ASTER

Agroecology-inspired Strategies and Tools to Enhance Resilience and ecosystem services in Tomato crop

DELIVERABLE N.	**1. 1**
DELIVERABLE NAME	**Common Protocols for Research Activities (CPRA)**
DELIVERY DATE	**31 July 2022**
PARTICIPANT	**P1**
RESPONSIBLE	**EMILIO GUERRIERI**
DISSEMINATION LEVEL	**PUBLIC**

ASTER
Agroecology-inspired Strategies and Tools to Enhance Resilience and ecosystem services in Tomato crop

CHARACTERIZATION OF SOIL MICROBIAL BIODIVERSITY

- **Soil sampling.** If it is possible, it would be desirable to choose fields previously cultivated with tomato plants and managed both conventionally and agro-ecologically. Soil samples will be collected before planting (T0)(task 2.1), at flowering (T1), at fruiting (T2) and at harvest (T3) (task 2.2).
  At T0, based on the field size, 1 sampling point/1000 m^2 will be collected at 20-30 cm depth (e.g. if field size is 5000 m^2, five sampling points will be identified using a W or Z scheme). Anyway, if some partners will use fields of smaller dimensions, soil sampling methods will be made according to W or Z scheme in order to have a sufficient number of samples for the analyses. With all the collected soil samples a pool will be made and then 5 sub-samples will be prepared and analyzed for the study of biodiversity (culturable bacteria, AMF spores, etc. and soil biodiversity by pyrosequencing using Illumina platform).
- **Selection and propagation of microorganisms.** Each partner will select the best microorganisms (AMF and/or PGPR) to be employed for the field experimentation, by previous pot experiments in semi-controlled conditions (without soil sterilization). These microorganisms could be newly isolated from field soils or already available from the partner' collections. Selected bacteria will be characterized in UNIUPO and USMBA laboratories for their PGPR traits (phosphate solubilization, siderophore production, antibiotics resistance, IAA production, ability to grow at different temperatures, resistance to drought stress). Bacteria and fungi showing the more interesting ability in supporting plant growth will be maintained and propagated according to the used protocols by the different partners.

P2 UNIUPO PROTOCOLS for task 2.1

Soil microbiota: Protocol for AM fungi

(from Cesaro et al., 2020; 2021 doi: 10.3389/fmicb.2021.676610)

DNA will be extracted using the Power Soil R DNA Isolation Kit (MO BIO) following the manufacturer's instructions. Then, DNA will be employed as template for a hemi-nested PCR using LR1 and FLR2primers for the first amplification and LR1 and FLR4 primers tagged with Multiplex Identifier sequences for ILLUMINA Pyrosequencing for the second one. In particular, the FLR2

primer used in the first reaction is specific for fungi, while the FLR4 primer used in the second reaction is specific for Glomeromycota.

The reactions will be performed in a Techne thermocycler at the following conditions: initial denaturation 94 °C for 5 min; then 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min or 5 min (for the first and the second amplification, respectively) for 30 cycles; finally, an elongation step at 72 °C for 5 min. Each reaction mixture (20 µl) will be contained five microliters of genomic DNA diluted at different levels (1:10, 1:50, 1:100 - for the first amplification) or the first PCR products diluted 1:100 and 1:500 (for the second amplification), dNTPs 500 µM, MgCl2 1.5 mM, 2 µl PCR buffer 10×, 500 nM of both forward and reverse primers and 0.4 U of Taq DNA Polymerase.

The products of the second PCR (size 700-bp) will be used for pyrosequencing (Illumina analyses – this last part of the protocol has not yet been fully finalized for fungi)

Soil microbiota: Protocol for bacteria (P2)

(from Bona et al., 2021 doi.org/10.3390/microorganisms9071359)

The genomic analysis of the soil microbiome will be performed on soil samples. The genomic DNA will be extracted using the DNeasy® PowerSoil® Kit (Qiagen, Milan, Italy), starting from 0.25 g of soil following the manufacturer's instructions and quantified by a fluorometric method according to the Qubit® 4.0 Fluorimeter protocol. The preparation of the bacterial 16S DNA libraries will be performed using the Microbiota solution B kit (hypervariable regions V3-V6) provided by Arrow Diagnostics srl. (Genoa, Italy), according to the manufacturer's instructions. The amplicon pool will be processed using the MS-103-1003-MiSeq Reagent Nano Kit v2 (500-cycles) kit, supplied by Illumina Inc., using the Phix as internal standard. Bioinformatic analysis workflow is proposed in this paper by the authors for the first time. Obtained raw sequences were processed with the new software MicrobAT (Microbiota Analysis Tool) v. 1.1.0 provided by UPO-SpinOff (SmartSeq srl, Novara, Italy). The software specifies the Phylum, Class, Order, Family, Genus and Species of the bacteria found in the samples and provides reports of the user-selected comparisons. MicrobAT is based on the RDP database, and it does not produce OTUs (operational taxonomic units). In particular, obtained sequences, after being filtered for length and quality (data quality evaluation), will be aligned against the RDP database and assigned to a specific species if they meet the following criteria: query coverage ≥80% and similarity ≥97%. From MicrobAT three files can be generated, which will be used for statistical analyses regarding variations within the bacterial communities using the Microbiome-Analyst software (Comprehensive Statistical, Visual, and Meta-Analysis of Microbiome data; https://www.microbiomeanalyst.ca, accessed on 13 May 2021). Before data analysis, a data integrity check was performed.

First data filtering will be used in order to identify and remove features that are unlikely to be useful when modeling the data. Features having low count and variance can be removed during the filtration step while those having very few counts are filtered based on their abundance levels (minimum

counts 10) across samples (prevalence). Data rarefaction and scaling based methods deal with uneven sequencing depths by bringing samples to the same scale for comparison.

Biodiversity indices (from Bona et al., 2021 doi.org/10.3390/microorganisms9071359)

Alpha diversity is characterized by the total number of observed species (richness) and by Shannon and Simpson indexes that, along with the number of species (richness), consider also the abundance of organisms (evenness) to describe the actual diversity of a community. Alpha diversity analysis will be performed using the phyloseq package. The results will be plotted across samples and reviewed as box plots for each considered group (plant species, sampling site and climatic zone).

Beta diversity analysis is used to compare the diversity of composition between the sampled bacterial communities. This method compares the changes in the presence/absence or abundance of the present species and summarizes these into how 'similar' or 'dissimilar' the samples are. Each sample gets compared to every other sample generating a distance matrix. The distance between samples will be measured using Bray-Curtis distance and Principal Coordinate Analysis (PCoA) will be used to visualize these matrices in 2 plot where each point represents the entire microbiome of a single sample.

Plant growth promoting trait of bacteria

(from Massa et al., 2020, doi.org/10.1016/j.apsoil.2020.103507)

Siderophore production will be assessed on universal Chrome Azurol S (CAS) agar (Schwyn and Neilands, 1987). The bacterial strains will be inoculated at the center of each plate and incubated at 28 °C for seven days. Siderophore production will be indicated by a halo of color change from blue to orange on the CAS medium and was measured in triplicate as the ratio between the two diameters of the halo and the two diameters of the colony.

Phosphate solubilization will be evaluated according to Goldstein (1986), using two different media: one containing dicalcium phosphate (DCP) (NH_4Cl 4.25 g L^{-1}, NaCl 0.85 g L^{-1}, $MgSO_4$ $7H_2O$ 0.85 g L^{-1}, glucose 8.5 g L^{-1}, K_2HPO_4 2 g L^{-1}, $CaCl_2$ $2H_2O$ 4 g l−1, agar 17 g L^{-1}) and one containing tricalcium phosphate (TCP) (NH4Cl 5 g l−1, NaCl 1 g l−1, $MgSO_4$ 7 H_2O 1 g L^{-1}, glucose 10 g L^{-1}, Ca3(PO4)2 40 g l−1, agar 20 g l−1). The strains will be inoculated in the center of each plate and incubated at 28 °C for 15 days. DCP solubilization was indicated by a clarification halo around the colony and TCP solubilization will be identified by colony growth on the medium.

Indole-3-acetic acid (IAA) production was quantified according to De Brito Alvarez et al. (1995). The bacterial strains were inoculated on nitrocellulose disc placed on TSA, containing 10% of L-tryptophan (5 mM), and incubated at 28 °C for three days. The membrane was then stained with the Salkowsky's reagent (FeCl3 2% in perchloric acid 35%): the development of a red/pink halo around

the colony indicated a positive reaction. The bacterial inocula were produced using an overnight culture on TSA medium, suspended in MgSO4 0.1 M and adjusted to 108 CFU/ml after optical density assessment (λ 600 nm).

