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ITINERIS

D6.7 (B13, ACTIVITY 6.09, CNR-IPSP-NA) LONG TERM, DISTRIBUTED PHEN-ITALY INFRASTRUCTURE READY AND OPERATIONAL FOR STATE-OF-THE-ART PLANT PHENOTYPING EXPERIMENTAL

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1. INTRODUCTION

EMPHASIS is a distributed research infrastructure, included in the ESFRI roadmap in 2016, aimed at promoting the development, integration, and access to individual infrastructures operating in Europe in the field of plant phenotyping. Plant phenotyping is considered a cornerstone of the new green revolution, enabling the rapid selection of productive, adaptive, and resilient plants that ensure adequate yields and sustainability of natural resource use, thereby addressing major global challenges of food security and environmental sustainability. Currently, EMPHASIS is in the implementation phase led by the Interim General Assembly (IGA), with CNR participating as a scientific partner, alongside representatives from the Ministry of University and Research (MUR). Since 2021, within EMPHASIS, negotiations have been underway leading to the establishment of the European legal entity EMPHASIS-ERIC, which will enable the full operation of the infrastructure starting from 2025-26. To implement EMPHASIS activities at the national level, the Joint Research Unit (JRU) "Phen-Italy" was established in 2017, representing the Italian node of EMPHASIS. The JRU Phen-Italy consists of 14 partners: the National Research Council (CNR) (coordinator), the Lucanian Agency for Agricultural Development and Innovation (ALSIA), the Council for Agricultural Research and Economics Analysis (CREA), the University of Tuscia, the Polytechnic University of Marche, Alma Mater Studiorum University of Bologna, the University of Basilicata, Sant'Anna School of Advanced Studies in Pisa, CIHEAM - Mediterranean Agronomic Institute of Bari (CIHEAM - Bari), the University of Bari, the University of Padua, the University of Naples Federico II, the University of Turin, and the Edmund Mach Foundation. The JRU aims to synergistically connect groups involved in high-intensity plant phenotyping measurements, with particular reference to those of agricultural and food interest, and to create a single Italian point of contact to better coordinate activities and requests from Italian, European, and international users. In the frame of WP6, the OBJ4: Sustainable Agriculture and environmental biotechnology, the RI EMPHASIS aim to develop services and technological tools for the sustainable use, valorization and optimization of agriculture and forestry resources for low impact bio-based processes, to mitigate climate change fostering a green transition to circular economy.

2. ACTIVITIES

The objective is to enhance the capabilities of two prominent southern high-throughput phenotyping platforms to efficiently capture and promptly provide non-invasive assessments of plant structure, function, and environmental interactions in near-real-time, ensuring the utmost accuracy in measurements. This enhancement will enable precise crop evaluation across diverse climatic conditions in authentic agronomic settings, utilizing cutting-edge digital phenotyping equipment to enhance statistical significance in genotype screening. To achieve this goal, controlled environment phenotyping facilities will be upgraded with: 1) sensors integrating 3D imaging with multispectral technology to capture high-quality, precise digital parameters in real-time, and 2) a walk-in phytotron equipped with an XYZ robot, facilitating the movement of a multi-sensor platform toward the plants at specified intervals for data acquisition. While the field phenotyping infrastructure will utilize a gantry crane-based robotic high-throughput platform, autonomously navigating along steel rails and continuously capturing imagery with a diverse array of cameras and sensors. Plants will grow in a system of individual pots placed on scales where irrigation is automated with high accuracy and reliability and loss of weight of every pot is automatically recorded with high precision allowing to compute transpiration dynamics and water use efficiency for every single plant. These controlled-environment and field phenotyping setups will be complemented by state-of-the-art metabolomics and volatilomics equipment, creating high-quality, unique platforms for selecting crop genotypes to address climate change adaptation and mitigation.

