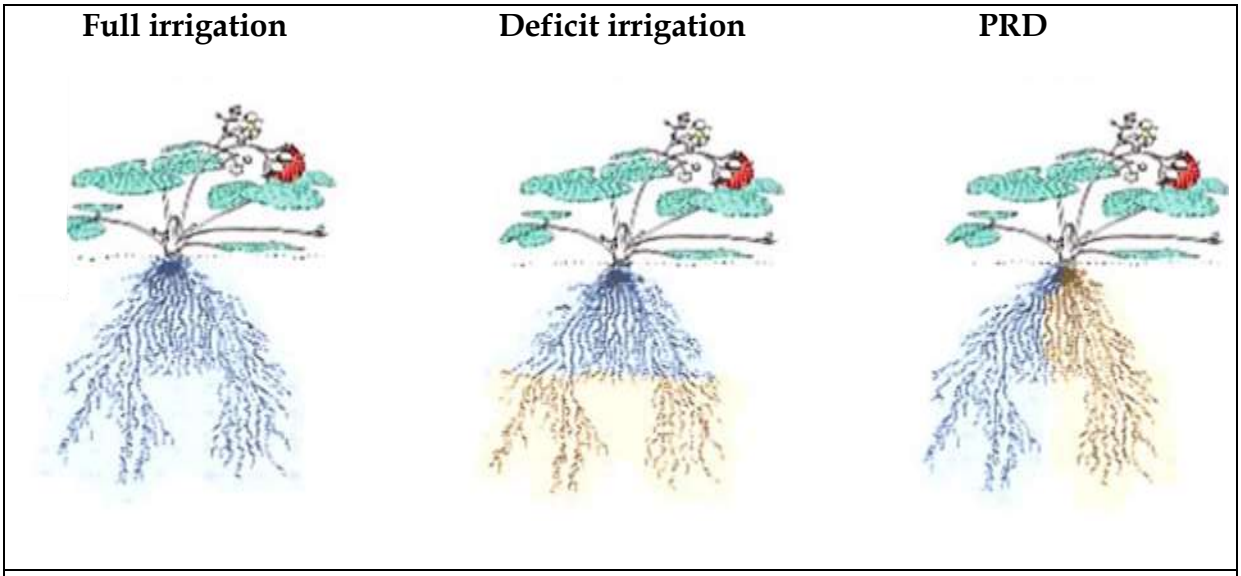


Fig. 1. Schematic of the irrigation pattern in FI, DI, and PRD (After Davies and Hartung, 2004)

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)

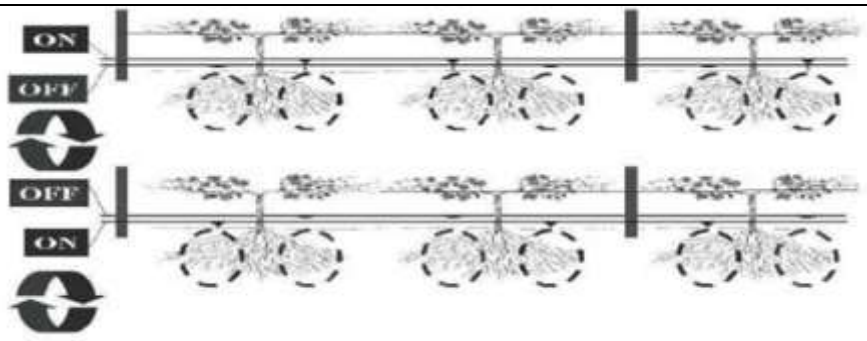


Fig. 2. Partial root zone drying using two above-ground drip lines in a vineyard

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).

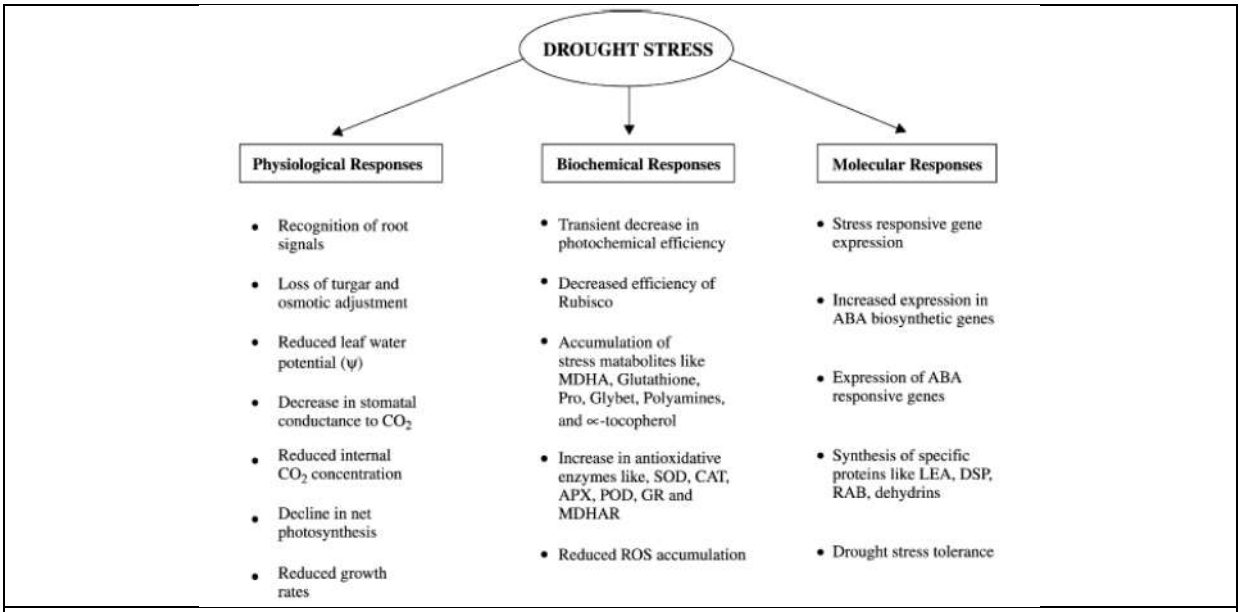


Fig. 3. Physiological and molecular bases of drought stress tolerance (After Shao *et al*., 2008)

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

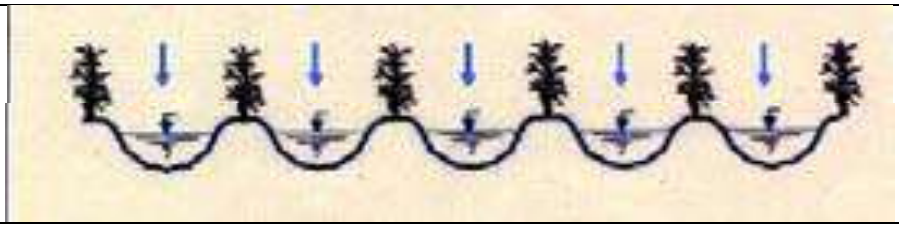
PRD and RDI could be applied in the field by different irrigation methods including:

- 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)

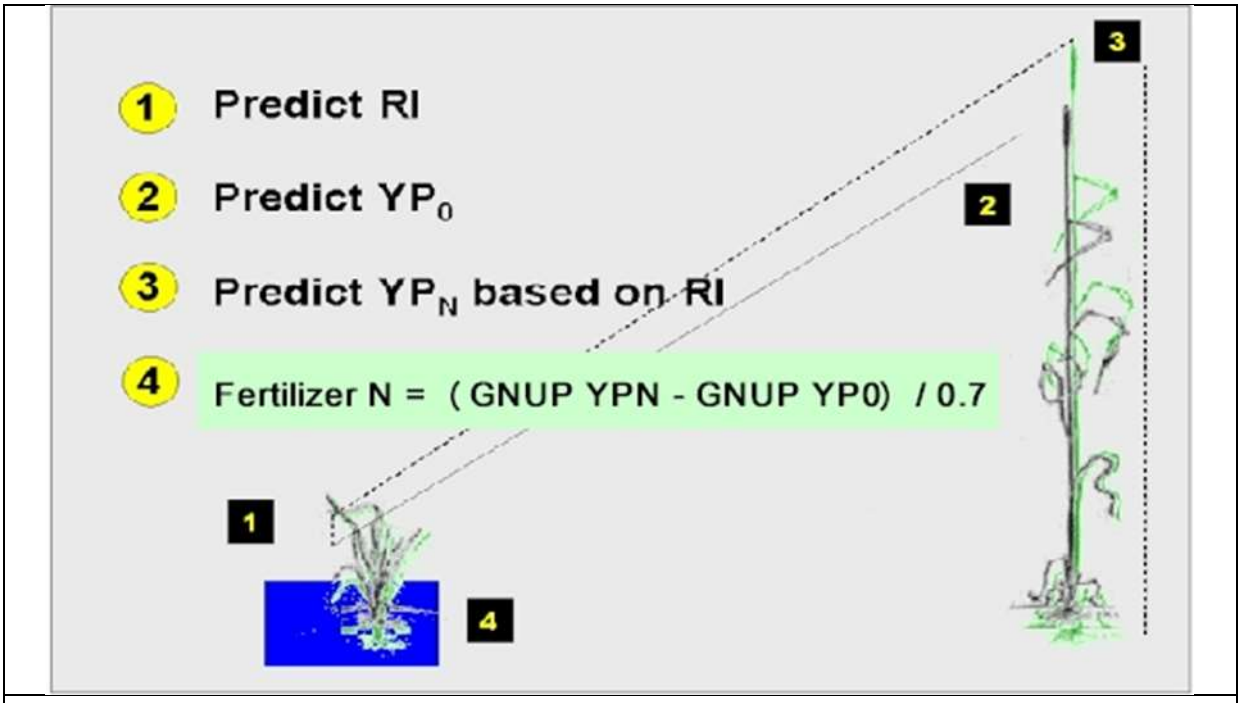

Fig. 1. Nitrogen fertilization algorithm Pictorial representation

(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.

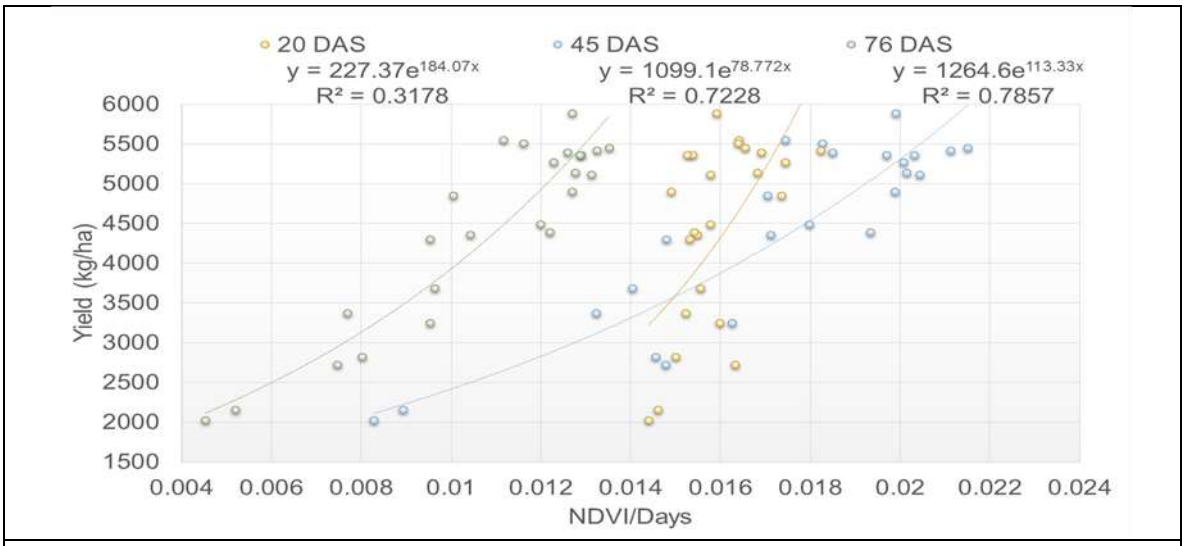

Fig. 2. Yield prediction equations

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.

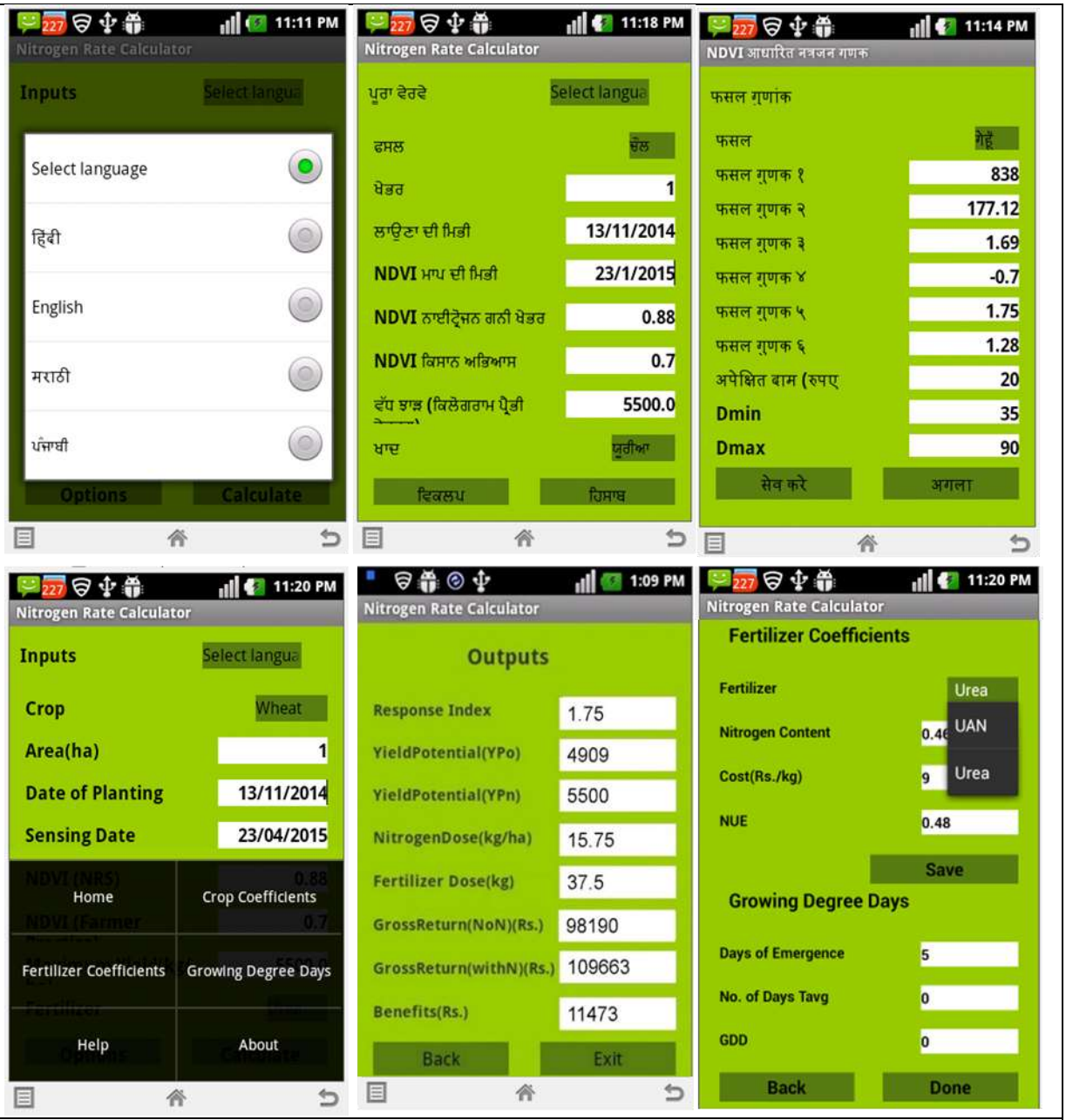

Fig. 3. Screen shots of the Multilingual Nitrogen fertilizer dose Calculator (Android App ver.1.02)

