«VALorization of Mediterranean small-scale FARMs by cropping wild UnExploited species»

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Deliverable D.6.3.1

Handbook

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D.6.3.1 Handbooks

In this handbook, the protocols for soil sampling and analyses that were performed in the context of Valuefarm regarding the activities of Wp2 and WP3 are presented.

Analyses performed by Bergische Wuppertal University (BUW; Germany)
Soil sampling and preparation

A soil sample should be composed of several sub-samples representing a seemingly uniform area or field with similar cropping and management history. Soil samples can be taken any time that soil conditions permit, but sampling directly after fertilization or amendment application should be avoided. The soil-fresh sample received in the laboratory should be analyzed directly after sampling for determination of nitrate, nitrite and ammonium. The soil-fresh samples received in the laboratory should be dried in the air and then passed through the 2 mm sieve.

Determination of basic soil properties

Soil properties of the soils should be determined according to the standard test methods for soil analyses (e.g., Sparks et al., 1996). Soil particle size analysis can be performed by the pipette method according to the method of Gee and Bauder (1986). Soil pH and salinity can be measured in soil suspension 1:1 (soil: water) using pH –meter and EC-meter, respectively. Organic matter content can be determined by the Walkley–Black method. Total and organic carbon content in soil can be measured using TOC-analyser (e.g., TOC-VE, Shimadzu, Kyoto, Japan). Soil cation exchange capacity (CEC) can be determined by saturating the soil with 1M-ammonium acetate at pH 7.0. Total calcium carbonate equivalent (TCCE) could be determined using a Collins calcimeter and active calcium carbonate equivalent (ACCE) using 0.2 N potassium permanganate solutions. Dissolved concentrations of soil anions can be determined using an ion chromatograph (e.g., Personal IC 790, Metrohm, Filderstadt, Germany). Total free Fe-Al-Mn oxides can be extracted with 3Msodium citrate+1M sodium bicarbonate+1 g sodium dithionite (CBD) in a water bath heated at 85 °C (Mehra and Jackson, 1960). Amorphous Fe-Al-Mn oxides can be extracted with 0.175 M ammonium oxalate+0.1 M oxalic acid adjusted to pH 3.0 according to Loeppert and Inskeep (1996).

Extraction and analyses of total nutrients and metal content

The pseudo-total contents of soil elements (e.g., Ag, Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, P, Pb, S, Sb, Se, Sn, Tl, V, Zn) can be exacted using the microwave digestion (e.g., microwave Milestone MLS 1200 Mega, Germany) according to the USEPA standard method (USEPA 3051a, 2007). Briefly, a sub-sample (0.6 g) of the sieved soil can be digested in the microwave with concentrated acids and then diluted for 50 ml

with acids, and then analyzed using ICP-OES (e.g., Ultima 2, Horiba Jobin Yvon, Unterhaching, Germany) or atomic absorption. Data quality control shuld be evaluated with the parallel extraction of soil certified reference materials (e.g., BRM No. 9b, 10a, 12, and 13, Federal Institute for Materials, Research and Testing), and the extraction recovery should be calculated. The quality control should be addressed also with blanks and triplicate measurements.

Extraction and analyses of potential available form of nutrients and metal content

Ammonium bicarbonate-diethylene tri-amine penta acetic acid (AB-DTPA)-extractable concentrations can be used as an availability index of the soil nutrients and trace elements (e.g., Ag, Al, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Ni, P, Pb, S, Sb, Se, Sn, Tl, V, Zn). The "potentially available" form of these elements in the soils can be extracted with 1M ammonium bicarbonate (NH_4HCO_3) + 0.005M diethylene tri-amine penta acetic acid (DTPA) solution according to Soltanpour and Schwab (1977). The "mobile" fraction of these studied elements can be extracted with 1 M NH_4NO_3 (Deutsches Institut fur Normung, 1997). The elements concentrations can be measured by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Ultima 2, Horiba Jobin Yvon, Unterhaching, Germany). Beside the AB-DTPA, available P in soils can be extracted in alkaline soils as per the method of Olsen et al 1954) using 0.5 M NaHCO3 1:20 soil-solution ratio) and then determined colorimetrically using the ammonium molybdate-ascorbic acid method described by Murphy and Riley (1962). More details about soil analyses could be found in the books of the standard test methods for soil analyses (e.g., Estefan et al., 2013; Sparks et al., 1996).

