D 7.2 Production of materials for improved phenotyping training

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ABSTRACT (FOR DISSEMINATION)	The document provides information on the potential and available techniques for Advanced Phenotyping. It also highlights the range of sensors that can be used to assess phenotypic traits in both wheat and potato. It is divided into 5 chapters and provides general background knowledge and information for researchers and plant breeders participating in the Advanced Phenotyping training events under ECOBREED. The training events will highlight

	the potential for improved phenotyping capabilities in terms of digital, thermal, and hyperspectral imaging, when where and how they can be used effectively.
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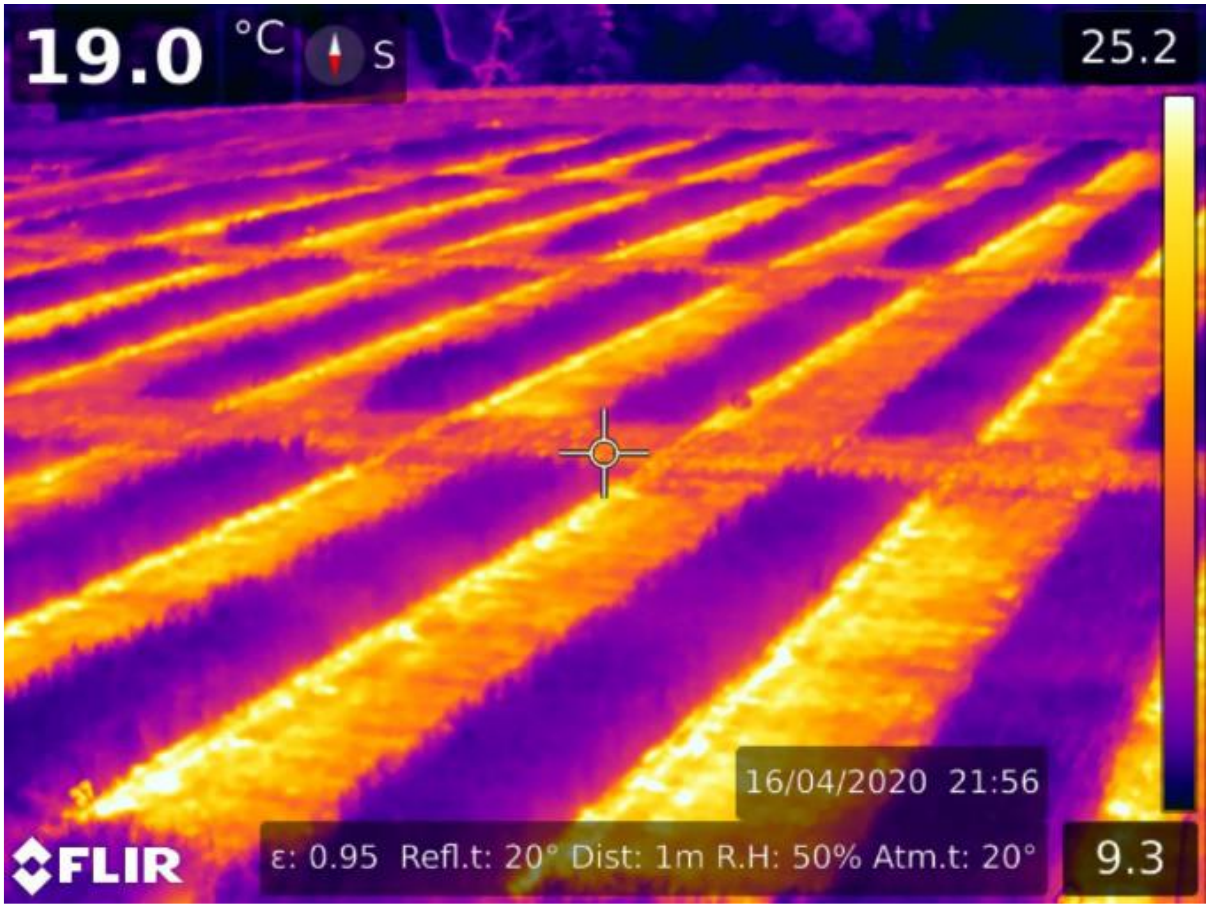
ECOBREED Advanced Phenotyping Training Course



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SUMMARY

ECOBREED's activities aim at increasing the competitiveness of organic farming. The main and innovative elements are the development of improved genotyping, evaluation of advanced phenotyping and farmer-participatory breeding, identification, selection and breeding of target traits suitable for an organic production environment to increase the availability of organic seed and varieties in Europe.

Establishing a link between genotype and phenotype is of great importance for the identification of breeding traits. In the last decade, many efficient, automatic, and accurate technologies and platforms have been developed that can capture phenotypic data. Combined with a range of other technologies, from sensors to information technology, data science and bioinformatics, morphological and physiological phenotypic traits of interest can be assessed non-destructively and repeatedly in whole populations. Therefore, **researchers and plant breeders** need to be trained in the **use and application of Advanced Phenotyping** capabilities related to **digital, thermal, and hyperspectral imaging** and know **when, where, and how** to use them effectively.

Advanced Phenotyping will be addressed in ECOBREED project though (i) the establishment of an integrated field phenotyping platform able to screen wheat and potato varieties/populations for canopy traits and resistance to biotic and abiotic stresses and (ii) the provision of training courses to stakeholders (young researchers, breeders, and farmers) from experienced researchers. With the current training materials, we aim to provide a general introduction to the high throughput tools available and technological advancements that are taking place to support the training events taking place in Slovenia, Austria, Czech Republic and the UK.

The training material is divided into five chapters to understand the application of advanced phenotyping in the breeding process and to explain the potential of the available techniques. It also highlights the range of sensors that can be used to assess phenotypic traits in crops. In the first chapter, the possibilities of using advanced phenotyping in crop studies are analysed. As mentioned earlier, plant phenotyping is the comprehensive evaluation of a wide range of plant traits such as morphology, phenology, biotic stress, quality traits and others. The phenotyping system enables accurate and reproducible data sets due to non-destructive data acquisition and minimises labour and cost. We can perform this phenotyping in controlled environment platforms and in the field. The most developed phenotyping platforms and the advantagesand disadvantages of the

different systems are listed. In the second chapter, the nondestructive analysis of growth and physiology by automated imaging is described. An important part is the discussion of imaging systems used in controlled environments, with emphasis on field-based systems. The third chapter describes nondestructive analyses of plant growth. As mentioned earlier, automated systems are ideal for various forms of phenotyping. Modern handheld devices are advantageous and provide us with a short answer to physiological questions. Finally, in the fourth chapter, thermal sensors for the analysis of absorption, reflection, and fluorescence (related to stress responses) are presented. Thermal sensors can be used to detect abiotic and biotic plant stress and to screen indirect yield and quality traits.

During the training events resident researchers as well as invited speakers will introduce different types of sensors that can be used for phenotyping and give presentations on phenotyping, data collection, and information management to stimulate broad discussion. Panel discussions will engage participants in an open dialogue to frame anticipated research activities and related challenges in data collection, data management, and access to equipment and facilities for plant science researchers.

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INTRODUCTION

In the context of climate change, food security and increasing restrictions on the use of agrochemicals there is a need to improve resource use efficiency and address crop nutrition and protection challenges through a combination of soil management, plant breeding and agronomic innovations. On the other hand, given the complete ban on the use of chemosynthetic pesticides and fertilisers, organic farmers rely mainly on agronomic practises and resistant/resilient varieties. Integrating agronomy and plant physiology with functional genomics could reveal the regulatory control of gene expression and support the development of functional molecular markers for plant breeding.

Thanks to rapid developments in plant molecular biology and molecular breeding techniques, the genomes of an increasing number of plant species have been sequenced, identifying thousands of genes that influence important agronomic traits. For crop species with extensive genotyping knowledge (e.g., wheat, potato, and soybean), **Marker Assisted Selection (MAS)** enables rapid identification of traits of interest in organic agriculture that can be targeted in the production of elite varieties. There is considerable potential for increased use of MAS, particularly for rapid introduction of disease resistance genes and other traits such as nodulation in soybean, grain quality in wheat, resistance to variegation in wheat, cadmium accumulation and tolerance to *Sclerotinia sclerotiorum* in soybean, and late blight in potato, all of which are important targets of the ECOBREED project. In order to map phenotypic traits to their underlying genetic variation, the integration of phenomic and genomic datasets using bioinformatics is required. Conventional crop phenotyping methods are labour intensive, time consuming, subjective, and often destructive to plants. Therefore, current genome sequence information has been underutilised to understand the complex traits of traits with multiple genes. This is due to the **lack of phenotypic data and phenotypic analysis, which has become an important limiting factor in plant breeding**.

Over the past decade, many efficient, automated, and accurate technologies and platforms have been developed that can capture phenotypic data. Combined with a range of other technologies, from sensors to information technology, data science and bioinformatics, morphological, physiological, and phenotypic traits of interest can be assessed non-destructively and repeatedly across entire populations. There is therefore an urgent need to train researchers and plant breeders on when, where, and how to use these platforms effectively.

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Advanced phenotyping will be used in the ECOBREED project by: **(i) establishing an integrated field phenotyping platform capable of screening wheat and potato varieties, breeding lines and populations for canopy traits and resistance to biotic and abiotic stresses; and (ii) conducting training courses for researchers, breeders, farmers, etc.** ECOBREED partners UNEW and KIS have developed high-throughput phenotyping methods using state-of-the-art image sensing technology in the form of Infra-red thermography (IRT). They were the first to use this technique under replicated field conditions to measure plant stress and for early detection of plant diseases. They have also collaborated on the development of an integrated system combining thermal and hyperspectral imaging techniques.

With the current training materials, we aim to provide a general introduction to the high-throughput tools available and the technological advances of the last decade in all Advanced Phenotyping training sessions that will support the workshops in the Czech Republic, Austria, Slovenia, and the UK. During the workshops, KIS will provide training on RGB, spectral imaging and data sets, while UNEW will provide training on RGB, thermal/spectral imaging and data analysis. The workshops are divided into three parts. The first part will provide a general introduction to the background of phenotyping and the tools and platforms available. The second part will include live demonstrations of these platforms in the field and in the greenhouse. Participants will also have the opportunity to collect data in real time. The third part will focus on the analysis of datasets and their use using real-time scenarios. As mentioned above, the aim of the training program is to inform, educate and train **different stakeholders** (e.g., **young researchers, breeders, and farmers**) on the use of improved phenotyping and to contribute to the transfer of the developed technologies into commercial practice. The level of knowledge of the participants and their expected learning outcomes will be assessed in advance in order to conduct the workshops at an appropriate level.

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1. Application of high-throughput phenotyping in crop studies

In recent years, many approaches have been discussed and incorpo rated into the ever-improving highly automated, nondestructive phenotyping of plants. Plant phenotyping involves the comprehensive evaluation of a wide range of plant traits such as architecture, phenology, pest/disease resistance, yield, and quality traits. The detailed and specific phenotyping strategies are required for genome-wide association studies and enable high-resolution linkage mapping studies and the application of genomic selection models for plant advancement.

Improving the correctness and throughput of phenotypic evaluation at all biological levels - phenomics, metabolomics, and genetics - are the main goals of modern phenotyping. The phenotyping system enables accurate and replicable data sets due to non-destructive data acquisition and minimises labour and costs due to its:

- automation,
- improved data integration
- remote sensing capabilities

Advanced Phenotyping platforms produce significantly more data than conventional phenotyping approaches and they need special systems for data management, access and storage. New statistical tools are needed to extend experimental design and make greater use of data integration and the derivation of biologically significant signals from experimental and environmental noise. In the field of phenotyping, there are two major challenges:

- Analysis of a large quantity of genetic lines and
- Replication of measurements of dynamic traits (i.e. traits whose phenotype changes during the growth period)

In most cases, the following detection systems are used in the field and in laboratory platforms:

- (semi)-automatic evaluation of morphometric parameters using RGB image analysis,
- chlorophyll a fluorescence kinetic imaging,
- hyperspectral or multispectral analysis of the light spectral reflectance thermal (IR) imaging,
- environmental monitoring systems and
- soil status monitoring systems.

