LIFE FROSTDEFEND

LIFE20 CCA/GR/001747

DC1.1 Technical guide for sample collection

and measurement techniques

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NATIONAL CENTRE FOR SCIENTIFIC RESEARCH "DEMOKRITOS"
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Deliverable DC1.1

Technical guide for sample collection

Project Number	**LIFE20 CCA/GR/001747**
Project Title	**Forecasting and protecting fruit crops from frost damage**
Project Acronym	**LIFE FROSTDEFEND**
Action	**C1. Database construction of key frost event indicators**
Deliverable	**DC1.1 Technical guide for sample collection**
Beneficiary	**AUA**
Date	**12/2021**

Table of Contents

Summary

The objective of Action C1, "Database construction of key frost event indicators," is to construct a comprehensive database containing biological, atmospheric, meteorological, and phenological data, which will be used to derive the empirical forecast algorithm. Sample collection (filters and leaves) during the field campaigns is one of the most crucial tasks that must be carefully assessed before initiating the experimental procedure. Possible mishandling of the samples or misoperation of the samplers may result in incomplete databases. To minimize these risks, a technical guide was prepared and presented to the technical staff of ACUA. This document serves as a manual for sample collection and instrument operation, particularly for gravimetric samples for air filter sampling.

The Technical Guide for sample collection is a document of the LIFE FROSTDEFEND project, delivered in the context of the Action C1 - Database construction of key frost event indicators.

1 Sampling Protocol for Fresh Leaf Collection

1.1 Introduction

This procedure defines the methodology for the collection of representative fresh leaf samples from the pilot plots. Samples will be collected weekly from the plots and then sent to the Agricultural University of Athens for further analysis (quantification of bacterial population).

1.2 Instruments and Materials

- Zip-lock plastic dag
- Pruning scissor
- Sprayer containing 70% ethanol

1.3 Methodology

Each experimental plot will be sampled from four individual randomly selected trees (biological replicate) and new vegetation is being removed using an alcohol autoclaved pruner. Autoclaved pouches are filled with the removed vegetation of approximately 150 grams/ pouch and samples are being sent to the AUA using iceboxes and refrigerated transportation vehicles.

Steps to follow:

1. Use a 3L ziplock plastic bag to store the samples

2. Open the bag without touching the interior, place it over the tip of a shoot with leaves

3. Cut the shoot with an ethanol and flame-sterilized pruning scissor

4. Repeat the procedure once more with a sample from a different tree using the same bag; Each sample consists of two pooled sub-samples.

5. Seal the bag and place it into a container .

6. Repeat the procedure taking a second sample from 2 different trees in the orchard.

7. Ship samples within 6 hr to the AUA for further analysis.

Samples are being processed during the next 4 hours post sample collection following the procedure described in 3.

2 Sampling Protocol for Filter Sample Collection

2.1 Introduction

This procedure defines the methodology for the air filter sample collection as deployed in the field. Particulate matter (PM) and airborne bacteria are collected on a filter material for a 24 hr period, at a controlled flow rate, by means of custom made gravimetric samplers (Environmental Radioactivity Laboratory, NCSR-D), designed to fullfil the requirements according to CEN EN 12341.

The main features of the custom-made samplers are summarized below:

- Measurement of the temperature of the sampled filters directly inside the magazine

- Measurement of the Pressure (Pup) in the filter (Pressure drop)
- Measurement of the atmospheric Pressure (Patm)
- Data storage on memory card
- Impactor inlets with exchangeable jets (PM10)
- Use of filters with diameters of 47 mm
- Two valve
- One screen: Siemens

2.2 Technical Characteristics

Inlets and flow rate

- PM10 Inlet for flow rate of 2,3 m³/h (CEN EN 12341)
- Flow rate deviation from the set point: < 2 %
- Sampling time minimum 1 h – maximum 96 h per filter
- Silicone grease and cleaning materia

Power supply

- Two valves, 230-240V, 50/60Hz
- Two general switches for choosing the side of sampling: SVN411, 230V, 16A
- Fuse for filter change SBN125, 25A

- ***Consumption***

- approx. 450 VA (2.3-m³) resp.
- Filter diameter 47 mm Diameter of active filter area approx. 40 mm

- ***Dimensions***

- Width 482 mm
- Depth 310 mm
- Height with inlet 1,58 m
- Weight approx. 80 kg
- Noise level according to DIN 2058
- Pressure sensor: MEAS

- ***Filter material***

- Nuclepore Track-Etch Mebrane
- Pore size 1.0 µm
- Filter diameter 47 mm

- **2.3 Methodology**

Below the routine procedure to follow before and after filter sampling is described.