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Analyses performed by Consejo Superior de Investigaciones Científicas (CSIC; Spain)

Tailored compost application

It was produced at CEBAS-CSIC and once matured, it was packed and sent to fields. The raw materials were spent mushroom compost mixed straw at ratio 1:1, they showed initial moisture of 70% and it was decreased till 50% and after this moment the temperature was increased to reach 65ºC that was decreased by turning the pile during different times till once after turning, the pile temperature did not increase more than 5ºC from the atmospheric temperature. Then piles were opened and inoculated with Trichoderma harzianum T78 from Trichosym (Symborg) to reach the level of 10(5) ufc per gram of composting material and it was left during 2 months to finish the composting process. Before packing, the compost was sieved through 50 mm mesh. The moisture to be packed was 35%.

Establishment of the bacterial cultures

The plant growth promoting rhizobacteria *Bacillus subtilis* and *Pseudomonas* sp. wil be cultivated in CEBAS (CSIC). The rhizobacteria will be grown in a liquid nutrient medium composed of yeast extract, peptone and sodium chloride (Yeast extract peptone - YEP) for 2 days at room temperature on a Heidolph Unimax 1010 shaker. The bacterial culture will be centrifuged at 2287 *g* for 5 min at 2 °C, and the sediment will be resuspended in sterilized tap water. The cells concentration of the bacterial suspension will be 10^7 CFU ml^{-1}.

The rhizobacteria dose per inoculation will be correspond to 10^{10} CFU $plant^{-1}$.

Establishment of the fungal cultures

The plant growth promoting fungal Trichoderma harzianum and Aspergillus niger will be cultivated into a solid bran matrix CEBAS (CSIC). Once they will ready, they will be mixed with bentonite and dry prepared to be used to reach the average concentration between 10^8 and 10^9 colony forming units (CFU). The fungal powder will be fridge maintained (4ºC) till be used. It will be also recommended that once the container bag will be opened the fungal powder should be used in less than one month to avoid the

less of bioactivity. It is also recommended to not expose it before using to temperatures higher than 30ºC, trying to be maintained at shadow.

The application dose that it will be recommended to be applied will depend on the size of the experiments and it will be between 1-10 kg per hectare, that it would means that the amount reached in the soil round the rhizosphere would be expected to be $10^4 – 10^5$ CFU per gram of soil. The application can be done by spreading the fungal powder before planting to the whole surface, or it could be also concentrated in the hole where the seed of the transplant is going to be sowed. In case that the experiment will be carried out in pots, the soil or substrate will be weighted and its correspondence amount of fungal powder will be applied.

The rhizobacteria dose per inoculation will be correspond to 10^{10} CFU plant^{-1}.

Mycorrhizal preparation

1. Preparation of mycorrhizal inoculum (Establishment of the AM fungal cultures)

Single species cultures of the different AM fungal morphotypes were established in pots using a mixture of sterile soil/vermiculite (1:1 (v/v)) as growing substrate and *Sorghum bicolor* L. and *Trifolium repens* L. as host plants. About 30 apparently healthy and viable spores of each AM fungal morphotype were used as inoculum by placing them close to the plant root system. Once multiplied and checked for purity, the different AM fungal isolates were used as AM fungal inoculum after growing for a period of 6-8 months. Then, the aerial portion of the plants was removed, and the substrate, containing infected roots, extraradical mycelium, and spores, was utilized as the inoculum.