Intrinsic antibiotic resistance will be determined using disc diffusion method, swabbing the bacterial suspensions on solid YMA medium, putting the antibiotic discs in the middle of the plates and incubating them at 28 °C for 48 h. The sensitivity to the following antibiotics will be tested: ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), kanamycin (30 µg), neomycin (30 µg), rifampycin (30 µg) and streptomycin (10 µg)

(from Novello et al., 2022 - doi.org/10.3390/microorganisms10050890)

ACC assay will be performed on bacterial strains in order to test their 1-aminocyclopropane- 1-carboxylic acid (ACC) deaminase capability using the method described by Penrose and Glick. Briefly, bacterial cells were grown for 24 h at 28 C in tryptic soy broth (TSB medium) and then in Dworkin and Foster (DF) salts minimal medium added of 45 µL 0.5M ACC (Sigma Aldrich, Milan, Italy) solution. The bacteria were first washed with 0.1M Tris-HCl, pH 7.6, suspended in 0.1 M Tris-HCl, pH 8.5, and then in toluene (Sigma Aldrich, Milan, Italy). An aliquot of the cells treated with toluene was stored for protein assay. The remaining aliquot was incubated at 30 C for 15 min in the presence or the absence of ACC. A total of 1 ml of 0.56 M HCl will be added to the suspension and centrifuged at 16,000x g at room temperature for 10 min. Then, 1 ml of the supernatant will be vortexed together with 800 µL of 0.56 M HCl and 300 µL of the 2,4-dinitrophenylhydrazine reagent. After 30 min of incubation at 30 C, 2 mL 2 N NaOH will be added, and the absorbance of the mixture was measured at 540 nm. Bacteria able to synthesize ACC deaminase and to produce alpha-ketobutyrate and its mutant, unable to synthesize this enzyme, will be used as positive and negative controls, respectively.

Qualitative screening of enzymes (P12, P13)

Enzymatic activity will be evaluated by measuring the halo of hydrolysis zone around the disc inoculated as follows: _ (no change): isolates showing no enzyme activity, + (halo zone less than 10 mm): isolates showing very low enzyme activity, ++ (halo zone of 10-30 mm): isolates showing low enzyme activity, +++ (halo zone of 40-60 mm):isolates showing high enzyme activity, ++++ (halo zone of 70 mm and above): isolates showing very high enzyme activity.

Phosphate solubilization

Phosphate solubilization will be screened qualitatively according to the method described by Gupta et al. (1994) on the solid medium Pikovskaya agar containing tricalcium phosphate as insoluble inorganic phosphorus source. Assay will be based on inoculated 6mm agar disc cut from 5 day-old fungal culture of each strain into medium and incubated at 26±2oC. Clear zone around fungal colony

indicated positive solubilization of mineral phosphate (Nooriand Saud 2012).The solubilization index (IS) will be calculated by the ratio of the total diameter (halo+colony) and the colony diameter (Premono et al.1996).

Pectinase production:

Pectinase screening will be carried out on solid medium according to the method described by Hankin and Anagnostakis (1975). Pectinase Agar Medium containing: (Citrus Pectin 10g, $(NH_4)2HPO_4$ 3g, MgSO4 0.1g, Agar 20g, Distilled water 1000 ml), pH will be adjusted to 5.5. After three days of incubation, the plates will be flooded with iodine potassium –iodine solution (Iodine 1g, Potassium iodine 5g in 330 ml distilled water). A clear zone around the colonies indicates positive pectinase activity (Gueyeet al.2018).

Amylase production

Amylase production will be determined by using amylase detection medium (Abe et al. 2015) (Yeast extract 3 g, Peptone 5 g, Amidon soluble 2 g, Agar 20 g, Distilled water 1000 mL). After incubation at 28ºC for 72 hours, the plates will be flooded with 5 ml of iodine solution (1% KI; 0.5% I_2 (v/v)) for 15 minutes. Amylase production will be assessed by the formation of a light yellow halo around colonies.

Lipase production

Qualitative lipase production will be evaluated on culture medium containing Tween 20 (polyoxyethylene-sorbitan-monolaurate) as a lipid substrate (Abe et al. 2015). Medium used containing (Tween20 10 mL, Peptone 10 g, NaCl 5g, $CaCl_2 \cdot 2H_2O$ 0.1g, Agar 20 g, Distilled water 1000 mL). Tween 20 will be autoclaved separately and added to the sterile medium. Discs of 6 mm of Trichoderma cut from 5 day-old fungal culture of each isolate will be inoculated in the center of the medium and incubated at 26 ± 2°C for 5 days.The presence of opaque precipitate or an opaque white halo around the colonies refers to the production of lipases.

Screening for qualitative PGPF activity of Trichoderma species

Hydrogen cyanide (HCN) production

HCN production will be screened qualitatively according to the method of Bakker and Schipper (1987). Trichoderma isolates will be inoculated on solid medium containing Tryptic Soya Agar (TSA) supplemented with 4.4 g/L of glycine. A Whatman filter paper impregnated by alkaline picric acid solution (2.5 g of picric acid; 12.5 g of Na_2CO_3; 1000 mL of distilled water) will be placed under the top cover of each plate. The plates will be incubated at 26±2°C for 7 days. A change in the filter paper color from yellow to light brown, brown or reddish brown implies the production of HCN (MeeraandBalabaskar2012).

Nitrogen fixation activity

In order to evaluate nitrogen-fixing ability, medium lacking nitrogen (KH_2PO_4 0.2 g, $CaCO_3$ 5 g, $MgSO_4 \cdot 7\ H_2O$ 0.2 g, NaC1 0.3 g, Mannitol 10 g, $CaSO_4 \cdot 2H_2O$ 0.1 g, agar 20 g and 1000 mL of distilled water (pH 7.2) will be used. Discs of 6 mm from pure culture will be inoculated in the center of a Petri dish containing nitrogen-fixing medium. After 3 days of inoculation, test of nitrogen-fixing ability will be considered positive if the colony can grow normally on the selective medium (Zhang et al. 2017).

Ammonia production

Production of ammonia will be tested in peptone water according to the method of Bakker and Schipper (1987). Broth culture containing 10 ml peptone water will be inoculated and incubated at 28oC for 72 h. Later, 1mL of Nessler's reagent will be added to each tube. Development of color from yellow to brownish orange was recorded as a positive test for ammonia production.

Siderophore production

Siderophores production will be evaluated according to the method of Hoyos-Carvajal et al. (2009) on agar medium containing 8-hydroxyquinoline (50 mg L^{-1}) as chelator. Growth of strains tested on this medium after 5 days of incubation at 26±2°C will be recorded as a positive result for siderophores production.

Quantitative estimation of gibberellins and indole-3-acetic acid (IAA) by the colorimetric assay

- ***Dosage of gibberellins***

Isolates will be screened for gibberellic acid production on medium containing (Yeast extract 5g, Sucrose 30g, Sodium nitrate 30g, Magnesium sulphate 0.5g, Potassium chloride 0.5g, Ferrous sulphate 0.01g, Dipotassium hydrogen phosphate 1g). Each isolate will be grown on this broth medium for 5 days at 28 ºC. After incubation period, the fermented media will be centrifuged, and the supernatant will be used. The gibberellins will be estimated calorimetrically by standard method (Holbrook et al. 1961; Sharma et al. 2018). 2 mL of zinc acetate reagent (21.9g zinc acetate + 1 mL of glacial acetic acid and volume will be made upto 100 mLwith distilled water) will be added to 15 mL of supernatant. After 2 minutes, 2 mL of potassium ferrocyanide (10.6% in distilled water) will be added and centrifuged at low speed (2000 rpm) for 15 minutes. To 5 mL of supernatant 5 mL of 30 per cent HCl will be added and mixture will be incubated at 20oC for 75 min. For blank 5 mL of 5 per cent HCl will be used. Absorbance will be read at 254 nm and the concentration of gibberellins will be calculated by preparing standard curve by using gibberellic acid as standard.

- ***Dosage of indole acetic acid (IAA)***

Isolates will be screened for their efficient ability to produce Indole Acetic Acid (IAA) using L-tryptophan as precursor according to the method of Bric et al. (1991). The essays will be done in liquid media, which will be supplemented with 1 g L-1 of L-tryptophan. The culture will be incubated in a shaker with 150 rpm at 28°C. After 3 days of incubation, production will be qualitatively estimated by colorimetric assay. The filtrates were centrifuged and then 2 mL of each supernatant will be added

to 2 mL of Salkowski reagent (1mL of ferric chloride (0.5M) will be added to 50mL of perchloric acid (35 %). The development of a pink or red color revealed the IAA production; the absorbance will be measured at 530 nm using a spectrophotometer (ZUZI spectrophotometer model 4201/50).