1.1 Controlled environment platforms

In many indoor facilities, systems consist of regulated irrigation and nutrient regimes controlled by automated weighing systems and environmental controls in imaging.

Control and programming of the platform systems, as well as data analysis, are performed with sophisticated and user-friendly software packages. These newly developed phenotyping systems have tools to estimate many photosynthetic parameters, e.g., RGB systems can estimate plant morphology, IR thermal cameras can assess stomatal conductance, and hyperspectral imaging systems can assess metabolomics of experimental plants at different growth stages.

The first major automated phenotyping platforms for a controlled environment were built in Australia (Australian Plant Phenotyping Facility in Adelaide and the CSIRO facility in Canberra). However, in the last decade, the development of phenotyping platforms has been concentrated mostly in Europe, including two main commercial developers of phenotyping systems - Lemnatec (Germany) and Photon Systems Instruments - PSI (Czech Republic).

1.2 Field based phenotyping platforms

Phenotyping in the field is a critical component for understanding the evolution and improvement of plant traits. It is the understanding of the interaction between genetic and environmental factors that helps us understand critical plant production and stress-related traits. High-throughput field phenotyping methods help us to increase selection efficiency for breeding traits by accounting for variability in management and environmental factors. There are several approaches to field phenotyping, ranging from point sensors that can be hand-held or in the form of fixed systems in the field, vehicle-mounted and powered platforms, or aircraft-based systems that can be either unmanned or manned, to the use of satellite-based systems for continuous monitoring. The most developed phenotyping platforms (up to 2018) are listed in Table 1.

Table 1. Automated and semi-automated high-throughput plant phenotyping platforms

Location / Producer	Platform	Features	URL
PSI	PlantScreen^{TM}	Conveyor phenotyping system in controlled environmental conditions with analysis of chlorophyll fluorescence, kinetic and thermal imaging, morphometric and RGB analysis, and hyperspectral and NIR imaging, uses an automated weighing and watering system	http://www.psi.cz
LemmaTec	Scanalyzer^{3D}	Comprehensive non-destructive 2D-3D assessment of plant physiological traits in controlled environmental conditions	http://www.lemnatec.com
INRA	Phenopsis	Specific platform for phenotyping *Arabidopsis* plant growth under controlled environmental conditions.	http://bioweb.supagro.inra.fr
INRA	Phenoscope	Automated phenotyping device to handle and monitor hundreds of individual's pots.	http://www.phenoscope.versailles.inra.fr
INRA	Phenodyn	Temporal of hundreds of monocot crop species.	http://www.phenome-fppn.fr
INRA	Phenoarch	Automated platform based on a LemmaTec system to analyze the genetic determinants of plant responses to environmental conditions.	http://www.phenome-fppn.fr
Phenospex	FieldScan	Phenotyping under field- or semi-field conditions that is designed to screen large populations.	http://www.phenospex.com
WPS	WSP	Fully automated digital phenotyping system using high-throughput RGB sensors.	http://www.wps.eu
Keygene	PhenoFab^{R}	Greenhouse service operation that combines phenotyping technology with trait interpretation to exploit phenotypic variation.	
Jülich Plant Phenotyping Centre	Growscreen	Non-invasive method designed to quantity shoot morphometrical and	http://www.fz-juelich.d

Table 1. Automated and semi-automated high-throughput plant phenotyping platforms

Location / Producer	Platform	Features	URL
		functional parameters and root architecture.	
Wageningen UR	PhenoBot	Autonomous mobile robot with camera promises to output direct registered depth and color image for morphometric analysis.	http://www.wageningenur.nl
Wiwam	Wiwam Conveyor	Integrated robotic system for phenotyping of larger plants with automated irrigation and measurement of a variety of plant growth parameters at regular time intervals.	http://www.wiwam.be
Australian Plant Phenomics Facility	PlantScan	Provides non-destructive analyses of plant morphology, structure and function by using high-resolution cameras with cutting-edge information technology.	http://www.plantphenomics.org

Ground-based field sensing platforms allow plot-level data capture both at organ and canopy level which along with **global positioning systems** (GPS) enable navigation and spatial analysis. There are some fixed systems available in the form of field scanners (for example systems at Rothamsted Research, Zurich field phenotyping platform, Arizona field scanner system etc). Similarly, mounted platform systems or technology (which can be in form of tractor mounted or adapted with another mobile system) is not a suitable fit for all systems and must be adapted depending upon the crop and planting specifications along with accounting for inconsistencies and plant characteristics variability. Table 2 provides some advantages and disadvantages of different systems (adapted from Deery et al., 2014).

Table 2 Advantages and disadvantages of different platforms (adapted from Deery et al., 2014)

Platform Type	Disadvantages	Advantages
Fixed systems	Generally expensive; can only monitor a very limited number of plots	Unmanned continuous operation; after-hours operation (e.g., night-time); good repeatability
Permanent platforms based on cranes, scaffolds or cable-guided cameras	Limited area of crop, so very small plots; expensive	Give precise, high resolution images from a fixed angle
Towers/cherry-pickers	Generally varying view angle; problems with distance (for thermal), bi-directional reflectance distribution function (BRDF), plot delineation, *etc.*; difficult to move, so limited areas covered	Good for the simultaneous view of the area; can be moved to view different areas
Mobile in-field systems	Generally take a long time to cover a field, so subject to varying environmental conditions	Very flexible deployment; good capacity for GPS/GIS tagging; very good spatial resolution
Tractor-boom	Long boom may not be stable	Easy operation; constant view angle; wide swath (if enough sensors are mounted as on a spraying bar); mounting readily available (needs modification)
Manned buggies	Requires a dedicated vehicle (expensive)	Flexibility with the design of the vehicle (e.g., tall crops, row spacing); Constant view angle; very adaptable
Autonomous robots	Expensive; no commercial solutions available; safety mechanisms required	Unmanned continuous operation; after-hours operation (e.g., night-time)
Airborne	Limitations on the weight of the payload depending on the platform; a lack of turnkey systems; spatial resolution depends on speed and altitude	Can cover the whole experiment in a very short time, getting a snapshot of all of the plots without changes in the environmental conditions

Table 2 Advantages and disadvantages of different platforms (adapted from Deery et al., 2014)

Platform Type	Disadvantages	Advantages
UAVs	Limited payload (weight and size); limited altitude (regulations) and total flight time (hence, total covered area); less wind-affected than blimps; regulatory issues depending on the country	Relatively low cost compared with manned aerial platforms; GPS navigation for accurate positioning
Manned aircraft	Cost of operation can be expensive and may prohibit repeated flights, thereby reducing temporal resolution; problems of availability	Flexibility with the payload (size and weight); Can cover large areas rapidly

2 Non-destructive analyses of growth and physiology by automated imaging

The system for automated non-invasive analysis of above-ground plant performance may be based on different technical solutions or measured signals. In the following sections, the most important/developed sensors that have been used for Advanced Phenotyping will be introduced. The systems highlighted below are systems which can be adapted and used for different imaging platforms depending upon the spectral and spatial resolution of the sensors. The major discussion below is mainly done keeping in mind the basis for controlled environment imaging systems with highlights about changes for field-based systems.

2.1 Visible RGB imaging of plant above-ground biomass

In most cases, the main observed trait in plant biology is above-ground growth and biomass production. In addition to numerous secondary traits describing the morphology of shoots, the primary and universal trait is the size (volume, fresh mass, dry mass) of biomass and the rate of biomass formation. The standard way to assess biomass production is via destructive sampling, by a simple weighing of the fresh (FW) and dry (DW) mass. However, this can be done only at the end-point analyses. Similarly, leaf area and consequently the plant growth rate are usually determined by manual measurements of the dimensions of plant leaves, or by passing harvested leaves through a leaf area machine. Such measurements are highly time consuming and thus cannot be used for large scale experiments involving a large number of genotypes. Therefore, controlled system plant phenotyping facilities prefer to evaluate the growth rate using imaging methods which employ digital cameras with subsequent software image analysis.

This enables a faster and more precise determination of the leaf area and other parameters called the projected area. In general, non-invasive techniques of shoot growth determination have proven very reliable and high correlations between the digital area and the shoot fresh, or dry weights, respectively, have been reported in Arabidopsis, tobacco and different crop species. Similarly, other common growth parameters such as stem length, number of tillers and inflorescence architecture can be assessed non-destructively and manually, but again the time requirements, limit the number of plants analysed. High-throughput approaches for analyses of these rather species-specific traits would

be very valuable however with the exception of Arabidopsis the range of accessible solutions has been rather limited.

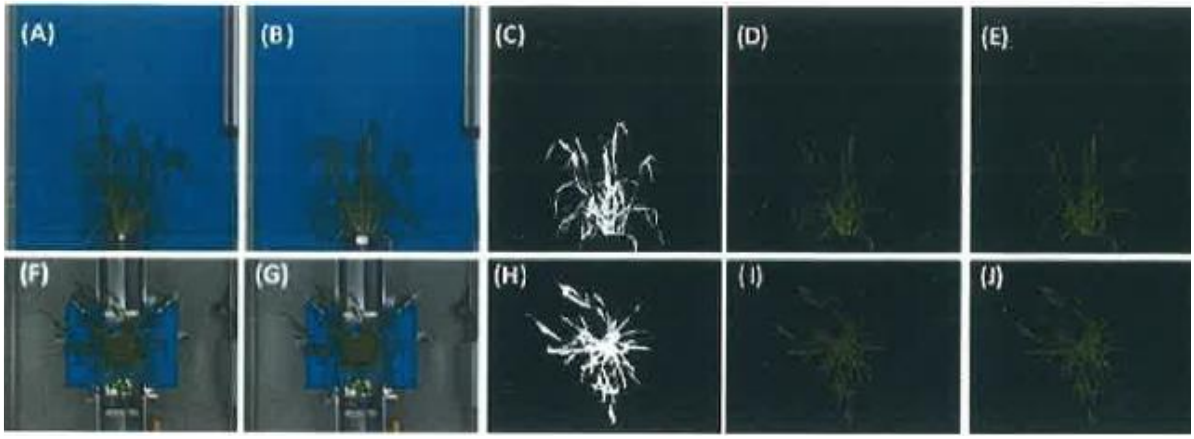

Fig. 1. Top and side view of a wheat plant. The original figure from two different angles from the side (A, B) and top (F, G) was segmented and the background, including pot and substrate was automatically identified during image processing by the system (C, H), then removed, so the final image for the numerical analysis conists only from the plant parts on the black background (D, E, I, J). Source: PlantScreen® phenotyping system (PSI, Czech Republic) and Slovak PlantScreen (SUA Nitra, Slovakia).

Current phenotyping platforms perform the procedures for capturing and automatically analysing images of above-ground growth. The images are acquired from the top view and/or the side view, and multiple views are analysed when the plants are rotated by a fixed angle. An example of top and side view image is shown in Fig. 1.

As an example, the RGB imaging system of PlantScreen® phenotyping system (PSI, Czech Republic) enables the researcher to calculate automatically a set of parameters using different modes of view. From the image captured from the top (top view), the following parameters can be calculated:

- Area (pixel count / mm^2)
- Perimeter (pixel count / mm)
- Roundness
- Compactness
- Eccentricity
- Rotation mass symmetry (RMS)
- Slenderness of leaves
- Color index
- Leaf tracking and leaf analysis

Using the series of side-view images, the following parameters can be calculated:

- Plant height (pixel count/mm)
- Growth width (pixel count/mm)
- Area (pixel count/mm^2)
- Perimeter (pixel count/mm)
- Compactness
- Number of leaves
- Leaf angle

A more complex procedure is the use of a combination of top and side view, which enables estimation of the following growth traits:

- Total biomass
- Leaf movement
- Relative growth rate

Similar to PlantScreen, studies are also evaluating the use of field, tractor or buggy mounted systems in the field for organ level and plant based traits. This includes the parameters mentioned above. Similar object in motion combined with photogrammetry based approaches are being used from a UAV based systems using RGB cameras to understand different canopy traits (height, canopy cover etc). The benefit being that the UAV based approaches give us canopy trait characteristics for a large number of varieties under differing field/ environmental conditions.