2. Inoculation with mycorrhizal inoculum

In pot experiments, we applied 5% of the total substrate volume as mycorrhizal inoculum. To do this, we first filled the pot with 2/3 of the corresponding total substrate volume, then added the mycorrhizal inoculum and lightly mixed it with the top layer of the substrate (2-3 cm deep). Finally, we applied the remaining substrate along with the plants.

In the field experiments, we applied 20 g of mycorrhizal inoculum to each plant. To do this, we first made the hole where we would plant the seedling, and in that hole, we applied the inoculum, mixing it lightly with the soil. After that, we planted the seedling.

Experimental sampling

1. Standard experimental sampling

Once the experiment is completed, sampling is carried out. Before harvesting, plant irrigation is stopped one day in advance. Two types of samples are then taken: aboveground and rhizospheric.

For the aboveground sample, the stem is cut at the collar, and the height and fresh weight are measured. The plant is then divided into two parts. One part is frozen at -80°C for further analysis, while the other part is weighed and subjected to a drying process in an oven at 60°C for a period of 4 to 8 days, until a constant weight is achieved. The dry part is used to determine the percentage of plant dry matter and estimate the total dry weight from the total fresh weight. Finally, the dry part is ground in a ball mill for nutrient analysis.

$$DM\ (\%) = \frac{DW\ (g)}{PFW\ (g)}\ x\ 100$$

$$TDM\ (g) = DM\ (\%)\ x\ TFW\ (g)$$

Where:

- DM = Dry Matter
- DW = Dry Weight (After drying)
- PFW = Partial Fresh Weight (Before drying)
- TDM = Total Dry Matter
- TDW = Total Fresh Weight

For the rhizospheric sample, the plant was first removed from the pot extract the pot´s substrate on a new filter paper for each sample. The substrate was removed to obtain

the root system and adhered rhizospheric soil. The root was placed in a large plastic bag and gently shaken to obtain the rhizospheric soil. Then, the soil was sieved using a 2mm mesh sieve and divided into two parts. One part was stored at -20°C for subsequent DNA sequencing, while the other was stored at 4°C for chemical and biochemical analysis.

The root was washed to remove any remaining substrate with running water and dried slightly with a paper towel to remove excess water. Then, the fresh weight was recorded and dried at 60°C in an oven for 4 to 8 days until a constant weight was achieved to calculate the percentage of dry weight and total dry weight.

2. Alternative experimental sampling

2.1 Field experiments

In the case of experiments carried out under field conditions, the sampling was adapted to the available conditions. First, the soil had to be moist to facilitate the collection of rhizospheric soil. The plants were cut at 8 cm above the ground, and each plant was individually placed in plastic bags and processed in the laboratory in the same way as in the pot experiments. Regarding the soil, the rhizospheric part was extracted by pulling the remaining stem upwards, recovering as much rhizospheric soil as possible, and shaking the root in a bag (Figure 1). When little soil remained attached to the roots upon extraction, it was collected from the field using a gardening shovel. The soil obtained was processed as in the pot experiments.

Figure 1: Plants extracted for rhizospheric soil sampling

2.2 Succesive sampling

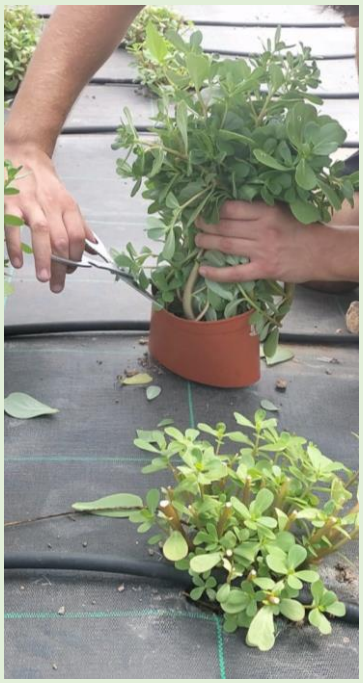

Figure 2: Cutting of plants with a mold at the height of 8 cm

In some experiments, we sampled multiple times, both the aboveground plant parts and the soil. For the aboveground part, taking advantage of the regrowth capacity of purslane, we were able to make several cuts to the plants in some experiments. To do this, depending on the experiment, we determined the minimum height at which, when cutting all plants to the same height, there were always axillary buds to facilitate regrowth. Then, we prepared a mold to ensure that all plants were cut at the same height by slightly stretching the stems upwards to avoid leaving excessively long, inclined stems (Figure 2).