Extraction of IAA

Since isolate showing high IAA production, they will be subject to liquid-liquid extraction of IAA. Fermentation was performed in liquid medium supplemented with 1 g L^{-1} L-tryptophan. Extraction will be carried out according to the method of Mohite (2013) with few modifications. After three days of fermentation, the fermentation broth will be centrifuged at 9000 rpm for 15 min at 4°C. The supernatant obtained will be acidified and adjusted to pH 2.5. The supernatant will be collected and mixed with ethyl acetate (1:2). The funnel containing ethyl acetate and supernatant will be vigorously shaken several times. Then, organic phase was separated from aqueous phase. The supernatant will be extracted three times with ethyl acetate. The organic layers will be combined and evaporated by a Rotavapeur at 50°C with a slight rotation. Extracts containing IAA, will be filtered through a 0.22 µm Millipore membrane and stored at −20 °C for high-performance liquid chromatography (HPLC) analysis.

Thin layer chromatography (TLC)of IAA

TLC slide will be prepared with silica gel G and calcium carbonate with a solvent system containing Propanol: Water (8:2). The extracted sample and standard IAA (10mg100mL-1) will be spotted on TLC plate. Chromatogram will be developed with the Salkowski's reagent (Chunget al. 2003). Rf value calculated as the ratio between the distance run by the compound and distance run by the solvent front.

Quantification of IAA by high-performance liquid chromatography

The production of IAA will be analyzed by HPLC (Agilent 1200), with a C18 column (4.6×150 mm). Solvent A (methanol) and solvent B (water with 1 % (v/v) acetic acid) (A/B=60:40) at a flow rate of 1 mL min^{-1}. IAA was separated at 254nm-280nm byUV-VIS detector and a column temperature of 30°C. A volume of 20 µL was injected for each analysis. IAA levels were quantified based on a standard curve first established in the same conditions.

P4 ISA-PT, protocols for task 2.1

Trichoderma (P4, P11)

(from Mistry and Bariya 2022). Isolation and Identification of *Trichoderma* Spp. from different agricultural samples.

1. For the isolation of Trichoderma, soil samples can be taken from the depth of 15–20 cm.

2. Collect soil sample in sterile sample collection container by using sterile spatula.

3. Sample must be brought immediately to laboratory and stored at 4 °C until further use.

4. Suspend 1 g soil sample into 9 mL of sterile distilled water contained in a conical flask to gain 10^{-1} soil suspension.

5. Then gently shake soil suspension for 5–10 min.

6. Use sterile distilled water to obtain 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} dilutions of soil suspension (Fig. 1).

7. Inoculate 100 µL soil suspensions of each dilution into sterile petri plates containing agar media (RBA or TSM).

8. Spread the inoculum with the help of sterile glass spreader.

9. Incubate all the plates at 28 ± 1 °C in BOD incubator for 5–7 days.

10. Transfer Trichoderma colonies onto fresh PDA plates to obtain pure culture.

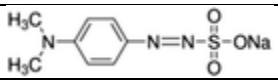
Table 1 Composition of Rose Bengal agar (RBA)

Sr. No.	Compound	Formula	Concentration (g/L)
1	Potassium dihydrogen phosphate	KH_2PO_4	1.0
2	Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	0.5
3	Peptone		5.0
4	Dextrose	$C_6H_{12}O_6$	10.0
5	Rose Bengal		0.35
6	Agar		15.0

Make up to 1000 mL with distilled water

Table 2 Composition of Trichoderma selective media (TSM)

Sr. No.	Compound	Formula	Concentration (g/L)
1	Dipotassium hydrogen orthophosphate	K_2HPO_4	0.9
2	Magnesium sulfate	$MgSO_4 \cdot 7H_2O$	0.2
3	Potassium chloride	KCl	0.15
4	Ammonium nitrate	NH_4NO_3	1.0
5	Dextrose	$C_6H_{12}O_6$	3.0
6	Rose Bengal		0.15
7	Agar		20.0
8	Chloramphenicol	$C_{11}H_{12}Cl_2N_2O_5$	0.25

9	p-dimethylaminobenzenediazo sodium sulfonate		0.3
10	Pentachloronitrobenzene	$NO_2C_6H_4Cl$	0.2

Add 960 mL of distilled water

AMF morphological charachterization (P4, P11)

Extraction of Spores (https://invam.wvu.edu/methods/spores/spore-extraction)

In all cases (including field soils), it is critical that a representative sampling of roots is included in the sample because some species in all genera except *Gigaspora* produce intraradical spores.

The sample is placed in water in a Waring Blender and blended at high speed for approximately five seconds (alternatively soil and root clumps can be ground in a mortar). The purpose of this step is to break up root fragments and release spores and also to separate spores from hyphal aggregates attached to roots or in the soil (especially those of species with thick-walled hyphae). Longer blending times do not affect spores, but can break up roots to the extent that more fine organic detritus likely will accompany spores in the final extraction prep.

The blended material is immediately poured through two sieves. Most sand remains in the blender. The bottom sieve has openings of 38, 45, or 53 µm (any of these three works well for most species, although there are some small hyaline glomoid species that require the 38 µm sieve). It captures the majority of spores. At our institute, the sieves used are of 140 and 50 µm respectively.

The top sieve generally has 500 µm openings. It captures roots, large debris, and really large spores or sporocarps. Despite the amount of organic material that might be present, spores are large enough to be easily detected after the material from this sieve is transferred to a petri dish.

Generally, we do not process material in the top sieve through a centrifugation step because of the amount of organic detritus. It is washed, transferred to a large petri dish, and viewed through a stereomicroscope just to verify that no spores are present. The material on the bottom sieve is collected in a 50-mL beaker with a rubber policeman and then transferred into 50-mL tubes containing a 20/60% gradient of table sugar (because its cheap) and water. These tubes are centrifuged (approx. 960 x g) for 2-3 minutes in a swinging-bucket rotor in a tabletop centrifuge. At the end of the run, soil and other sense particulates are pelleted in the bottom of the tube. Spores and fine organic detritus is suspended in sucrose (now somewhat blended).

The supernatant in each tube is quickly decanted into smaller sieves—either commercial stainless steel ones made by Tyler or homemade units of two types: (i) made from plastic vials with base removed and nylon mesh held in place by the plastic cap (with center removed using a heated cork borer) and (ii) nylon mesh glued to the rim of thick-walled PVC pipe.

The material collected on these smaller sieves is washed for 1-2 minutes under tapwater and transferred to a glass petri dish. Spores are collected manually using an extruded 9-inch glass pipette to separate from organic material.

Once this is done, they can be stored at 4°C for up to 30 days (checking weekly for parasitized spores which then are immediately removed).

The final (and most laborious) step is to separate spores of each morphotype (to mount and preserve, inoculate plants, extract DNA, perform germination assays, etc.). Some taxonomic expertise is needed here, but if the spores are in good condition, then anyone with observation skills can make the separations and initiate monospecific cultures. Identification to species can be another matter. Inoculated plants (leek, maize or sorghum) will yield enough fungal material for inoculation/analysis after six months.

Beauveria

Selective medium (https://doi.org/10.1186/s41938-018-0054-z)

The selective DOC2-PDA medium for EPF was prepared [0.2 g CuCl2, 2 mg crystal violet, 39 g PDA (Potato Dextrose Agar), 1000 ml distilled water, pH 4.0 with HCl] according to Shin et al. (2010). A 0.2 g of soil sample was placed in a 1.5 ml micro tube with 1.3 ml of 0.02% Tween-80 solution and was vortexed for 15 min. The resulting suspension was serially diluted (10−1) and plated on DOC2-PDA medium. After incubation for 6 days at 25 °C, the putative EPF were selected by morphological characteristics (aspects of the colonies, such as color, diameter, and mycelia texture). The fungus, B. bassiana was identified mainly based on the morphological characteristics of reproductive structures according to the taxonomical key of Samson et al. (1988). Baiting using *Tenebrio molitor* and *Galleria mellonella* (https://doi.org/10.3897/mycokeys.38.26970)

EFFECT OF BENEFICIAL MICROBES ON TOMATO NUTRITION (P15)

- **Field Experiment**: the selected microorganisms (max 3 different treatments) will be applied in the field on tomato plants, choosing the cultivar generally planted by farmers, in order to assess the main plant growth parameters, photosynthetic performance and mineral uptake.

During the experimentation, the rhizosphere soil and tomato roots will be collected at flowering (T1), at fruiting (T2) and at harvest (T3), to study the microbiota associated with tomato roots and to assess the AMF root colonization, respectively.