Canopy cover estimates can be estimated automatically from images using simple threshold and segmentation based analysis available in many image processing programmes. This can help estimate the leaf area index (LAI) and light interception. Further approaches can be used or have been developed to extract traits like shape, compactness etc.

2.2 Chlorophyll fluorescence imaging

The technical development and release of commercially available devices have led to a wide expansion of practical applications of chlorophyll fluorescence in plant biology, stimulating the progress in photosynthetic research and crop science. The routines for distinguishing of different fluorescence quenching were developed, leading to important discoveries on the excess light energy dissipation and partitioning of the light energy between photochemical and non-

photochemical processes. Although the different technical solutions enabled measurement of chlorophyll fluorescence operating efficiencies, most of the results have been obtained thanks to development of pulse amplitude modulation (PAM) fluorometers, using saturation pulse method.

Since its introduction more than 30 years ago, the saturation pulse (SP) method employing the PAM technique has become a common way to assess the photosynthetic electron transport in plant tissues or other photosynthetically active samples. The parameters derived from chlorophyll a fluorescence measurements based on the PAM method provides information about the fluxes of energy originating from the de-excitation of chlorophyll molecules in photosystem II (PSII) in plant chloroplasts, by non-invasive assessment of almost any plant. The parameters of chlorophyll fluorescence analysis can be calculated from quite a few fluorescence intensities, obtained either in dark-adapted or light-exposed plants. The principle of the modulation technique lies in measurements of the rise of total fluorescence in response to a measuring pulse. Thanks to using the increment of fluorescence instead of the total values, the fluorescence parameters can be determined even under conditions of actinic light. Different light environments lead to different states of PSII de-excitation fluxes, which are reflected in changes of fluorescence intensities.

The measurement (Fig. 2) was done on dark-adapted sample (kept for 20 minutes in darkness before measurements). After modulated measuring, light was turned on (ML ON), the fluorescence signal increased, reaching the value of minimum fluorescence in the dark-adapted sample, Fo. Then, the short saturation pulse (SP, intensity 10,000 umol nr^2 s^{-1} for i second) was applied, which led to the fluorescence intensity increase to the maximum value, Fm, followed by the decrease back, almost to the Fo level. In the next step, non-saturating actinic light was turned on, leading to a steep increase of fluorescence signal (F'), reaching the maximum at given actinic light intensity (Fp) followed by the gradual decrease, reaching after a few minutes the steady-state value of actinic-light influenced fluorescence signal (Fs'). The same saturation pulse applied in the sample exposed to actinic light led to an increase of fluorescence signal (peak), reaching the maximum fluorescence in light-adapted state, Fm', followed by a fall back to the Fs' level. In order to obtain minimum fluorescence of the light adapted sample (Fo'), a short period of far-red excitation was applied, leading to a decrease of FS' signal to Fo' value. Individual fluorescence intensities (Fo, Fm, Fp, Fs', Fm', Fo')

represent the variables used for calculation of all fluorescence parameters (quantum yields, quenching parameters, etc.) derived from modulated chlorophyll fluorescence measurements.

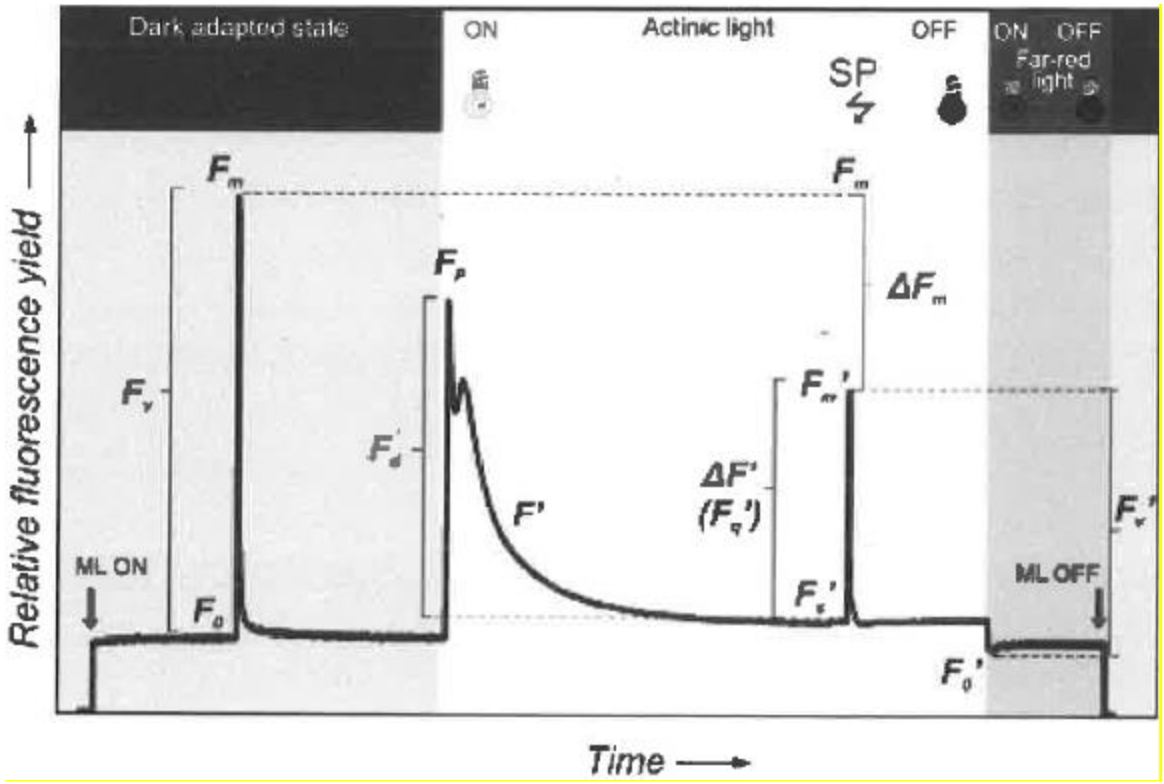

Fig 2. The principle of the fluorescence measurement using the saturation pulse method with quenching analysis by Kalaji et al. (2017).

Most of the instruments for chlorophyll fluorescence analysis integrate the signal of the measured area. Anyway, advances in the technology of imaging detectors, LED light sources and processing of the data enable construction of systems for chlorophyll fluorescence imaging, which provides spatial resolution to the fluorescence records. These systems represent probably the most useful innovation of the technique of chlorophyll fluorescence, with universal applicability. Fluorescence imaging devices have been constructed for their use at the microscopic, plant, leaf and organ level or for remote sensing of chlorophyll fluorescence. The use of a sensitive camera to detect the fluorescence signal may be useful to observe photosynthetic responses at the subcellular level, in plant cells, tissues, leaves, or other plant organs, as well as at the whole plant level. In addition to integrated data, the images provide precise visual information about photosynthetic performance at the different levels of organisation. The fluorescence imaging technique makes possible observation of the spatial and

temporal heterogeneities resulting from variation in internal and/or environmental factors on photosynthesis over the large observed area. The conventional point measurements can just barely (or not at all) detect the heterogeneities easily detectable using chlorophyll fluorescence imaging.

Chlorophyll fluorescence imaging techniques can be also used for some special applications. The responses of stomata, especially the heterogeneity and dynamics of stomatal opening and distribution can be well identified. In addition to numerical assessment, the images can be created in false colour palates to encode the areas differing in stomatal opening with different colours in a sufficient pixel resolution of the images. Such images correspond to topological maps identifying the heterogeneity of the values of the measured parameters across the sample. Therefore, imaging techniques can help to avoid the imprecision typical of point measurements of chlorophyll fluorescence, which is responsible for many false or inaccurate results. The imaging technique can be easily used for analysis of different protocols, such an induction curve or light curve, including the calculation of quenching parameters and parameters of energy partitioning in the dark- or light-adapted state. The visible spectra of PAR are commonly used for excitation of chlorophyll fluorescence, but application of other spectra (mostly UV-radiation) is also possible, providing a specific type of information, different from PAR excitation. Commonly, three types of light have to be used, i.e. pulse-modulated measuring light, actinic light for continuous light exposition and saturating light pulses. Like point measurements, the five key fluorescence levels are used to calculate fluorescence parameters for each measured pixel: Fo, F_0', Fm, Fm' and Fs'. Technical limitations in some of devices disable the direct Fo' measurement by far red light; therefore, Fo' must be calculated using the formula. The commercially available fluorescence imaging devices provide full operating possibilities, including programming of any common light curve, induction curve and recovery) or user-defined protocols. Typical sets of fluorescence parameters that can be achieved using automated chlorophyll fluorescence imaging are shown in Table 3. The sensitivity of the chlorophyll fluorescence imaging to the stress effects was previously documented by many studies. Drought stress led to the heterogeneous distribution of values of chlorophyll fluorescence parameters on the leaf surface.

Table 3. Fluorescence parameters provided automatically by the FlourCam® imaging system as a part of automated phenotyping facility at SAU Nitra.

Fluorescence parameter	Definition
F, F'	Steady state fluorescence emission from dark- or light-adapted leaves, respectively
Fo, Fo'	Minimal chlorophyll fluorescence intensity measured in the dark- or light- adapted state, respectively
F_m, F_m'	Maximal chlorophyll fluorescence intensity measured in the dark- or light-adapted state, respectively
F_v, F_v'	Variable chlorophyll fluorescence (F_m-Fo) measured in the dark- or light-adapted state, respectively
F_q'	Difference in fluorescence between Fm' and F'
Fv/Fm	Maximum quantum yield of PSII photochemistry measured in the dark-adapted state
F_p	Peak fluorescence during the initial phase of the Kautsky effect
Rfd	Fluorescence decline ratio in steady-state (F_p - F')/F'
Φ_{PSII}	PS II operating efficiency; effective quantum yield of photochemical energy conversion in PSII (Fq'/F_m')
NPQ	Non-photochemical quenching (F_m/F_m') -1
qL	Fraction of PSII centers that are 'open' based on the lake model of PSII (F_q'/F_v')(Fo'/F')
ETR	Electron transport rate

Plant breeding represents one of the desired future applications of fluorescence imaging, such as high-throughput screening of cultivars resistant to biotic and abiotic stresses. To date, evaluation of disease or stress resistance in breeding programs is usually done by visual assessment by experienced breeders. In addition to being time consuming, this approach can lead to biases between experimental replicates and assessments by different experts. Advanced high-throughput phenotyping tools are needed to reduce time and improve objectivity. A key advantage of chlorophyll fluorescence imaging is that it can be used to screen large numbers of plants in a short time. Furthermore, it can be integrated into robots for automated analyzes.

In addition to previously mentioned examples, the imaging was used to show the effects of herbicides or the herbicide induced accumulation of reactive oxygen species (ROS) in plant tissues. Chlorophyll fluorescence imaging also identified heterogeneities caused by chilling stress, induction of photosynthesis, wounding, fungal diseases, viral infections, nutrient stress, senescence, drought, and ozone

stress. Chlorophyll fluorescence imaging enables a study of the interactions between leaf structural properties and environmental conditions, directly related to photosynthetic assimilation. The challenge using fluorescence imaging is to process all the data collected in a scientifically meaningful way. As an example of a possible solution, the data can be analysed by frequency distribution parameters.

There are also several limitations of chlorophyll fluorescence imaging. For example, for reliable imaging measurements, it is critical that the whole sample area is illuminated homogeneously - this is, however, very difficult to achieve in larger plots. Moreover, the position of leaves (leaf angle, distances of the leaf from the light sources) can cause large heterogeneity of illumination of samples. Whereas the values of the maximum quantum yield of PSII photochemistry can be correct (except the parts of plants in which the incident light of saturation pulses will be below the saturating level), the values of efficient quantum yield of PSII photochemistry (<&PSII) and ETRpsn can be partially over-estimated in the positions with lower incident actinic light intensities. Despite some risks, chlorophyll fluorescence imaging represents an emerging technique with a high potential for practical use.