In the case of multiple soil samples over time, different methods are used for pot and field experiments. For pot experiments, a lanceolate tube is used to extract 25-30 grams of rhizospheric soil without cutting the plant (Figure 3). The extracted soil is kept at 4°C.

For field experiments, due to the highly rocky terrain, extracting deep soil with this system is difficult. Therefore, for experiments where soil sampling at different time points is needed, enough plants are planted to obtain at least 4 destructive samples at each time point as described previously.

Figure 3: Soil sampling using a lanceolate tube.

Plant analyses

Dry plant tissue nitrogen was determined using a TrueSpec CN Analyzer (LECO, St. Joseph, MI, USA). Other elements such as P and K were determined by ICP/OES (Thermo Elemental Co. Iris Intrepid II XDL).

The percentage of mycorrhizal root colonization was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), according to Phillips and Hayman (1970). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse, 1980).

Soil analyses

1. Chemical analyses
    a. Organic carbon (OC) and matter (OM)

We used the Walkley and Black (1934) method. Using 0.5 g of dry soil in a 500 ml Erlenmeyer. We apply 5 ml of potassium dichromate $K_2Cr_2O_7$ 1N and manually mixed. Then we apply 10 ml of sulfuric acid H_2SO_4 96%. After 30 minutes, we apply 100 ml of milliQ water to stop the reaction. After cooling, we apply 5 drops of ferroin and titrate with ferrous sulphate $FeSO_4$ 0.25 N.

The percentage of organic carbon is calculated with the following expression:

$$Organic\ Carbon\ (\%) = \frac{(N \cdot Vml)K2Cr2O7 - (N \cdot Vml)FeSO4 \cdot 0.003 \cdot 100}{Dry\ Soil\ Weight}$$

Where:

- N = Normality used
- Vml = Volumen used

The organic matter percentage is obtained by multiplying the organic carbon by the empiric factor 1.724

    b. pH and conductivity (EC)

Soil pH and conductivity were determined at a soil-to-water ratio of 1:2.5 and 1:5, using 10 g and 8 g of dry soil, respectively. pH and conductivity were measured after agitation (230 rpm for 30 minutes).

c. Available phosphorous (AP)

We used the "Olsen" method. 2 g of dry soil is mixed with 40 ml of a sodium bicarbonate (NaHCO3) solution 0.5 M, pH 9.5. After 30 minutes of agitation at 135 rpm, the tubes are centrifuged at 2500 rpm for 15 minutes. 1 ml of the supernatant is pipetted into a new 10 ml tube. We added to that tube 4 ml MilliQ water, 0.25 ml H_2SO_4 2.5 N and 1 ml of B reagent.

After a brief mixture we measure the supernatant colour in a spectrophotometer at 882 nm.

Reagent:

- Reagent A: 12 g of ammonium molybdate $(NH_4)_6Mo_7O_2 – 4H_2O$ + 0.2908 g of antimony tartrate $K_2(SbO)_2C_8H_4O_{10}$ * 3 H_2O in 850 ml of sulfuric acid 5N. Mix and dilute to 2L with milliQ water.
- Reagent B: 1.056 g of ascorbic acid in 200 ml of Reactive A

d. Total nitrogen (TN) and total carbon (TC)

TN and TC were determined using an elemental CHNS-O analyser (EA – 1108, Carlo Erba, Barcelona, Spain).

e. Total elements (K, P, Ca, Mg…)

The total elements concentrations were determined using inductively coupled plasma-mass spectrometry (ICP-MS) with an ICAP 6500 DUO instrument (Thermo Fisher Scientific, Hayward, California, USA).