Mycorrhizal degree in tomato root (P2, P11 can also measure this parameter)

Sixty randomly chosen 1 cm long root pieces from each plant will be clarified in 10% KOH in a water bath at 60 C for 20 min. Then, the samples were stained with 1% lactic blue (methyl blue 1% in lactic acid). The excess dye was removed with a series of lactic acid washes. Finally, the samples were stored at 4 °C for 24 h in lactic acid. The following day, the obtained samples were mounted on a slide and observed under an optical microscope: two slides for each plant were prepared (30 root pieces for each slide). According to Trouvelot et al. (1986), each root piece was attributed to a class based on the mycorrhizal.

For this parameter, INAT will use the method of MGonigle et al., 1990.

Plant growth and physiological parameters

Plant biomass: For each plant, fresh weights (FW) of root and shoot will be recorded at harvest. Then, each shoot and root system will be dried in oven at 60◦C for three days and dry weight was (DW) recorded (UNIUPO, USMBA, INAT).

Leaf chlorophyll and total carotenoid concentrations (UNIUPO, INAT) Chlorophyll a and b and carotenoid concentrations will be determined according to Porra et al. 2002 using a 0.1–0.5 nm resolution range spectrophotometer. Briefly, 0.02 g of fresh leaves from each plant were kept in the dark at 4 C in N, N-dimethylformamide (1.5 mL) for a week, i.e., until complete pigment extraction. The concentrations of chlorophylls and carotenoids were evaluated spectrophotometrically using the following wavelengths: 663.8 nm, 646.8 nm, and 480 nm.

Chlorophyll pigments content (USMBA)***:*** The chlorophyll content in leaves will be determined using the DMSO method as described by Burnison (1980). To this end, sliced 20 mg of leaf tissue (flag leaf) was placed in a vial containing 7 mL DMSO. Mixtures were incubated in a glass tube at 65 °C with regular shaking intervals for at least 60 minutes (tissues became colorless). Absorbance was read at two wavelengths 663 and 645 nm, using a UV-VIS spectrophotometer. The content of chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Chl T) was calculated using the following equations. The content of each phlorophyll fraction (Chl a, Chl b, and Chl T) was then expressed as mg per g of leaf fresh weight (FW).

Chl a (mg/L) = (0.0127 × A663) - (0.00269 × A645)

Chl b (mg/L) = (0.0229 × A645) - (0.00468 × A663)

Chl T (mg/L) = (0.0202 × A645) + (0.00802 × A663)

Chlorophyll concentration will be analyzed by INAT according to Pompelli et al., 2012.

SPAD index (USMBA, INAT): Chlorophyll content was measured using Chlorophyll meter SPAD-502plus (SPAD KONICA MINOLTA, made in Japan)(Arenas-Corraliza et al., 2019).

Photosynthetic activity (in vivo measurements of the Chl a fluorescence by Quantum yield of dark-adapted leaves (Fv/Fm) was measured with chlorophyll fluorometer (OS-30p, opti-sciences USMBA**)** or Handy-PEA (UNIUPO).

Element Concentrations by ICP-OES/ICP-MS/ICP-AES Analysis in different plant organs (UNIUPO, USMBA, INAT)

The dried shoot and root samples of each plant were finely powdered for ICP–OES/ICP-AES analysis and digested following the method of Ene-Obong et al. with some modifications, i.e., 5 mL of 65% nitric acid (HNO3) were added to 0.1 g of dried material. The obtained samples were digested in a CEM MARS microwave (CEM corporation, Cologno al Serio, BG, Italy). An inductively coupled plasma with mass spectrometry (ICP-MS) X Series II system (Thermo Electron Corporation,Waltham, USA) were used for the different mineral analysis

Leaf total soluble sugars content (TSS) (USMBA)

The extract of total soluble sugars (TSS) was prepared following the method of Erice et al. (2007). Briefly, 100 mg fresh leaf was mixed with 5 mL of phosphate potassium buffer (50 mM, pH 7.5) and centrifuged 15 min at 10000 rpm. The supernatant was used as the extract of TSS.

TSS content was measured using the method of Yemm and Willis. ('1954). Briefly, 0.1 mL of supernatant was added to the anthrone reagent, and the mixture was heated at 90 °C for 10 min, and the absorbance at 625 nm (Spectrophotometer JASCO V-730, made in Japan). TSS was measured using a standard curve of D-glucose.

Soluble proteins content (USMBA)

Leaf sample (200 mg) was homogenated on ice in 2 mL of refrigerated phosphate sodium buffer (100 mM, pH 7.5). The supernatant taken after centrifugation (8000 rpm/15 min) was considered as proteins extract. Soluble proteins content was determined photometrically (Spectrophotometer JASCO V-730, made in Japan) following the method described by Lowry et al. (1951) using a standard curve of Bovine Serum Albumine (BSA).

Relative water content (RWC) (USMBA)

Leaves Relative Water Content (RWC) was estimated following the Weatherlay's method (Barrs and Weatherley, 1962) using formula:

$$\text{RWC}\ (\%) = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgor weight} - \text{Dry weight}} \times 100$$

Electrolyte leakage (EL) (USMBA)

Electrolyte leakage (EL) was evaluated using the technique of Szalai et al. (1996)**.** Briefly, leaf samples were emerged in distilled water overnight at room temperature, then, electrical conductivity was noted (EC1). The samples were incubated at 95 °C for 10 min and the electrical conductivity was measured (EC2). EL was calculated using the formula:

$$EL\% = (EC1/EC2)*100$$

Proline content (USMBA)

Proline content was evaluated following the method described by Bates et al. (1973). Briefly, 200 mg of leaf sample was homogenized with 4 mL of sulfoscalicylic acid (3%) and centrifuged at 10000 rpm for 10 min. 2 mL of supernatant was mixed with 2 mL of glacial acetic acid and 2 mL of Ninhydrin reagent was added to the mixture. The set was incubated 1 h at 100 °C. Proline was extracted in toluene and the absorbance was measured at 520 nm. Proline content was measured using a standard curve of L-proline.

Hydrogen peroxide (H_2O_2) content and lipid peroxidation (USMBA)

250 mg of leaf sample was homogenized with 5 mL of Trichloroacetic acid (TCA) 0.1%. The supernatant was taken after centrifugation (10000 rpm/5 min) and considered as the extract of H_2O_2 and malondialdehyde (MDA).

To quantify H_2O_2 content, 0.2 mL of extract was added to 0.8 mL of phosphate potassium buffer (10 mM, pH7), then, 1 mL of KI (1M) was added to the mixture. The set was incubated 10 min at room temperature, and the absorbance was measured at 390 nm (Spectrophotometer JASCO V-730,

made in Japan). H_2O_2 content was measured using a standard curve of H_2O_2 (Jana and Choudhuri, 1981).

Lipids peroxidation was evaluated by quantifying leaf MDA content. It was estimated using the method of Heath and Packer. (1968). Briefly, 1 mL of the extract was added to 4 mL 20% TCA containing Thiobarbituric acid 0.5 %. The set was heated at 95 °C for 10 min. After centrifugation (10000 rpm /5 min), the absorbance was noted at 532 nm and 600 nm (Spectrophotometer JASCO V-730, made in Japan). MDA content was estimated using the extinction coefficient of MDA at 532 nm which is 155 $mM^{-1}.cm^{-1}$.

Antioxidant enzyme activities (USMBA)

Around 500 mg of leaves samples was ground with 4 mL of 50 mM phosphate potassium buffer (pH 7) in an ice-cold mortar. The mixture was centrifuged at 20 000 rpm / 30 min at 4 °C. the supernatant was considered as the enzymes extract.

Catalase (CAT; EC 1.11.1.6) activity was measured photometrically following the method described by Beers and Sizer. (1952). Briefly, the reaction was started by adding 50 µL of enzymes extract to the assay solution containing phosphate potassium buffer (50 mM, pH 7) and H_2O_2 (10 mM), the mixture was incubated 5 min at room temperature and the absorbance was measured at 240 nm, CAT activity was calculated using molar extinction coefficient ε=43.6 $M^{-1}.cm^{-1}$.

Ascorbate peroxidase (APX; EC 1.11.1.11) was determined photometrically using the method of Nakano and Asada. (1981). Briefly, the reaction was started by adding 50 µL of enzymes solution to the assay solution containing phosphate potassium buffer (50 mM, pH 7), H_2O_2 (1 mM), EDTA (0,2 mM), and ascorbic acid (0,5 mM), after 1 min at room temperature, the absorbance was measured at 290 nm. APX activity was calculated using molar extinction coefficient ε=2.8 $mM^{-1}.cm^{-1}$.