The cropping systems experiments carried out at plot and field levels across different circular sites yield extensive environmental, agronomic, and cultivation data. Nonetheless, this data has never been standardized or made readily accessible via a database that can be easily and freely accessed by the entire scientific community. The controlled environment platform will be integrated with a walk-in phytotron accommodating a XYZ robot bringing multi-sensorics platform. The acquisition of proximal and remote sensing data will allow to optimizing management of the experimental sites. The acquisition of these new technologies will serve to strengthen the existing controlled-environment and field platforms with state-of-art nondestructive plant phenotype facilities, based on robots and image analysis, aiming at the development of phenotyping pipelines to assess crop properties across scales with different levels of precision from lab to field combined with central access to coordinated information systems for data management and storage.

At the moment, the following instruments have been ordered and purchased, as reported in Table 1.

Table 1 – WP6 – OBJ4 acquired equipment.

Infrastructure	*Equipment acquired*	*Status acquisition*	*Assigned location*
EMPHASIS	VOCUS-S for Volatile Organic Compounds (VOC) detection	Purchased	Portici (NA)
EMPHASIS	Growth Capsule - Phenotyping phytotron	Contract signed	Portici (NA)
EMPHASIS	High-speed field phenotyping platform	Purchased	Portici (NA)
EMPHASIS	Scale/lysimeter system	Contract signed	Portici (NA)
EMPHASIS	Software for analyzing phenotyping data	Purchased	Portici (NA)
EMPHASIS	CIRAS-4 Portable CO2/H2O Gas Analysis	Purchased	Portici (NA)

3. PLANT PHENOTYPING INFRASTRUCTURES AT CNR-IPSP-NA

3.1 Volatile organic compounds (VOC) infrastructures

At CNR-IPSP-NA (Portici) we had available instruments for the detection of volatile organic compounds (VOCs):
-the mass spectrometer **PTR-Qi-TOF-MS** from Ionicon (Figure 1A);
-the mass spectrometer **Vocus-Ci-TOF** (Chemical Ionization Time-of-Flight Mass Spectrometer) from Tofwerk installed on June 2024 (Figure 1B);
- gas chromatography **GC system** from Aerodyne installed on July 2024. The GC is an accessory for the mass spectrometer Vocus-Ci-TOF, (Figure 1B).

The mass spectrometers available at CNR-IPSP-NA (Portici) measures gas-phase compounds in ambient air and headspace samples by using chemical ionization to produce positively charged molecules, which are detected with a time-of-flight (TOF). This ionization method uses a gentle proton transfer reaction method between the molecule of interest and protonated water, or hydronium ion (H3O+), to produce limited fragmentation of the parent molecule. If the proton affinity (PA) of the molecule in the sample is greater than the PA of H_2O, there will be a proton transfer and a detectable ion will be produced.
Some examples of molecules with PAs > H_2O are:

- alkenes such as C_3H_6
- alkanes such as n-Octane n-Decane and n-Dodecane
- cycloalkanes
- alkynes
- isoprene and atmospheric reaction products.

The PTR-MS consists of three main parts:
1. The ion source where the H_3O^+ ions are produced.
2. The drift tube where the air samples are introduced, and the analyte molecules are ionized by reaction
with H_3O^+.
3. The TOF mass detector where the ion signal is generated.

The mass spectrometers infrastructures available offer some several significant advantages for the plant phenotyping:
- not invasive and not destructive procedure (no sample preparation required);
- real-time analysis (on line) measurement of the VOC released from the samples of interest;
-untargeted monitoring of VOC emissions, by detecting the entire set of volatiles compounds emitted from the samples (volatilome);
-qualitative and quantitative analysis;
-high mass resolution at low detection limits;
-high sensitivity, with the possibility to detect trace amounts of VOCs, often at sub-parts-per-billion (ppb) or even parts-per-trillion (ppt) levels.
In addition, the advantages of the Vocus-Ci-TOF, in comparison to the PTR-TOF-MS, are that it allows the detection of a broader range of chemical compounds (i.e. volatile organic compounds and highly oxidized compounds) by using negative reagent as ions source.