2. Physical analyses

Texture determination was realized according to Gee and Bauder (1986) using the Bouyoucos tube method, which is based on the gravity sedimentation of soil particles in a glass tube. Weight 50 g of dry and sieved soil and add 400 ml of distilled water and 10 ml of the sodium hexametaphosphate solution. Mix the solution for 8 to 24 h in a swing shaker. After that, bring the solution to a 1 L graduated cylinder and bring the

solution up to 1 L and mix vigorously for 1 m. When the mixing is finished, a densimeter is immediately inserted. After 40 seconds, measure the density (1D) and the temperature (1T). After that, after that, let it rest for 2 hours and measure again density (2D) and temperature (2T).

Use the following formulas to determine the texture:

$$Clay\ \&\ Silt\ \% = \frac{100(1D + (1T - 20) \cdot 0.36)}{P}$$

$$Clay\ \% = \frac{100(2D + (2T - 20) \cdot 0.36)}{P}$$

$$Silt\ \% = Clay\ \&\ Silt\ \% - Clay\ \%$$

$$Sand\ \% = 100 - Clay\ \&\ Silt\ \%$$

Where:

- Clay & Silt % = The percentage of clay and silt in the soil
- Clay % = The percentage of clay in the soil
- Silt % = The percentage of silt in the soil
- Sand % = The percentage of sand in the soil
- 1D = First density measurement
- 2D = Second density measurement
- 1T = First temperature measurement
- 2T = First temperature measurement
- P = Weight of soil used (50 g)

To determine the texture of the soil, apply de results obtained to a texture triangle (Figure 4)

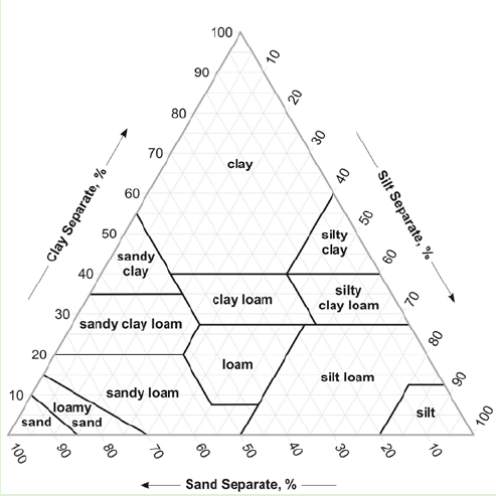

Figure 4: Texture diagram triangle

3. Biological analyses
    a. β – glucosidase activity (β – GLC)

β – glucosidase activity was determined as described by Eivazi and Tabatabai (1988). We used 0.5 g of non – dryed soil, in 50 ml Falcon tubes in triplicates per sample (1 control (C) and 2 substrates (SS1 and SS2)). We added 2.5 ml and 2 ml of MUB – HCl pH 6 to C and SS. Mix for 10 minutes at 37ºC and then, added 0.5 ml of PGN to the SS tubes. Agitate for 1 hour at 37ºC. Add 2 ml of Tris/NaOH 0.1M pH 12 to stop the reaction, and then 0.5 ml $CaCl_2$ to cause precipitation. Then, centrifuge for 3000 rpm for 15 minutes and measure the nitrophenol in the supernatant in a spectrophotometer at 400 nm. If measure is higher than 3, dilute 1:3 with milliQ water. The measurements of the control should be subtracted from those obtained in the samples.

Also, prepare 2 blank tubes; Control Blank (CB) and substrate blank (SB). CB: 2,5 mL MUB pH 6 + 2 mL Tris/NaOH 0,1 M pH 12 + 0,5 mL $CaCl_2$. SB: 2,0 mL MUB pH 6 + 0,5 mL PGN + 2 mL Tris/NaOH 0,1 M pH 12 + 0,5 mL CaCl2.