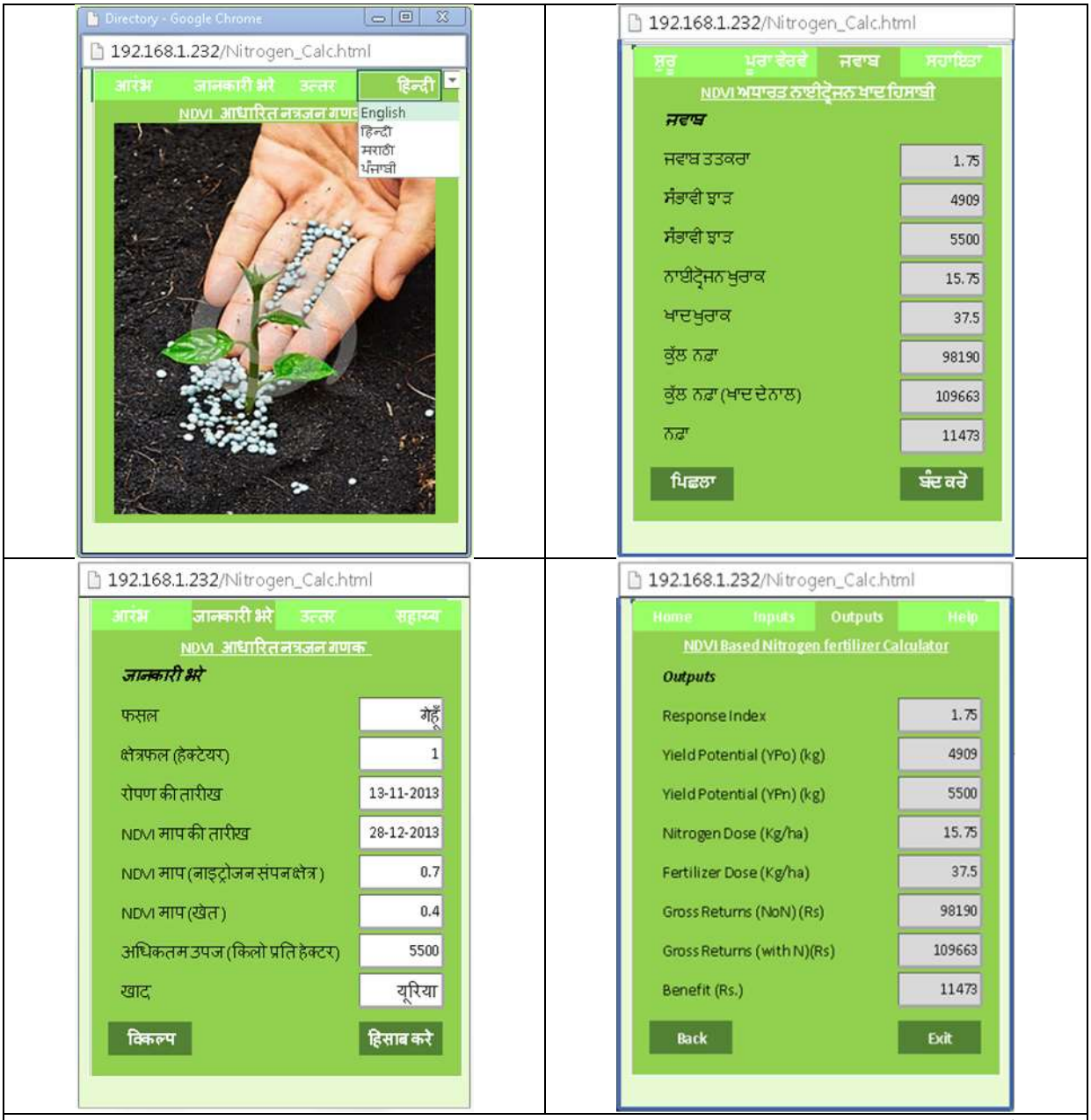

Fig. 4. Screen shots of the Multilingual Nitrogen fertilizer dose Calculator (Web App)

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.

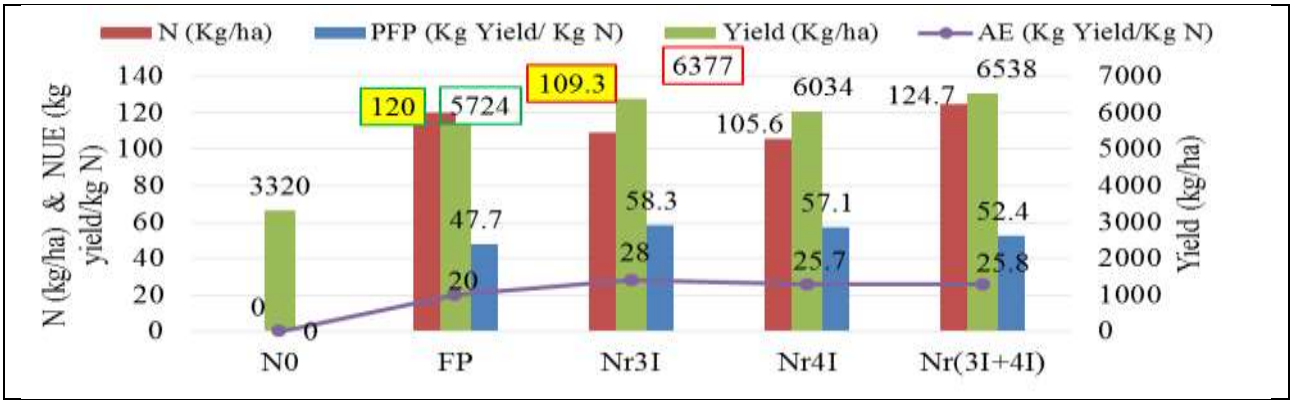

Fig. 5a: Yield and N use efficiencies for Paddy crop using NDVI based recommendation

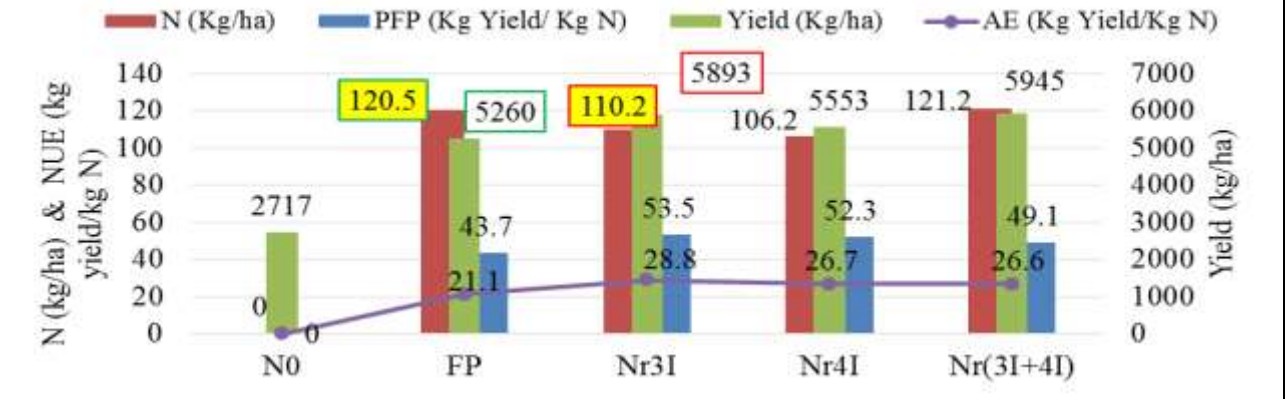

Fig. 5b: Yield and N use efficiencies for Wheat crop using NDVI based recommendation

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: Multi-omic approaches for assessment and alleviation of edaphic stresses synergizing with integrated farming

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Abstract

Edaphic stresses like salinity, acidity, alkali/sodicity, nutrient deficiency/excess, low or high organic matter content, shallow basaltic soils, soil moisture deficit, water logging, persistent bioaccumulative toxicants, persistent organic pollutants and poor water quality have gradually increased, which are the major limitations to food production in crops, horticulture, livestock and fishes. In the present review, assessment of priority edaphic stressors and their mitigation using multi-omics synergizing with integrated farming are discussed.

Introduction

Integrated farming

It is necessary to improve productivity in the agricultural sector by capitalizing on high value agriculture and generating income from allied activities. The farmers concentrate mainly on crop production which is subjected to a high degree of uncertainty in income and employment to the farmers. In this contest, it is imperative to evolve suitable strategy for augmenting the income of a farm. Integration of various agricultural enterprises viz., cropping, animal husbandry, poultry, fishery and integrated agri-aquaculture have great potentialities in the agricultural economy. In this direction, implementation of improved technology interventions in field crop, horticulture, livestock, poultry, fisheries and integrated agri-aquaculture for improving the livelihood of resource poor farmers is indispensible. Field and horticulture crops, livestock, poultry and fisheries activities not only provide additional income to the farmers, but also create employment opportunities in the rural areas for whole year. The integrated farming system approach introduces a change in the farming techniques for maximum production in the cropping pattern and takes care of optimal utilization of resources. The farm wastes are better recycled for productive purposes in the integrated system. A judicious mix of agricultural enterprises like dairy, poultry, piggery, fishery,

sericulture etc. suited to the given agro-climatic conditions and socio-economic status of the farmers would bring prosperity in the farming.

Aquaculture is one of the most important economic venture in many countries across the world. However, intensive aquaculture results in a rise in nutrient concentrations, then leads to serious eutrophication in freshwater, brackishwater and marine waters. Nitrogenous toxicant such as ammonia and nitrite are abiotic stresses in aquaculture. Elevated level of ammonia, coupled with poor water quality and bacterial pathogen, can cause deterioration in fish health and adversely affect fish production due to stress. Bacterial pathogens such as Vibrio harveyi in brackishwater shrimp and Aeromonas hydrophila in freshwater fish are biotic stresses, which can adversely effect aquaculture productivity. Due to environmental concerns associated with the accumulation of contaminants in food products and water supplies there is a great need to develop safe, convenient and economically feasible methods for decontamination. Ameliorating conditions of abiotic and biotic stresses is an important aspect in context of sustainable agricultural production.

Edaphic stresses

Millions of hectares of land throughout the world are too saline to produce economic crop yields, and more land becomes nonproductive each year because of salt accumulation. Salinity problem in agriculture are usually confined to arid and semiarid regions, whereas sodic soils tend to dominate in semi-arid and sub-humid regions. In India, such lands constitute about 6.75 m ha (saline 2.92 m ha and alkali/sodic 3.83 mha) of land area. Development of salinity and sodicity not only reduces crop productivity but also limits crop choice. Even though closely related and having many characteristics in common, soil salinity and sodicity are two different problems, which should be dealt differently and require different management practices. In contrast to saline soils, sodic soils have excess exchangeable sodium percentage (ESP). In order to improve and sustain high agricultural productivity, saline and alkali/sodic environment need to be modified to suit the available plants.

Among the persistent organic compounds (POPs), pesticides belonging to the class organophosphate, organochlorine, carbamate, neonicotinoids, and pyrethroid are commonly used in agriculture. Excessive and indiscriminate use of pesticides has resulted in contamination of water resources, air and soil, which pose major health problems to human beings affecting the nervous, endocrine, reproductive and immune systems. Besides, these compounds adversely affect to a variety of organisms that include living soil biota along with beneficial arthropods and soil microbes, fish, birds, animals, plants, and crop production as well. Characteriation and remediation of soil and water of a region is an important aspect in context of

sustainable agricultural production. There are many physico-chemical and biological approaches to remove POPs from the ecosystem, among them most promising is biodegradation. Bioremediation is one of the most rapidly growing areas of environmental biotechnology. The biochemical importance of bioremediators indicates a need for devising more reliable methods for identification of environmentally and agriculturally important microorganisms. Multi-omics such as genomics, metagenomics, metabolomics, proteomics, transcriptomics, and bioinformatics can be used as innovative polyphasic tools for enhancing potential applicability, predictability and reliability of microbes mediated bioremediation of agricultural soils, which in turns, may help in facing the challenges of environmental pollution. The use of native bacteria could be advantageous to degrade POPs in the contaminated environment with the result of formation of completely non toxic end products, which can be beneficial for human health perspectives. In situations, where indigenous degraders cannot rapidly degrade recalcitrant chemicals, bioaugmentation involving the addition of indigenous laboratory grown microorganisms capable of biodegrading the target contaminant or serving as donors of catabolic genes may be the only means for successful bioremediation. The biochemical importance of bioremediators indicates a need for devising more reliable methods for identification of environmentally and agriculturally important microorganisms. Adoption of genomics and ecogenomics based techniques have led to the realization that microbial populations and processes in and around the natural environments are much more diverse than our expectation. However, despite their key involvement, the potential roles of these tiny unseen microbiota in the transformation of xenobiotics and catabolic pathways remain poorly understood. Functional genomics offer novel information about their enzymatic system. Both aerobic and anaerobic routes and degradation pathways of POP's at the molecular and genetic levels to enhance biodegradation are required to be studied in a holistic manner.

Pollution stress stimulate genetic adaptation in microorganisms and assist in evolution of diverse metabolic pathways for their survival on several complex organic compounds. Diverse microorganisms, harboring numerous plasmids and catabolic genes, acclimatize to these environmentally unfavorable conditions Microbes constitute a huge reservoir of enzymes for the diagnosis of pollution and for bioremediation and more than 99% have never been cultured. Microbial metagenome constitutes the largest genetic reservoir with miscellaneous enzymatic activities implicated in degradation. This techniques provides the particularly exciting potential to mine functional genes for novel enzymes. The latest advances in functional metagenomics aimed at the discovery of enzymes capable of degrading various types of pollutants. The field of environmentalbioremediation has been ameliorated by exploiting diverse bacterial detoxification genes. The exact genetic

mechanisms of microbes for bioremediation of toxic compounds may be characterized by examining the uncultured microbes. Metagenomics can help in mining of novel catabolic genes for utilization in enhanced biodegradation.

Novel nanoparticles and its role in environmental remediation is the subject of extensive research. Nanomaterials have increasingly been used in water treatment because of economical and environmental viability and wider availability. Nanotechnological interventions for input use efficiency are required for mitigation of abiotic and biotic stresses in agriculture.

In conclusion, bioremediation synergizing with integrated farming has tremendous potential to alleviate the edaphic stresses in agriculture. Suitable and proper strategies can bring noticeable advantages of mitigation of abiotic stresses in agriculture in a sustainable and eco-friendly manner. In addition, involvement of multi-omics may advance the mitigation techniques.

Chapter 12: RNAi mediated abiotic stress tolerance in crop plants

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Abstract

RNA interference (RNAi) is a promising functional genomics toll for down-regulation of gene expression in precise manner without affecting the expression of other genes. RNAi phenomenon involves small interfering RNA (siRNA) or short hairpin or microRNA (miRNA) to repress the expression of sequence-specific gene at post-transcriptional and translational level. This technology has been extensively used for elucidating function of genes, to alter the gene expression in plants for enhancing crop yield, nutritional quality improvement and for increasing productivity by manipulating the gene involved in biomass, grain yield and for developing resistance against various biotic (bacteria, fungi, viruses, nematodes, insects) and abiotic stresses (drought, salinity, cold, etc.). Here, we are describing the mechanism of RNAi mediated gene silencing and application of RNAi silencing technique in elucidating function of genes associated with abiotic stress tolerance in crops.

Key words: siRNA- Small interfering RNA, miRNA- Micro RNA, RNAi- RNA interference, Drought Stress, Salinity Stress, Abiotic Stress tolerance, Dicer, Argonaute, Gene silencing

Introduction

Globally, crop yield is negatively affected by various unfavourable environmental factors such as drought, salinity and cold and high temperature. Therefore, there will be high demand of crops maintaining yield stability under various abiotic stress conditions. Drought and salinity stress tolerance and adaptation in crop plants have been improved by genetic engineering of various transcription factors, genes associated with signalling and biosynthetic pathways, compatible solutes and accumulation of antioxidants (Pradhan *et al*. 2015). Several genes associated with metabolic pathways have been functionally elucidated to understand stress tolerance mechanisms and for developing abiotic stress tolerance in crop plants (Singh *et al*. 2008). It is utmost important to elucidate role of transcription factors or genes by genetic manipulation for higher yield and also yield stability under various abiotic stress conditions. Several researchers are trying to identify and characterize various genes associated with plants response to drought and salinity stress by using

genomics, transcriptomics, proteomics and metabolomics approaches. Therefore, it is essential to know the exact role of specific small RNA followed by genetic manipulation for the crop improvement.