2.3 Spectral Sensing

Spectral remote sensing methods assess changes in plant spectral signatures, due to disease, abiotic stress, or ontogenetic development, within and outside the visible part of the electromagnetic spectrum. Plant optical properties are characterised by three processes: (1) transmission through the leaves and stems, (2) absorption by chemicals inside tissue (e.g. metabolites, water, pigments, proteins, cellulose, lignin), and (3) reflectance from inside tissue structures and from a leaf surface. Plant spectral signatures are therefore always a complex combination of these three processes. Most optical sensors do not measure plant physiological parameters, but instead measure the sum of reflectance from various plant tissues and metabolic products. Spectral imaging sensors can obtain the spectral absorption and reflectance characteristics of crops, which can be used to monitor the crop planting area and crop growth, to evaluate the biological and physical characteristics of a crop, and to predict crop yield. Plants have different defense mechanisms for preventing entrance and colonisation by pests and pathogens, such as induction of hyper-sensitive reactions, production of

antimicrobial metabolites and proteins and plant tissue structure. These changes lead to highly specific changes in reflectance. A simple examplified form, Spectral reflectance from plant or crop tissue is inversely related to the chlorophyll content and relies on the interaction when light penetrates tissue, where it can be absorbed, reflected from the surface or transmitted through the leaf and is dependent leaf pigment content. In a healthy plant, the maximum absorption spectrum is generally found in the blue spectral region (400-500 nm and the red spectral region of chlorophyll band (660-680 nm) and hence reflecting most green and infra-red light, making it appear green to the human eye. In the case of stress scenario, nitrogen deficiency reduces leaf chlorophyll concentration leading to lower light absorption and higher reflectance in the visible or infra-red range. Thus these spectral signatures can help us understand the reflectance mode for different crops, different varieties and different stress scenarios. The most commonly used sesning systems include, Multispectral and hyperspectral imaging sensors which can be deployed under controlled, fixed and mobile field based and UAV based systems to obtain and characterise crops and varieties based on their spectral signatures.

2.3.1 Multispectral Imaging/sensing

Multispectral imaging sensors are defined as hardware that are capable of sensing and recording radiation from invisible as well as visible parts of the electromagnetic spectrum, which have been widely used for crop phenotyping due to the advantages of low cost, fast frame imaging and high work efficiency; however, they are limited by the low number of bands, low spectral resolution, and discontinuous spectrum

2.3.2 Multispectral and Hyperspectral imaging of light reflectance

The development of new, more accessible cameras and sensors has enabled the spread of use of hyperspectral imaging of light spectral reflectance from remote sensing applications into plant phenotyping. The absorption of light by endogenous plant compounds is used for calculations of many indices which reflect the composition and function of a plant. A typical example is the normalized difference vegetation index (NDVI), which was originally developed to estimate plant chlorophyll content. Another example is the photochemical reflectance index (PRI), which can be used to estimate photosynthetic efficiency. The absorption by compounds (e.g. chlorophylls, carotenoids, anthocyanins,

water, lignin, etc.) at a given wavelength can be used for direct estimation of their concentrations in the plant. For practical reasons, measurement of absorbance is replaced by measurements of reflectance. Simple NDVI analysis can be carried out using low cost multispectral cameras.

These are sensors that are capable of sensing and recording radiation from invisible as well as visible parts of the electromagnetic spectrum, which have been widely used for crop phenotyping due to the advantages of low cost, fast frame imaging and high work efficiency; however, they are limited by; low number of bands, low spectral resolution and discontinuous spectrum.

A slightly more sophisticated range of these sensors include hyperspectral sensors which have more narrowly placed wavebands for higher spectral resolution. Depending on the measured wavelengths of reflected signal, various detectors are used. The most frequent are the VNIR (350-1200 nm) detectors, less abundant are the SWIR (short-mid wavelength infra-red region; 1000-2500 nm) detectors. Both wavebands are valuable for plant phenotyping. The reflectance signal can be detected at selected wavelengths or separated spectral bands (so-called multispectral detection). The whole spectral region can also be measured even for each pixel when cameras are applied and the hyperspectral imaging is carried out (Fig. 4). Whereas the hyperspectral imaging in the VIS-NIR spectral region is used for evaluation of several indices as mentioned above, the SWIR spectral region is mainly used for estimation of a plant's water or lignin content. Despite the many indices that have been defined so far, based on the reflectance measurements, it is difficult to assess them accurately. For this reason, critical revision of all reflectance indices is needed to evaluate which of them provide the required information in the best way.

In recent years, hyperspectral imaging (HSI) has been widely accepted as a non-destructive, rapid and safe method of qualitative analysis of plants. Spectral data can present much information about the object state. Usually, the dataset is very large and needs to be analysed by appropriate multivariate and machine-learning methods. The investigated spectral parameters of leaf tissue are estimated non-destructively and interpreted with various methods, e.g., principal component analysis.

The following HSI applications are the most frequent:

- Analyses of leaf pigments (chlorophyll, carotenoids, and anthocyanins).
- Spectral reflectance is also known as a fast method for determining nitrogen levels in plants. The general principle of spectral analysis involves reflectance values measured at different wavelengths. Nitrogen content is predicted from linear dependence of reflectance and reference values of leaf nitrogen content.
- Hyperspectral and fluorescent imaging provides a means to directly and non-invasively detect and quantify secondary metabolites such as flavonoids and terpenoids.
- Hyperspectral imaging has shown high effectiveness for assessing fruit and vegetable quality and their safety regarding surface defects, contamination, starch index, bruising, sugar content, freeze damage, firmness and bitter pits. Defect detection with HSI analysis is based on identifying the spectral trait wavelengths for the defect using these spectral parameters.
- HSI was found to be useful for automatic detection of pest and disesae infections
- Classification models have been established to detect insect damage. Fruit analysis after hyperspectral imaging has shown better detection ability compared to the standard conventional visual investigation.
- The moisture content and surface colour are also needed for estimation of fruit and vegetable quality.
- HSI has been used to classify crop seeds including maize, barley, rice, oat, soybean and wheat for the presence of weed seeds.
- HSI at the leaf level is proven to be relevant for the estimation and quantification of pest and fungal invasion. As a non-destructive diagnostic tool HSI has high potential.

3. Non-destructive analysis of growth and physiology by fast manually operated techniques

Although automated systems represent the best choice for phenotyping, an alternative, cheaper way to assess specific traits related to plant phenotype and physiological responses are the modern hand-held tools with low cost and low labour demand. We propose some of them, which are routinely performed at SUA Nitra.

3.1 Fluorescence excitation ratio method to assess flavonoid, anthocyanin, chlorophyll and nitrogen content of plants

Chlorophyll a fluorescence represents the re-emission of light absorbed by photosynthetic pigments with emission spectra in the red to far-red region (600-800 nm), with peaks at ~680 and -730 nm. Although the emission of chlorophyll fluorescence is directly related to the photochemical activity running on the thylakoid membranes in the chloroplast (see Fluorescence Imaging section), the fluorescence signal is strongly influenced by optical properties of plant tissues not directly related to photochemical processes. It was shown, however, that adjustment of leaf optical properties is not purposeless, but usually serves as a protection of photosynthetic structures. Thus, in addition to others, an important defence mechanism against the deleterious effects of solar radiation involves synthesis of relatively stable compounds that serve as light screens and/or internal traps. Depending on the concentration in cells and tissues, protective compounds reduce the fraction of radiation absorbed by light-sensitive cell components thereby diminishing light-induced damage. Probably the most important compounds providing the passive photoprotection (screen) in plants are flavonoids and anthocyanins.

The phenolic compounds, (flavonoids, hydroxycinnamic acids) have absorption maxima in the UV part of the spectrum. The flavonoids are located either in the epidermal vacuoles, cell walls, or dissolved in the epicuticular wax. They have absorption maxima around 260 nm (isoflavones, flavanones), 320 nm (hydroxycinnamic acids), 260 nm and 340 nm (flavones) or 360 nm (flavonols), although the relative importance of the different phenolic compounds as a UV-screen remains an open question.

Based on the strictly UV-absorbing properties, the effects of phenolic compounds on visible light-induced chlorophyll fluorescence is negligible, whereas their

presence strongly suppresses chlorophyll fluorescence emission under UV excitation. This phenomenon has been successfully applied for estimation of transmittance of UV radiation by chlorophyll fluorescence. As experiments have confirmed that the phenolic compounds in the epidermis are responsible for most of the UV-absorption of the leaf, the ratio of visible light-excited to UV-excited chlorophyll fluorescence can serve as an indirect measure of the content of UV-absorbing phenolic compounds in leaves, as shown by the model presented (Fig. 3).

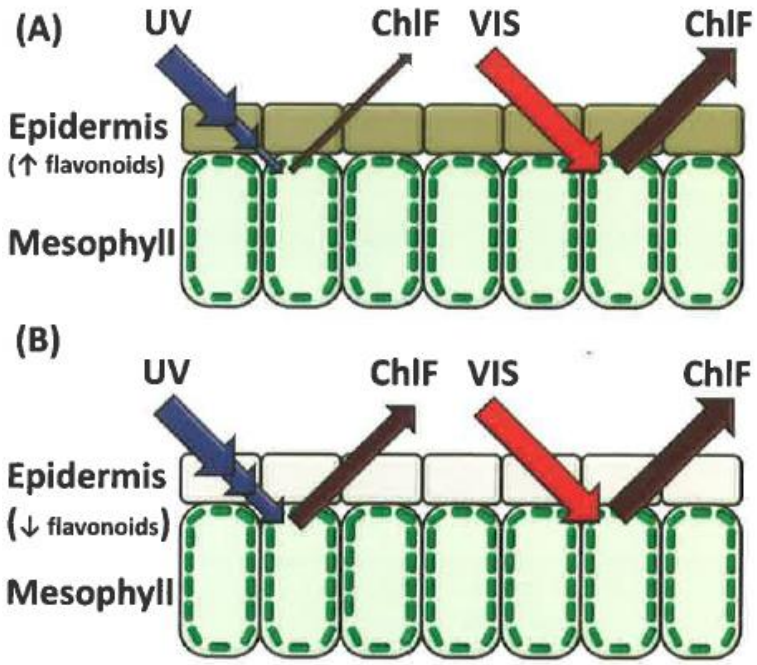

Fig. 3. Schematic diagram (Sytar et al., 2016) of the adaxial part of a leaf cross-section illustrating the principle of the chlorofyll fluorescence (ChlF) method for assessement of content of UV-absorbing compounds in a sample with (A) high and (B) low flavonoid content. The thickness of the beams indicates relative intensity.

In parallel, anthocyanins are water-soluble vacuolar pigments of higher plants. They are responsible for the red/purple coloration of plant tissues, especially in fruits but can also occur in plant leaves. In many cases, significant accumulation of anthocyanins is induced because of environmental stresses such as low temperature, nitrogen and phosphorus deficiencies, UV-B stress, drought, pathogen infections, or due to toxic effects. Anthocyanins absorb strongly in the green region of the spectrum. The spectral band around 550 nm (green) is sensitive to anthocyanin content. Thus, similarly to flavonoids, the ratio of red (or blue) light-excited to green light-excited chlorophyll fluorescence can serve as an

indirect measure of anthocyanin content in plant samples, as shown in the model (Fig. 4).

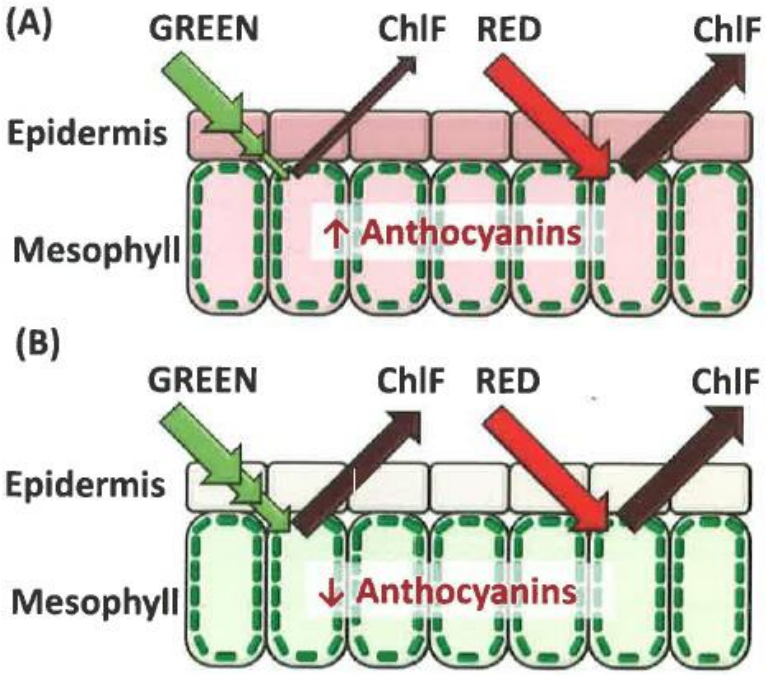

Fig. 4. Schematic diagram (Sytar et al. 2016) of the adaxial part of a leaf cross-section illustrating the principle of the chlorofyll fluorescence (ChlF) method for assessement of content of anthocyanins using simultaneous green and red excitation in the sample with (A) high and (B) low anthocyanin content. The thickness of the beams indicates relative intensity.