Also, prepare a calibration line with p – nitrophenol (PNP) measuring 0, 20, 50, 100 and 200 ppm (mg/L) with 2,5 mL MUB pH 6 + 2 mL Tris/NaOH 0,1 M pH 12 + 0,5 mL $CaCl_2$ at 400 nm.

$$\beta - \text{GLC} = \frac{C \cdot V}{Pm \cdot G \cdot T}$$

Where:

- β – GLC = β – glucosidase activity
- C = p – nitrofenol measured with the calibration line
- V = Dilution factor applied if used
- G = Estimated dry soil used.
- T = Incubation time (1h)
- Pm = Molecular weight of p – nitrophenol (139)

Reagents:

- MUB – Stock: 12.2 g of tris(hydroxymethyl)aminomethane (THAM), 11.6 g of maleic acid, 14 g of citric acid and 6.28 g of boric acid dissolved in 488 ml NaOH 1M and bring the solution up to 1 L.
- MUB – HCl pH 6: Add 500 ml HCL 0.1 M to 200 ml MUB Stock and adjust the pH to 6.
- PGN 25 mM: Add 0.377 g of 4-Nitrophenyl-beta-D-glucopyranoside to 50 ml of MUB HCl 6 pH
- Tris/NaOH 0.1M ph 12: Add 12.2 THAM to 800 ml H_2O and adjust pH to 12 with NaOH 0.5. Bring the solution up to 1 L with water.
- p- nitrophenol stock solution: Add 250 mg of p- nitrophenol to 250 ml of water.

b. Phosphatase activity (PHO)

Phosphatase activity was determined according to Tabatabai and Bremner (1969). We used 0.5 g of non – dried soil, in 50 ml Falcon tubes in triplicates per sample (1 control (C) and 2 substrates (SS1 and SS2)). We added 2.5 ml and 2 ml of MUB – HCl pH 6 to C and SS. Mix for 10 minutes at 37ºC and then, added 0.5 ml of PGF to the SS tubes. Agitate for 1 hour at 37ºC. Add 2 ml of Tris/NaOH 0.1M pH 12 to stop the reaction, and then 0.5 ml $CaCl_2$ to cause precipitation. Then, centrifuge for 3000 rpm for 15 minutes and measure the nitrophenol produced in the supernatant in a spectrophotometer at 400 nm. If measure is higher than 3, dilute 1:3 with milliQ water. The measurements of the control should be subtracted from those obtained in the samples.

To determine alkaline phosphatase, use MUB – HCl to pH 9

Also, prepare 2 blank tubes; Control Blank (CB) and substrate blank (SB). CB: 2,5 mL MUB pH 6 + 2 mL Tris/NaOH 0,1 M pH 12 + 0,5 mL $CaCl_2$. SB: 2,0 mL MUB pH 6 + 0,5 mL PGN + 2 mL Tris/NaOH 0,1 M pH 12 + 0,5 mL CaCl2.

Also, prepare a calibration line with p – nitrophenol (PNP) measuring 0, 20, 50, 100 and 200 ppm (mg/L) with 2,5 mL MUB pH 6 + 2 mL Tris/NaOH 0,1 M pH 12 + 0,5 mL $CaCl_2$ at 400 nm.