RNA interference (RNAi) phenomenon involves suppression of the gene expression by degrading the specific messenger RNAs. The phenomenon of RNAi involves small non-coding RNAs called small interfering RNA (siRNA), short hairpin RNA (shRNA) and microRNA (miRNA) that are the cleavage product of dsRNA. The mRNA degradation process is triggered by the introduction of double stranded RNA (dsRNA) which is further cleaved by the enzyme dicer (Kumar *et al.* 2012). The phenomenon of RNAi, in addition to small non-coding RNAs, also involves a RNA-induced silencing complex (RISC) (Redfern *et al*., 2013; Wilson and Doudna, 2013) and Argonaute proteins (AGOs) (Ender and Meister, 2010; Riley *et al*., 2012). The phenomenon of gene silencing was discovered accidentally in petunia flowers where expression of *chalcone synthase*, pigment producing gene, resulted in variegated flowers instead of expected deep purple colour. Since, the expression of both the transgene and the homologous endogenous gene was suppressed, the phenomenon was termed co-suppression (Napoli *et al*., 1990; Campbell, 2005). RNAi technology can be used to identify and functionally characterize thousands of genes within any genome which can be exploited for crop improvement (Younis *et al*. 2014). The RNAi technology has been employed successfully in improvement of several plant species by enhancing tolerance to abiotic stress (Ali *et al*. 2010).

Mechanism of Post Transcriptional Gene Silencing through RNAi Pathway

There are two small RNA in the RNAi pathway, a small interfering RNA (siRNA) and a micro RNA (miRNA) (Figs.1, 2) The miRNA are similar to siRNA in many respect as they originate from double stranded structure, the size of the miRNA is 20 to 24 bp and both are proceed by DICER or DICER like enzyme (DCL1, DCL2). RISC uses as both target sequence and they direct post transcriptional gene silencing. They differ from each other in their origin. The miRNA is derived from genomic DNA, while siRNA is generated by chopping of dsRNA into smaller segment. Active miRNA has two phases that is primary miRNA (pri-miRNA) and pre-miRNA. Both pri and pre-miRNA are characterized by a hairpin structure. Processing of miRNA occurs of both at the nuclear and cytoplasmic levels (Williams *et al*. 2004). Once a miRNA gene is transcribed, the transcript will form a roughly 42 to 60 bp long hairpin structure with two arms of approximately the same length. Out of these, one of the strands produces active miRNA via DICER. RNA interference pathway majorly follows four common steps such as i) cleavage of dsRNA by dicer, ii) entry of SiRNA into RISC complex, iii) silencing complex activation, iv) mRNA degradation (Ali *et al*. 2010). In the first step of RNAi, the introduced dsRNA in the

cell, which is perfectly homologous in sequence to the target gene, is recognise by DICER enzyme (Figure 1). Dicer enzyme further processes the dsRNA into dsSiRNA of 21 - 25 nucleotides. Then, the SiRNA produced by the dicer are incorporated into multicomponent nucleus complex into the RNA induced silencing complex, which is inactive in this form to conduct RNAi. The next step involves unwinding of the SiRNA by a helicase and further remodelling of the complex to create an active form of the RISC. RISC is a ribonucleoprotein complex and its two important components are the single stranded SiRNA and the Argonaute family protein (Kumar *et al*. 2012). The next step is degradation of mRNA. The active component of an RISC are endonuclease called argonaute protein which cleave the target mRNA strand complementary to their bound SiRNA, therefore argonaute contribute "silencer" activity to RISC. When the dsRNA chopped by the dicer produced the small siRNA, in which one strand is known as guide strand binds that the agronaute protein and directs gene silencing. After the cleavage is complete, the RISC departs and the siRNA can be reused in a new cycle of mRNA recognition and cleavage (Figs 1, 2).

Functional Characterization of Genes employing RNAi technology

Considerable progress has been made in developing genomic resources for plants such as soybean, chickpea, pigeon pea, peanut rice, wheat, maize, barley, grape and jowar. A large number of genes have been identified, most with unknown function. Therefore, a major research priority in the post-genomic sequencing era is determining the function of these genes (Wesley *et al*., 2001). The primary tool for dissecting a genetic pathway is the screen for the loss of gene function, disrupting the target pathway. Modern biotechnology has enabled the elucidation of gene function through the systematic modification of gene expression followed by quantitative and qualitative analyses of the gene expression products. The modulation of gene expression can be achieved by the integration of foreign DNA sequences in the plant genome, leading to either overexpression or gene silencing. Gene silencing is currently achieved through interference RNA (RNAi), a process of sequence-specific, post-transcriptional gene silencing initiated by double-stranded RNA that is homologous in sequence to the target gene (Figs. 1 , 2). Overexpression and silencing are complementary strategies to functionally characterize genes.

Role of RNAi in Abiotic Stress

Stress is usually defined as an external factor that exerts a disadvantageous effect or harmful effect on the plant. Abiotic stress causes the serious damages to the plant by negatively affecting its growth and yield. It has been estimated that nearly 70% of crop yield is reduced due to the abiotic stress (Younis *et al*. 2014). Plants are subjected to many types of fluctuations in the physical environment. In general,

various types of strategies have been used to avoid the fluctuation by the animals but plants are not able to avoid because of their sessile nature. Therefore, plants have adapted numerous physiological, biochemical and metabolic approaches for tolerating the abiotic stress. Abiotic stress are classified into the following major categories such as drought, salinity, heat, cold and oxidative stress. Classical techniques of breeding crop plants with enhance tolerance to abiotic stress have until now achieved inadequate success. Therefore, transgenic technology is one of the numerous tools offered improvement in modern plant breeding programme. Identification of candidate gene through functional genomics programmes discovered multiple gene families which regulates the abiotic stress tolerance phenomena and high production. Therefore, researchers are trying to incorporate the candidate gene or multiple numbers of genes to express ectopically for crop improvements (Younis *et al*. 2014). Now a days, RNAi technology has been evolved a modern approach for gene function analysis and in translational research programme. Recent findings suggest the RNAi is playing an important role in abiotic stress stimulation in different crops (Fig. 2). RNAi technology may be a substitute of complex molecular techniques because of containing several benefits, its specificity and sequence based gene silencing. Due to this property, RNAi has been effectively utilized for incorporating desired trait for abiotic stress tolerance in various plant species (Jagtap *et al*. 2011). RNAi technology may be a substitute of complex molecular techniques because of containing several benefits like its specificity and sequence-based gene silencing. This ability of RNAi has been efficaciously utilized for incorporating desired traits for abiotic stress tolerance in various plants species.

Role of siRNAs in abiotic stress tolerance

The first evidence that siRNAs are involved in abiotic stress responses in plants was provided by Sunkar and Zhu (2004). Nat-siRNAs, which is derived from a natural cis-antisense transcript pairs of SRO5 and P5CDH genes, demonstrated an important role of nat-siRNAs in osmoprotection and oxidative stress management under salt stress in Arabidopsis (Borsani *et al*. 2005). Under high salt stress, the 24-nt nat-siRNA corresponding to the SRO5 mRNA is produced; this nat-siRNA targets the P5CDH mRNA for degradation and leads to the production of a population of 21-nt natsiRNAs. Downregulation of P5CDH leads to proline accumulation, which is an important step contributing to the plant's ability to tolerate excess salt (Borsani *et al*. 2005). However, reduced P5CDH activity also leads to the accumulation of P5C (a toxic metabolic intermediate) and ROS; that accumulation is probably countered by the SRO5 protein through direct detoxification activity in the mitochondria (Borsani *et al*. 2005). The initial induction of SRO5 mRNA by salt stress may be mediated by oxidative stress that is an inevitable consequence of salt stress. Thus, the SRO5-

P5CDH nat-siRNAs together with the P5CDH and SRO5 proteins are key components of a regulatory loop controlling ROS production and salt stress response (Borsani *et al*. 2005). Abiotic stress responsiveness has also been observed in a pool of Triticum aestivum small noncoding RNAs (Yao *et al*. 2010). In wheat seedlings, cold, heat, salt, or drought stresses substantially change the expression of four siRNAs: siRNA002061_0636_3054.1 is strongly downregulated by heat, salt, and drought; siRNA 005047_0654_1904.1 is greatly upregulated by cold stress and downregulated by heat, salt, and drought; siRNA080621_1340_0098.1 is slightly upregulated by cold and downregulated by heat but not by salt and drought; and siRNA007927_0100_2975.1 is downregulated by cold, salt, and drought but not by heat stress (Yao *et al*. 2010). TasiRNAs are a specialized class of siRNAs that are generated by miRNA processing of a TAS gene transcript, resulting in the production of 21-nt RNAs that are phased with respect to the miRNA cleavage site. Four families of TAS genes have been identified in Arabidopsis, with TAS1 and TAS2 transcripts recognized by miR173, TAS3 recognized by miR390, and TAS4 targeted by miR828 (Allen *et al*. 2005). TAS1, TAS2, and TAS3 tasiRNAs all showed increased expression in hypoxia-treated samples in Arabidopsis). These changes in tasiRNA levels are reflected in the changes to TAS-targeting miRNAs; both miR173 and miR390 showed increased expression. Most pentatricopeptide (PPR) repeat-containing proteins (PPRs) targeted by hypoxia-responsive small RNAs are from the P subfamily, which is predicted to localize to the mitochondria. The downregulation of these PPR genes may either protect mitochondria during hypoxia or simply reflect a decreased requirement for these gene transcripts. Stress responses in plants also involve novel long non-protein coding RNAs (npcRNA). In Arabidopsis, salt stress resulted in a dramatic increase in npcRNA60 and npcRNA536 and a decrease in npcRNA72 and npcRNA82 accumulation (Ben *et al*. 2009). In the same study, phosphate deprivation caused substantial upregulation of npcRNA43 and npcRNA536, substantial downregulation of npcRNA33, slight upregulation of npcRNA60, and slight downregulation of npcRNA311 (Ben *et al*. 2009). In Craterostigma plantagineum, an endogenous siRNA has been identified that is induced during dehydration and that may contribute to desiccation tolerance (Furini *et al*. 1997).

Role of microRNAs in abiotic stress tolerance

Micro RNAs has an important gene expression regulator during plant abiotic stress (Gupta *et al*. 2014). miRNA under specific conditions can regulate the expression of specific target gene. In abiotic stress condition, the plant after signal perception, the abiotic stress responsible miRNA gene undergoes transcription by RNA Polymerase II enzyme into primary miRNA (pri-miRNA), the miRNA is proceed by dicer like DCL 1 into a miRNA duplex. The miRNA is then exported into the cytoplasm from

the nucleus The mature miRNAs are incorporated into RNA induced silencing complex (RISC), where the mature single stranded miRNA guides the RNA silencing activity of AGO1 to partially complementary mRNA (Fig. 2). The microRNA then targets the abiotic stress responsive mRNA and that causes translation repression and mRNA degradation (Ding *et al*. 2009). The function of miRNAs (microRNA) in relation to abiotic stress like oxidative stress, cold, drought, and salinity were reported by Sunkar and Zhu (2004) in Arabidopsis plants under various abiotic stress and confirmed miR393 was sturdily up-regulated when exposed to higher salinity levels, dehydration, cold, and abscisic acid (ABA). Additionally, miR402, miR319c, miR397b, and miR389a were controlled by abiotic stress under varying levels in Arabidopsis.

Role of MicroRNAs in drought stress tolerance

Now days, miRNAs have been emerged as key modulator in drought avoidance and also in drought tolerance by controlling of the drought responsive gene(s). It has been reported that drought induced miRNAs downregulate their target mRNA, which results in production of non-functional protein in response to drought stress. In contrast, the downregulation of few miRNA lead to accumulation of their target mRNA which have positive effect to stress adaptation (Ding *et al*. 2009). The miRNA expression profiling has been done in many plants such as Arabidopsis, populus trichocarpa, Oryza sayiva under drought stress. The miR169, miR 396, miR171, miR319, miR393, miR156, miR158 known to be drought responsive (Younis *et al*. 2014, Liu *et al*. 2008). The role of miR159 was found to be induced by ABA and drought treatment in germinating Arabidopsis seeds (Reyes and Chua 2007). In Arabidopsis, miR159 mediates the cleavage of MYB101 and MYB33 transcripts (Reyes and Chua 2007, Abe *et al* 2003). Its role in ABA signalling and their mechanism are evaluated by over expressing miR159 which suppressed MYB101 and MYB33 mRNA level. So, the transgenic plant over expressing MYB101 and MYB33 were hypersensitive to ABA (Ding *et al*. 2009, Reyes and Chua 2007) and improved osmotic stress tolerance. MYB functions as a positive regulator of ABA signalling and miR159 which probably play a key role in ABA response in Arabidopsis plant (Ding *et al*. 2009). Interestingly, it has been reported that miR167 was downregulated by treatment of ABA in rice seeding (Liu *et al*. 2009), in contrast, its expression was upregulated by drought stress in Arabidopsis (Liu *et al*. 2008).

In relation to drought responses, miR169 and miRNA393 genes have been observed in rice crop which were stimulated under drought conditions (Zhao *et al*. 2007). Among genetically engineered plants the rice exhibited gene expression of RACK1 inhibition caused by RNAi, which explained the potential role of RACK1 to drought stress in rice crop. The transgenic rice was observed with a superior level of tolerance in contrast to non-transgenic rice plants (Jian *et al*. 2010). Analysis of

miRNAs and genome sequencing profiling were executed in drought-studied rice at a various range of growth stages, from tiller formation to inflorescence, utilizing a microarray platform. The results suggested that miRNAs such as miR1126, miR1050, miR1035, miR1030, miR896, miR529, miR408, miR156, miR171, miR170, miR168, miR159, miR397, miR396, miR319, miR172 and miRNA1088 were involved in down regulation in response to drought stress (Liu *et al*. 2008). In contrast, miRNAs such as miR1125, miR159, miR903, miR169, miR901, miR171, miR896, miR319, miR395, miR854, miR851, miR474, miR845, and miRNA1026 were found in up-regulation under drought stress. Few miRNAs gene families, like miR319, miR896, and miR171 were recognized as both up- and down regulated groups133. In P. vulgaris, miR2119, miR1514a, and miRS1exhibted a gentle but obvious increase in accretion upon drought treatment, on the other hand, the accumulation was higher for miR2118, miR159.2, and miR393 in reaction to the identical treatment. In recent studies, miRNA expressing patterns of drought tolerance wild emmer wheat in relation to drought-stress explored by utilizing a plant miRNA microarray platform (Kantar *et al*. 2010). At the same time, up regulation throughout drought stress in maize crop has been studied by miR474, which interact with proline dehydrogenase (PDH).

Role of microRNAs in salinity, cold and heat stress tolerance

Many regulated miRNAs have been reported in salinity stressed plants. In Arabidopsis, miR397, miR156, miR394, miR158, miR393, miR159, miR319, miR165, miR171, miR167, miR169, miR168, and miR398 were up-regulated in reaction to salinity stress, whilst the accumulation of miR398 was reduced (Liu *et al*. 2008). In P. vulgaris, it was reported that increment in accumulation of miR159.2 and miRS1 with the addition of NaCl3. In *P. trichocarpa*, miR171l-n, miR530a, miR1446a-e, miR1445, and miR1447 were down regulated; on the other hand, miR1450 and miR482.2 were up-regulated in salt stress period (Lu *et al*. 2008). Recently, a research investigation was carried out by using microarray to elucidate the miRNA profile salinity- tolerant and a salt-sensitive line of maize; the findings indicated that members of the miR396, miR156, miR167, and miR164 groups were down-regulated, while miR474, miR162, miR395, and miR168 groups were up-regulated in saline-stressed maize roots. miRNA in wheat showed variant expression in heat stress response. In wheat, 32 families of miRNA distinguished, among them 9 identified miRNAs were supposed heat responsive. For instance, miR172 was distinctly decreased, while miRNAs including miR827, miR156, miR169, miR159, miR168, miR160, miR166, and miR393 were found to be up regulated in response to heat stress (Xin *et al*. 2010).