- ***Preparation of filter holders***

Filters and filter holders should always be handled with care to avoid possible contamination. The assembled filter holders and filters are sterilized in an autoclave prior to installation in the air sampler.

- Clean the filter holders if necessary using ethanol;
- Check if the O-rings are still in good shape, else replace.
- Place the filters in the appropriate holders.
- Place the filters holders in the transport ziplock plastic bags.

Figure 1: a) Filter holder, b) filter holder and filter cassete, and c) filter material (Nuclepore) for PM_{10} sampling

- ***Clean the PM10 head***

Before operating, the sampling head must undergo periodic maintenance. Remove accumulated PM from the primary deposition area in the inlet with brush or ethanol and dry. Using a general-purpose cleaner and paper towel, clean the internal wall surface of the PM10 head. Then, apply a thick layer of silicone grease on the impaction surface of the PM10 head to minimize the particle bounce off effect.

- ***Installation of the filter holders***

The sampler can take two filter holders (Figure 2a,b), one on each side (Figure 2c), positions 1) and can be programmed to automatically change the filter after the pre-selected sampling time e.g. 24 or 48 hours. However, for the purposes of the project only one side will be operated and the sampler will be manually stopped and started by the operator.

Figure 2: Flow direction control sytem

To connect the sample holder with the system, the lower point of the holder needs to be connected with the valves (**Figure 2, positions 1**). The upper point of the sample holder needs to be connected to the sample inlet. Thus the air flow passes from the inlet

through the filter holder. The air flow is generated by the pump that is connected in position 2, Figure 2.

During sampling both filters holders must be connected to positions 1, Figure 2. Sampling takes place from one of the two possible positions 1, Figure 2 (right and left) at each time e.g. if the right valve is open the left valve is closed, and the sampling takes place in the right filter holder; at the next sampling day the opposite happens.

Please note that the tubing that connects the inlet with the filter holders should be kept as short as possible to minimize sample losses.

- ***Installing the pump***

The pump needs to be connected using suitable tubing (air tight that can withstand the pressure that is created) with position 2, Figure 2. In Figure 3 a possible way of connecting the pump to the rest of the system is presented.

Figure 3: Pump for air sampling

- ***Flow rate***

The flow rates are controlled by means of a temperature – and pressure - compensated orifice plate flow meter according to Bernouli's law and by conversion into operating – m^3/h resp. standard- m^3/h (Nm^3/h:273K and 1013mbar) according to the law of Boyle-Mariotte. The deviation of the controller is <2% from the set point under field operating conditions. The pressure behind the filter (Pup) and the ambient pressure (Pamb) are stored in the memory card; From the pressure drop created, the volume flow rate can be calculated.

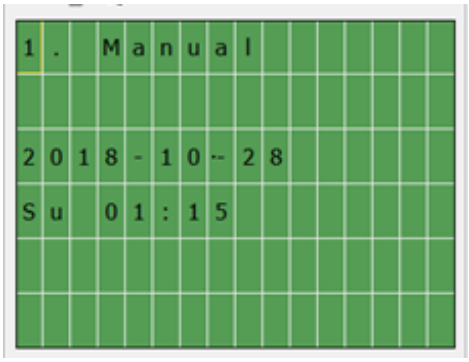
Once every three months the flow rate has to be measured by means of a certified flow meter. The flow meter has to be connected in between the filter holder and the inlet of the pump.

- ***Programming the sampler***

On the display the following parameters are shown during sampling:

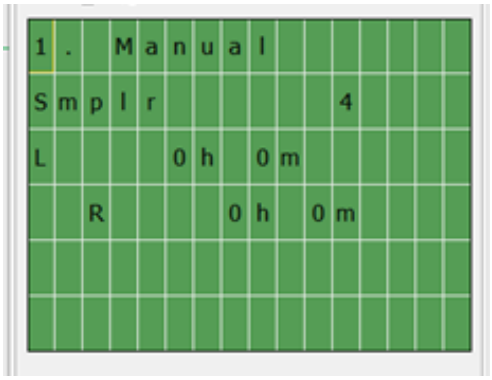
First screen: Selected Program and Date

In this screen the sampling protocol (Programm) that is selected (first line), the date (third line) and time (forth line) and the sampling cycle day are presented.