Calculate the phosphatase activity with the formula:

$$\text{PHO} = \frac{C \cdot V}{Pm \cdot G \cdot T}$$

Where:

- PHO = Phosphatase activity
- C = p – nitrophenol in samples measured with the calibration line
- V = Dilution factor applied if used
- G = Estimated dry soil used.
- T = Incubation time (1h)
- Pm = Molecular weight of p – nitrophenol (139)

Reagents:

- MUB – Stock: 12.2 g of tris(hydroxymethyl)aminomethane (THAM), 11.6 g of maleic acid, 14 g of citric acid and 6.28 g of boric acid dissolved in 488 ml NaOH 1M and bring the solution up to 1 L.
- MUB – HCl pH 6: Add 500 ml HCL 0.1 M to 200 ml MUB Stock and adjust the pH to 6.
- MUB – HCl pH 9: Add 500 ml HCL 0.1 M to 200 ml MUB Stock and adjust the pH to 9.
- PGF 25 mM: Add 0.46383 g of p – nitrophenyl phosphate to 50 ml of MUB HCl 6 pH
- Tris/NaOH 0.1M ph 12: Add 12.2 THAM to 800 ml H_2O and adjust pH to 12 with NaOH 0.5. Bring the solution up to 1 L with water.
- p- nitrophenol stock solution: Add 250 mg of p- nitrophenol to 250 ml of water.

c. Dehydrogenase activity (DHA)

Dehydrogenase activity was determined according to Trevors at al. (1982) modified by García et. al. (1993). We weighted 0.5 g of undried soil in 50 ml Falcon tubes in triplicates per sample (1 control (C) and 2 substrates (SS1 and SS2)). We added 0.2 ml of milliQ water to control tubes and 0.2 ml of INT to substrate tubes. Mix well and incubate in darkness for 20 hours. Add 9.8 ml of methanol to stop the reaction and mix. Centrifuge at 3000 rpm for 15 minutes. Measure the INTF produced in the supernatant at 490 nm in a spectrophotometer against a calibration blank.

Prepare a calibration line with 0, 12, 24, 36, 48 and 60 ppm (mg/L) of INTF.

Calculate the dehydrogenase activity with the formula:

$$\text{DHA} = \frac{C \cdot V}{Pm \cdot G \cdot T}$$

Where:

- DHA = Dehydrogenase activity
- C = INTF measured in samples with the calibration line
- V = Dilution factor applied if used
- G = Estimated dry soil used.
- T = Incubation time (20h)
- Pm = Molecular weight of INTF (471.3)

Reagents:

- INT 2-p-Iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium 0.4%: Weight 0.2 g of INT in a flask with 45 ml of water, dissolve with ultrasound for 2 – 3 hours and then, add water up to 50 ml.
- INTF Iodonitrotetrazolium formazan 60 ppm: Dissolve 0.015 g of INTF with 200 ml of methanol and bring the solution up to 250 ml with methanol.

d. Urease activity (URE)

Urease activity was determined using the method described by Kandeler and Gerber (1988) modified by Kandeler et al. (1999). We weighted 0.5 g of undried soil in 10 ml tubes in triplicates per sample (1 control (C) and 2 substrates (SS1 and SS2)). We added 0.75 ml of milliQ water to control tubes and 0.75 ml of urea 79.9 mM to substrate tubes. Mix the tubes in vortex and incubate for 1.5 hours at 37.5ºC gently agitating the tubes. Add 6.75 ml of KCl 2M to all tubes and agitate for 30 minutes. Centrifuge at 2500 rpm for 10 min.

Pipette 1ml from the tube to a new one and add 3.5 ml of milliQ water. Add 0.25 ml of EDTA. Mix the tubes in vortex and then, after 30 minutes, add 0.25 ml of sodium hypochlorite buffer (Make sure that the buffer has no precipitated). Vortex and incubate at 37ºC for 1 h and measure the N – NH_4^+ produced at 667 nm.

Also prepare 2 blanks:

Control blank (CB) 0.75 mL of Milli-Q water + 6.75 mL of 2 M KCl + 3.5 mL of Milli-Q water, 0.25 mL of EDTA, 1 mL of nitroprusside salicylate, and 0.25 mL of sodium hypochlorite buffer.

Substrate blank (SB) 0.75 mL of Urea + 6.75 mL KCl 2 M) + 3.5 mL of Milli-Q water + 0.25 mL of EDTA + 1 mL of Nitroprusside salicylate + 0.25 mL of sodium hypochlorite buffer.