On the other hand, one disadvantage of mass spectrometers is the limited capability to distinguish between isomers, but the presence of a GC, upstream to the Vocus, offers the possibility to discriminate and to identify individual compound even if they are similar in structure (structural isomers)

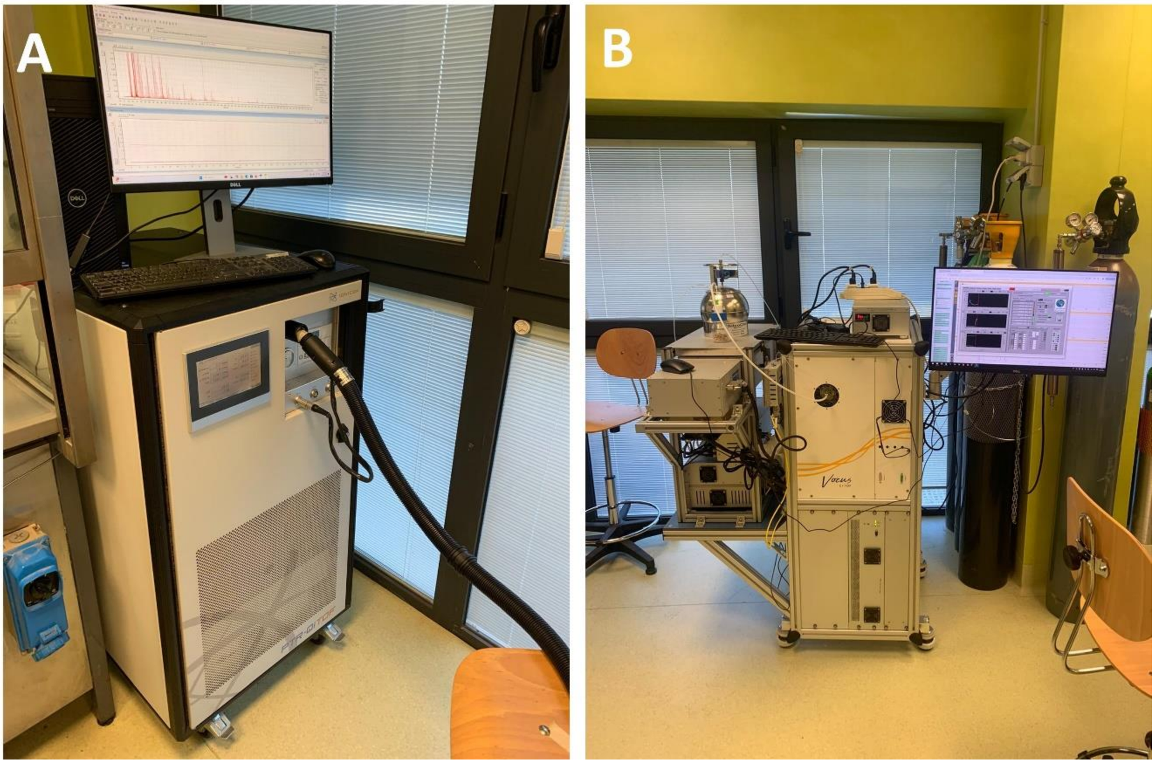

Figure 1. Infrastructures for the detection of VOC at CNR-IPSP NA (Portici). A) PTR-Qi-TOF-MS. B) Vocus Ci-TOF accessorized with the Aerodyne GC (mounted on the left of the Vocus-Ci-TOF).

3.2 Data acquisition

We use three software licensed by Ionicon for the settings of the PTR-Qi-TOF-MS: the PTR Manager, the TPS-Controller and TofDaq viewer. The PTR Manager allows to control the ion production in the drift tube and to monitor temperatures, flow and pressure controllers, turbo pumps, and the instrument's overall operational state. It also allows the controls regarding the data acquisition settings, including the averaging time and length of data records. The TPS-Controller, conversely, is used to control the settings of the TOF detector. Two software licensed by Tofwerk are used in the operation of Vocus.ci-Tof: Vocus Trak and TPS to control the settings regarding the ion source, the reaction chamber (drift tube) and TOF as well the length of the data records.
One software licensed by Aerodyne, GC-aerodyne, is used to control the setting for GC and to perform simultaneous measurements with Vocus-Ci-TOF.

3.3 Data collection

Our work was focused on the acquisition of phenotyping data that will be shared through the computational infrastructures under development within ITINERIS framework. In particular, we collected data about different case studies and from different matrices, including plant leaves, seeds to understand the effect of the environment on the emission of volatile compounds.