Second screen: Sampling Side

Describes the side of sampling (L for left and R for Right) and the duration of sampling for each side. In Figure a case that a sampling of 0 hours and minutes for both sides has taken place is presented.

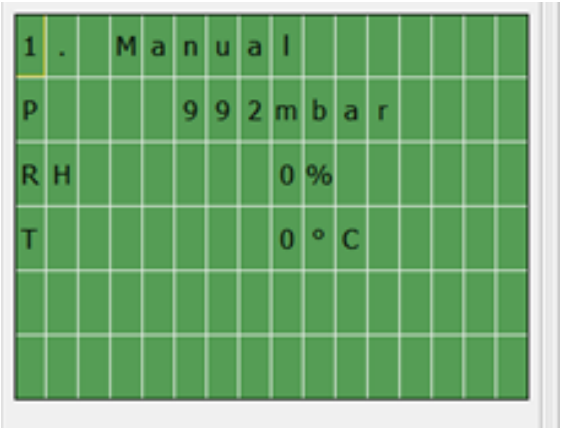


Third screen:

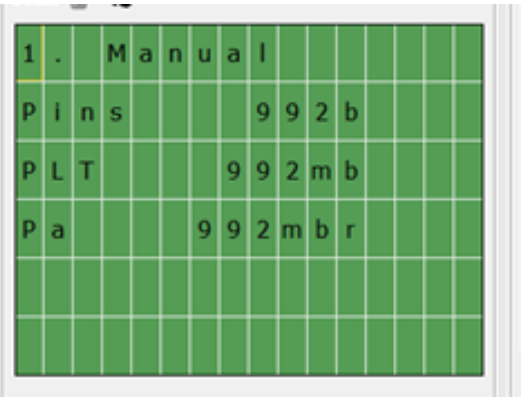
First line: Pressure in the filter (mbar)

Second line: Relative Humidity % (RH) (humidity sensors are optional and not included in every version of the sampler)

Third line: Ambient temperature (T)



Fourth screen:



First line: Sampling Programm

Second line: Pressure in the filter (mbar)

Third line: Pressure Leak Test

Forth line: Atmospheric Pressure (mbar)

Sampling protocol: Manual

For starting the operation of the system and Program selection for the first time, the buttons L and R need to be simultaneously pressed for more than 5 seconds. By doing this action Screen 1 (Programm menu) appears (Figure 4).

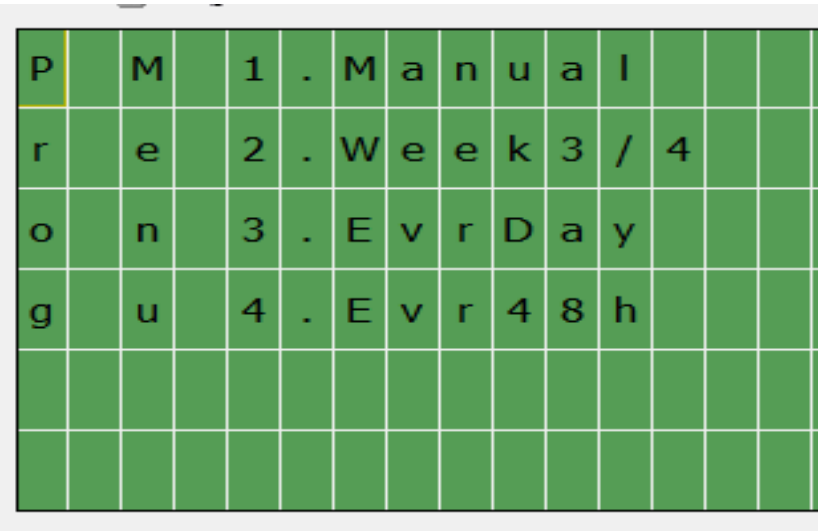


Figure 4: Screen 1. Programm menu

To select a programm, one of the two buttons (L and R) be pressed (Figure 5). Each time one of the two buttons is pressed twice a different programm is selected.