Conclusion and Future Perspectives

RNA interference technology involving siRNA and miRNA have emerged as an attractive tool used by plant biologists not only to decipher the plant function but also to develop plants with improved and novel agronomic traits by manipulation of both desirable and undesirable genes. A complete understanding of the actions of small RNAs depends on the identification of the target genes. Identification of entire sets of miRNAs and siRNAs and their targets will lay the foundation that is needed to unravel the complex miRNA and siRNA-mediated regulatory networks controlling development and other physiological processes. Given that miRNAs and siRNAs are crucial components of in gene regulatory networks, we believe that a complete understanding of the functions of miRNAs and siRNAs will greatly increase our understanding of plant tolerance to biotic and abiotic stresses. The regulatory role of miRNAs in plants is definitely a subject that will require much more investigation in plant biology. Several miRNAs have been determined to be commonly involved in drought and salinity stress responses and also plant development. The miRNAs regulate numerous transcription factors in response to different stresses. For many drought and salinity stress related genes, miRNAs function as critical post-transcription modulator for their expression. Although a number of drought associated miRNAs have been identified, their precise role remains to be verified. Additional strategies need to be employed to investigate the functions of miRNAs and their associated signalling pathways and gene networks under both drought and salinity stress.

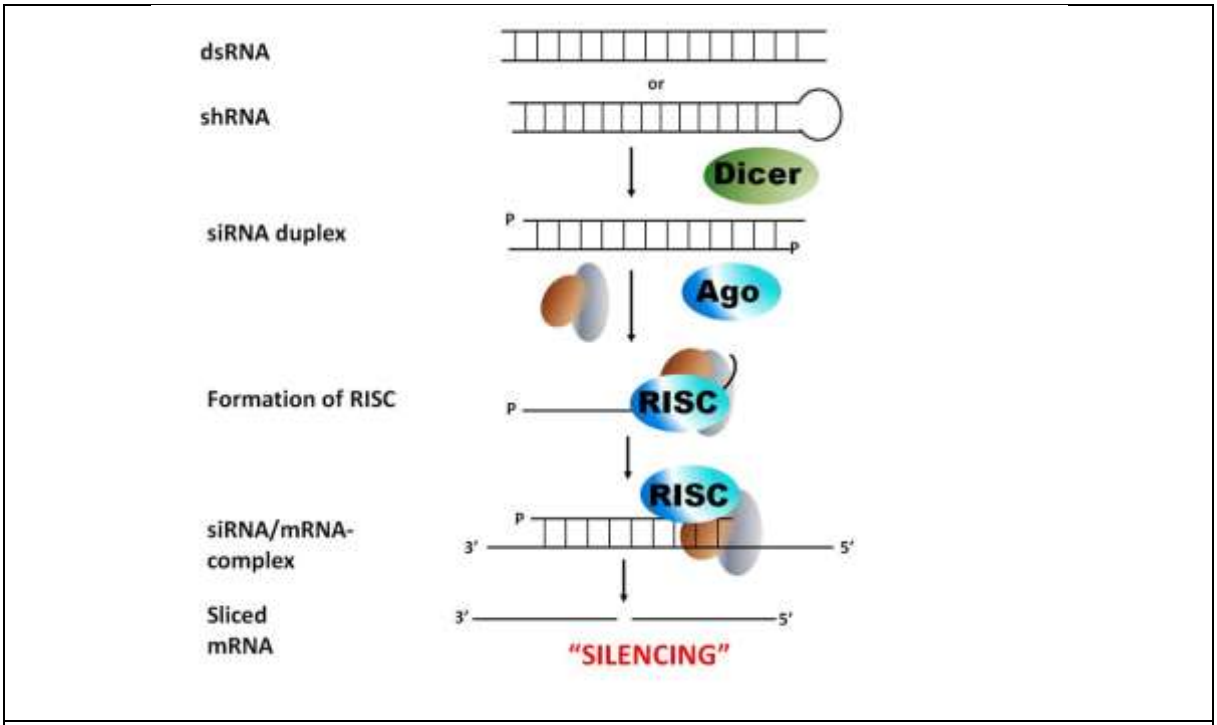

Fig. 1. siRNA/shRNA mediated RNAi gene silencing.

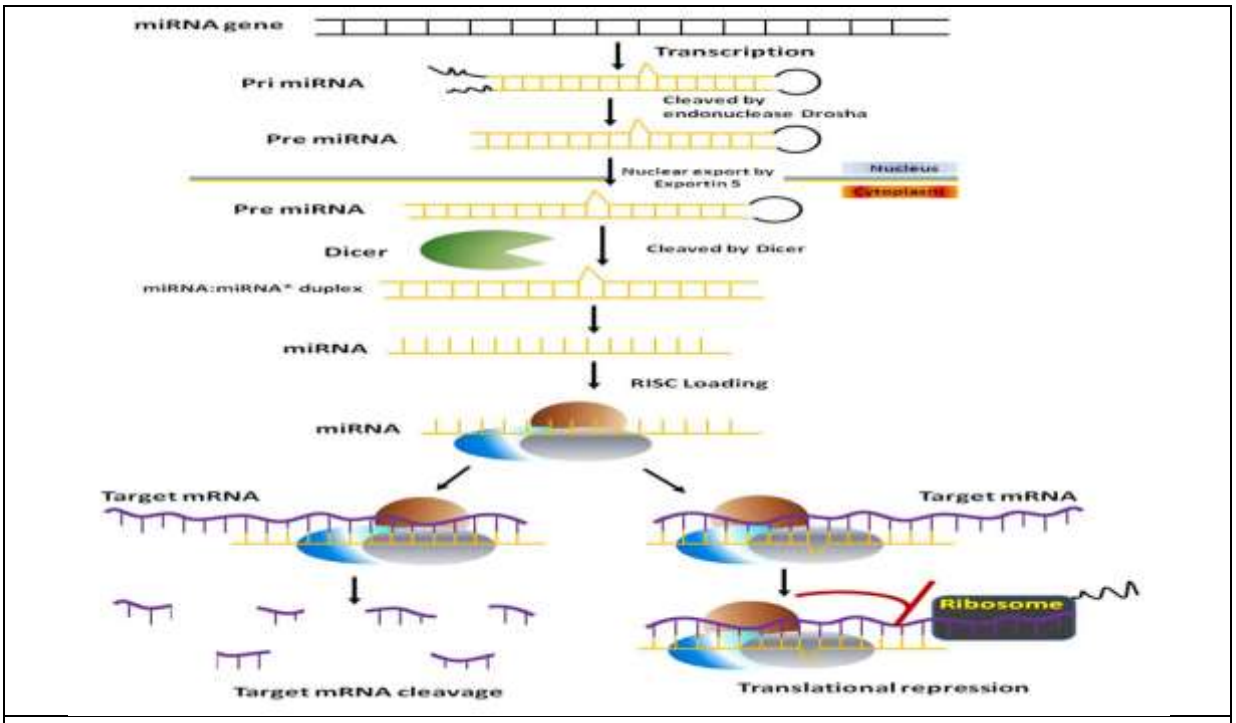

Fig. 2. miRNA mediated RNAi gene silencing.

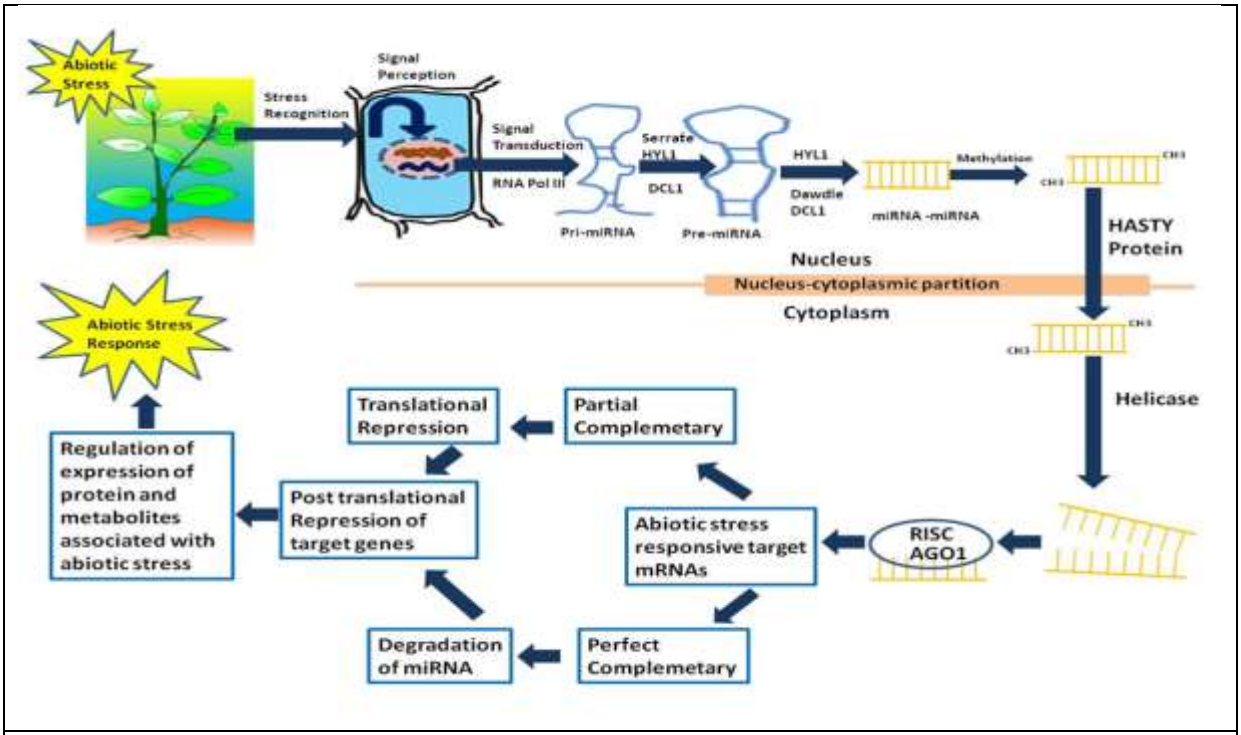

Fig. 3. Role of RNAi in abiotic stress tolerance

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Chapter 13: 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
Hyperspectral imaging	Near-infrared instruments, spectrometers ,hyper spectral camera	Crop vegetation cycles, indoor time series experiments	Leaf and canopy water status; Leaf and canopy health status; panicle health status; leaf growth; Coverage density	Wheat (Moshou *et al*., 2005)	Controlled 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 14: 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.

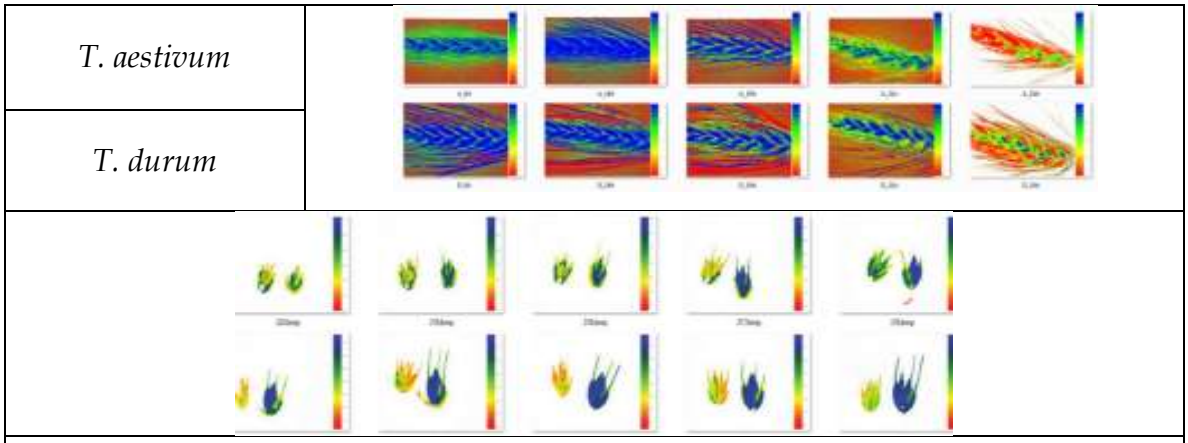
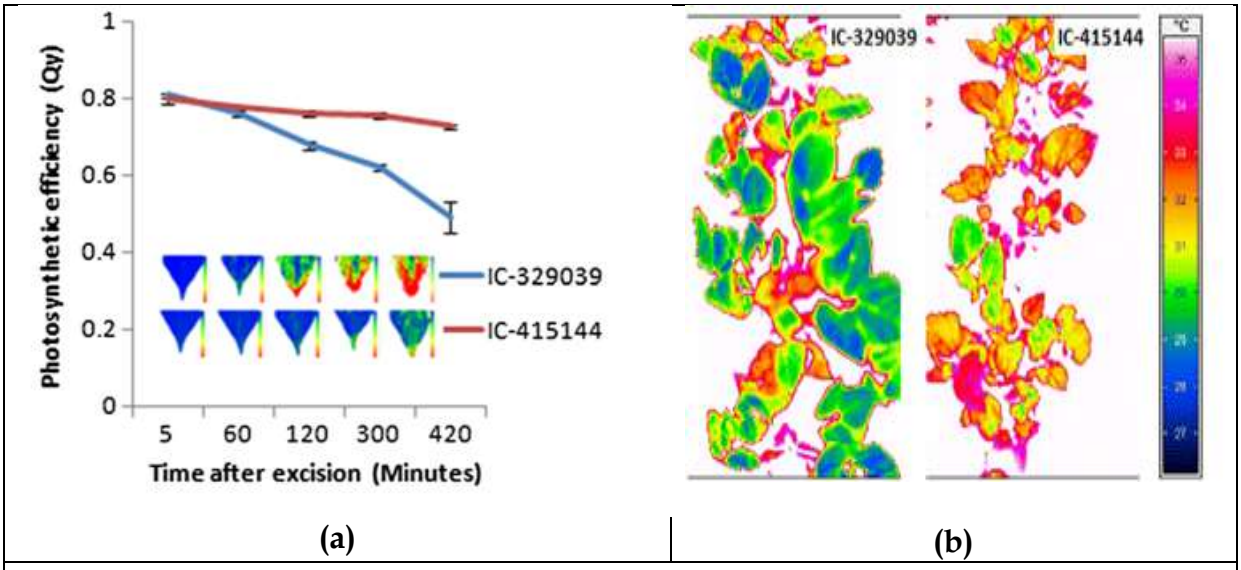


Fig. 1. Chlorophyll fluorescence imaging to assess desiccation tolerance

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



(a) (b)

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 15: 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).

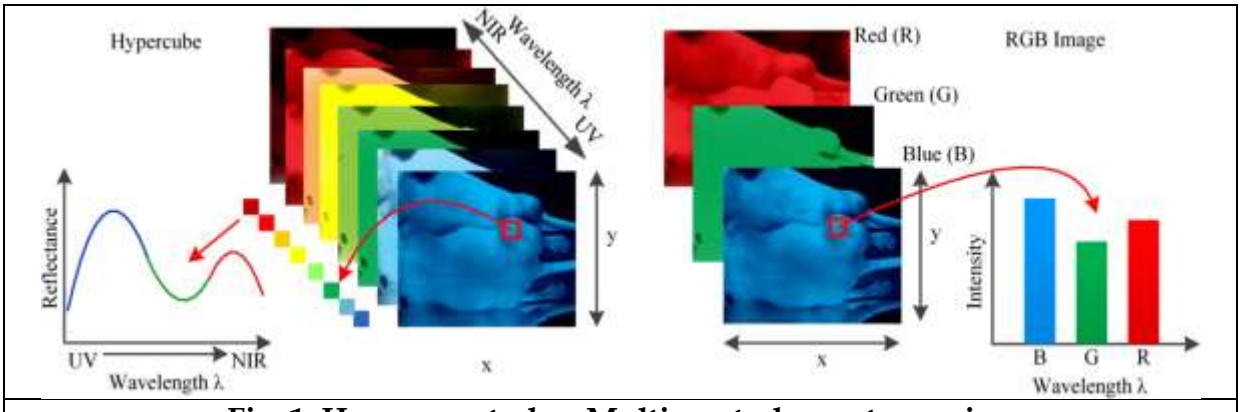

Fig. 1. Hyper-spectral vs Multispectral remote sensing

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).