In previous decades, numerous studies have examined and confirmed the possibility of using the chlorophyll fluorescence signal in the estimation of phenolic and anthocyanin contents. In addition to self-constructed devices or standard fluorometers combined with external light sources and filters, which have been used in the majority of studies, factory-made special devices for this purpose have also been introduced.

The research group of Z. Cerovic (France) developed several devices using the principle of multispectrally induced chlorophyll fluorescence described above. In principle, they introduced two types of devices: leaf clip-based instrument (commercially available under trademark Dualex, Force-A, France) as well as a non-contact type of instrument (under trademark Multiplex, Force-A, France) (Fig. 5).

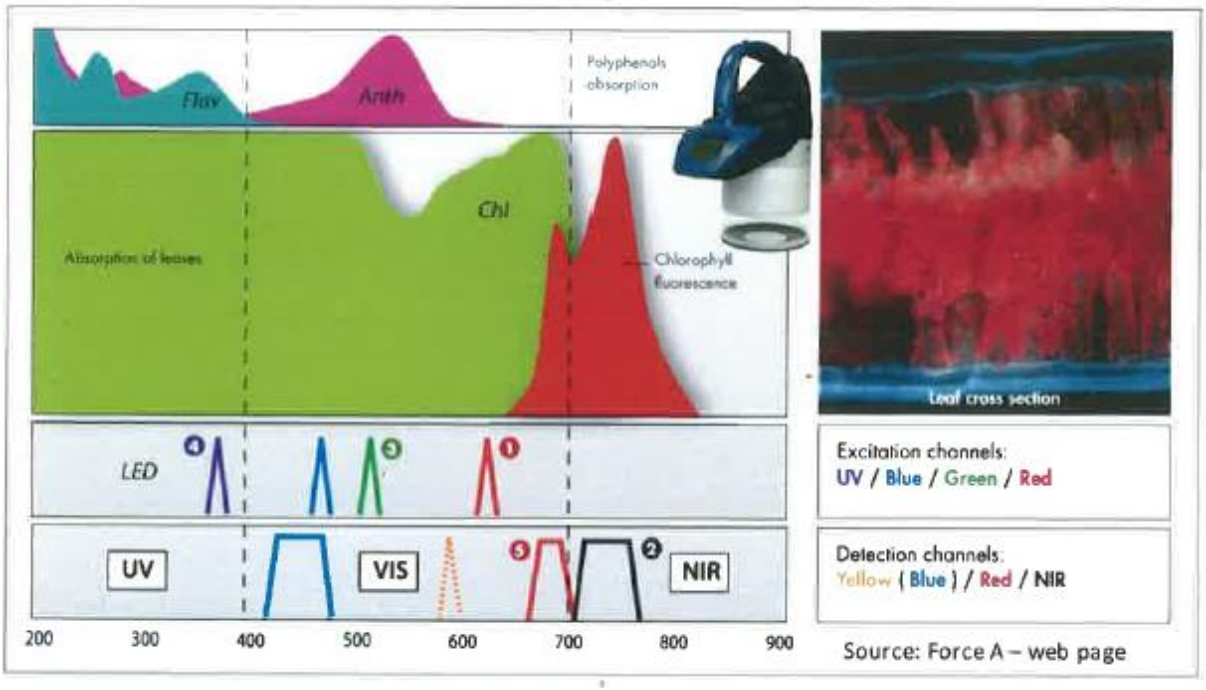

Fig. 5. Scheme of the wavelengths emitted by the LED-units of Multiplex-3 device (Force-A, France), and detected by 4 detector units of the device, which serve for estimation of flavonoid, anthocyanin, chlorophyll and nitrogen contents in leaves and/or fruits.

While the Dualex system measures only two signals (UV and VIS-light induced chlorophyll fluorescence), several kinds of Dualex are produced, specialised for estimation of UV-absorbing compounds (flavonoids), anthocyanins or chlorophylls. In contrast, the Multiplex system measures simultaneously different fluorescence signals after excitation under several spectral regions of light (UV, blue, green, red excitation); thus, this system enables estimation of flavonoid, anthocyanin, chlorophyll cotent together with other information from a single measurement. Thanks to the fact that the Multiplex system does not need any leaf clip, it can be used for measurements even with objects other than flat leaves, e.g. fruits, stems, flowers, etc. This makes this system especially useful for special applications, potentially also in automated systems (as it needs no direct contact with plants).

Moreover, the ratio of fluorescence signal measures in parallel at different wavelengths can serve to estimate the chlorophyll content and in combination with the FLAV signal, provide an estimate of nitrogen content, as SFR decreases and FLAV increases in nitrogen deficient conditions, hence, the ratio NBI =SFR/FLAV represent a good and reliable indirect estimate of the level of nitrogen nutrition.

In summary, the Multiplex-3 device (Force-A, France) measures:

- Total flavonoid content determined as the **FLAV index,** derived from UV absorption properties of flavonoids.
- Total anthocyanin content determined as the **ANTH index,** derived from green-light absorption properties of anthocyanins.
- Total chlorophyll content determined as **SFR index** derived from red/far-red fluorescence,
- Estimate of the level of nitrogen nutrition as **NBI index** (nitrogen balance index), Based on SFR and FLAV indices.

3.2 Analysis of fast fluorescence kinetics by the JIP-test

As the methods based on saturation pulse analysis are relatively time-consuming, a big effort has been applied to develop a more efficient way of measurement of photosynthetic performance and environmental effects. In recent decades, an exponential increase in studies applying fast fluorescence kinetics has been observed. Chlorophyll fluorescence induction represents a plot of measured fluorescence intensity as a function of time of continuous illumination (Fig. 6).

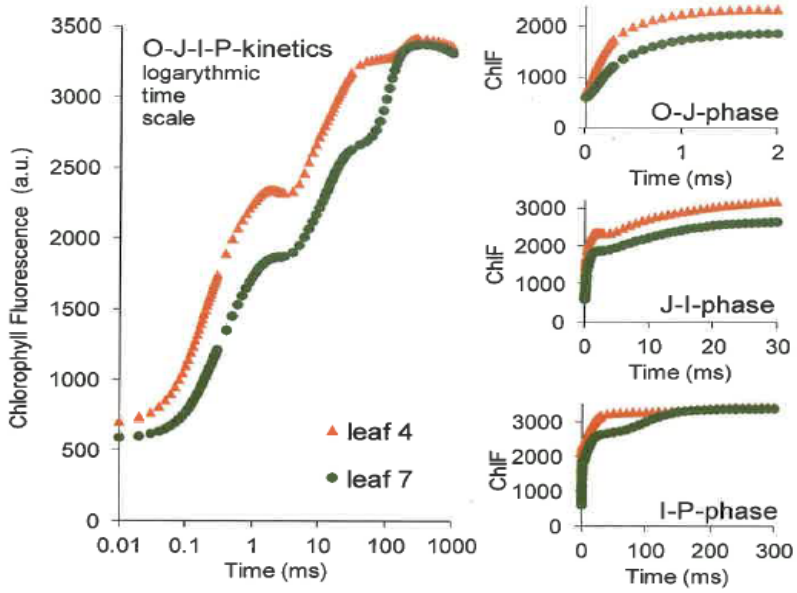

Fig. 6. Examples of O-J-I-P-curves recorded in two different leaves of barley (*Hordeum vulgare* L. cv. Kompakt) at the post-anthesis stage. The leaves are numbered in the order in which they appeared. Leaf 7 represents a penultimate leaf (the second leaf from the top, well exposed to sun), leaf 4 (an older leaf, below the others) was almost completely shaded inside the canopy. The main graph (left) shows the entire O-J-I-P-kinetics plotted on a logarithmic time scale. The small graphs (right) show individual phases plotted on a regular time scale: O-J phase in time o -2 ms, J-I phase in time 2-30 ms, and I-P phase in time 30-300 ms. Data is published in Zivcak et al. (2017).

Such a curve recorded under continuous light has a fast (less than one second) exponential phase, and a slow decay phase (duration of a few minutes). The rise has a typical polyphasic shape, clearly evident when the curve is plotted on a logarithmic time scale, or if the individual steps are plotted separately, at different time resolution (Fig. 8). The shape of OJIP-transient is sometimes denoted as a 'fingerprint' of a sample of a given physiological status; any deviation of the curve indicates photochemical changes at the thylakoid membrane level. The analysis of OJIP curves taking the theoretical assumptions and probabilities derives different photosynthetic parameters for the dark adapted state of the photosynthetic systems. The nomenclature for 'OJIP' is as follows: O for origin or Fo level measured at 50 us (or less) after illumination, J and I represent intermediate states measured after 2 ms and 30 ms, respectively, and P is the peak or Fp = Fm (maximal fluorescence). This is valid only if sufficient light intensity is used. In heat-stressed samples, another peak occurs between Fo and Fj at app. 300 us, which is usually called K-step; therefore, some authors call fast chlorophyll fluorescence induction the OKJIP-curve or transient curve. The OJIP curve from Fo to Fm is correlated with the primary photochemical reactions of PS II and the fluorescence yield is controlled by a PS II acceptor quencher (the primary quinone acceptor, QA). Thus, the OJIP transient can be used for estimation of the photochemical quantum yield of PS II photochemistry, and electron transport properties. OJIP fluorescence curve analysis can be used to monitor the effect of various biotic and abiotic stresses, and photosynthetic mutations affecting the structure and function of the photosynthetic apparatus. There are several groups of parameters derived from the fluorescence rise. In addition to the basic fluorescence values and fundamental parameters, such as Fo, Fm, Fv/Fm (similar to the saturation pulse method), there is also a group of parameters derived from the JIP-test, introduced by Strasser and Govindjee (1992). We can divide it into the fluorescence parameters derived from the data extracted from OJIP transient and the biophysical parameters calculated using the previous group of fluorescence parameters. In plant stress research there are several possible ways of interpreting the data. A multiparametric approach is based on the visualisation of data e.g. by spider plots or pipeline models. On the other hand, the model offers the integrative parameters enabling simple assessment of the status and vitality of the photosynthetic apparatus, which are sensitive and created mostly for

possible practical applications in pre-screening or selection in research and breeding programs.

From numerous JIP-test parameters, for practical applications in crop research the **Performance Index (PI)** was also introduced. This complex parameter integrates several independent structural and functional properties of the photochemistry, reflecting the functionality of both photosystems II and I and providing quantitative information on the current state of plant performance under stress conditions.

However, the current level of knowledge does not entitle us to draw further conclusions about photosynthetic performance based on fast chlorophyll fluorescence only. Even usefulness of the fast chlorophyll fluorescence for leaf photosynthetic performance testing could be proven in the future, more probably, the method will remain mostly a tool for assessment of the stress effects on the photosynthetic functions. In this respect, the availability of user-friendly portable fluorometers for high-frequency record of OJIP-transient and the useful software for the analysis of experimental data, make the JIP test derived from the fast chlorophyll fluorescence attractive even for users without a deep knowledge of photochemical processes at the thylakoid membrane level. As we have mentioned above, the small and portable devices allow efficient data records even under field conditions. The chlorophyll fluorescence induction kinetics contains valuable information about the photochemical efficiency of primary conversion of incident light energy, electron transport events, and related regulatory processes. These issues can be deciphered using advanced mathematical models based on the analysis of fluorescence curves, providing many fluorescence parameters.

