**HEATSTOP PROJECT**

**Report 2nd year: November 2023 – November 2024**

**M1 – EFFECTIVE PROJECT MANAGING**

Both RUs are collaborating to the achievement of the first milestone, M1 – EFFECTIVE PROJECT MANAGING, that will be completed at the end of the project, as scheduled in the GANTT. A meeting took place on 16/10/2024 as a part of the A1.1: Administrative project coordination, during which the two groups planned the research activities envisaged by the HEATSTOP project. The financial matters are being addressed in compliance with MUR requirements, through a constant dialogue between the University administrations and the RUs. The two RUs are both independently and conjunctly addressing all the technical aspects of the project realisation, as part of the A1.2: Technical project coordination, that will be in place for the whole duration of the project, with a bimonthly evaluation of the progresses made, summarised in this report. As part of D1.2 Mid-term project review report, the unit coordinators are thrilled to be able to state that at the end of the current four-month period, including the fifth and sixth two-month periods, the activities and objectives achieved by HEATSTOP are in line with expectations, nevertheless the initial delay. All necessary precautions have been taken to respect the timeframes indicated in the GANTT, among these continuous exchanges of reflection between the members of the research units took place successfully. A mid-term project review report has been produced collecting all the information relative to the project to date.

**M4 – EVALUATION OF MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES IN POLLEN UNDER HS**

For the activity A4.1: Evaluation of pollen productivity in three time points after HS induction (BIM4), around 30 plants per rapeseed cultivar were cultivated from seed to the rosette stage during the third bimester, vernalized for 40 days from the third to the fourth bimester, and eventually grown in standard conditions until reaching the phenological stage GS61. At this stage, half of the individuals from one cultivar was randomly selected for the heat stress imposition (HS plants), while the others were assigned to the control group (CT plants). Only for one group, the treatment lasted ten days, in which CT plants remained in standard conditions, while HS plants underwent a gradual increment of temperature (+2.6°C per hour), from 22°C to 35°C, remained at 35°C for four hours, and then gradually returned to 22°C with a temperature decrease of -2.6°C per hour. An estimation of the total pollen productivity was conducted at the end of the flowering stage. While CT Dariot plants did not produced pollen due to its sensitivity to abiotic stress environment and this allowed us to focus the study on Phoenix variety. CT Phoenix produced on average more pollen (350.3 mg) than HS Phoenix (70 mg). Freshly and stressed collected pollen grains have also been photographed by UB at 20x e 100x magnification and measured with ImageJ to assess morphological differences in HS and CT pollen as part of A4.2: Determination of morphological and physiological signals of stress induction in pollen. For this activity, UB has also micrographed air-dried and rehydrated HS and CT pollen grains by Scanning Electron Microscopy (SEM), and it was set up an Environmental Scanning Electron Microscope (ESEM) protocol to observe the micromorphology of fresh and rehydrated pollen grains without altering their shape (D4.1: Spreadsheet with morphological and physiological data on pollen from ST and CT plants). To obtain a better visualization of the pollen grain, an ESEM (Thermo Fisher Scientific) was used. This microscope is known for its high resolution and its ability to observe fine structural details, even on complex biological samples. This type of microscope is particularly useful for studying surfaces and morphologies, thanks to its advanced technology that uses an electron beam to produce high-definition images.

Pollen samples from Phoenix control and stressed as well as Dariot control, fresh and hydrated, were comparatively analyzed. The grains were applied to a conductive carbon adhesive support, mounted on special aluminum stubs, and then inserted into the microscope chamber. The optimal parameters were set as follow: chamber pressure, electron beam intensity, and electron current. A GSED (Gaseous Secondary Electron Detector) was used to acquire the images, capable of capturing the signals of the secondary electrons emitted by the samples hit by the beam. In low-vacuum mode, the specific parameters to obtain images at different magnifications were as follows:

* Acceleration voltage (HV): 10,000 V
* Current: 0.34 nA
* Pressure: 1200 Pa
* Detector: GSED

These settings allowed for the capture of detailed images of the pollen morphology, highlighting the surface features with high precision. This third cohort allowed us to collect the necessary quantities of pollen to perform (A4.3: PS isolation) for CT Phoenix and HS Phoenix. Three subsamples of pollen for one group and treatment were rehydrated. The hydrated pollen (HP) germinated, and PS were isolated by differential centrifugation obtaining three fractions: intact germinated pollen grains (GP), PS, and EVs-free germination medium (EVF). This procedure was repeated for each activity that requires PS and EVF. A4.4: PS measurements: EVs present in the PS fraction were measured and quantified by Nano particle Tracking Analysis (NTA); visualized with the Zetaview and subsequently stained with FM4-64. Total proteins will be then isolated and quantified for all three fractions and immunoblotted to probe for EVs molecular markers.