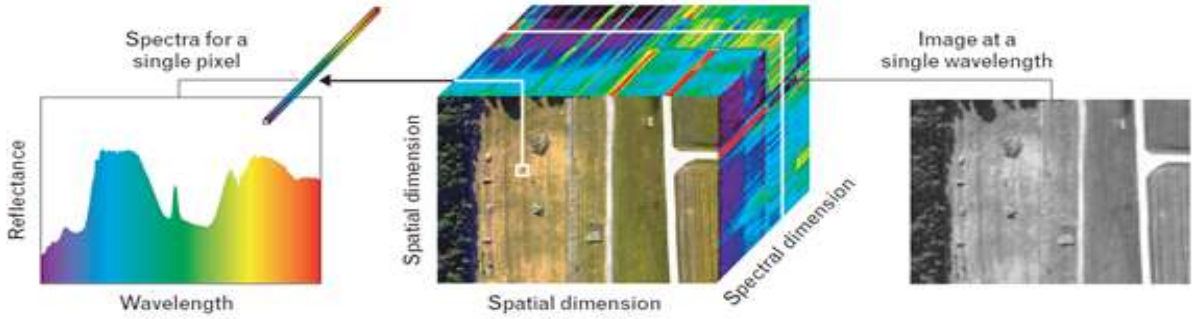

Fig. 2. Three dimensions of hyperspectral cube

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

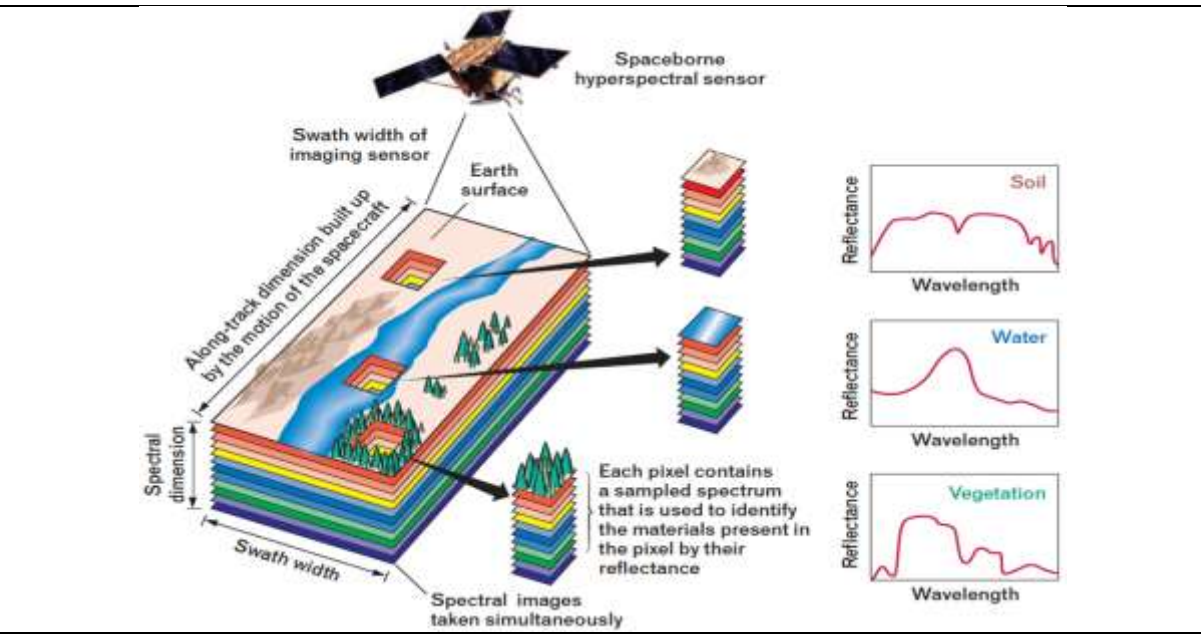

Fig. 3. Concept of data cube generated by a spaceborne hyperspectral imager

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).

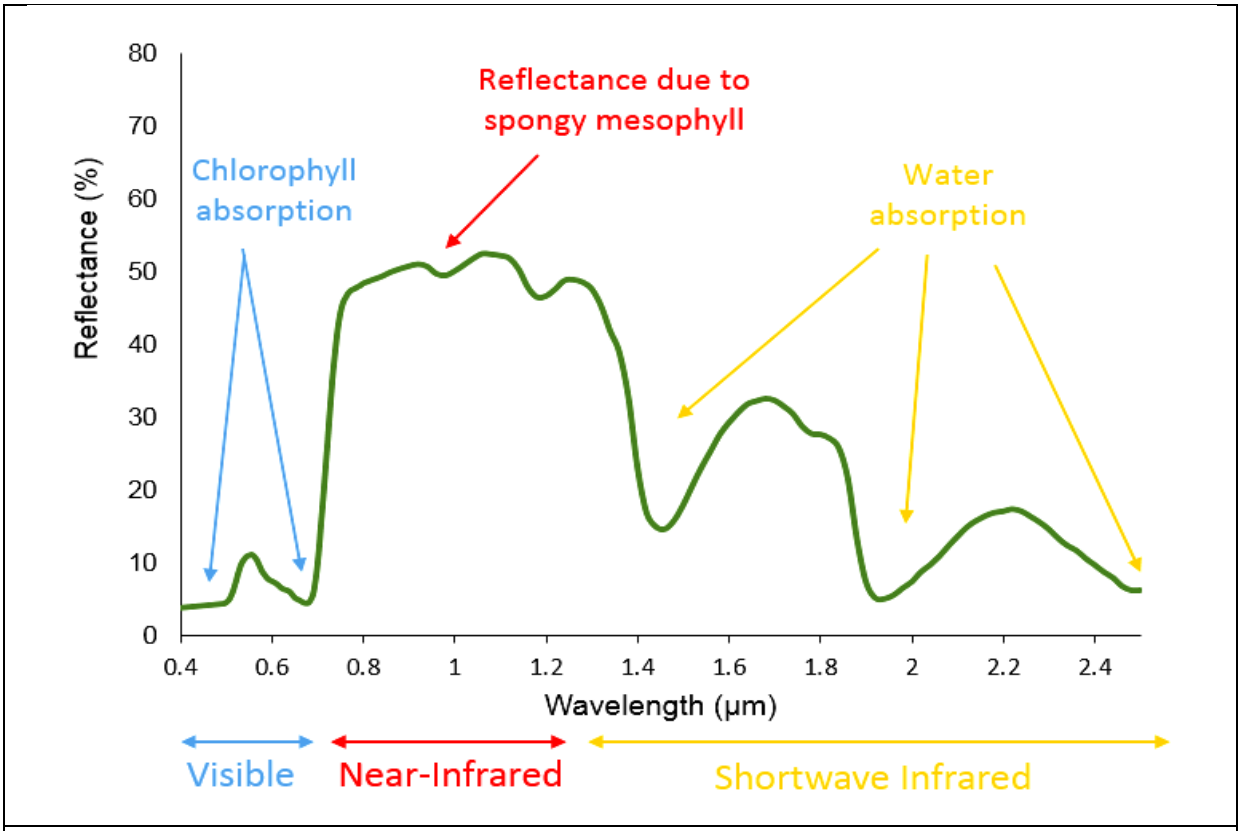

Fig. 4. Spectral bands response in vegetation

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).

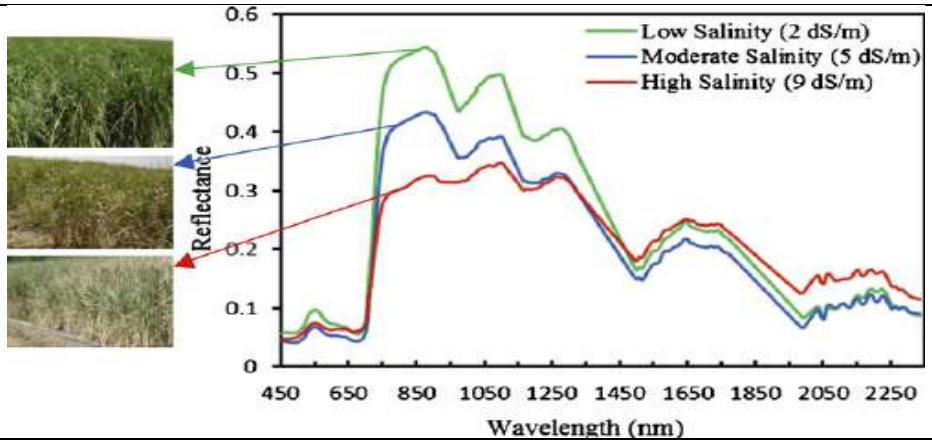

Fig. 5. Spectra of salinity stress response

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.

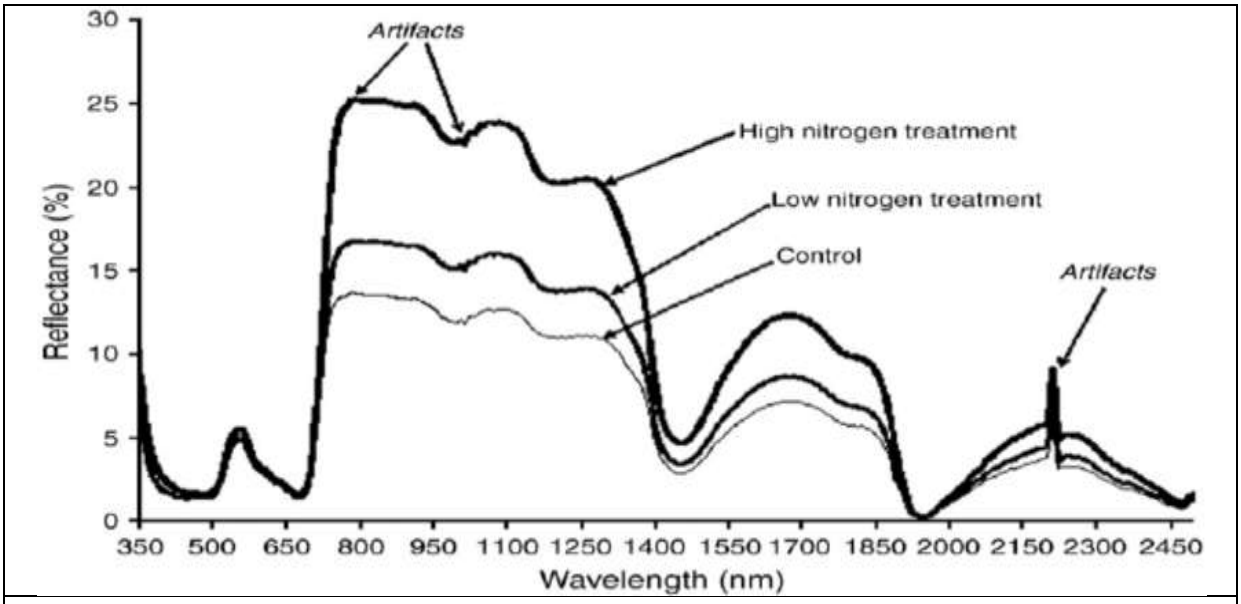

Fig. 6. Spectral response of plant at different nitrogen level

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 16: Phenotyping for root in-situ: Constraints and promises

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Roots are vital to plants for accomplishing numerous processes which includes nutrient and water uptake, storage functions, anchoring and mechanical support and as the major interface between the plant and different biotic and abiotic factors in the soil environment, but its measurements are limited under field conditions. The crop management practices can affect root architectural development but at what extent it can influence the yield and quality of the produce, often we do not know. It is the integrated action of shoots and roots that determines productivity. 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	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

flow meter			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

1. 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).

2. 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 "*Hands on mini-rhizotron technique for in-situ root phenotyping*".

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).

3. 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: Hands on mini-rhizotron technique for in-situ root phenotyping

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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.

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

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

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. . However, tubes with greater length (180 cm) are also can be utilized.

Fig. 2. Rhizo-tube 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. 1). A wooden platform for guiding the motorized root auger can be used while making the holes at 45^0 angles (Fig. 2). 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.

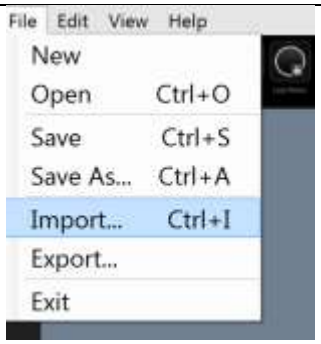
Analysis of in-situ root images with CI-690 RootSnap! Software:

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.

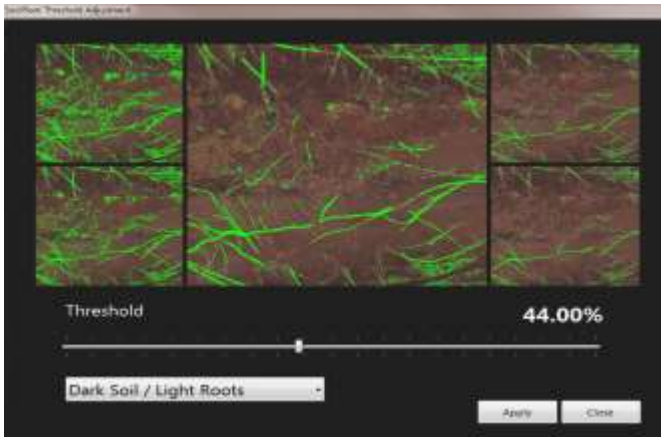
Import an Image

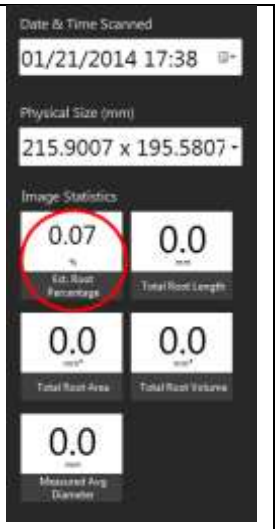
- To import an image, select <File> <Import > or press Control + I
- Browse the computer or enter the file name of the image you wish to import.



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.





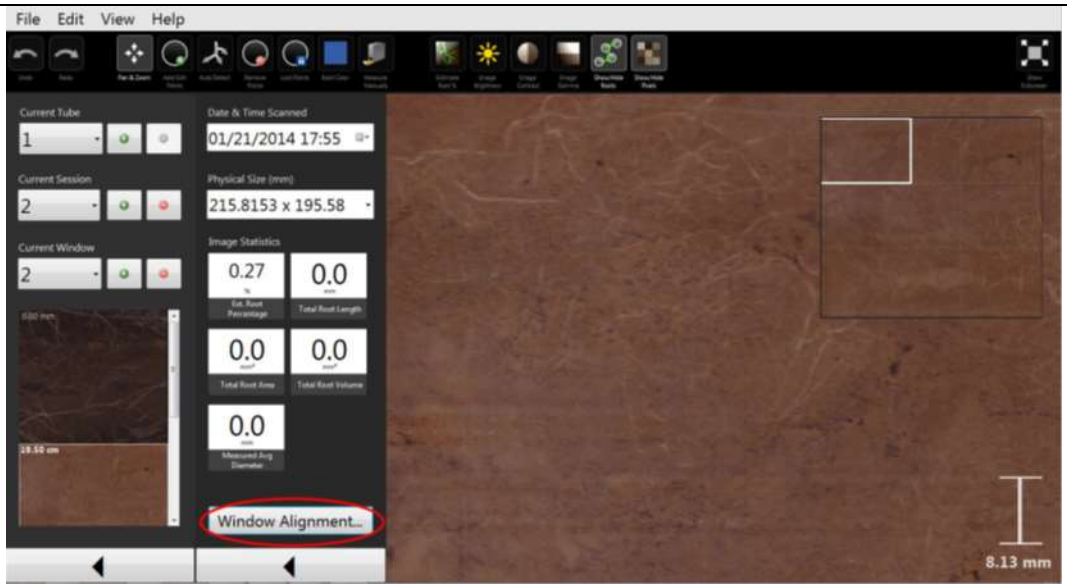
Window Alignment

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.