Case study 1: Tomato Plants and biotic stress.
We analyzed the volatilome profiles of tomato plants subjected to pathogen (fungus) attack by comparing the response of two genotypes, a mutant and a wild-type. We measured the VOC emitted from tomato leaves by analyzing four different conditions: no_primed_no_stress, Primed_no_Stress, Primed_stress; no_Primed_no_Stress, no_Primed_Stress for each genotype. We acquired data in two times: on March and on April 2024 in Portici (NA) (Figure 2).

Case study 2: Tomato Plants and abiotic stress.

a) We studied plant-plant communication in response to drought stress. Two tomato genotypes were used, including a wild-type and a mutant. Pre-stressed plants were used as emitter plants and four conditions were evaluated for each genotype: primed-stressed, primed-not stressed and not-primed not-stressed and not-primed stressed. The volatile emissions were evaluated from leaves and were measured in three temporal points: September 2023, October 2023 and November 2023. We performed the experiment in Metaponto (Basilicata region) (Figure 2).
b) We setted the experiment in Portici (NA). We studied the plant-plant interaction in response to drought stress by analyzing the effect of three different synthetic volatiles as priming VOCs. One experiment was setted in July, the other two experiments in October 2024 (Figure 2).

Case study 3: Seeds and germination.
We analyzed the change in volatile fingerprint different accessions of four different species of legumes (soy, pea, fenugreek and cicerchia) subjected to different treatments (hydropriming and artificial aging) affecting the seed germination. One treatment was related to see the effect of the water the second treatemt was related to see the effect to high temperature. Two accessions were analyzed for each legume species. The measurements were performed on April 2024 in Portici (NA) (Figure 2).

During May and June 2024 we were also involved in setting the VOC sampling protocol and workflow for data analysis for a dataset aimed to characterize the volatilome profiles of tomato fruits coming from a population composed of about 200 individuals. We also setted a sampling protocol for data of volatiles emitted from dispensers embedded with a specific volatile with the aim to set up a protocol to follow and quantify the concentration of the volatile along the time.