Figure 5: Control board

The screens that represent each Programm are presented in Figure 6.

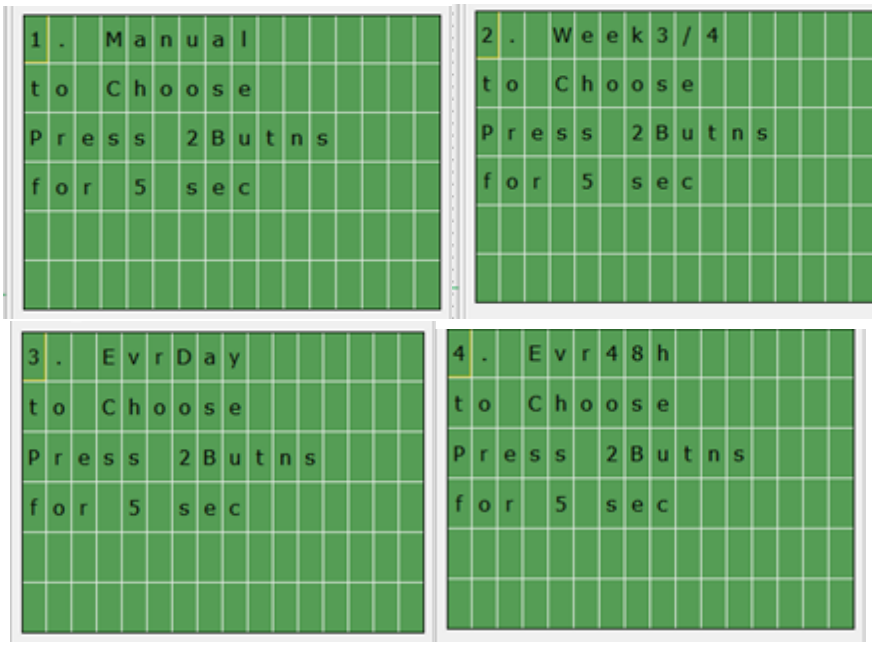


Figure 6: Screen 2 Programms

Program "Manual"

This Program is not referring to a certain sampling protocol and it is defined by the user. You select the side of sampling and the hours (**24 hr**). Sampling starts when the switch "F/P change" is open and the side that will be used for sampling is selected with L (Left) and R (right) buttons. A red light turns on at the side the sampling will initiate at the "side sampling" (Figure 5). Figure 7 presents the different screens during the operation

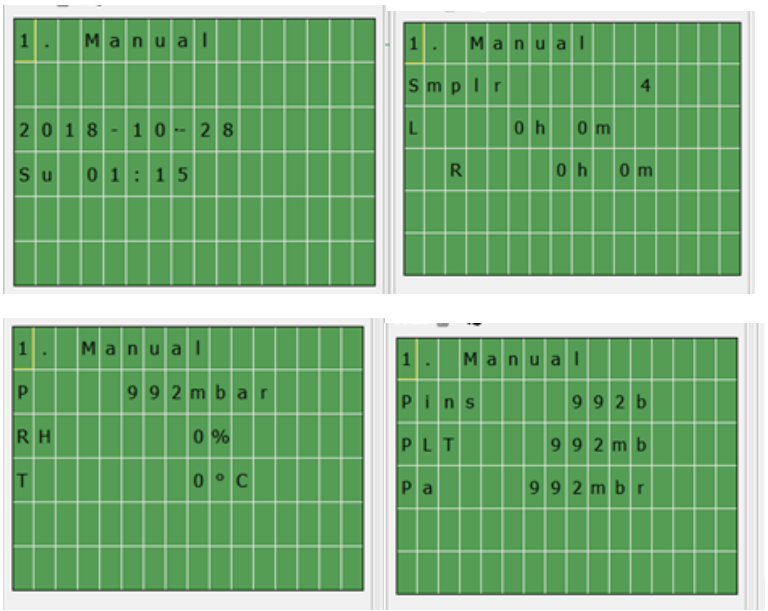


Figure 7: Different Screens that are referring to the Program “Manual”.