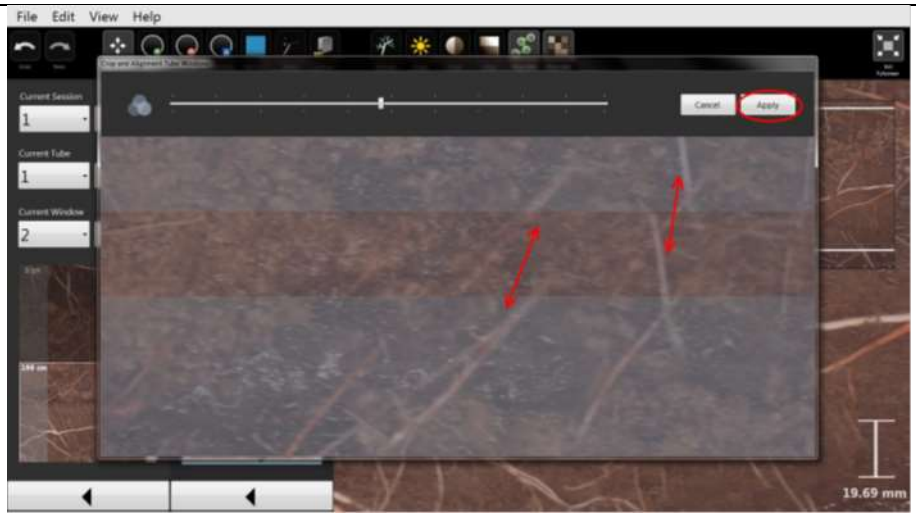
To align window images:

- 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

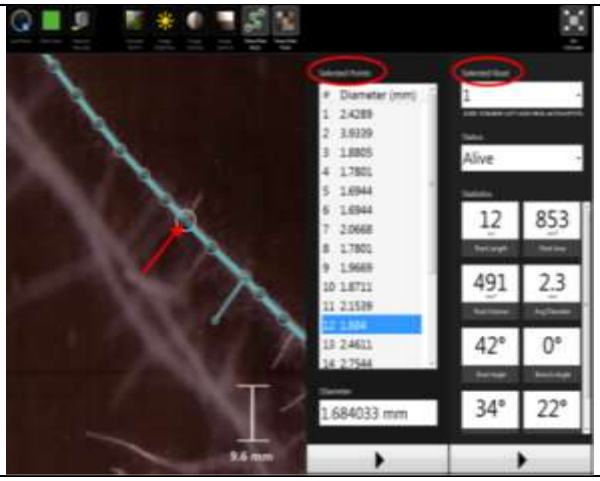
Select a window image to start mapping. Follow the steps as given below for mapping the roots:

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.

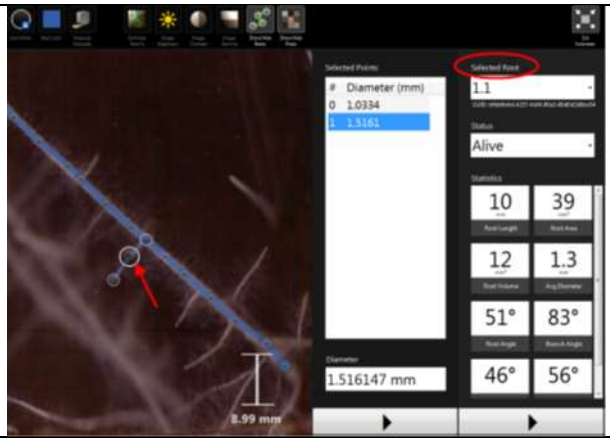
Tool bar options

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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CI-690 Rootsanp manual, https://www.cid-inc.com/plant-science-tools/root-measurement-with-minirhizotron/ci-600-in-situ-root-imager/

Chapter 18: Crop water production functions and foliar application plant bio−regulators for enhancing productivity and quality of major crops

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Abstract

Defining use of plant bio−regulators and supplemental irrigation on crop yield formation is essential for optimising irrigation and mitigating the impacts of water stress under water scarce conditions. Therefore, field experiments were conducted in last five years (2012−17) to evaluate the interactive effects of plant bio−regulators (PBRs) and supplemental irrigation on growth, yields and water productivity of major crops (wheat, sorghum, soybean, onion and eggplants). Potential PBRs included: salicylic acid, sodium benzoate (SB), thio−urea (TU), potassium nitrate (KNO3), gibberellin (GA3), ortho-silicic acid (OSA) were applied exogenously at 3−4 critical growth stages of specific crop as main plot treatments. Line−source sprinkler system (LSS); a unique sprinkler system designed to apply seven variable levels of irrigation water (IW) ranged between 0−1.0 times the CPE (cumulative open pan evaporation) as subplot treatments. The application of PBRs mitigated water stress and significantly improved yields, water productivity and water use. PBRs maintained higher leaf water content, lower canopy temperature, modulated the stomatal opening and ultimately the source–sink relations thereby improving the yield and productivity under deficit irrigation. Particularly PBRs like thio-urea (10 mM), sodium benzoate (100 mg L-1), KNO3 (1.5%) and salicylic acid (10 µM) were found effective to mitigate water stress in wheat, sorghum, onion and eggplant, respectively. Thus relative response of PBRs is highly specific environment conditions and varies with crop to crop. PBRs also helped to improve significantly physicochemical and functional quality characteristics viz., rehydration ratio, protein content, total soluble sugar, total phenolics content and pyruvic acid in water deficits. It is concluded that conjunctive use PBRs along with supplemental irrigation present viable option for improving crop and water productivity under the conditions of deficit irrigation.

Introduction

Almost all crops/vegetables are most vulnerable to abiotic stresses caused due to negative impact of various natural factors viz., extreme temperature, wind, soil moisture, drought and salinity. Water stress and moisture availability in soil exert great influence on plant growth through direct and indirect effects viz., root development, vegetative growth, uptake and mobilization of nutrients. It also affects turgidity, normal metabolism, cell division and enlargement which influence overall crop growth response. Bio−regulators (PBRs) play an important role to control the physiological metabolic activities in crops under water stress conditions. The research needs to be focused on increasing water use efficiency and control of metabolic activities in crops through use of PBRs under varied irrigation water regimes in different crops cultivated in water stressed regions. For this purpose, creation of large number of water levels which vary systematically from one end of single plot to the other are needed. The line source sprinkler technique facilitates the application of progressively decreasing amounts of water at increasing perpendicular distance from the line source (Hank, 1980). Therefore present research aimed to develop of crop water functions and impact of bio−regulators and supplemental irrigation on productivity and post-harvest qualities of different crops grown under different water stressed regions using line source sprinkler plot irrigation system.

Crop yield is primarily water-limited in arid and semi-arid regions. Under limited irrigation water, reduction in grain yield and its quality due to restricted water availability depends on degree, duration and timing of imposed water deficit. Many studies on plant responses to water deficits (stress) were carried out by investigators concerned with agricultural production, environment and resources, and macroscopic physics of soil, plant, and atmospheric water. As expected, the physiological and metabolic aspects of these studies were often weak and, on the other hand, studies carried out by metabolism-oriented biologist's frequently slighted important physical facets. Nevertheless, laudable investigations, especially during the last few years, have been sufficient to warrant optimism about substantial progress in the near future. Therefore, well understanding of plant responses to the interactive effect of water and nutrients deficits, how these deficits may theoretically affect plant processes using PBRs are the needs of the future line of research.

The beneficial role of PBRs in enhancing the crop yields through the regulation of physiological processes and plant–water relations has recently been elaborated through several reports (Khan *et al*., 2015; Srivastava *et al*., 2016; Wakchaure *et al*., 2016a&b). Though the most of PBRs have been tried under pot or controlled conditions, those reported for their viability include salicylic acid (Fayez and Bazaid, 2014); sodium benzoate (Beltrano *et al*., 1999; Kumar *et al*.,2014); thiourea (Bhunia *et al*., 2015; Wakchaure *et al*., 2016) and potassium nitrate (Gimeno *et al*., 2014). Nevertheless, there is general lack of information on the relative responses of PBRs under field conditions (Wakchaure *et al*., 2016a&b). So, the other objective was to evaluate the effectiveness of selected PBRs on growth, yield and water productivity of under variable water deficits in semi-arid Deccan Plateau of India.

Salient research findings

1. Wheat (HD−2189) response to PBRs under varied water deficit

The interactive effect of irrigation regimes and PBRs on grain yield and water productivity of spring wheat (Triticum aeastivum L) were evaluated during three years (2012−15) using LSS (Fig.1). PBRs applied through exogenous sprays included: 10 mM thio-urea (TU), 10 uM salicylic acid (SA), 15 g L−1potassium nitrate (KNO3), 25 ppm gib-berellic acid (GA3), 8 ppm ortho-silicic acid (OSA) at crown root initiation (CRI), flag leaf and seed milking stages and control (no PBR). Seven irrigation levels were generated through a line source sprinkler system (LSS) viz., application of irrigation water (IW) equalling 1.0, 0.85, 0.70, 0.55, 0.40, 0.25 and 0.10 times the CPE (cumulative open pan evaporation). The maximum yield obtained with PBRs varied between 4.11–4.46 Mg ha−1 at IW: CPE 0.85 against 4.09 Mg ha−1without PBR. While the yield decline equalled 0.35–0.42 Mg ha−1for every 0.1 IW: CPE for PBRs against 0.43 Mg ha−1without PBR (. The overall improvement in grain yield and total biomass with PBRs ranged between 5.9–20.6% and 4.8–15.3%, respectively. Specifically TU and SA showed a major role under medium (IW:CPE 0.40–0.69) and severe (0.10–0.39)stress conditions in terms of maintenance of leaf water content, modulating the stomatal opening and better water usage and thereby improved yield by 0.41–0.88 Mg ha−1 The maximum water productivity ranged between 1.20–1.35 kg m−3 with different PBR's while it was 1.18 kg m−3 without PBR and the latter could be achieved with 19–56% lesser irrigation water with PBRs. Overall conclusions are that the effects of deficit irrigation could be substantially enhanced in terms of grain yield and water productivity when used conjunctively

with PBRs like TU and SA. Thus for integrating PBRs with supplemental irrigation, large scale testing is required for defining their economic spray schedules under water scarcity conditions (Wakchaure *et al*., 2016a).

Fig. 1. Experimental setup and evaluation of PBRs using line source system (LSS)

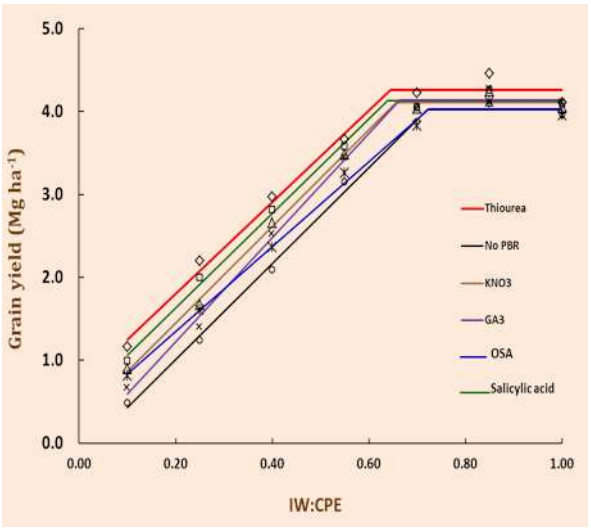

Fig. 2. Relative response of PBRs to grain yield of wheat

2. Effect of plant bio-regulators on growth, yield and water production functions of sorghum [Sorghum bicolor (L.) Moench]

Effect of plant bio-regulators (PBRs) and supplemental irrigation on growth and grain yield of sorghum [*Sorghum bicolor* (L.) Moench] was evaluated (Fig.3) during two years (2015−2016). Exogenous application of PBR's included: 10 µM salicylic acid (SA), 100 mg L−1 sodium benzoate (SB), 500 ppm thiourea (TU), 1.5% potassium nitrate (KNO3) at seedling elongation (20 DAS), reproductive (50 DAS) and panicle emergence (75 DAS) stages and control (no spray of PBR). The maximum grain yield (3.60–3.88 Mg ha−1) was obtained at IW: CPE 0.80 and declined @ 0.43–0.49 Mg ha−1 for every 0.1 IW: CPE for PBRs and the corresponding values were 3.49 and 0.53 Mg ha−1 without PBR. The application of PBR's mitigated water stress and improved gain yield, straw yield and water productivity by 6.8–18.5%, 5.7−14.7% and 1.16−1.41 kg m-3, respectively (Fig.4). SA was more effective under moderate (IW: CPE 0.79–0.50) while SB and TU were better under severe water deficits (IW: CPE 0.49–0.05). Thus SB and TU present viable option to reduce

water use by 25.2-49.7% under the conditions of deficit irrigation (Wakchaure *et al*., 2016)

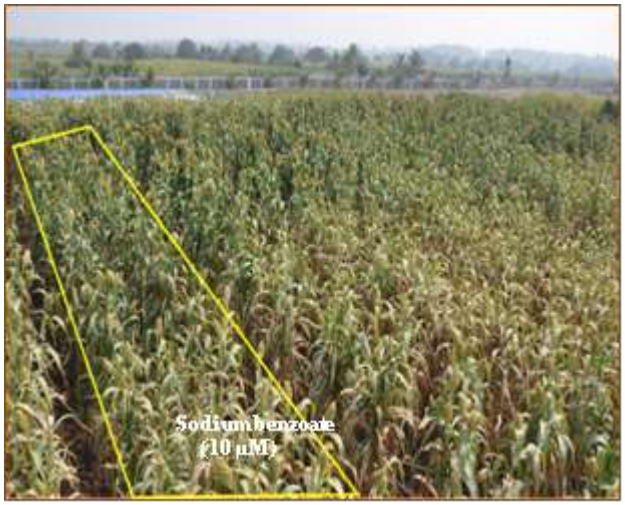

Fig. 3. Sorghum response to PBRs

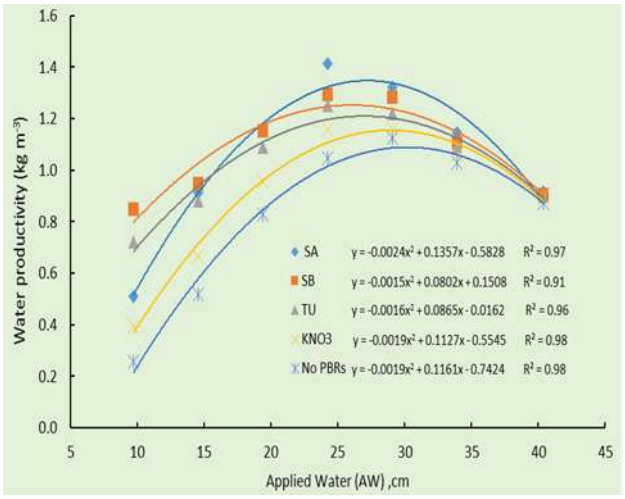

Fig. 4. Water productivity as expressed as the function of PBR's at various quantities of applied water (AW)