They can be divided into:

- Parameters directly derived from fluorescence data (Fo, Fm, Fv, Area),
- Specific quantum yields, i.e. energy fluxes per absorbed light spectra (TRo/ABS, DIo/ABS, ETo/ABS, ETo/ABS)
- Energy fluxes per active reaction centre (ABS/RC; TRo/RC; ETo/RC, DIo/RC)
- Energy fluxes per excited cross section: (ABS/CS; TRo/CS; ETo/CS, DIo/CS)
- Density of reaction centres (RC/ABS; RC/CSo; RC/CSm)
- Probabilities of electron transport between individual steps of the electron

transport chain

- Performance indices and driving forces (PI_{ABS}, PI_{TOT}, df)

All parameters are precisely and simply defined, and they can be used to characterise the status of PSII photochemistry, which reflects the effects of external factors and the status (vitality) of plants.

3.3 Analysis of leaf chlorophyll content using chlorophyll meters

Chlorophyll content represents an important indicator of plant health status and basic information of the limitations of photosynthetic capacity. The main methods for determination (HPLC, spectrophotometric) are destructive. An alternative way is the use of chlorophyll meters, which have been used successfully in many species to estimate leaf chlorophyll, and allows measuring of chlorophyll content on the same leaf over time. The readings from chlorophyll content meters can be also used to predict the nitrogen status of leaves and hence the efficiency of fertiliser uptake. The meters/devices that calculate chlorophyll content indices (e.g. SPAD value, CCI index) are based on measuring the reflectance, absorbance or fluorescence at particular wavelengths. The most common hand-held chlorophyll absorbance meters, of which several are commercially available, measure absorbance by the leaf at two different wavelengths of light: red and near infra-red. The red light is strongly absorbed by chlorophyll. The second is a 'reference wavelength' necessary to adjust for differences in tissue structure.

It was shown that all the chlorophyll meters available on the market are useful, but precision is not always the same in all conditions; especially, the fluorescence-based instruments have some limits. The values of different types of devices should therefore not be combined and compared.

4. Thermal sensors

The sensors mentioned above work mainly with the phenomena of absorption, reflection, and fluorescence, but one of the other powerful tools for phenotyping, especially for traits related to stress responses, especially water, is the use of thermal imaging.The approach works on the basis of simple physics phenomena of evaporation causing cooling in plants, which interact with their environment through interface of "stomata", maintaining a carbon-water and energy exchange balance with adaptability to ever-changing conditions. Thus stomata play an important role in plant adaptation and growth by balancing the need to minimise water loss while maintaining photosynthetic gains. Evaporative cooling through transpiration is a major component of the leaf energy balance and thus any stomatal closure in response to drought stress, therefore, will be manifest as a warmer temperature, so that thermal imaging can be used to quantify stomatal closure (Prashar and Jones 2016).

Similar to spectral reflectance sensing, thermal sensing suffers from difficulties of background interference such that techniques are necessary to obtain a pure signal from the canopy only. These can include the overlaying of spectral images or extraction of canopy variation or thresholding. Various automated or semi-automated methods have been proposed and are used for canopy temperature extraction. Examples of use of thermal sensing include from plant level to canopy level and also include crops in various categories from small grain cereals to broad leaf crops like maize and potatoes and even fruit trees (Prashar et al., 2013; Prashar and Jones 2014). Infra-red thermograpy can allso be used for plant stress detection:

1. Abiotic stresses
    a. Drought
    b. Salinity
    c. Heat and frost
2. Biotic stress
    a. Stem and foliar pathogens
    b. Soil-borne pathogens
3. Screening for indirect yield and quality traits
4. Dynamic and spatial variation in stomatal conductance

5. Potential use of sensors for non-destructive crop phenotyping of wheat and potato traits required in varieties suited to organic agriculture

In addition to the many crop traits that are also important in conventional farming, varieties for organic farming must deliver sufficiently high and stable yields with minimal use of external resources (e.g. no use of chemosynthetic fertilisers and chemical crop protection inputs) as well as to deliver crops of high technical and nutritional quality. In brief they must have additional features such as: (i) resistance to soil and seed-borne diseases, (ii) rapid canopy development, (iii) high weed suppression and tolerance, (iv) lodging resistance at greater plant heights, (v) increased nutrient use efficiency (e.g. extensive root systems and promotion of symbiosis with soil microorganisms); as well as (vi) various crop quality related traits (e.g. grain protein content). Organic seed and planting material are multiplied or propagated for at least one generation under organic management conditions. They originate from varieties that are a result of breeding programmes using permitted breeding techniques such as: Hybrids; DNA marker assisted selection; Meristem culture etc.

Specific breeding goals for organic cereal varieties include: **(i)** resistance or tolerance against seed borne (such as *Tilletia tritici*) and foliar diseases (such as *Septoria tritici*); **(ii)** ability to suppress weeds (traits such as: long straw; early plantophile growth habit; good crop establishment and rapid early growth (until shoot extension); high tillering ability and good ground cover in tillering stage; (iii) increased yield stability and grain quality (such as protein, HFN etc.) under different conditions (nutrient uptake efficiency, resistance to abiotic stress factors etc.). Specific breeding goals for organic potato varieties include: (i) adaption to organic fertilisation (animal manure, composts) with traits such as: Rapid root growth and adequate root system architecture; high nutrient uptake and use efficiency (canopy coverage); rapid juvenile development; good growth vigour; (ii) resistance to diseases such as Phythophtora, Rhizoctonia, silver scurf, scab as well as several viruses; (iii) yield stability and tuber quality (high dry matter, good cooking and baking quality, good storability).

A range of different types of sensors are currently available to use in Advanced Phenotyping in order to identify growth traits throughout the season, including traits not visible to the human eye, and can combine measurements of different phenotypes by using mobile platforms of multiple sensors. For example, UAV

based sensors, in combination with advanced image processing routines, can automatically detect single plants and determine germination rates and timing in the field. Automated field phenotyping approaches can also detect seed emergence under extreme climatic events such as frost or heavy rain, offering opportunities to directly select for establishment traits suited to agronomic practices. After germination canopy biomass and stem elongation are typically determined with non-destructive measures of the canopy structure and destructive biomass measures to extract growth rates. Unfortunately, there are no widely available and sufficiently precise methods to determine total canopy biomass non destructively.

Growth rates as well as canopy structural measurements such as canopy height can be derived with the use of UAVs equipped with various sensors and by subtracting background soil information. A key challenge is that canopies close; as a result, most phenotyping efforts have developed approaches with a top-of-canopy view, which has obvious limitations for lower leaves or crops hidden from the sensor's field of vision. Research is also ongoing in order to automatically differentiate between crops and weeds which are a main challenge especially in organic systems. Plant photosynthesis rates are associated with yield and biomass and photosynthetic traits remain key targets for breeding. Fluorescence-based methods that measure the efficiency of photosynthetic light reactions have become popular. However at present there are no fast and reliable methods available to detect photosynthetic carbon-uptake rates in large-scale field phenotyping.

Detection of biotic stresses such as diseases are important for breeding programs but challenging for automated field phenotyping. Disease symptoms are scored using expert knowledge, and the precision required for optical measurements remains a challenge. The most promising approaches combine high-precision optical sensors (with the use of multispectral or hyperspectral cameras) with advanced feature extraction methods, such as adaptive machine learning or linear and non-linear regression models.

Abiotic stresses, like biotic stresses, are diverse in type, space, and time in the crop cycle. Notable successes in the identification of abiotic stress tolerance were achieved within pre-breeding programs using non-destructive canopy temperature and normalized difference vegetation index (NDVI) sensors. A

challenge for Advanced Phenotyping is that field crops express multiple stresses as a single symptom which might be caused by either biotic or abiotic stressors. Multispectral or hyperspectral methods may provide solutions.

Advanced Phenotyping approaches have recently focused on traits associated with final yield and crop quality. For example, grain moisture, protein content, test weight, starch, oil content etc could be measured automatically by using spectral sensors mounted on combine harvesters. Furthermore UAV-based sensors-from-motion measurements could be used to detect homogeneity of ripening and lodging resistance. The information presented in Tables 4 and 5 sumarises the potential of digital, spectral and thermal sesors for use in the phenotyping of traits needed for 'organic' wheat and potato varieties.

Table 4. Potential use of sensors for non-destructive crop phenotyping of wheat traits required in varieties suited to organic agriculture

Winter wheat	Digital	Spectral	Thermal
Crop vigour			
Growth habit	🟢	🟢	🟢
Germination %	🟢	🟢	🟢
Tillering ability	🟢	🔴	🔴
Leaf Area Index	🟢	🟢	🟢
Green Area Index	🟢	🟢	🟢
Plant height	🟢	🟢	🟢
Crop phenology			
Days to maturity	🔴	🟡	🟡
Days to anthesis	🔴	🟡	🟡
Yield related			
Yield	🔴	🔴	🔴
Harvest Index (HI)	🔴	🔴	🔴
Ear number per m2	🟡	🔴	🔴
Number of grains per ear	🔴	🔴	🔴
Disease %	🟢	🟢	🟢
Grain quality			
Protein %	🔴	🟢	🔴
Hectolitre weight	🟢	🔴	🔴
Hagberg Falling Number	🔴	🟡	🔴

🟢 Applicable 🟡 Potential 🔴 Not applicable

Table 5. Potential use of sensors for non-destructive crop phenotyping of potato traits

Potato	Digital	Spectral	Thermal
Crop vigour			
Growth habit	🟢	🟢	🟢
Leaf area index	🟢	🟢	🟢
Plant height	🟢	🟢	🟢
Crop phenology			
Days to maturity	🟢	🟢	🟡
Yield related			
Yield	🟡	🟡	🟡
Harvest index (HI)	🟡	🟡	🟡
Tuber number	🟢	🟡	🟡
Average tuber weight	🟢	🟡	🟡
Disease %	🟢	🟢	🟢
Tuber quality			
Dry matter %	🔴	🔴	🔴
Tuber size	🟢	🟢	🟢
Skin colour	🟢	🟡	🔴
Eye depth	🟡	🟡	🔴
Cooking/baking quality	🟡	🟡	🟡

🟢 Applicable 🟡 Potential 🔴 Not applicable

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Appendixes

Appendix 1: Training Course Curriculum

Advanced training on Plant Phenotyping Technologies

Introduction

This training module advanced phenotyping was prepared for the activities of the support of an extensive training program that will facilitate rapid technology transfer from the project into commercial practice. As the title suggests, the course is focused on technical procedures in improving phenotyping management of crop improvement for breeding in organic agriculture. A practical workshop and demonstration experiments will also be organised to pass on the experience of using the various technologies made available by plant phenotyping experts. This module follows the order of the course, which is divided into discussions, lectures (Part 1), and practical hands-on sessions (Part 2).

Training objectives

The objectives of the training will be to present the evolving areas of automated sensor-based plant phenotyping technologies and to bring together experts working in these interdisciplinary fields. The course will be a seminar based that will cover a wide range of technologies available for plant phenotyping to train early-stage researchers, scientists, and breeders professionals in good practices for effective the transfer of technologies developed into commercial practice.

Specific objectives are designed:

- To enhance the use of standard protocols for research and breeding operations;
- To develop a practical understanding of the capabilities of different sensors
- To provide a vehicle to initiate collaboration and co-operation between researchers, breeders and farmers;
- To equip participants with essential knowledge in advanced phenotyping and database management;

D 7.2 Production of materials for improved phenotyping training

- To acquaint participants with morphological/physiological techniques to characterize and evaluate genetic material.