- ***Download data***

In order to read the stored data or change the Micro SD card we have to stop the sampler from running. **Warning: If the following procedure will not be followed the card will not be recognised and no data will be logged after this time!**

Step 1. Stop the program

Press the down button from the LOGO module.and you will get the screen below:

Then press the ESC button and you will ge to the screen below:

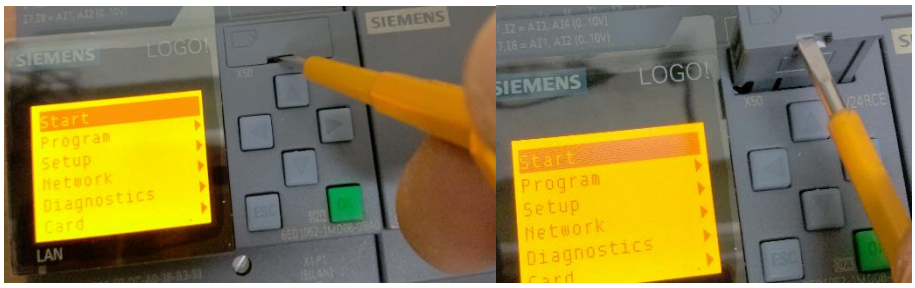
Then press ok button and you will get the screen below after you choose yes

Then press ok and you will get the screen below:

Step 2. Remove the Micro SD card

You can now remove the microSD card with the following procedure:

Use a screwdriver very carefully to take out the card drawer.



Then you can remove the card from the left side:

Each time the sampler is stopped by this procedure, a new data file is created in the memory card. When the data is downloaded the card needs to be put back. Using the

arrows the option “start” needs to be selected. The program that was selected before the stop is restored. To continue sampling the “F/P change” switch is turned on.

- ***Documentation and forms***

The following information has to be recorded before (start) and after (stop) sampler operation

- Name of operator
- Model/serial number of the sampler
- Date, time and Location
- Place
- Notes/Comments
- Filter code

ΕΚΕΦΕ ΔΗΜΟΚΡΙΤΟΣ

ΙΝΣΤΙΤΟΥΤΟ ΠΥΡΗΝΙΚΩΝ & ΡΑΔΙΟΛΟΓΙΚΩΝ ΕΠΙΣΤΗΜΩΝ & ΤΕΧΝΟΛΟΓΙΑΣ, ΕΝΕΡΓΕΙΑΣ & ΑΣΦΑΛΕΙΑΣ

ΕΡΓΑΣΤΗΡΙΟ ΡΑΔΙΕΝΕΡΓΕΙΑΣ ΠΕΡΙΒΑΛΛΟΝΤΟΣ

ΠΡΩΤΟΚΟΛΛΟ ΔΕΙΓΜΑΤΟΛΗΨΙΑΣ	Αρ. Έκδοσης: 1,1	Σελ. 1 από 2

Sampler: Custom 3
Τοποθεσία Δειγματοληψίας:
Υπεύθυνος Δειγματοληψίας:

Filter code	Date/ time start	Date/ time end	Duration	Pup start	Pup end	RH%	To C	Patm	Patm end	side	notes

Δ-10-ΕΝ-2

Pup, Pamb, RH% and T are monitored and stored in the memory card.

3 Quantification of the total bacterial population and ice-nucleation activity

3.1 Introduction

The protocols in this section describe the procedure for analysis of leaf and air filter samples from all experimental plots (commercial and high-altitude) in a lab.

3.2 Microbiological instruments and materials

- Laminar flow hood
- Autoclave
- 20-200-1000 μL pipettes
- Sterile pipette tips
- Sterile eppendorf tubes (1,8 or 2 mL)
- Vacuum pump
- Water filtration unit (or similar)
- Cooling bath with ability to set for ramp temperatures from 0 to -12°C
- Paraphin-coated aluminum foil or pan with flat surface for ice nucleation assays
- Sterile water
- Nutrient Agar microbiological medium (commercially available)
- King's B microbiological medium containing proteose peptone No3, glycerol, dipotassium hydrogen phosphate, magnesium sulfate heptahydrate, and bacteriological agar
- Standard plastic petri dishes (sterile)3.2.2 Additional instruments and materials for molecular biology
- Quantitative PCR unit
- Minifuge for 1,8 or 2 mL Eppendorf tubes
- Nanodrop device for estimation of DNA concentration and purity
- DNA extraction kit for environmental samples (DNeasy Power Soil, Qiagen)
- Quantitative PCR kit (commercially available)
- DNA electrophoresis tray and power supply
- Agarose
- Tris base
- Boric Acid
- NaOH
- EDTA
- Ethidium bromide

Figures 8 and 9 show the main equipment available at AUA for microbiological analyses.