Guiacol peroxidase (POX; EC 1.11.1.7)) activity was determined according to the method of Ubarnek et al. (1991). The reaction mixture contained phosphate potassium buffer (50 mM, pH 7), EDTA (0.1 µM), Guaiacol (5 mM), and hydrogen peroxide (15 mM). the assay was started by adding 50 µL of enzymes crud to 2 mL of assay solution. The absorbance was measured at 470 nm after 1 min of the incubation at room temperature. The activity of POX was calculated from the rate of formation of guaiacol dehydrogenation product (GDHP) using the extinction coefficient of 26.6 mmol/L/cm,

TESTING THE EFFECT OF BENEFICIAL MICROBES ON TOMATO PROTECTION

(P1, P4, P6, P8, P9, P11, P13, P14, P15)

The effect of root colonization on plant protection will target abiotic and biotic stresses.

DROUGHT

In a set of 100 plants 50 will be “colonized” by selected symbionts and 50 will act as controls. In each group of 50 plants, 25 will be used controls irrigated (NS) and maintained in a well-watered state (at container capacity). The remaining 25 plants were subjected to a WS treatment. For the WS treatment, irrigation was withheld about 6 to 7 weeks after fungal inoculation, and severe WS was achieved in about 3 weeks (petiole water potential less than 21 MPa). To allow the collection

of data from a sufficient number of replicates, five randomly chosen NS plants and five WS plants for each condition were subjected to the physiological measurements. The experiment was repeated 5 times using 10 plants, five NS and five WS, for each condition (50 plants total).

PESTS

Targeted pests include: aphids *Tuta absoluta, Bemisia tabaci, Tetranychus urticae, Aculops lycopersici, Phenacoccus solenopsis*

Direct protection against these pests will be assessed by calculating the development and fecundity rates of 20-50 female individuals (e.g. intrinsic rate of increase).

For aphids, *B. tabaci* and *T. absoluta* indirect protection in terms of attraction of natural antagonists will be assessed in wind tunnel and olfactometer bioassay followed by the collection and characterization of Volatile Organic Compounds by headspace air entrainment and GC/MS.

PATHOGENS

The development of selected pathogens on colonized plants will follow the sampling procedure as indicated below.

CHARACTERIZING INSECT BIODIVERSITY TARGETING HYMENOPTERA

ASTER
Agroecology-inspired Strategies and Tools to Enhance Resilience and ecosystem services in Tomato crop

Objective

Assessing the level of aboveground biodiversity in tomato crops by targeting the hyper-diverse group of Hymenoptera (including pollinators and biocontrol agents that are leading actors of ecosystem services) ex-ante and ex-post the definition and implementation in the field (WP5, task 5.1) of the ASTER model of crop management. Hymenopterans have been chosen as indicators of the functional biodiversity (biological control and pollination) associated to the ASTER model of crop management vs conventional management of open field tomato.

Hymenoptera biodiversity will be assessed primarily in open field tomato crops. In Countries where the research activity will focus exclusively on greenhouse tomatoes, the Hymenoptera biodiversity will be assessed outside the greenhouses.

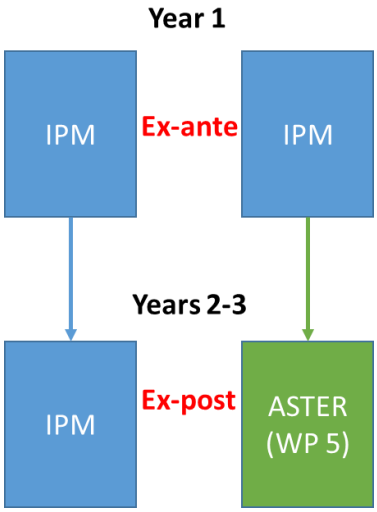
Participants of task 3.1	Country	Open field	Greenhouse
P1 CNR-IPSP	Italy	X	
P4 ISA-PT	Portugal		X
P5 IVIA	Spain	X	X
P6 DUTH	Greece	X	
P7 AUTH	Greece	X	
P9 ISUBU	Turkey	X	
P10 ISA-CM	Tunisia	X	X
P11 INAT	Tunisia	X	X
P12 UBMA	Algeria	X	
P14 ENAM	Morocco	X	

Sampling plan

In each of the eight Countries participating in ASTER, at least two replications of the field protocol to collect Hymenoptera will be set up to obtain results of statistical significance. For each replication, the Hymenoptera biodiversity will be compared between a conventional tomato field and a tomato field where the ASTER model of crop management (based on in-crop and border-crop management) will be applied. The tomato fields will be selected in WP5 – task 5.1. In total, the collection of insects will be run in at least four fields per Country.

Fields of each pair (replication) should have similar soil, climatic, other ecological and agricultural (e.g. crop rotation) properties that should be constant for three years.

The ex-ante collection will be conducted during year 1 in both fields under conventional management. The ex-post collection will be conducted during years 2 and 3 by comparing the ASTER model of crop management vs the conventional tomato management.



For each pair (replication) of tomato fields four (4) collections of Hymenoptera/year will be conducted during the cultivation season:
- spring (post-planting);
- early summer (flowering-fruit development-beginning of harvest);
- mid summer (harvest);
- end summer-early autumn (end of harvest).

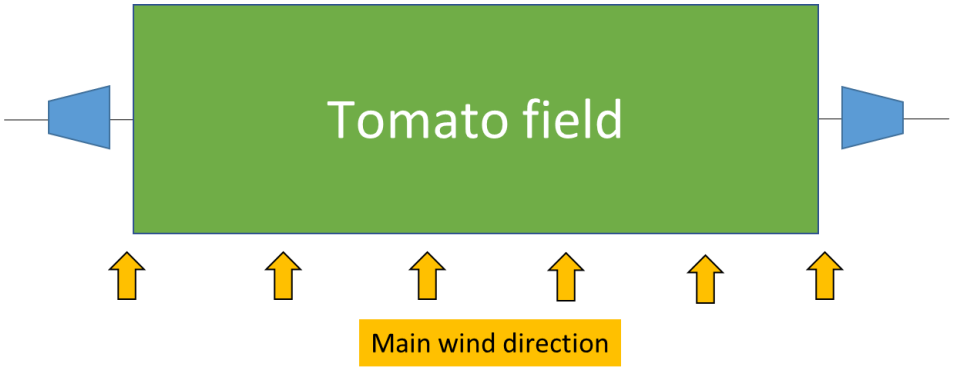
Two complimentary sampling methods will be adopted, one for Hymenoptera in general (Malaise traps) and one specific to pollinators, mainly Apoidea.

Hymenoptera collection by Malaise traps (“short-term sampling”)

Malaise traps allow the collection of flying insects, including Hymenoptera. Traps should be placed in the field perpendicularly to the main wind direction. Two traps will be installed for each tomato field, each at one head. Overall, at least eight (8) Malaise traps will be installed in each Country.

ASTER
Agroecology-inspired Strategies and Tools to Enhance Resilience and ecosystem services in Tomato crop

collection bottle + ethanol 70-90%



The “short-term sampling” protocol will be used to minimize the impact of traps on beneficial arthropods. This protocol consists of a collection period of three (3) days, replicated four times during the cultivation season as specified in the “sampling plan” paragraph.

Malaise traps with the following sizes will be used: cm 183 x 122, maximum height 2 m, 500 ml collection bottle.

Monitoring Apoidea biodiversity

This activity consists in a differential sampling strategy aiming to evaluate the ecosystem services of wild pollinators (mainly Apoidea) in the tomato field (conventional vs ASTER management). Three sampling methods will be used.