LECTURE TOPICS

***PART 1**. Discussion and Lectures*

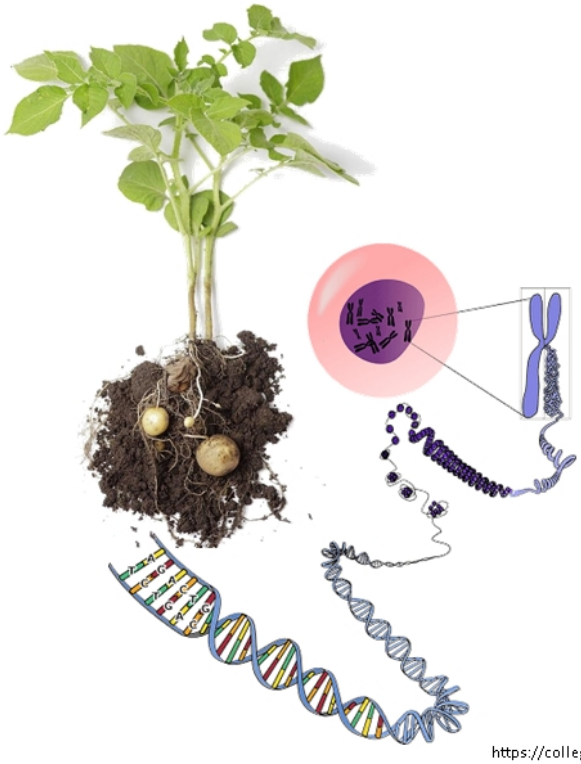
- Introduction to Ecobreed conventional phenotyping
- Introduction to Advanced Phenotyping
- Sensor technologies
- Application of high-throughput phenotyping in crop studies
- Non-invasive crop plant phenotyping
- Environmental characterisation
- Data analysis and modelling
- The potential use of sensors for non-invasive crop phenotyping

***PART 2.** Practical work*

- Demonstration activities: high-throughput phenotyping
- Drone based systems
- Interactive discussion

Appendix 2: Lecture slides for plant phenotyping techniques

What is Plant PHENOMICS-PHENOTYPING ?

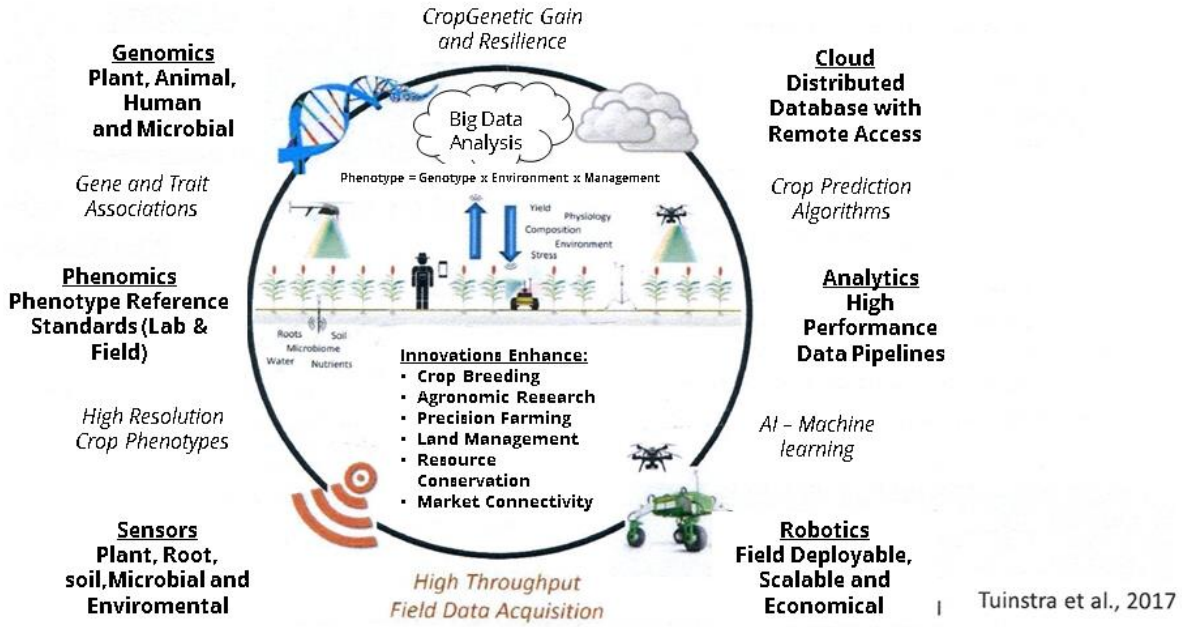


Phenome=Genome X Environment

- **Genomics is accelerating gene discovery but how do we capitalise on these data sets to establish gene function and development of new genotypes for agriculture?**
- **High throughput and high resolution analyses capacity now the factor limiting discovery of new traits and varieties**

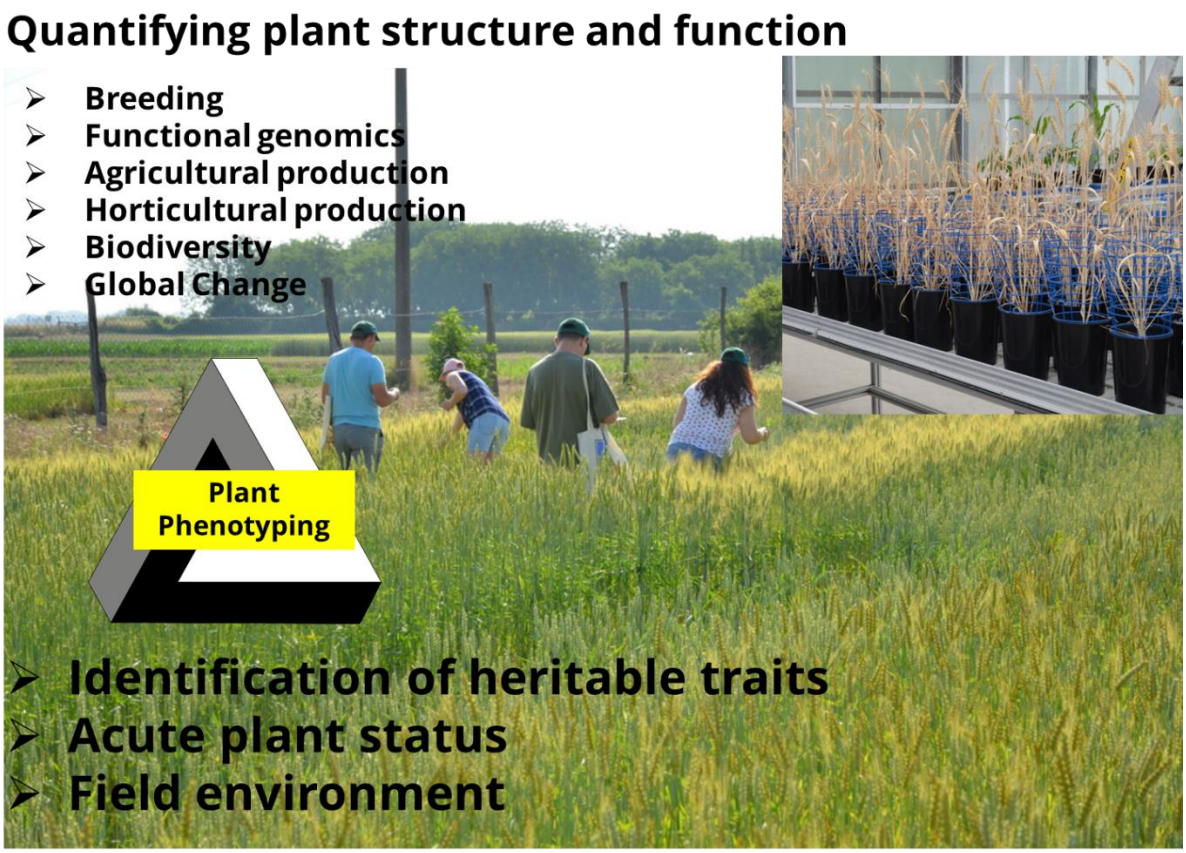
https://es.gardenmanage.com/statuses/1000264090.html
https://collegian.com/2015/03/csu-research-team-awarded-200000-to-create-genome-institute/

Recent phenotyping: The convergence of BIOLOGY x ENGINEERING X COMPUTER SCIENCE

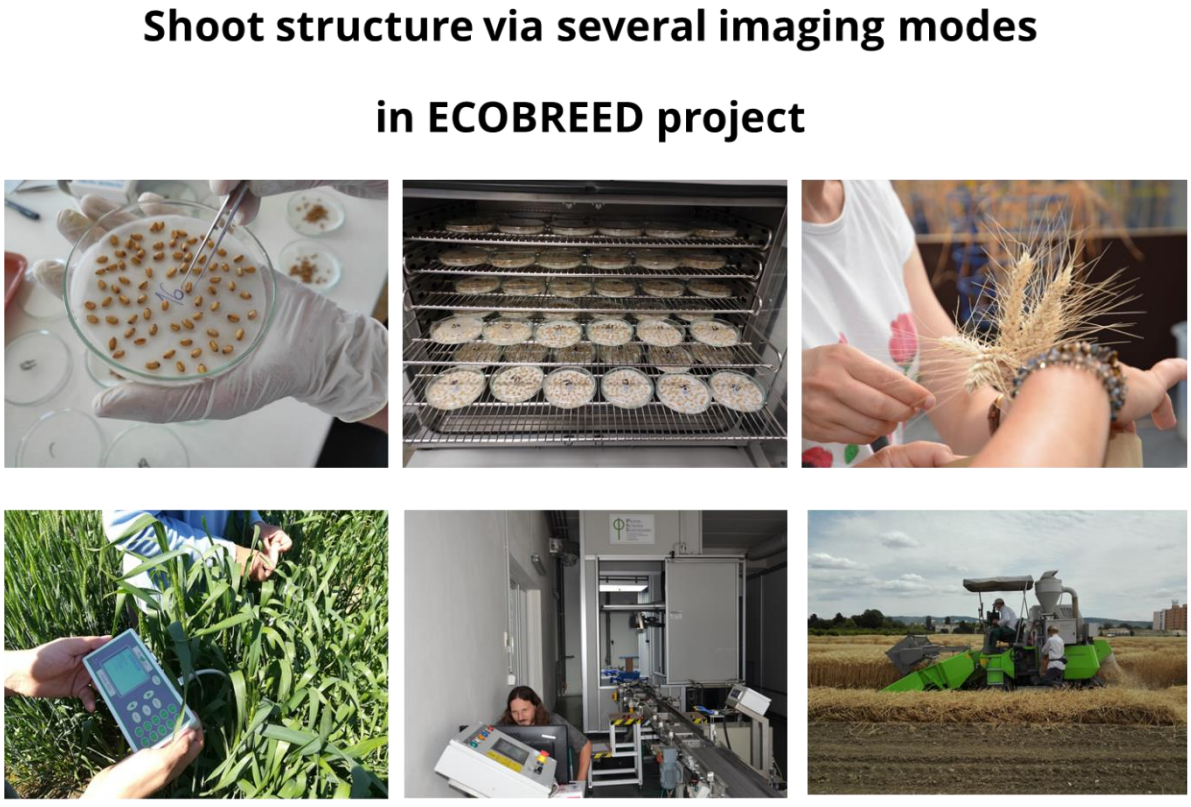


Tuinstra et al., 2017

http://docplayer.net/29027381-Characterization-challenges-and-uses-of-sorghum-diversity-to-improve-sorghum-through-plant-breeding.html



Source: NPPC, 2020



Source: NPPC, 2020

Manual phenotyping

Disadvantages:

- Slow and expensive
- Variation between observers
- Sometimes destructive

https://www.delta-t.co.uk/; https://agfax.com/2019/06/21/corn-soybeans-do-you-know-your-crop-growth-stages/; https://www.barnowltrust.org.uk/sitemap/galleries/field-vole-holes/field-vole-holes_hole-survey-quadrat/

Automated systems: Phenotyping by Image Analysis

Automated RGB image analysis

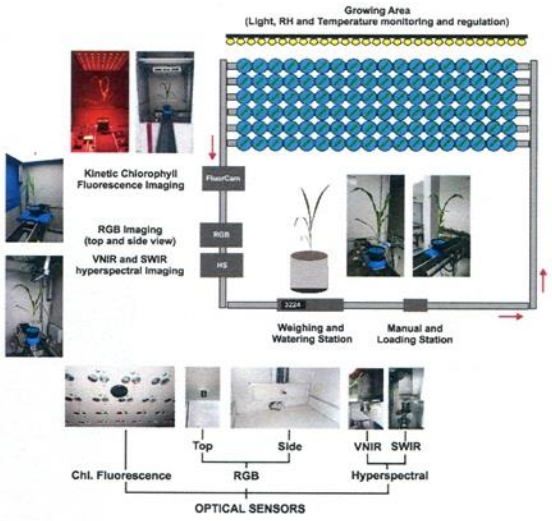
Phenotyping of *Lycopersicum*

- **Most image analysis systems for automatic phenotyping bring the plants to the camera.**

https://psi.cz/; http://plantphenotyping.com/products/plantscreen-modular-system/#gallery