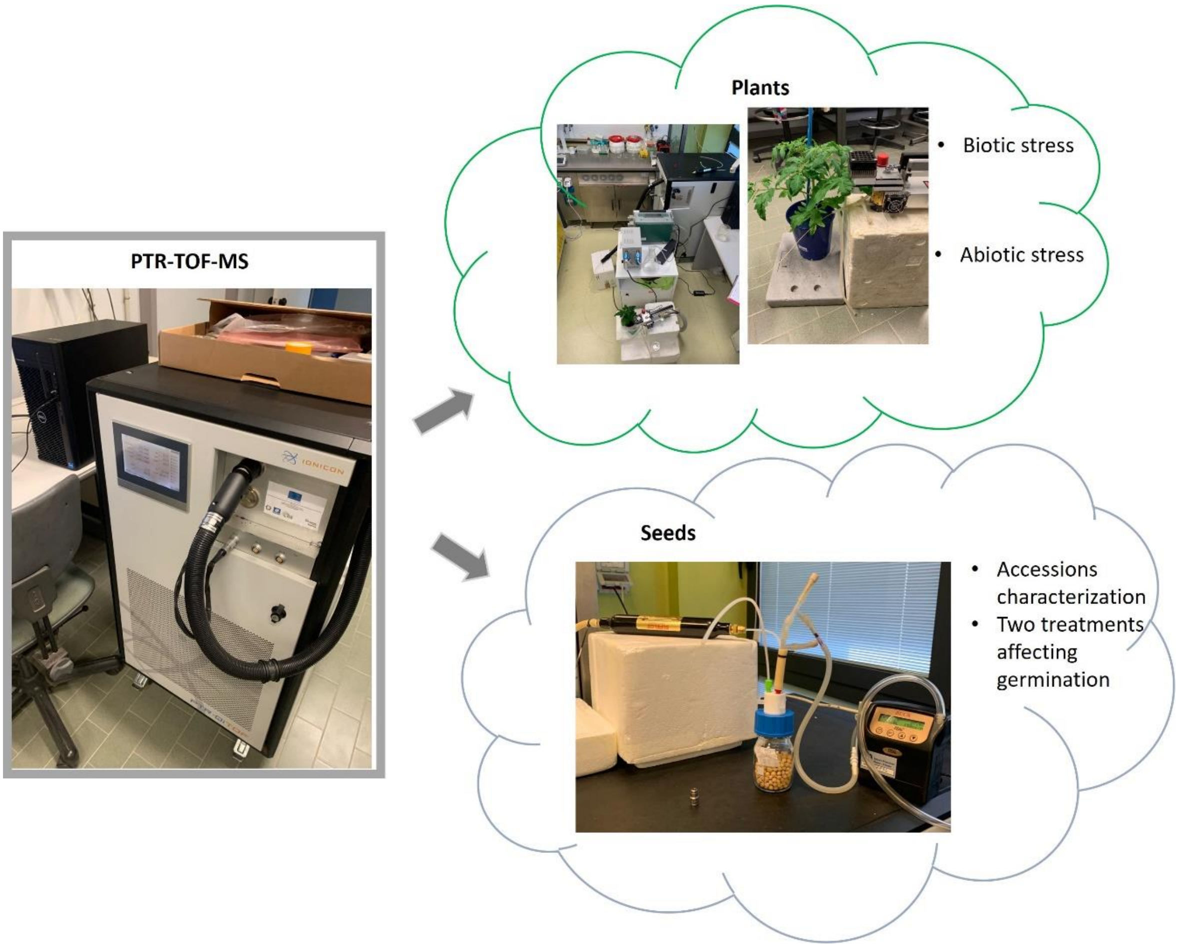

Figure 2. Images from the experiments on tomato leaves and legume seeds during the acquisition with PTR-Qi-TOF-MS.

3.4 Volatile Organic Compounds (VOCs) data pre-processing

The volatiles mass raw spectra acquired from each datasets were analyzed by using the following pipeline:

-mass calibration, to ensure an ion's exact mass reflects the mass identified by the instrument. We use a three-point mass calibration choosing masses covering from the lower to the higher range of the spectra.

- baseline correction and peak detection;

-peak alignment, to align the detected peaks across different samples;

-intensity normalization;

-quantification of volatiles organic compounds;

-putative identification of detected volatiles compounds by searching in literature and by consulting library ad hoc for mass spectrometers data;

The volatiles raw spectra pre-processed resulted in data matrices reporting the identification and quantification of VOCs as well as their putative annotation.

3.5 Volatile Organic Compounds (VOCs) statistical analysis.

The data matrix (rows = number of samples, columns = number of identified peaks) constructed after the pre-processing step was ready for statistical analysis. Thus, multivariate analysis and data mining methods were used for data exploration and visualization. Preliminary analysis was carried out by employing principal component analysis (PCA). HeatMap was used to visualize volatiles emission profiles along the conditions. The analysis of variance (Anova) was used to identify significant statistical differences among the conditions of interest.

3.6 Field plant phenotyping platform

The high-speed field phenotyping platform consists of a rover equipped with IMAGING fluorescence technology (FluorCam FC 1300-R, PSI) designed for the estimation of photosystem II (PSII) activity under field conditions. The platform is highly versatile and engineered for the rapid integration of additional digital sensors. These include, for example:

- Thermal and multispectral cameras, which measure foliage temperature and estimate indices based on visible and infrared spectra.
- A hyperspectral camera, enabling the detailed study of vegetation indices across a wide range of spectral bands.
- A LIDAR system, which facilitates the creation of three-dimensional vegetation models.

These tools collectively enable advanced monitoring and analysis of plant responses to abiotic stresses, making the platform a powerful asset for precision agriculture.

In addition, the platform incorporates the CIRAS-4 Portable CO_2/H_2O Gas Analysis System, a cutting-edge device capable of simultaneous analysis of gas exchange and chlorophyll fluorescence. This instrument serves as a "ground truth" tool, providing point-specific measurements that act as a reference standard for evaluating the accuracy of biometric and vegetation indices derived from proximal or remote sensing data.