1 - PAN-traps

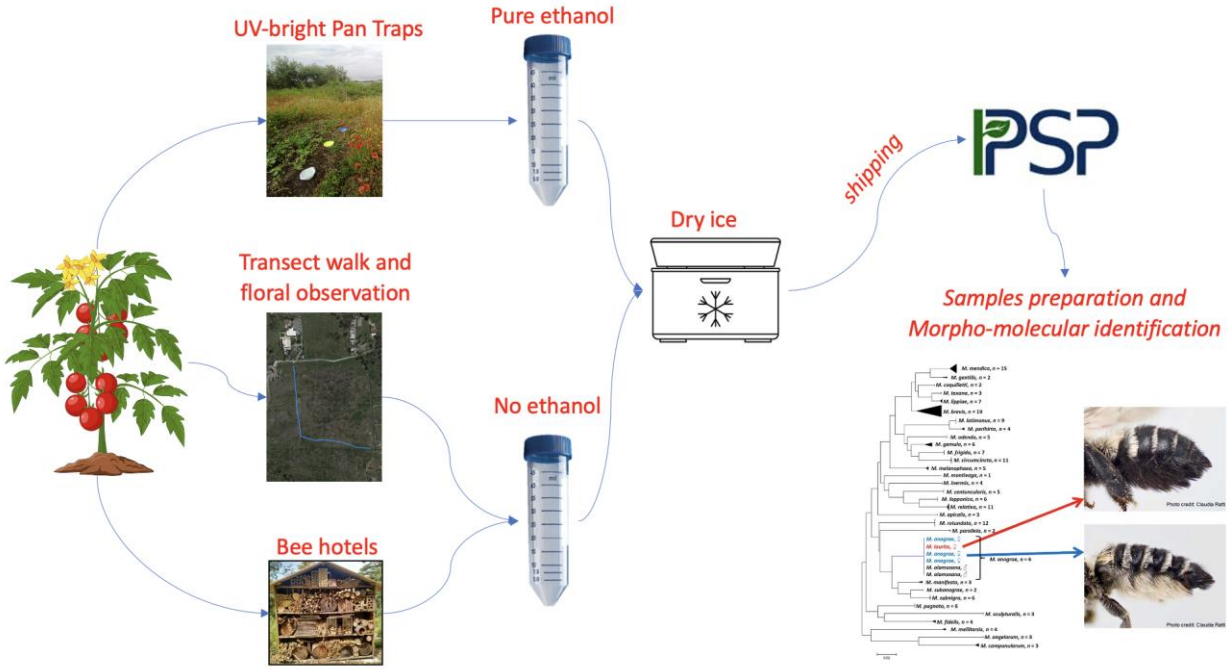
Pan traps are a standard passive sampling method for sampling insects of the Apoidea family. It consists of a set-up of yellow, white, and blue PAN traps, which represented prevailing floral colors in the study regions, to account for the different color preferences of bee species. The traps are 500-mL polypropylene soup bowls painted with UV-bright yellow, white, and blue paint. The PAN traps will be mounted on a wooden pole at vegetation height, filled with 400 mL of water and a drop of detergent, and left active for 48 hours. A total of 3 PAN traps (yellow, blue, and white) will be installed for each field (ex-ante and ex-post) during the first, second and third year in the middle of the crop field. The sampling plan follows the one for the parasitoids: spring (post-planting); early summer (flowering-fruit development-beginning of harvest); mid-summer (harvest); end summer - early autumn (end of harvest). The collected specimens will be temporarily stored at -20°C in pure ethanol (by discarding the water), in Falcon tubes until pinned for identification (see the Workflow for Apoidea collection).

2 – Transects

For the standardized transect walks, a permanently marked corridor of 500x4 mt will be established on the study sites. The bees within the corridor will be collected with an entomological net for each subunit separately during a 50-min walk covering the length of the subunit. Wild plant species visited by bees during the transect walk will be classified in the field or collected for subsequent species determination. For each field (ex-ante and ex-post) during the first, second and third year, one transect will be designed and georeferenced by following the crop edge. The transect should be the same with a tolerance of a few meters from one year to the next. The sampling plan follows the one for the parasitoids: spring (post-planting); early summer (flowering-fruit development-beginning of harvest); mid summer (harvest); end summer-early autumn (end of harvest). Species will be kept in a Falcon tube (with no buffer) for subsequent identification (see the Workflow for Apoidea collection).

3 – Bee Hotels

Bee Hotels are artificial wooden nests that provide winter refuges for wild bees and other small Arthropods. They will be filled with wood trunks, vegetable straws and removable cardboard tubes, of about 20-50 cm long and 3-15 mm diameter holes in it. One bee hotel will be installed in each field (ex-ante and ex-post) on the crop edge before the first year of tomato blooming. At the end of each season (e.g., autumn), cardboard tubes with cocoons will be collected and kept in the laboratory for the subsequent adult emergence. Specimens will be kept in a Falcon tube (with no buffer) for subsequent identification (see the Workflow for Apoidea collection).



Workflow for Apoidea collection

Management of material collected by Malaise traps

After the 3-days collection, the bottle containing the insects in ethanol must be closed with its cap, refilled with ethanol, and labelled. Labelling is important to avoid loss of information. The label must include the following data:

NATION: LOCATION NAME
GPS COORDINATES (centesimal grades) + ALTITUDE
DATE OF COLLECTION (three days)
NAME OF COLLECTOR (INSTITUTION)

ITALY: Battipaglia (Salerno) 40.609237 N, 14.980987 E, 70 m asl 24-27.vi.2022 coll. L. IODICE (CNR-IPSP)

Example of label

Two labels/bottle (1 label attached externally + 1 label dipped in the ethanol), printed or written in pencil, should be used. Collection bottles will be stored in a freezer at -20°C until the examination (in loco or to be sent).

Sorting the Hymenoptera collected by Malaise traps

Upon receiving the bottles, taxonomists must sort all HYMENOPTERA (and possibly DIPTERA SYRPHIDAE) from each sampling bottle, placing them in smaller containers (e.g., Falcon tubes) containing ethanol and labeled (2 labels/tube as above) as follow:

HYMENOPTERA (or SYRPHIDAE)
NATION: LOCATION NAME
GPS COORDINATES (centesimal grades) + ALTITUDE
DATE OF COLLECTION (three days)
NAME OF COLLECTOR (INSTITUTION)
NAME OF TAXONOMIST (INSTITUTION)

HYMENOPTERA ITALY: Battipaglia (Salerno) 40.609237 N, 14.980987 E, 70 m asl 24-27.vi.2022 coll. L. IODICE (CNR-IPSP) sorted E. GUERRIERI (CNR-IPSP)

Example of label

All vials must be stored in a freezer at -20°C until further sorting into morphospecies.

Management of Apoidea

All Apoidea samples (with pure ethanol from PAN traps or with no buffer from transects and bee hotels) will be kept in a falcon tube at – 20 °C.

Sorting of Apoidea

All tubes must be labeled with the mandatory information, including: Country, location, field, date, and type of collection (pan trap, transect, bee hotel).

Hymenoptera identification

Morphological identification of the specimens collected will be performed at least at the genus level. Specimens will be separated in morphospecies, and identity will be determined using

taxonomic keys and by comparing the collected insects with reference material stored in main EU collections (e.g., Natural History Museum of London).

Taxonomic identity of uncertain morphospecies will be confirmed by sequencing genes of taxonomic value (COI, 28S, ITS1, ITS2) and using BLAST and phylogenetic analysis. To associate voucher specimens to DNA sequences, non-destructive protocols of DNA extraction will be adopted to preserve the morphology of the individuals that will be kept in public collections of ASTER partners or sent to relevant museum collections.

In addition, parasitoid wasps emerged by specific insect pests (e.g., aphids, whiteflies, *Tuta absoluta*, leafminers, etc.), sampled in task 5.1 to assess their population density and biological control level, will be also identified.

The measure of Hymenoptera biodiversity

Biodiversity indexes, a quantitative measure of species diversity in a given community, will be used to measure the impact of the ASTER model of tomato crop management on functional biodiversity, compared with conventional crop management.

The following indexes will be evaluated:
- Shannon diversity index [H'], measures diversity in terms of richness (number of species);
- Simpson's Diversity index [D'], measures diversity in terms of dominance (number of individuals per species).

$$\text{Shannon Index (H)} = -\sum_{i=1}^{s} p_i \ln p_i$$

p = proportion (n/N) of individuals of a given species i (n) divided by the total number of individuals found (N);

s = number of species.

$$\text{Simpson Index (D)} = \frac{1}{\sum_{i=1}^{s} p_i^2}$$

In addition, the impact of ASTER on Hymenoptera taxonomic composition (e.g. relative abundance of different families), functional composition (relative abundance of functional groups (e.g. parasitoids of Lepidoptera, of aphids, of whiteflies, etc.), ecosystem services of the wild pollinators (species or morphospecies checklist, nesting activity and plant visited) will be evaluated.