PLANTSCREEN™ phenotyping platform in SUA

- ❑ **PlantScreen™ conveyor modular system**
- ❑ **Capacity** for **108 plots,** 6 lines, 18 transportable discs per line (max plant height 1200 mm)
- ❑ **Total area** of cultivation 24 m²
- ❑ **Precision regulation of environmental conditions** (PPFD, Temp, Relative Humidity)
- ❑ Automatic **weighing and watering**
  - ❖ **Five optical sensors:**
  1. **Chlorophyll a fluorescence (PAM system**
  2. **RGB top view**
  3. **RGB side view (differing side angels)**
  4. **VNIR hyperspectral imaging**
  5. **SWIR hyperspectral imaging**



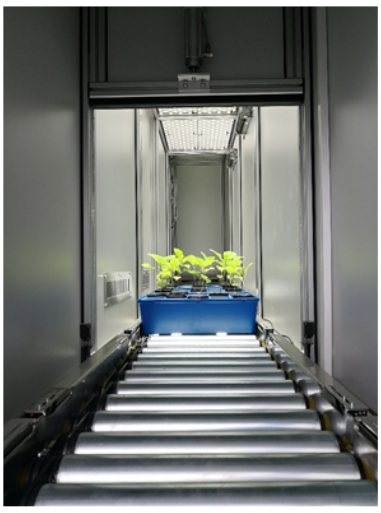
https://www.agrobiotech.sk/; https://educons.edu.rs/wp-content/

LIGHT AND DARK ADAPTATION TUNNEL (SUA)

Light and dark adaptation tunnel is used for plant acclimation prior physiological phenotyping.
Multichannel LEDs with programmable interface for defining desired light regime and spectral quality.
Precise setting of light intensity in smooth steps, with the maxima of 1.500 µmol $m^{-2}s^{-1}$.

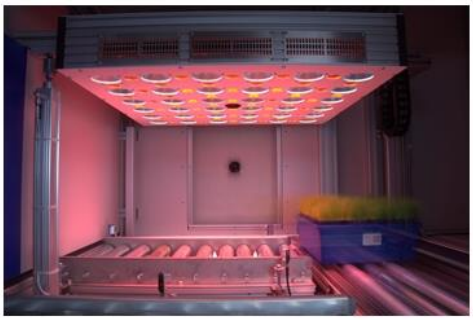
Characterization:

- ✓ **Localized before fluorescence imaging unite**
- ✓ **For dark and light adaptation of plants**
- ✓ **Artificial light intensity up to 1.500 µmol m^{-2} s^{-1}**
- ✓ **Cool white LED**
- ✓ **Red LED**
- ✓ **Infrared LED**



ttp://plantphenotyping.com/products/plantscreen-modular-system/#gallery; https://educons.edu.rs/wp-content/

FLUORESCENCE IMAGING UNIT



TECHNICAL SPECIFICATION

- ✓ **Image Sensor:** ½ ´´ format, mono, 7.4 x 5.9 array
- ✓ **Effective Pixels size:** 720 x 560, 8.6 x 8.3 µm pixels
- ✓ **Frame rate:** 50 fps
- ✓ **Dimensions (w x h x d):** 70 x 150 x 60 mm
- ✓ **Lens Mount:** CS-Mount
- ✓ **Lens type:** Lensagon CY0314
- ✓ **FC panel area (h x w):** 800 x 800 mm

Light sources panel:

1. **Pulse-modulated short duration flashes (620 nm)**
2. **Actinic light: two types:** Cool white (maximal intensity 500 µmol $m^{-2}s^{-1}$, red -orange light (620nm) (maximal intensity 300 µmol $m^{-2}s^{-1}$)
3. **Saturating light pulse:** cool white, maximal intensity 300 µmol $m^{-2}s^{-1}$
4. **Additional light: FAR (735nm)**

https://psi.cz/; http://plantphenotyping.com/products/plantscreen-modular-system/#gallery; https://educons.edu.rs/wp-content/

RGB IMAGING UNIT

RGB TECHNICAL SITUATION

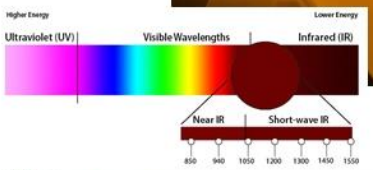
- ✓ **Sensor:** CMOS (MT9P031STC), ½ ´´ format (Aptina)
- ✓ **Camera type:** UI-5480SE-C/M
- ✓ **Spatial resolution (h x w):** 2560 x 1920
- ✓ **Pixel resolution:** 5 MPx
- ✓ **Effective pixel site:** 2.2 µm per pixel
- ✓ **Depth of image:** 12 bit
- ✓ **Scan area (w x h): 710** x 550 and 1030 x 1500 mm

https://qubitphenomics.com/; https://psi.cz/; http://plantphenotyping.com/products/plantscreen-modular-system/#gallery; https://educons.edu.rs/wp-content/

VNIR AND SWIR HYPERSPECTRAL IMAGING UNIT

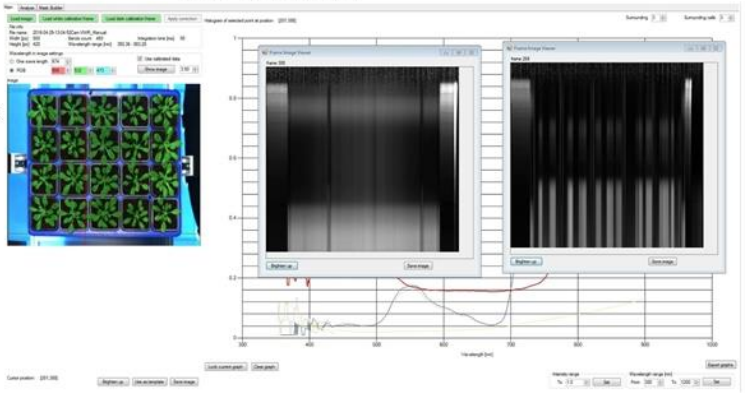
TECHNICAL SPECIFICATION VNIR

- ✓ Spectral range: 340 – 900 nm
- ✓ Band size: 400 nm
- ✓ Entrance slit width: 25 um
- ✓ Dispersion per pixel: 0,2 nm/pixel
- ✓ Wavelength resolution: FWHM 0.8 nm
- ✓ Detector: Silicon
- ✓ Spatial resolution: 480 pixels
- ✓ Spectral resolution: 640 pixels
- ✓ Image frequency: 12 – 60 fps
- ✓ Dynamic range: 68 db



TECHNICAL SPECIFICATION SWIR

- ✓ Spectral range: 900 – 1700 nm
- ✓ Band size: 600 nm
- ✓ Entrance slit width: 25 um
- ✓ Dispersion per pixel: 0,95 nm/pixe
- ✓ Wavelength resolution: 1.2 nm
- ✓ Detector: InGaAs
- ✓ Spatial resolution: 480 pixels
- ✓ Spectral resolution: 640 pixels
- ✓ Image frequency: 50 fps
- ✓ Bit depth: 16 bit



https://psi.cz/; http://plantphenotyping.com/products/plantscreen-modular-system/#gallery; https://educons.edu.rs/wp-content/

WEIGHING AND WATERING SYSTEM

- ✓ High-precision (±2.0 g) irrigation for programmable delivery of water (and nutrient)
- ✓ Watering to exact volume and predefined weight of pot

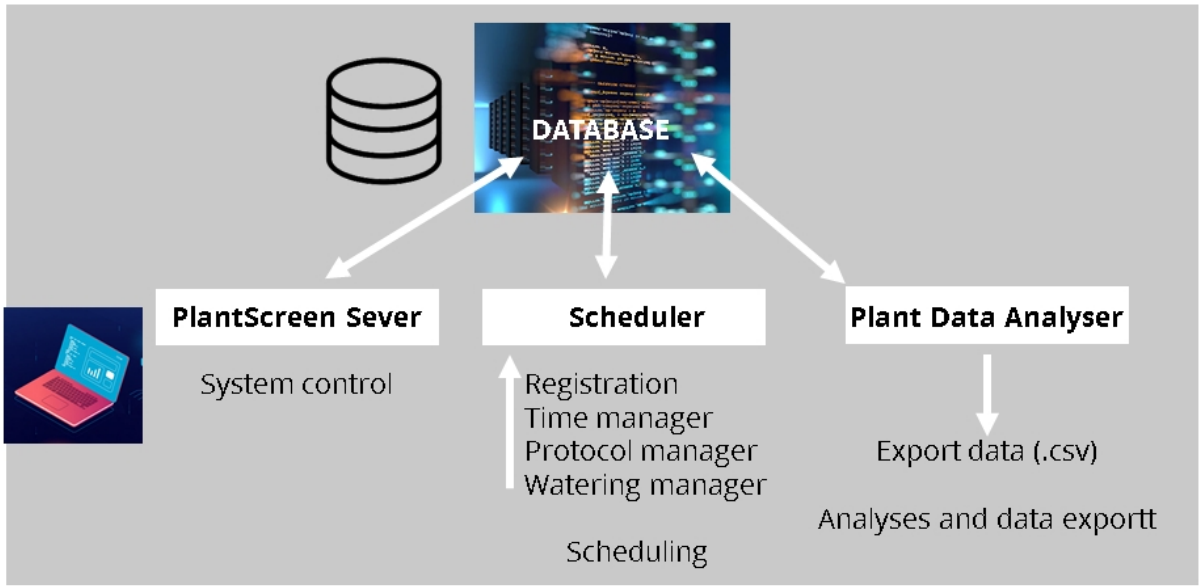
The automated watering and weighing, together with estimated shoot FW and DW, allows the estimation of daily water consumption (or evapotranspiration, ET) and water use efficiency (WUE) of plant.
Consider two consecutive days i and i+1.

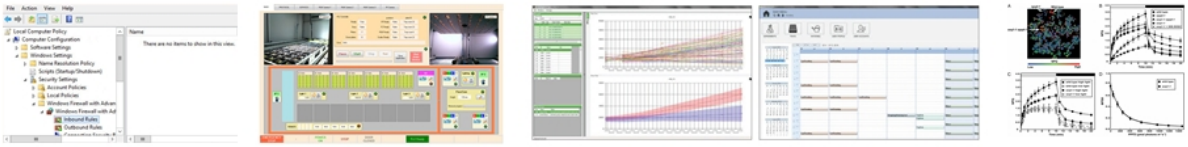
$$ET=[(W_{(I,after)}-Fw_i)-(W_{(i+1,before)}-FW_{i+1})]/T$$
$$WUE=(DW_{I+1}-DW_I)/ET$$

where $W_{(I,after)}$ is the total pot weight after water application for Day i; $W_{(i+1,before)}$ is the total pot weight before water application for Day i+1; FW_i and FW_{i+1} is shoot fresh weight estimated at the point of imaging for Day i and i+1, respectively; and DW_i and DW_{i+1} is shoot dry weight estimated at the point of imaging for Day i and i+1, respectively. T is the time interval and is equal to 1 day (daily pot weighing following the same schedule every day).

https://psi.cz/; https://www.agrobiotech.sk/; https://educons.edu.rs/wp-content/

SOFTWARE CONTROL OF PLANTSCREEN™





https://psi.cz/; http://plantphenotyping.com/products/plantscreen-modular-system/#gallery; https://qubitphenomics.com/; https://educons.edu.rs/wp-content/; https://www.dataversity.net/in-memory-database-architecture-overview/

Bottleneck Phenotyping

Key Messages

Plant phenotyping has become a bottleneck for progress in basic plant science and plant breeding Novel opportunities for phenotyping develop from interdisciplinary approaches of plant scientists, (bio)informatics, sensors, and environmental sciences and simulation.

Phenotyping needs to integrate activities for establishing mechanistic, high-throughput, and field-based platforms.

Phenotyping facilities need to be available for the diverse user community.

https://repository.cimmyt.org/; https://www.frontiersin.org/; https://psi.cz/; https://www.mdpi.com/journal/agronomy