Together, these two advanced technologies form a state-of-the-art research platform for high-throughput field phenotyping. This platform is currently employed at the Plant Phenomics Research Infrastructure of IPSP-Research Unit hosted by Metaponto (ALSIA-Centro di Ricerca Metapontum Agrobios, S.S. Jonica 106 Km 448.2, 75012 Bernalda, MT). It is actively utilized in the experimental fields managed by IPSP for ongoing phenotyping research activities.

The platform plays a pivotal role in supporting experimental studies in plant phenomics, with a particular emphasis on developing a comprehensive field phenotyping system tailored to meet the needs of digital agriculture. This innovative infrastructure is instrumental in advancing the precision and scope of phenotyping methodologies, facilitating breakthroughs in plant science and sustainable agricultural practices.

The integration of these advanced tools and technologies highlights its immense potential to enhance precision farming and deepen our understanding of plant responses to diverse environmental conditions. This includes investigating plant reactions to both biotic and abiotic stresses, with the ultimate goal of identifying resistance mechanisms, adaptation processes, and effective methods for plant protection.

The platform's activities span a broad range of objectives focused on the enhancement of plants with agricultural and forestry importance, including:

- Harnessing natural antagonisms and developing biocontrol methods for plant parasites and weeds.
- Optimizing water use efficiency and contributing to the restoration of degraded lands.
- Improving both the quality and quantity of agri-food production.
- Selecting and promoting valuable plant germplasm.
- Characterizing and producing bio-molecules of agro-industrial relevance.
- Mitigating the impacts of global climate change, while fostering sustainable and environmentally friendly agricultural growth.

A significant focus is placed on plant phenomics approaches to advance scientific understanding of fundamental plant biology and ecology. These studies aim to unravel the mechanisms that protect plants from environmental constraints and address the challenges posed by global change. By combining cutting-edge technology with ecological insights, this research contributes to the advancement of sustainable agricultural practices and the development of resilient plant systems for the future.

4. CONCLUSIONS

Plant phenotypic data, specifically the emission of volatile organic compounds (VOCs), was meticulously acquired from leaves subjected to biotic and abiotic stressors, as well as from seeds treated under high-temperature and water conditions. This valuable data will be made accessible through the ITINERIS Hub, fostering collaborative research and innovation. To achieve this, a robust plant phenotyping infrastructure for VOC detection was established, marked by the acquisition and installation of advanced instruments such as the Vocus-Ci-TOF mass spectrometer and a state-of-the-art gas chromatography system. These technologies have significantly bolstered the capability to identify and quantify plant-emitted VOCs, enhancing the understanding of plant responses to diverse stressors and treatment regimens.

Moreover, the integration of a high-speed field phenotyping platform represents a significant leap forward in agricultural research. This platform, equipped with cutting-edge technologies such as the FluorCam FC 1300-R imaging fluorescence system, thermal and multispectral cameras, hyperspectral imaging, LIDAR, and the CIRAS-4 Portable CO_2/H_2O Gas Analysis System, provides unparalleled precision in monitoring plant responses to environmental conditions. Collectively, these instruments enable the simultaneous measurement of photosystem II (PSII) activity, thermal and multispectral indices, three-dimensional vegetation structure, and gas exchange parameters.

The advanced capabilities of this platform are pivotal in elucidating plant responses to abiotic and biotic stress factors. By delivering high-throughput and high-resolution phenotypic data, the platform facilitates the identification of resistance mechanisms, adaptive traits, and efficient methods for plant protection. These insights drive innovations in precision farming, sustainable agriculture, and climate-resilient plant systems.

Beyond stress response studies, the phenotyping platform supports diverse objectives such as enhancing natural biocontrol strategies against parasites and weeds, improving water use efficiency, restoring degraded ecosystems, and optimizing agri-food quality and yield. The platform also plays a critical role in the selection and promotion of valuable plant germplasm, the production of agro-industrially relevant biomolecules, and the mitigation of global climate change impacts.

By merging cutting-edge phenotyping technology with ecological research, the infrastructure addresses fundamental questions in plant biology and ecology. It advances the scientific understanding needed to tackle challenges posed by global environmental changes while promoting the sustainable and environmentally conscious growth of agriculture and forestry sectors. These efforts align with a broader vision of integrating plant science with digital agriculture, paving the way for transformative advancements in food security, ecological restoration, and agricultural innovation.