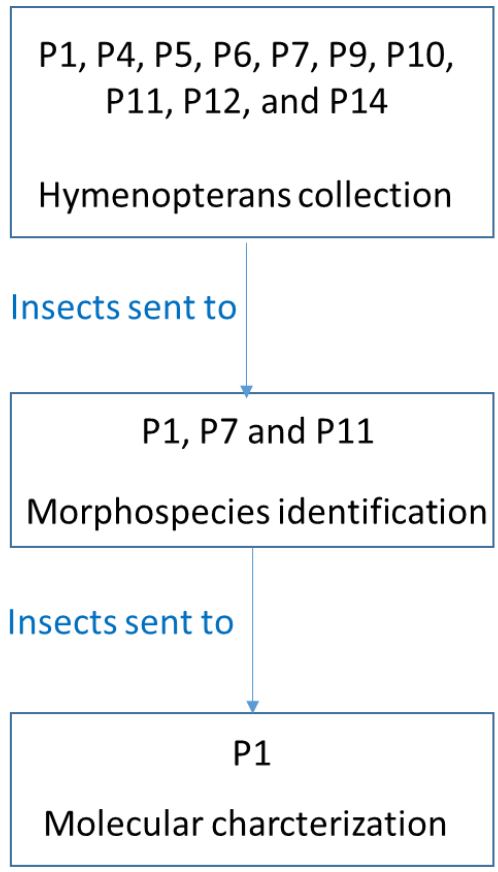
Distribution among the partners of the activities related to the task 3.1

Hymenopterans will be collected in each country. Two partners, namely P1 (CNR-IPSP) and P7 (AUTH) will be in charge for morphospecies identification. P1 and P7 will receive the material collected in other countries. Molecular identification of insects will be run in the laboratory of P1.

Participants of task 3.1	Country	Hymenoptera collection		Apoidea collection		Parasitoids identification		Apoidea identification	
		MT	S	AT	S	Morphology	Molecular	Morphology	Molecular
P1 CNR-IPSP	Italy	X	X	X	X	X	X	X	X
P4 ISA-PT	Portugal	X	X	X	X				
P5 IVIA	Spain	X	X	X	X				
P6 DUTH	Greece	X	X	X	X				
P7 AUTH	Greece	X	X	X	X	X		X	
P9 ISUBU	Turkey	X	X	X	X				
P10 ISA-CM	Tunisia	X	X	X	X				
P11 INAT	Tunisia	X	X	X	X	X		X	
P12 UBMA	Algeria	X	X	X	X				
P14 ENAM	Morocco	X	X	X	X				

Legend: MT = Malaise traps; S = Sorting (separation of Hymenoptera and Apoidea from the other insects collected); AT = trapping of Apoidea.

ASTER
Agroecology-inspired Strategies and Tools to Enhance Resilience and ecosystem services in Tomato crop



ASTER
Agroecology-inspired Strategies and Tools to Enhance Resilience and ecosystem services in Tomato crop

FIELD PLANT SAMPLING

Samplings will be performed on plant organs following the growth stage of the crop which should be recorded (i.e. vegetative growth, flowering, fruit formation, mature fruiting) and the target pest/pathogen. On average, weekly samplings will be performed by collecting leaves and flowers and inspecting sprouts, fruits and stems from 20 different plants randomly chosen. In each sampling date, 6 sampling units (organs) per plant or 120 plant organs per field will be sampled. Presence and abundance of targeted pests/pathogens and the relative antagonists (predators, parasitoids) will be recorded. The provisional list of targeted pests/pathogens in relation to the sampled organ includes:

SPROUTS

Pests: Aphids (*M. euphorbiae, M. persicae, A. solani*), whiteflies *(T. vaporariorum, B. tabaci), Phenacoccus solenopsis*

During sampling, young sprouts of the selected plants will be inspected and the number of aphids and whiteflies will be recorded.

Pathogens: Downy mildew (*P. infestans*); powdery mildew (*L. taurica*)

STEMS

Pests: *T. absoluta*

Pathogens: Downy mildew (*P. infestans*); powdery mildew (*L. taurica*) ; Phytophthora crown and root rot, Fusarium (*Fusarium oxysporum*) wilt, Verticillium wilt (*Verticillium* spp.)

During sampling, the stem of the selected plants will be inspected and infestation (*T. absoluta*), infection (Phytophthora crown rot, powdery mildew; downy mildew) as well as the number of mines by *T. absoluta* will be recorded.

FRUITS

Pests: *T. absoluta,* mites (*T. urticae* and *A. lycopersici*), *whiteflies*

Pathogens: Downy mildew (*P. infestans*), Botrytis *(B. cinerea)*

During sampling, 6 fruits per selected plant will be inspected and the number of infested (*T. absoluta,* mites, downy mildew; botrytis), *honeydew*-contaminated (whiteflies) *fruits* will be recorded.

LEAVES

Pests: Aphids (*M. euphorbiae, M. persicae, A. solani*), whiteflies *(T. vaporariorum, B. tabaci)*, *T. absoluta, L. trifolii*, mites (*T. urticae* and *A. lycopersici*)

Leaves form the lower, middle and upper part of the canopy will be picked, placed in plastic bags, stored in a polystyrene cooler box and transferred to the laboratory. The leaf samples will be further inspected under a stereomicroscope for the presence of the different pest species and their antagonists. More specifically, we will record: a) *T. absoluta & L. trifolii*: the number of infested leaves and mines with larvae, b) *T. vaporariorum & B. tabaci:* the numbers of eggs and nymphs, c) *T. urticae:* the numbers of eggs, juveniles and adults, d) *A. lycopersici:* the numbers of individuals and e) number of individuals of antagonists (i.e. syrphids, chrysopids, mirids, ladybeetles, predatory gall midge, parasitoids (parasitized nymphs of aphis and whiteflies) and phytoseiids).

Pathogens: Downy mildew (*P. infestans*), botrytis *(B. cinerea)*, powdery mildew (*L. taurica*) ; Fusarium and Verticillium wilts

Phytophthora crown and root rot, Fusarium and Verticillium wilts: All the plants in each plot will be regularly checked every week for disease symptoms; yellowing, wilting and/or stem necrosis disease incidence will be recorded. Disease severity will also be determined by using 0-5 scale where 0= healthy plant, 1= 1-30% wilting, 2= 31-50% wilting, 3= 51-70% wilting, 4= 71-90% wilting and 5= >90% wilting or dead plant (Gilardi et al., 2014). (Gilardi, G., Demarchi, S., Gullino, M.L., Garibaldi, A., 2014. Managing Phytophthora crown and root rot on tomato by pre-plant treatments with biocontrol agents, resistance inducers, organic and mineral fertilizers under nursery conditions. Phytopathologia Mediterranea, 53, 205-215.)

Downy mildew: Percentage of the leaf surface with downy mildew symptoms will be evaluated based on 0-5 scale where; 0= no disease, 1= up to 20% leaf area affected, 2= 21-40% leaf area affected, 3= 41-60% leaf area affected, 4= 61-80% leaf area affected, 5= more than 80% leaf area affected. Fruits will also be checked for disease symptoms and number of fruits showing disease symptoms among all fruits checked will be recorded (Sokhi et al., 1993). (Sokhi, S. S., Thind, T. S. and Dhillon, H. S. 1993. Late Blight of potato and tomato. Punjab Agricultural University, India. Directorate of Research Publication. pp. 19.

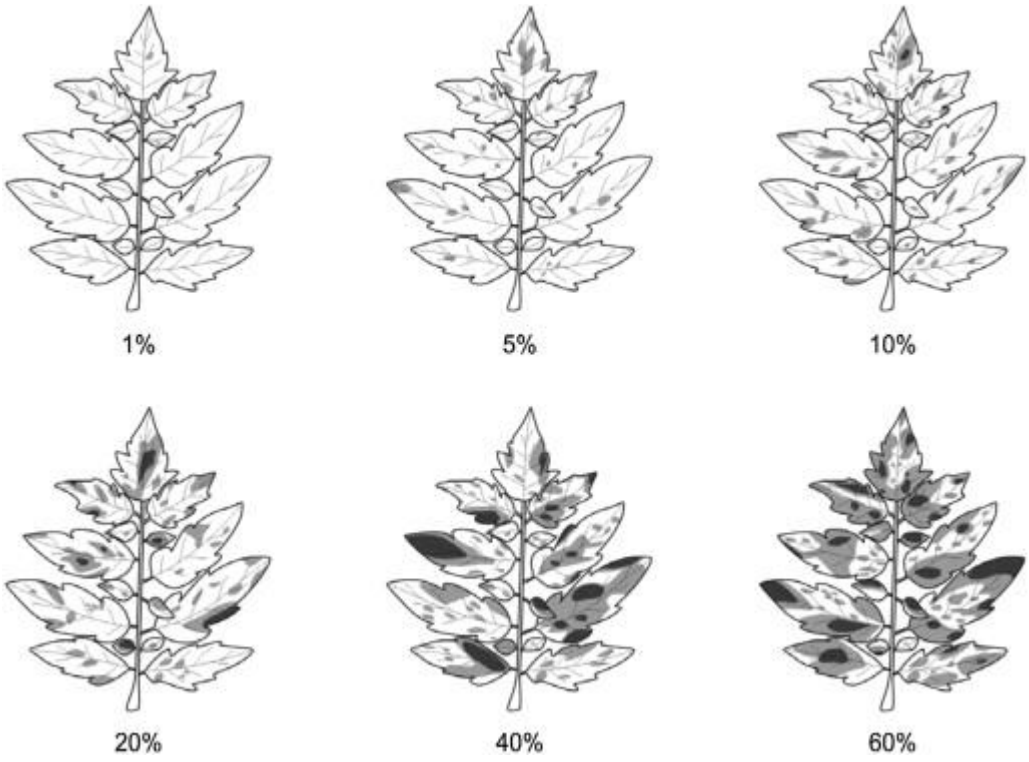
Powdery mildew: Percentage of the leaf surface covered with powdery mildew symptoms will be evaluated based on 0-4 scale where; 0= no visible pustule, 1= pustules on less than 10% of leaf surface, 2= pustules on less than 50% of leaf surface, 3= pustules less than 75% of leaf surface, 4= pustules on more than 76% of leaf surface (Toyoda, 2008). (Toyoda, H., 2008. An inoculation assay of tomato plants for screening resistance to Oidium neolycopersici: Tabulation of host responses and fungal growth on laboratory list of common and wild Lycopersicon species. Mem.Fac. Agric. Kagoshima. Univ., 41: 177-188.)