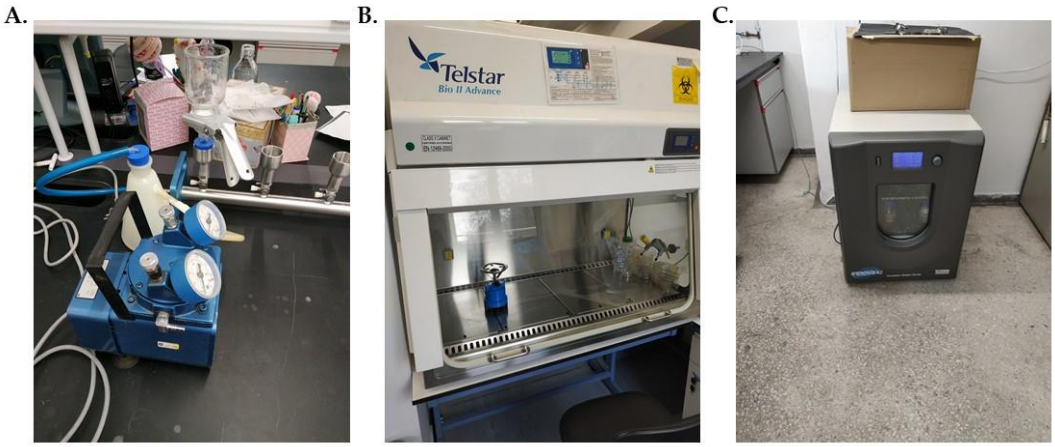


Figure 8. A. Vacuum pumps, **B.** Biological safety cabinets, **C.** Microbiological incubators.

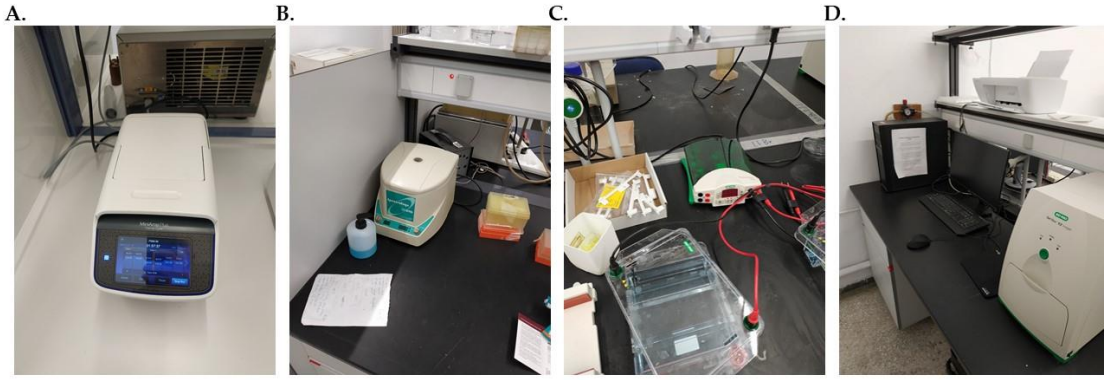


Figure 9. PCR machines, **B.** Microcentrifuges, **C.** DNA electrophoresis apparatus, **D.** DNA imaging.

3.3 Methodology

3.3.1 Microbiological analysis and ice nucleation assay

Upon arrival at the AUA, samples of leaves (3 biological replications) and filters from air samplers undergo weighing and aseptic removal from the collection bags and air sampler cartridges, respectively. Subsequently, they are transferred to sterilized bottles and filled with autoclaved water containing 0.05% v/v Tween-20. The filled bottles are then moved to rotary shakers to eliminate epiphytic material through a washing process. The washing procedure is conducted at 100 rpm for 30 minutes. Following the wash, the material is subjected to vacuum filtration using an autoclaved Nuclepore filter with a pore size of 0,45 μm, designed explicitly for microbiological applications. The filtered material is then re-suspended in 1 ml of sterilized double-distilled water to enable subsequent analyses.