**M5 – MIRNOME ANALYSIS**

The team of Tor Vergata RU (URT) has worked on the activity *A5.1: Isolation of MiRNA* with the aim to characterize the miRNome of hydrated pollen (HP), germinated pollen (GP), and pollensomes (PS) of the selected crop, both from stressed (HS) and control plants. Extracellular vesicles-free germination medium (EVF) was also analysed for the potential presence of miRNAs. The optimization of protocols achieved in the fourth bimester was useful to understand how to process the samples sent by the other RU, Alma Mater Studiorum University of Bologna (UB). In the highly specialized research areas for the analysis of biomolecules, miRNAs were purified from HP, GP, and PS, using the mirPremier microRNA Isolation Kit (Sigma-Aldrich, St. Louis, USA). In detail, pollen samples (20 mg each) were maintained at -80 °C. Then, they were crushed with liquid nitrogen and mortar directly in the Eppendorf and resuspended in the extraction buffer provided by the kit. PS, instead, were immersed in PBS 1X and directly subjected to the extraction. The steps applied subsequently were those described in the kit manufacturers’ instructions. Concentration and purity of the miRNA extracts were estimated by spectrophotometric analysis (NanoDrop 2000, Thermo-Fischer Scientific, USA). As scheduled in the GANTT, miRNAs isolation was completed within the sixth bimester. During the 6th bimester, the activity *A5.2 Library preparation and NGS analysis* started, setting the protocol for this specific step. The procedure will include the preparation of the indexed libraries with NEX SmallRNA Seq v3 (Perkin Elmer) according to the manufacturer’s instructions. Libraries will be quantified using the Tape Station 4200 (Agilent Technologies) and Qubit Fluorometer (Invitrogen Co., Carlsbad, CA) and pooled such that each index-tagged sample will be present in equimolar amounts for the sequencing, carried out using an Illumina Novaseq6000 System (Illumina) in a single-end format. This activity will end at the 8th bimonthly period; however, it is already clear from the analyses in progress that the possibility of obtaining significant results from all the samples (exposed to standard conditions or to heat stress, HS) is high, considering the quality of the extraction and the controls performed in the first phases of the protocol (*D5.1: Profiling of the miRNA spectra for each sample*).

**M6 - ANALYSIS OF THE PROTEOMIC PROFILE AND ENZYMATIC ACTIVITY OF PS AND EVF (BIM11)** that it is in the process of being planned.

**M7 – ANALYSIS OF THE LIPOPHILIC** **FRACTION**

This milestone consists in the characterization of the lipophilic profile of pollen secretome. After the chromatographic analysis (BIM7-9), the results will ascertain if lipophilic metabolites synthesized by pollen change between the first and the last stage of germination, and if specific compounds are accumulated in PS compared to those present in pollen. These following steps will aim at clarifying the release, and potential role, of lipophilic molecules in pollen-pistil interaction. Thus, during the 6th two-month period, for isolating the highest number of compounds, several organic solvents were tested on the samples, according to the literature and the expertise of the URT UR. The activity *(A7.1)* of *isolation of the lipophilic components* included, according to preliminary tests that the UR has performed, the resuspension of the samples in chloroform:methanol (2:1; *v:v*) for 20 minutes at room temperature and in the dark. Subsequently, the samples were centrifuged and the supernatant recovered and filtered. Initial screening biochemical analyses showed that hydrophobic molecular markers were successfully isolated from the samples (*D7.1: Report on the isolation of lipophilic metabolites from* *HP, GP, PS, and EVF for all samples*). In the next four months the investigation of the lipophilic fractions will be carried out by a GC-MS system.

**M9 – DISSEMINATION OF HEATSTOP MILESTONES AND RESULTS**

the whole project, as detailed in the GANTT. In particular, the teams are working on the activity A9.1: Dissemination to the public and the stakeholders. A website dedicated to the project (https://site.unibo.it/prinheatstop) was created by the two RUs on UniBo portal, depicting the milestones and the activities of the project, and portraying the team members. Social pages about the project have been created on Facebook, Instagram, and X platforms. (D9.1 Report on bimonthly visualizations and interactions generated by the website and the social networks). Currently, interactions have been minimal due to the low level of social media sponsorship.

The activities carried out in this two-month period did not cause damage (in accordance with the **DNSH principle**) to any of the six relevant environmental objectives (i.e., climate change mitigation, climate change adaptation, sustainable use and protection of water and marine resources, the circular economy including waste prevention and recycling, the prevention and reduction of pollution, the protection and restoration of biodiversity and ecosystems). Furthermore, the Project complies with the relevant EU and national environmental legislation and does not include so-called "brown" research activities in accordance with the EU Commission Communication 2021/C 58/01 "Technical guidance on the application of the DNSH principle".