3. Water production functions and impact of plant bioregulators on growth, yield and quality of Onion (cv. Bhima Kiran)

Water production functions and impact of plant bioregulators (PBRs) on growth, bulb yield and quality of onion (Allium cepa L.) was evaluated under water stress conditions (Fig.5.). The foliar application of PBR's included: 15 g L–1 potassium nitrate (KNO3), 10 mM salicylic acid, 500 ppm thiourea, 150 mg L–1 sodium benzoate, 25 ppm gibberellic acid (GA3) applied at 40, 60, 80 and 100 days after transplanting (DAT) and control (no PBR). Line source sprinkler system (LSS) was used to maintain seven levels of irrigation water (IW) equaling to 1.00, 0.85, 0.70, 0.55, 0.40, 0.25 and 0.10 times of cumulative open pan evaporation (CPE). The maximum bulb yield (49.7–51.8 t ha–1) was obtained at IW: CPE 1.00 for PBRs and corresponding value 48.8 t ha–1 without PBR. Application of PBR's also helped to mitigate the water stress through maintenance of leaf water content, modulating the canopy temperature and better water usage thereby improve bulb yield and water productivity. The physicochemical and functional quality characteristics of the onion bulb improved significantly with exogenous application of PBRs in water deficits. In conclusion, conjunctive use of PBR's like KNO3 and TU with supplemental

irrigation could sustainably enhanced yield and quality of onion grown in shallow basaltic soils and water scarce environment of Western India (Wakchaure *et al*., 2016)

Fig. 5. Intensity of water stress at 110 DAT of onion

4. Exogenous application of PBRs for enhancing water productivity of eggplant (cv. Panchganaga)

Exogenous sprays of PBRs viz., 15 g L^{-1} potassium nitrate (KNO_3), 10 mM salicylic acid (SA), 500 ppm thiourea (TU) and 100 ml L^{-1} microbial biopolymer (BP) were applied at vegetative, flowering, fruit formation and development stages of eggplant. The interactive effect of PBRs, BP and supplemental irrigation on yield formation was evaluated using line source sprinkler system (LSS) at seven levels of irrigation water (IW) equalling to 0.90, 0.75, 0.60, 0.45, 0.30, 0.15 and 0.0 times of cumulative open pan evaporation (CPE). Application of PBRs and biopolymer significantly improved marketable yield and water productivity over control (Fig.6) . PBRs like salicylic acid (10 µM) at higher and KNO_3 (15 g L^{-1}) at lower irrigation levels present viable option to mitigate water stress and reduce water use by 50 per cent. Nutritional quality (TSS, protein contents and antioxidant enzymes activities) of brinjal enhanced significantly with PBR's underwater deficits. Identified plants PBR's like KNO_3, SA help to mitigate water stress and can help to boost the productions vis-a-vis profitability of onion under water scarcity conditions. Similarly use of microbial biopolymer can be better alternative for chemical PBRs for enhancing yield

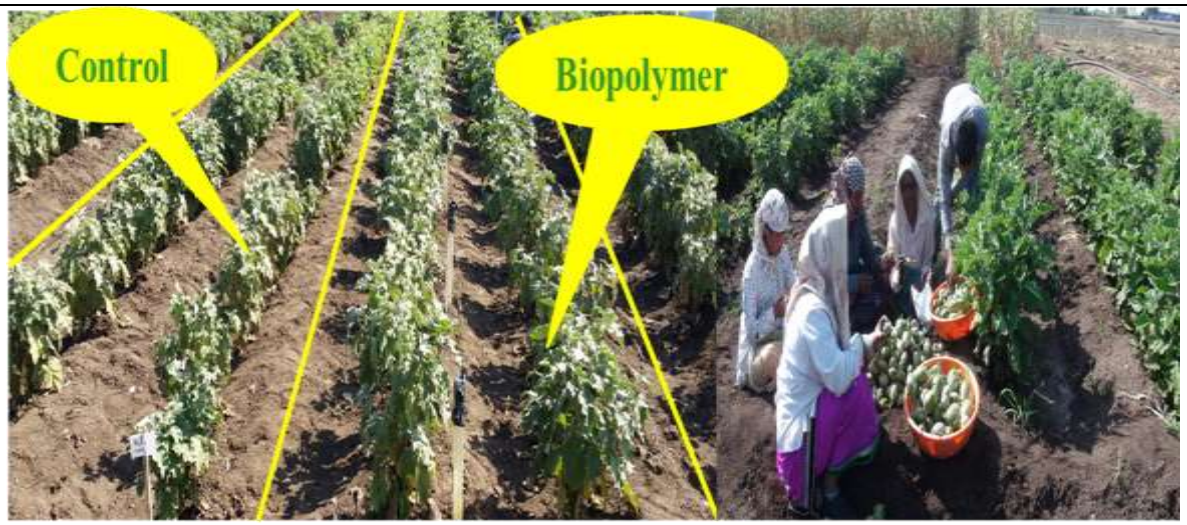

Fig. 6. Relative response of PBRs and biopolymer to eggplants

Conclusions

- Response of PBRs is highly specific environment conditions and varies with crop to crop
- PBR's like thiourea (10 mM), sodium benzoate (100 mg L^{-1}), potassium nitrate and salicylic acid (10 µM) helped to mitigate water stress for wheat, sorghum, onion and eggplants, respectively
- Nutritional quality (TSS, protein contents and antioxidant enzymes activities) of vegetable crop enhanced significantly with PBR's under water deficits
- Overall use of PBRs can help to boost the productions vis-a-vis profitability of crops under water scarcity conditions

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Chapter 19: 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 20: Plant phenotypic responses with the application of Plant Growth Regulators (PGR) in grapes

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Introduction

Plant growth regulators play an integral role in controlling the growth and development of plants. Some growth regulators fit the definition of a "classical" plant hormone i.e. an organic compound synthesised in one part of the plant and translocated to another part, where at low concentrations it elicits a physiological response (Salisbury and Ross, 1992). However, this definition is somewhat restrictive as ethylene has been shown to bring about a change in the same tissue and even in the same cell where it was produced (Chang and Stadler, 2001). Also, there are other compounds such as sucrose (Smeekens, 2000), and even inorganic compounds such as phosphates (Stitt, 1999) and nitrates (Sadka *et al*., 1994) that may fit the classical definition and can elicit physiological responses. There are five "classical" plant hormone categories; these are auxins, cytokinins, gibberellins, abscisic acid and ethylene. Other more recently recognised plant growth regulator compounds include brassinosteroids, salicylates, the jasmonates and polyamines. Plant hormones must meet three criteria to elicit a response. They need to be present in the correct location at the correct concentration, to be recognized and bound by a specific receptor molecule, and once bound to the receptor molecule, to trigger some metabolic change that results in amplification of the growth regulator signal.

Response of gibberellin for fruit development

Enlargement of berries in seedless fruit (Black Corinth and 'I'hompson Seedless) is one of the most significant responses of grapes to gibberellin. This might indicate that the native supply of gibberellin or related compounds in seedless fruit is low. The response of seeded berries to the regulator was much less than that of seedless. Perhaps seeds produce sufficient gibberellin or related compounds to result in almost maximum berry enlargement. Length of shoots and internodes was greatly increased by applications of gibberellin. The basal internodes of Zinfandel were elongated when shoots were sprayed at very young stages of development. At later

stages internodes farther toward the shoot tip were elongated, but basal ones were not. This would indicate that it is the young meristematic internode tissue that is most responsive to the regulator. Elongation of basal internodes would be very undesirable in both spur- and cane-pruned vines, as the vines would tend to grow rapidly out of shape. It is desirable to apply gibberellin before time of bloom in order to elongate cluster parts. Young clusters are very responsive to the regulator. Accompanying the elongation of cluster parts, however, is the development of shot berries, which are deleterious in table grapes but not necessarily a defect in wine grapes. However, proper concentrations and times of application may produce looser clusters with few or no shot berries.

The development of fruit is normally dependent on fertilization and in the absence of pollination the ovary will cease growth and senesce or abscise. However, there are several reasons why parthenocarpic growth – the development of fruit in the absence of fertilization – is desirable. In several fruit crops, particularly grapes and citrus, seedless varieties are more popular with consumers, while in aubergine (eggplant) the seeds impart a bitter taste and result in increased browning of the flesh. Difficulty in achieving pollination can also limit productivity: unfavorable environmental conditions can affect pollen production or the abundance of pollinating insects. While this can be circumvented by growing under glass and supplying the insect pollinators, this is an expensive solution. There is good evidence that in many species continued fruit growth depends upon hormonal signals, principally GA and auxin, originating from the developing seed. Parthenocarpy can thus often be achieved by application of auxin or GA to the unpollinated flower bud.

Role of abscisic acid (ABA) in grape berry ripening

Hormones control plant development by coordinating changes in the expression of numerous genes at crucial times in a tissue and organ-specific manner. They have been implicated in controlling various aspects of grape berry development, in particular, the important process of ripening and are used in some crops to control growth and ripening. Abscisic acid (ABA) is associated in grapevine with the response to water stress but may also have a role in berry ripening.

Research studies showed that ABA levels in Cabernet Sauvignon berries increase dramatically at veraison, consistent with it being involved either as a trigger for ripening or as a response to the increase in sugars that occurs at this time (Susan,

2016). Net ABA accumulation doesn't occur until veraison, the decrease in ABA concentration in the first phase of berry development being due to berry expansion. The decrease in ABA that occurs later in development is likely to be due to a combination of catabolism and sequestration into the bound form. The genes crucial to ABA synthesis, 9-cisepoxycarotenoid dioxygenase (NCED) and zeaxanthin epoxidase (ZEP) were expressed throughout berry development and no clear correlation was found between their levels and that of ABA. Results of laboratory studies (Susan, 2016) showed that isolated berries respond to the presence of sucrose through an increase in ABA biosynthesis pathway gene expression (NCED and ZEP).

Replicated field trials clearly showed that ABA treatments can be effective in significantly enhancing ripening when applied in at or near the end of the first period of berry expansion (Susan, 2016). Colour accumulation in the skins commenced earlier in ABA-treated fruit as did the increase in sugar levels. ABA treatment also advanced the timing of the second phase of berry expansion as it appeared to eliminate the lag phase of berry growth. Taken together these data demonstrate that ABA is likely to play some part in the control of berry ripening and can be used to advance the timing of ripening. Further investigation into the characteristics of ABA-treated Fruit will be needed to investigate the compositional character of treated fruit and to gauge its suitability for winemaking. An ability to control the timing of ripening may provide considerable benefits to the wine industry in terms of wine style/quality and for winery scheduling.

Impact of plant hormones including auxins, cytokinins, and gibberellins on physiological aspects of plants

A plant's sensory response to external stimuli relies on hormones, which are simply chemical messengers. Plant hormones affect all aspects of plant life, from flowering to fruit setting and maturation, and from phototropism to leaf fall. Potentially, every cell in a plant can produce plant hormones. The hormones can act in their cell of origin or be transported to other portions of the plant body, with many plant responses involving the synergistic or antagonistic interaction of two or more hormones. In contrast, animal hormones are produced in specific glands and transported to a distant site for action, acting alone. Plant hormones are a group of unrelated chemical substances that affect plant morphogenesis. Five major plant hormones are traditionally described: auxins, cytokinins, gibberellins, ethylene, and

abscisic acid. In addition, other nutrients and environmental conditions can be characterized as growth factors. The first three plant hormones largely affect plant growth, as described below;

Auxins

The term auxin is derived from the Greek word auxein, which means to grow. Auxins are the main hormones responsible for cell elongation in phototropism and gravitropism. They also control the differentiation of meristem into vascular tissue and promote leaf development and arrangement. While many synthetic auxins are used as herbicides, indole acetic acid (IAA) is the only naturally-occurring auxin that shows physiological activity. Apical dominance (the inhibition of lateral bud formation) is triggered by auxins produced in the apical meristem. Flowering, fruit setting and ripening, and inhibition of abscission (leaf falling) are other plant responses under the direct or indirect control of auxins. Auxins also act as a relay for the effects of the blue light and red/far-red responses. Commercial use of auxins is widespread in plant nurseries and for crop production. IAA is used as a rooting hormone to promote growth of adventitious roots on cuttings and detached leaves. Applying synthetic auxins to tomato plants in greenhouses promotes normal fruit development. Outdoor application of auxin promotes synchronization of fruit setting and dropping, which coordinates the harvesting season. Fruits such as seedless cucumbers can be induced to set fruit by treating unfertilized plant flowers with auxins.

Cytokinins

The effect of cytokinins was first reported when it was found that adding the liquid endosperm of coconuts to developing plant embryos in culture stimulated their growth. The stimulating growth factor was found to be cytokinin, a hormone that promotes cytokinesis (cell division). Almost 200 naturally-occurring or synthetic cytokinins are known, to date. Cytokinins are most abundant in growing tissues, such as roots, embryos, and fruits, where cell division is occurring. Cytokinins are known to delay senescence in leaf tissues, promote mitosis, and stimulate differentiation of the meristem in shoots and roots. Many effects on plant development are under the influence of cytokinins, either in conjunction with auxin or another hormone. For example, apical dominance seems to result from a balance

between auxins that inhibit lateral buds and cytokinins that promote bushier growth.

Gibberellins

Gibberellins (GAs) are a group of about 125 closely-related plant hormones that stimulate shoot elongation, seed germination, and fruit and flower maturation. GAs are synthesized in the root and stems apical meristems, young leaves, and seed embryos. In urban areas, GA antagonists are sometimes applied to trees under power lines to control growth and reduce the frequency of pruning. GAs break dormancy (a state of inhibited growth and development) in the seeds of plants that require exposure to cold or light to germinate. Abscisic acid is a strong antagonist of GA action. Other effects of GAs include gender expression, seedless fruit development, and the delay of senescence in leaves and fruit. Seedless grapes are obtained through standard breeding methods; they contain inconspicuous seeds that fail to develop. Because GAs are produced by the seeds and because fruit development and stem elongation are under GA control, these varieties of grapes would normally produce small fruit in compact clusters. Maturing grapes are routinely treated with GA to promote larger fruit size, as well as looser bunches (longer rachis), which reduces the incidence of mildew infection.

Accumulation of anthocyanins was enhanced by ABA treatment and suppressed by NAA and shading

Abscisic acid (ABA) used individually at a concentration of 5 to 25 mg/L slightly stimulated the accumulation of anthocyanins. The highest content of anthocyanins in the roots of the plants was obtained after treatment with ABA at 10 mg/L. The combined treatment with methyl jasmonate (20 mg/L) and ABA at 5 mg/L Resulted in increased content of anthocyanins in the roots, compared to the treatment with JA-Me alone. ABA at a concentration of 10 mg/L did not affect the accumulation of anthocyanins induced by JA-Me, but at 25 mg/L inhibited their accumulation in the root (Justyna *et al*., 2014). Jeong *et al*. (2014) shows that the juice of the ABA-treated grape 4 weeks after veraison showed a higher concentration of soluble solids and lower titratable acidity than that of the control, and the NAA-treated berries showed the opposite results. Shading of clusters did not significantly affect the concentration of soluble solids or the titratable acidity. There was no significant difference in the average weight and volume among the berries of four experimental conditions (data

not shown). The accumulation of anthocyanins in the berry skins started at veraison; it was enhanced by ABA treatment and suppressed by NAA and shading. The concentration of anthocyanins of ABA-treated berry skins decreased 6 weeks after veraison, which indicates that the ABA-treated berries were overripe at that period. Thus, ABA-treated berries ripened earlier than the control berries, but there was no significant difference in the maximum concentration of anthocyanins.

Role of plant hormones in plant defence responses

Plant hormones play important roles in regulating developmental processes and signaling networks involved in plant responses to a wide range of biotic and abiotic stresses. Significant progress has been made in identifying the key components and understanding the role of salicylic acid (SA), jasmonates (JA) and ethylene (ET) in plant responses to biotic stresses. Recent studies indicate that other hormones such as abscisic acid (ABA), auxin, gibberellic acid (GA), cytokinin (CK), brassinosteroids (BR) and peptide hormones are also implicated in plant defence signaling pathways but their role in plant defence is less well studied (Rajendra and Jonathan 2009).

Plant hormones regulate complex signaling networks involving developmental processes and plant responses to environmental stresses including biotic and abiotic stresses. Significant progress has been made in identifying the key components and understanding plant hormone signaling (especially SA, JA and ET) and plant defence responses. Several recent studies provide evidence for the involvement of other hormones such as ABA, auxin, GA, CK and BR in plant defence signaling pathways. Stoll *et al*. (2000) in grapevine found that the partial root drying had negative influence on the xylem cytokinin concentration concomitant with distinct increase in xylem-sap pH. In grape rootstocks, NaCl salinity caused reduction in root cytokinins content and the rootstocks with high cytokinin contents under salinity maintained high K-Na ratio and root-shoot dry mass ratio (Upreti and Murti 2010). They found significant alterations in root polyamines in grape rootstocks by salinity stress with tolerant rootstock showing greater increases in spermidine and spermine as well as ABA. The high increase in polyamine helped plants in maintaining high root-shoot biomass ratio and high K-Na ratio in the tolerant rootstock.