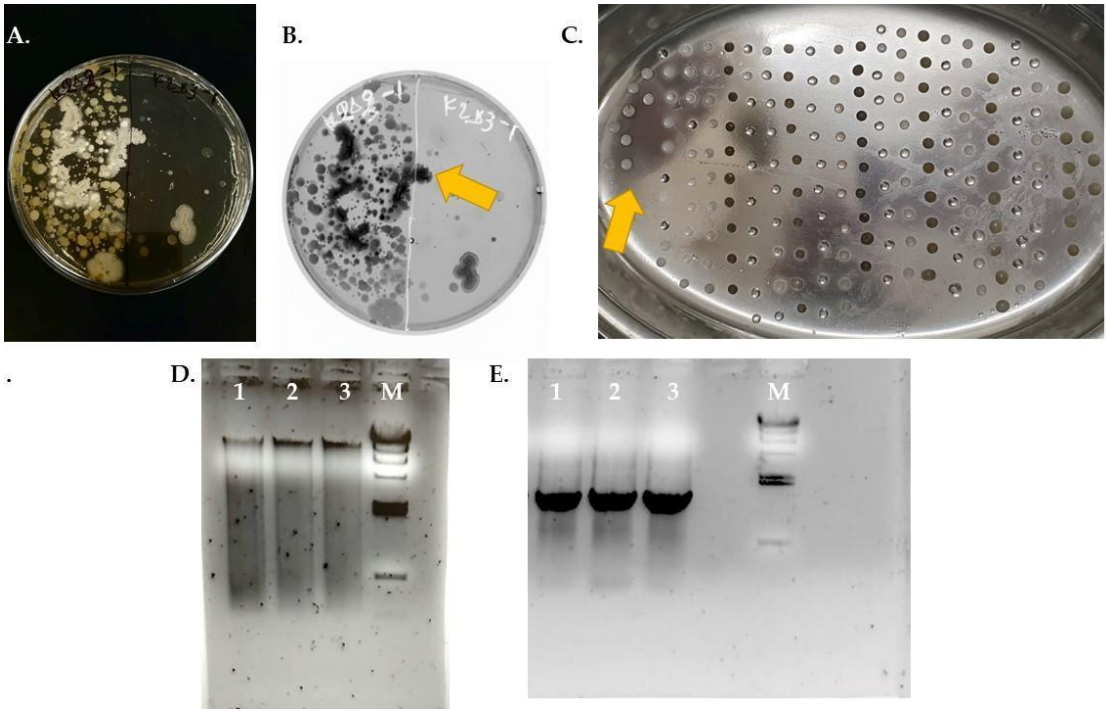


Figure 10: A. Routine microbiological analyses of epiphytic populations using King's B medium. **B.** Same analysis using UV imaging techniques to measure fluorescent bacterial colonies. **C.** Ice-nucleation assays performed in aluminum plates coated with 1% paraffin in xylene submerged in a water bath set at -6°C. **D.** Genomic DNA purification from concentrated epiphytic washates. **E.** 16S RNA PCR analysis of genomic DNA isolated from different samples of epiphytic washates.

Air filters are directly re-suspended in 0,5 ml sterilized double distilled water upon laboratory arrival to perform further analyses. 100 μl of each sample is used for

microbiological analyses in King's B and nutrient agar media to estimate the total epiphytic and fluorescent bacterial population. A series of 10-fold serial dilutions of leaf-washing concentrates is performed in sterile Eppendorf tubes containing 900 μL of sterile double-distilled water. From each dilution, 100 μL is spread onto one Nutrient Agar (for total culturable bacteria) and one King's B (Sands et al., 1980) (for fluorescent pseudomonads) plate. Plates are incubated at 28°C and colonies are counted after 48 to 72 hours . King's B plates are also examined under a UV lamp to visualize the typical fluorescent colonies of *Pseudomonas* sp.

Furthermore, 100 μL of each dilution is used for estimating ice-nucleation activity with Vali's droplet freezing method (reference) in a cooling bath set at -6°C: from each dilution, ten 10-μL droplets are placed on the surface of a paraphing-coated aluminum pan. Sterile distilled water control is also used. The pan is then placed on the surface of the cooling bath and the number of frozen droplets from each dilution is visually recorded after 3 minutes.