Botrytis gray mold: The severity of infection was rated on a 0-4 scale where; 0 = no infection, 1 = 5%, 2 = 25%, 3 = 50%, and 4 = infected leaf area about 75%-100% (Yıldız et al., 2007). (Yıldız, F., Yıldız, M., Delen, N., Coşkuntuna, A., Kınay, P., and Türküsay, H., 2007. The Effects of Biological and Chemical Treatment on Gray Mold Disease in Tomatoes Grown under Greenhouse Conditions. Turkish Journal of Agriculture and Forestry, 31, 319-325.)

The disease severity on the fruits will also be evaluated based on 0-4 scale where; 0 = no visible symptoms on the fruit (no infection); 1 = 1-25% of the fruit covered with slight necrotic and water-soaked lesion, 2 = 26–50% of the fruit covered with necrotic, white to gray mycelia and water-soaked lesion, 3 = 51–75% of the fruit necrotic with spore mass appeared and water-soaked, 4 = >76% of the fruit with soft, watery and decayed necrotic tissue (Yusoff et al., 2021). (Yusoff, S.F.; Haron, F.F., Asib, N., Mohamed, M.T.M., Ismail, S.I. 2021. Development of Vernonia amygdalina Leaf Extract Emulsion Formulations in Controlling Gray Mold Disease on Tomato (Lycopersicon esculentum Mill.). Agronomy, 11, 373. https://doi.org/10.3390/agronomy11020373

Disease severity ratings for each disease will be converted to a percentage disease severity index using the equation: $DSI = \sum (n.\ v)N.\ X \times 100$ where n is the infection class frequency, v is the number of each class, N is the number of observed plants and X is the highest value of the evaluation scale.

For *L. taurica* (possibly also for *P. infestans* and *B. cinerea*) the severity of infestation will be recorded according to the following diagrams (From *Lage, D.A., Marouelli, W.A., Duarte, H.S., & Café-Filho, A.C. (2015). Standard area diagrams for assessment of powdery mildew severity on tomato leaves and leaflets. Crop Protection, 67, 26-34*.)



Alternatively Disease severity will also be determined by: i) using 0-5 scale where 0= healthy plant, 1= 1-30% wilting, 2= 31-50% wilting, 3= 51-70% wilting, 4= 71-90% wilting and 5= >90% wilting or dead plant; ii) scanning the figure of infested leaves with image J software.

The area under the disease progress curve (AUDPC) may be calculated to describe the cumulative disease progress throughout the trial (Campbell and Madden, 1990). Campbell, C. L. and Madden, L. V. 1990. Introduction to Plant Disease Epidemiology. John Wiley & Sons, New York. 532 p.

Pests: Thrips (*F. occidentalis, T. tabaci)*

For the samplings, falcon tubes (50 ml) containing 70% alcohol will be used to sample flowers from the top one-third of the canopy. In each tube, 6 flowers will be collected (details in Funderburk, J., Martini, X., Freeman, J., Strzyzewski, I., Traczyk, E., Skarlinsky, T., Adkins, S. Sampling for Estimating *Frankliniella* Species Flower Thrips and *Orius* Species Predators in Field Experiments. *J. Vis. Exp.* (149), e59869, doi:10.3791/59869 (2019).) Samples will be further inspected under a stereomicroscope.

Trap samplings

Sticky traps will target thrips (blue) (4 traps per field, 10x25 cm, 20-30 adults, randomly selected, will be collected for species identification) and mealybug males (yellow).
Pheromone traps (1 per field) will target *T. absoluta*.

The following economic thresholds will be considered for each targeted pest/pathogen:

PESTS

1- Aphids, whiteflies, mites: 30% of leaves, 5% on fruits (with no antagonists)

2- Thrips: 20% on flowers (with no antagonists)

3- *Liriomyza sp.*: 30% on leaves with mines (with no antagonists)

4- *T. absoluta*: average of 2 mines on the leaves; 5% on fruits

PATHOGENS

On average 20% on leaves and 5% on fruits

Population densities of biological control agents will be determined by the methods given in WP2 before and after the trials.

Application of biological control agents: For ASTER plots, germination trays with tomato seedlings will be soaked in suspensions containing the spores or cells of the biological control agents or their combinations as determined in WP2, for 30 minutes before transplanting. Commercial products will be used in rates as recommended by the producer.

Mycorrhizal fungi: Inoculum will be first produced on a suitable plant in sand and spores will be extracted by wet sieving. Obtained spores will be applied at the stem base of the plants at a rate of 100-200 spores/plant, before transplanting (Ziane et al., 2017). (Ziane, H., Meddad-Hamza, A., Beddiar, A., Gianinazzi, S., 2017. Effects of Arbuscular Mycorrhizal Fungi and Fertilization Levels onIndustrial Tomato Growth and Production. . Int. J. Agric. Biol., 19: 341–347).

Trichoderma isolates: they will first be cultivated on PDA plates at 28±2°C for 7 days, then spore suspensions (10^7spore/ml) will be prepared by pouring sterile distilled water into plates and scraping conidia with a sterile spatula (Mwangi et al., 2022). (Mwangi, M.W., Monda, E.O., Okoth, S.A., Jefwa,

J.M., 2022. Inoculation of tomato seedlings with Trichoderma harzianum and Arbuscular Mycorrhizal Fungi and their effect on growth and control of wilt in tomato seedlings. Brazilian Journal of Microbiology 42(2), 508-513).

Bacillus sp.: Two loopfuls of bacteria from 3-day old cultures on tryptic soy agar (TSA) will be transferred to tryptic soy broth (TSB)medium and 24 h shaken culture will be incubated at 28±2°C. Inoculum concentration of 10^9 cfu/ml will be prepared with sterile distilled water before use (Utkhede and Koch, 2004). (Utkhede, R.D., Coch., C. 2004. Biological treatments to control bacterial canker of greenhouse tomatoes. BioControl 49, 305–313).

Seedlings will directly transplanted into the field soil to allow the natural infections of the pathogens.

At the end of the trial, after the determination of plant growth parameters at harvest, all plants in each plot will be up-rooted and the lower stem and tap root will be longitudinally sectioned for the examination of internal tissues for vascular wilt diseases. Each plant will be rated on a scale from 0 to 4 where: 0: healthy plant, 1: <25% vascular discoloration, 2: 26–50% vascular discoloration, 3: wilting with 51–75% vascular discoloration, and 4:76–100% vascular discoloration or dead plant (Abdel-Monaim, 2012). (Abdel-Monaim, M.F., 2012. Induced systemic resistance in tomato plants against Fusarium wilt disease. International Research Journal of Microbiology (IRJM) (ISSN: 2141-5463) Vol. 3(1) pp. 014-023)

Isolations will be made from the selected plants showing various disease symptoms in order to determine the related pathogens.

In order to check mycorrhizal fungi root colonization on plants with different applications, root pieces randomly taken from the harvested plants will be clarified with 10% KOH, stained with Trypan blue (0.05%) and colonization rates will be determined by the gridline intersect method of Giovanetti and Mosse (1980). (Giovannetti, M., & Mosse, B. (1980). An Evaluation of Techniques for Measuring Vesicular Arbuscular Mycorrhizal Infection in Roots. New Phytologist, 84, 489-500. http://dx.doi.org/10.1111/j.1469-8137.1980.tb04556.x)