Treatment of plants with some hormones results in the reprogramming of the host metabolism, gene expression and modulation of plant defence responses against microbial challenge. Depending on the type of plant–pathogen interactions, different hormones play positive or negative roles against various biotrophic and

necrotrophic pathogens. However, the underlying molecular mechanisms are not well understood and several questions remain to be answered. For example, how is the intracellular level of phytohormones regulated in response to various pathogens?

Plant hormone signaling pathways are not isolated but rather interconnected with a complex regulatory network involving various defence signaling pathways and developmental processes. To understand how plants coordinate multiple hormonal components in response to various developmental and environmental cues is a major challenge for the future. It is important to note that the type of interactions and plant responses to stresses vary depending on the pathosystem as well as the time, quantity and the tissue where hormones are produced. Another important question to answer is how different hormone-mediated developmental and defence-related responses are regulated in specific tissues and cell types? Most of the studies in understanding phytohormone signaling have been done using seedlings and there is limited study on mature leaves. More studies using mature leaves are necessary to understand the role of hormone signaling components involved in plant defence against various pathogens (Rajendra and Jonathan 2009).

In addition to the production of hormones by plants, several plant pathogens also produce phytohormones or their functional mimics to manipulate defence-related regulatory network of plants. Emerging evidence suggests that plant pathogens manipulate components of hormone biosynthesis and signaling machinery leading to hormone imbalances and alterations in plant defence responses. This is one of the strategies used by some pathogens to confer virulence and cause disease. However, we have very limited knowledge on how pathogen effectors confer virulence by modulating hormone signaling components. Recent global expression profiling studies in response to pathogen challenges are providing useful information about different components involved in the complex interactions between hormone-regulated defence signaling pathways. However, additional studies involving mature leaves and detailed time course experiment will be necessary to extend our understanding of the complex regulatory mechanisms operating between plant hormone signaling and plant defence responses. A better understanding of phytohormone-mediated plant defence responses is important in designing effective strategies for engineering crops for disease and pest resistance (Rajendra and Jonathan 2009).

Effect of auxins and indolebutyric acid (IBA) on grape root induction

Research showed that different auxin and planting bed treatments had a significant influence on grape rooting (Galavi *et al*., 2013). The maximum number of roots, root length, and root fresh and dry weight was obtained by applying 4000 mg/l IBA. The significant effect of planting bed treatments was found in studying traits, so that maximum number of roots, root length, and root fresh and dry weight was obtained in mixture of agricultural soils and sand planting beds. Studied traits significantly affected by an interaction effect of IBA and cuttings beds, so that maximum number of roots, root length, and root dry weight was obtained by using 2000 mg/l IBA + sandy planting bed, and maximum root fresh weight was obtained by using 4000 mg/l IBA + sandy planting bed (Galavi *et al*., 2013).

Conclusions

In conclusion, plant growth regulators (PGR) controls development by coordinating changes in the expression of numerous genes at crucial times in a tissue and organ-specific manner. In grapes, the largest berries on 'Thompson Seedless' were obtained with many repeated applications of GA confirming the efficacy as berry growth promoters in grapes. Pedicel thickness increased by treatment of GA3 and increased shatter incidence in 'Thompson Seedless', 'Redglobe' and 'Ruby Seedless'. Although auxin shows controls cell division during fruit set, it is thought to play an important role during the growth phase by influencing cell enlargement together with gibberellins. ABA is a plant growth regulator that has very important effects on a plants response to environmental stresses such as osmotic stress and temperature extremes. It also has a critical role in seeds involving their acquisition of desiccation tolerance and in preventing precocious germination. In grapes, three plant hormones have now been implicated in the control of berry ripening i.e. ethylene, the brassinosteroid castasterone and ABA. Endogenous plant growth regulators play an important role in regulating plant responses to abiotic stress by sensitizing growth and developmental processes. While the physiological and molecular mechanisms linked to the role of ABA and cytokinins in stress tolerance are well explained, there is growing interest to elucidate the associations of auxins, ethylene, gibberellins, brassinosteroids, and polyamines in stress tolerance mechanism and also on possible cross talk mechanism among different growth regulators during stress tolerance acquisition. Several studies have proved the hypothesis that use of auxin could be a useful on rooting grape cuttings in the greenhouse condition. In the current research

root fresh and dry weight, number of roots and root length significantly affected by growth regulators and types of planting bed. Agricultural soils as planting bed had a positive effect on rooting grape cuttings.

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Production, extraction and UHPLC based characterization of microbially derived plant growth hormones

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Abstract

Agriculturally important microorganisms are common members of rhizosphere, phyllosphere and endophytic community. It has been proved that microbes are being played an important role in development of adaptation to plants to extreme environmental conditions. This in fact, can be credited to unique traits expressed by the microbes includes production of plant growth hormones (PGRs), siderophores, and several other volatile organic compounds. Therefore, agriculturally important microbes have gained a rapid attention of scientific managers for their exploitation towards sustainable development of agriculture under changing agro-climatic conditions. Therefore, characterization of microbes for their plant growth promotional traits has become an important prerequisite to exploit them commercially for growth, establishment and development of crop plants in extreme conditions. To confirm the growth promotional traits of an organisms a sets of protocols and procedure are need to be followed. Keeping in view to this important aspect we proposed this chapter to describe the methods used for production, extraction of bacterially derived plant growth hormones and their characterization using UHPLC. This chapter also covered the major aspects to be considered while developing new operational methods for unknown compounds present in the samples.

Keywords: Plant growth hormones; UHPLC; extraction; XAD 16; indol acetic acid

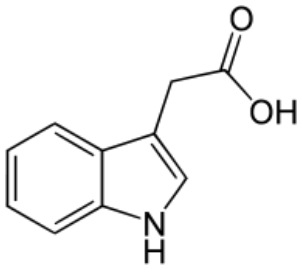
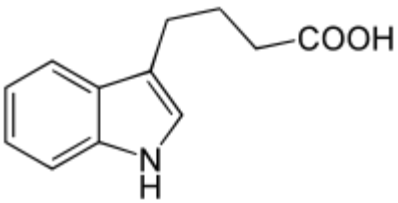
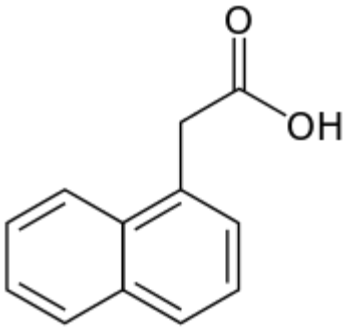
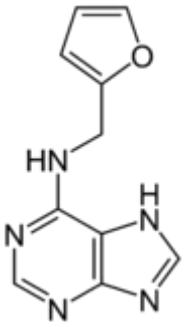
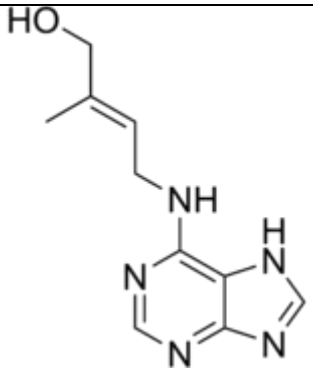
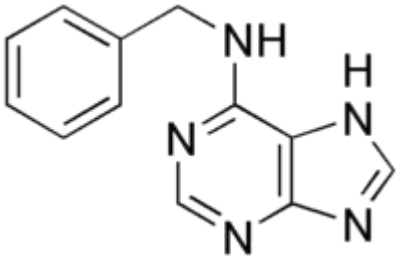
Introduction

Microbial biomolecules in plant growth promotion

Microbes including bacteria, fungi and actinomycetes known or well characterized for ability to promote plant growth collectively termed as plant growth promotion (PGP) and the microbes having this property are known as plant growth promoting microbes (PGPMs). Several mechanisms of microbial plant growth promotion have been put in elucidation, e.g. microbial production of plant growth hormones (PGHs), siderophores, volatile organic compounds (VOCs), fixation of atmospheric nitrogen, solubilization of phosphate, and mineral cycling, etc. To ensure the effective in-situ applicability of the microbial inoculants in agriculture a qualitative and quantitative estimation of leading class plant growth promoting biomolecules always remained central in light of implementing candidate microbes. An array of multifaceted, cutting edge technologies are in service in this era. The ultra-high pressure liquid chromatography (UHPLC) is one of such equipment, used to resolve the mixtures of biomolecules with high sensitivity. Therefore, this chapter is being proposed to describe various aspects pertaining to production, extraction, and estimation of bacterially derived PGHs under saline conditions.

Bacterial production of PGHs

Bacteria represent the major fraction of microbial population associated with soil and plants. The members of rhizosphere and phyllosphere community including pink pigmented facultative methylotrophic (PPFM) bacteria like *Methylobacterium*, species of *Pseudomonas*, *Rhizobium*, *Azotobacter*, *Pantoea*, *Enterobacter*, etc. are known to produce PGHs in varying quantities. The major PGHs producedinclude both auxins, cytokinins gibberelins (GAs), indole acetic acid (IAA), indole butyric acid (IBA), Zeatin, abscisic acid (ABA), BAP, Kinetin.

Auxins		
 **IAA**	 **IBA**	 **NAA**
Cytokinins		
 **Kinetin**	 **Zeatin**	 **BAP**

Detection and estimation of bacterial derivatives of PGHs: Traditional recipes for detection of microbial derivatives of PGHs appear principally based on the colorimetric reactions of the functional moieties harbored by PGHs. These methods make use of thin layer chromatography, colorimeter, etc. and developing reagents, e.g. salkovsky's reagent is extensively used for quantitative determination of IAA. However, the resolution of closely related structures remain curtained with this methods.

UHPLC mediated detection of PGHs: Liquid chromatic separation, and estimation of biomolecules have boosted the research in biomolecules several folds. Estimation of PGHs using HPLC/UHPLC have gained rapid attention owing to the high degree of accuracy and sensitivity.

a. Production and extraction of microbial derivatives of PGHs

Bacteria secrete the PGHs in surrounding environment during a particular growth stages. It is thus critical to pool the desired biomolecules from complex mixture of the bacterial growth medium. Further, the PGHs to be microbial metabolites, their production is extremely sensitive to the physicochemical environment maintained during growth conditions. Therefore, probability of reproducibility can be strengthened by creating favorable production conditions through controlled growth environment. The PGHs can be pooled using specified chemical environment and organic solvents like ethyl acetate, n-hexane, resins like XAD 7, XAD 2, XAD 16, etc.

b. Pre-analysis processing of samples

The solvent content from the pooled samples should either be evaporated or appropriate blank must be used otherwise during the analysis. It is always recommended to use mobile phase composition as diluent. The samples are then diluted to avoid the excessive loading. Similarly known concentration of standard PGHs also dissolved in appropriate diluent. All the samples and standards need to be filtered using 0.2µ filter. (The selection of filter again depends on constituents of sample. A filter should not retain any of the constituents of the sample, as it can potentially hinder with the results). Heat labile samples should be maintained on ice bath throughout.

c. UHPLC analysis

Selection of appropriate column is needful to ensure proper results. Variety of columns are available with different specifications (*see: point 'a' in section 'e'*). Use of optimum mobile phase, flow rate and the column temperature are also crucial factors for better resolution of constituents from the samples (*see: point 'b & c' in section 'e'*). Properties of the molecules from the sample are central for the selection of detector (*see: point 'e' in section 'e'*). The samples can either be injected manually or with the help of an auto-sampler. The injection volume typically range from 0.1-50 µL for analytical UHPLC. The samples injections are done in at least 5 replicates per sample.

d. Data analysis

HPLC generates huge data following the analysis. Subtraction of blank from the mean results help in reduction of probable noise arising due to mobile phase.

Peak area and height are considered for quantitative determination. The concentration of desired constituents are then calculated using standards.

e. Key notes

a. The column

Variety of column are available for HPLC analysis of different samples. Most crucial aspect need to be considered prior to analysis is knowledge of the sample; e.g. composition of the sample, the constituents to be resolved from the mixture of analytes, their chemical nature, structure, predicted number of constituents, expected concentration, etc. Additionally, goals of separation should also be clearly defined, e.g. whether maximum or partial resolution is needed, level of sensitivity, economy of the method, slow/fast analysis, etc. Prediction of such characters is important as it directly relates to composition of mobile phase and selection of column as well. Clearly defined goals facilitate the selection of most appropriate column that can achieve the job; e.g. shape and size of the particles in column, internal diameter, length and pore size of the column, desired surface area, carbon load, type of bonding –(monomeric/polymeric), etc can be determined more keenly. This approach can significantly reduce the time and efforts during optimization of method for a particular sample.

Most widely used column for HPLC analysis of biomolecules is **C18**. It has compatibility with diverse range of biological molecules. However, the compounds like sugar moieties can be better analyzed using amino columns. Therefore it is highly recommended to gain needful knowledge regarding the sample prior to proceeding for HPLC analysis. Selection of column with wide range of compatibility can suit better with complex mixtures like microbial secondary metabolites, etc.

Length of column also determines the retention time of analytes. It is thus needful to select the column of appropriate length, e.g. 50, 100, 150, 250 mm, etc. More complex analyte mixtures generally better analyzed using longer column length, e.g. 250 mm. Similarly, particle size of the column also is equally important; it typically ranges between 03-20 µm. Pore size, carbon load, bonding type, etc. can also be chosen similarly.

Columns have a typical pH range for optimal performance. The lower and upper pH limits of the column being used must always be considered while using mobile phases with specific pH; e.g. mobile phases containing different concentrations of formic acid, orthophospheric acid, trichloroacetic acid, etc. Exceeding the limits of pH tolerance of a column can directly affect the efficiency and life of column.

b. Mobile phase

Mobile phase constitutes an important part of successful analysis. In general, mobile phase may be a single solvent like water, methanol, hexane, etc. or it can be a defined mixture of water / buffer and miscible organic solvent like methanol, acetonitrile (ACN), etc. Composition of mobile phase e.g. ratio of organic: aqueous phase, pH, exert significant influence on retention of analytes. Thus, behavior of constituent molecules from the sample in mobile phase is another important aspect that should be addressed carefully. More specifically, the polarity of a molecule and mobile phase cumulatively determine its retention in the column. It is therefore clear that an unknown sample may demand trial and error attempts with different columns as well as mobile phases.

c. Flow rate

Retention of molecules is also significantly affected by flow rate of the samples. Flow rate is directly responsible for rise in pressure. Particle shape of the packing material in the column also has equivalent function in developing the pressure, e.g. spherical shape of the particles offer reduced back pressure when compared with that of the particles having irregular shape.

d. Column temperature

Temperature has great influence on kinetics of mobile phase, as well as behavior of the analytes. Trial and error are thus needed to discover the best separation temperature. Temperature limits again depend on column type and mobile phase composition. Overall, maintaining well defined temperature environment throughout the analysis yields highly reproducible results.

e. Detection and Detectors

Selection of right detector leads to detect the analytes with high sensitivity. Most commonly used detectors include photo diode array (PDA), refractive index detector, fluorescence detector, evaporative light scattering detector, etc.

1. **PDA Detector:** It is relatively more versatile and popular one, particularly due to ease of monitoring the response of analytes in both UV as well as visible range. It is more convenient with unknown analytes where it is easy to monitor the response at absorption maxima, and spectral properties of the analytes.

2. **Fluorescence detector:** The response of the compounds with known composition, on the other hand can be monitored using other detectors as well. For instance, IAA can also be monitored using fluorescence detector (fluorimetric detection) at excitation wavelength of 280 nm, and emission wavelength of 350 nm (λ_{ex} 280/ λ_{em} 350). This offers additional sensitivity and high degree of specificity. Similarly response of other analytes can also be monitored using literature-based data regarding their characteristic properties.