Using this protocol we were able to estimate: a) the average fluctuation of the total bacterial population (Figure 10A), b) the average fluctuation of the total fluorescent bacterial population (Figure 10B) and the average ice-nucleation activity of the compounds (biotic and abiotic) washed material from the leaves and the air filters (Figures 10C, D).

3.3.2 Molecular biology methods

DNA is extracted from all leaf and filter washates. After the microbiological analysis and the ice nucleation assay, the remaining quantity of leaf washates from all three biological replications are pooled. DNA extraction is performed using the Qiagen PowerSoil DNA extraction kit, according to instructions included. DNA concentration and purity is estimated in a Nanodrop device.

Quantitative PCR designated for ice nucleation genes of bacteria is performed in all samples according to Hill et al. (2014), as modified by AUA. The primer set of 3341fb and 3462r1, and the KAPA SYBR® FAST qPCR Kit were selected for 20μl reactions. Each reaction mixture contained 2μl of Template DNA, 0,3μl of the primer set, 0,4μl of High ROX 50X, 7μl of PCR-grade water and 10μl of 2X KAPA SYBR® FASTqPCR Master Mix. Cycling conditions were an initial denaturation at 95°C for 2 min, followed by 43 cycles of 94°C for 15 s, annealing and extension at 54°C for 30 s and 79°C for 3s, respectively, and then hold at 95°C for 1 s before signal acquisition.

Amplification is performed on the "Applied Biosystems QuantStudio 5 Real-Time PCR System" instrument. Two to four replicate reactions were used. Products were

confirmed by electrophoresis in 1.5% agarose gels in 1x sodium borate buffer (20x stock contained 47 g/liter boric acid and 8 g/liter NaOH) (to give a pH of 8.2) at 200 V for up to 35 min, using ethidium bromide for visualization. A 50-bp ladder was used for sizing. Some products were sequenced to identify the principal INA allele in the amplicon. Conveniently, there is a direct correlation between the number of INA genes in a sample and the number of INA bacterial cells.

For qPCR standards, we use DNA extracted from *Ps. syringae* Cit7 cells using the same kit as previously, from known numbers of cells (enumerated by dilution plating). This method, coupled with the ice nucleation assay, gives an accurate count of ice nucleation active bacteria in samples.

Routine microbiological analyses, ice-nucleation assays, genomic DNA extraction and 16S RNA PCR analyses are presented in Figure 8. With our standardized protocol we could isolate quantities of genomic DNA from epiphytic material (Figure D) sufficient to be used in PCR analyses (Figure E) for further metagenomic sequencing.

Metagenomic sequencing of DNA is performed on selected samples, those with high ice nucleation activity, at regular intervals during winter time (once or twice per month) and at longer intervals throughout the rest of the year.

References

1 CEN EN 12341: Ambient air - Standard gravimetric measurement method for the determination of the PM10 or PM2,5 mass concentration of suspended particulate matter.

2 Hill, T.C.; Moffett, B.F.; DeMott, P.J.; Georgakopoulos, D.G.; Stump, W.L.; Franc, G.D. Measurement of ice nucleation-active bacteria on plants and in precipitation by quantitative PCR. Appl. Environ. Microbiol. 2014, 80, 1256–1267.

3 Sands, D.C.; Schroth, M.N.; Hildebrand, D.C. Genus Pseudomonas in Laboratory Guide for Identification of Plant Pathogenic Bacteria; Schaad, N.W., Ed.; Bacteriology Committee of the American Phytopathological Society: St. Paul, MN, USA, 1980; pp. 36–44.

4 Möhler, O., Adams, M., Lacher, L., Vogel, F., Nadolny, J., Ullrich, R., Boffo, C., Pfeuffer, T., Hobl, A., Weiß, M., Vepuri, H. S. K., Hiranuma, N., and Murray, B. J.: The Portable Ice Nucleation Experiment (PINE): a new online instrument for laboratory studies and automated long-term field observations of ice-nucleating particles, Atmos. Meas. Tech., 14, 1143–1166, https://doi.org/10.5194/amt-14-1143-Vali, G. Quantitative Evaluation of Experimental Results and the Heterogeneous Freezing Nucleation of Supercooled Liquids. J.Atmos. Sci. 1971, 28, 402–409.

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