Prepare a standard curve with 0, 1, 1.5, 2, 2.5 ml of the standard ammonia solution.

Calculate the urease activity with the formula:

$$\text{URE} = \frac{C \cdot V}{Pm \cdot G \cdot T}$$

Where:

- DHA = Dehydrogenase activity
- C = N – NH_4^+ determined by the calibration line
- G = Estimated dry soil used.
- T = Incubation time (2 h)
- Pm = Molecular weight of INTF (471.3)

Reagents:

- Urea 79.9 mM: Dissolve 0.239 g of urea in 45 ml of milliQ water and bring up to 50 ml with the same water.
- KCl 2M: Dissolve 149 g of KCl in distilled water. Add 10 ml of HCl 1M and bring up to 1000 ml with distilled water.
- Sodium Nitroprusside – salicylate: Dissolve 2.96 g of NaOH and 9.96 g of $Na_2HPO_4 * 7H_2O$ in 60 ml of distilled water. Add 10 ml of sodium hypochlorite and adjust pH to 13 using NaOH 1M. Bring the solution up to 100 ml with distilled water.
- EDTA – Na solution 6%: Dissolve 6 g of EDTA – Na in 100 ml
- Ammonia standard solution (1000 µg N- NH_4^+ / ml): Dissolve 3.82 g of ammonium chloride in 1000 ml of distilled water.

4. DNA extraction and Illumina sequencing

Genomic DNA was obtained from rhizosphere soil samples by using the DNeasy PowerSoil DNA Isolation kit (Qiagen), following the manufacturer's instructions, using 0.25 g of rhizosphere sopil. The quality and quantity of the extracted DNA were evaluated using both electrophoresis and a Qubit 3.0 fluorometer. DNA from each sample was sequenced on the Illumina MiSeq platform at the genomics service of the Institute of Parasitology and Biomedicine "López Neyra" (CSIC), Granada, Spain. Prokaryotic libraries were generated using primers targeting the hyper-variable V3–V4 regions of the 16S rRNA gene, using the primer 341F (5′-CCTACGGGNBGCASCAG-3′) and 806R (5′-GACTACNVGGGTATCTAATCC-3′), while fungal libraries were constructed using primers targeting the ITS2 region using the primer pair ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and fITS7 (5′-GTGARTCATCGAATCTTTG-3′). PNA PCR clamps were utilized to minimize the amplification of plastid and mitochondrial DNA. The sequencing was performed using a paired-end 2x300bp (PE 300) strategy.

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Analyses performed by University of Thessaly (UTH; Greece)
Soil and plant analyses

1. Soil analyses

1.1 Sample preparation

Soil samples were put in paper bags and placed in an oven at 40-50 ˚C until totally dry. Then the samples were grinded in a porcelain mortar, sieved in a sieve with openings of 2 mm, and stored until further analysis. All measurements were performed according to Rowell (1994)

1.2 pH

For pH measurement, 10g of soil was weighed and placed in 50 mL falcon bottles. Afterwards 25 mL of distilled water was added and the samples were shaken for 10 minutes and allowed to rest at least for 30 minutes. The pH was measured with an electronic pH meter.

1.3 Electrical conductivity

For electrical conductivity measurement, 10g of soil and 50 mL of distilled H_2O were weighed in falcon vials and shaken for 20 minutes. Then the samples were measured with an electrical conductivity meter.

1.4 Organic matter

Soil organic matter was evaluated with wet oxidation. Firstly, 0.5 g of soil, 10 mL of 0,166 M $K_2Cr_2O_7$, and 10 mL of dilute H_2SO_4 were added at a beaker. The solution was kept to rest for 30 minutes to allow oxidation of soil organic matter to occur, and 200 mL of H_2O was added for filtration. Then 10 mL of H_3PO_4 and 5 drops of diphenylamine indicator were added. Then the $K_2Cr_2O_7$ left over from the oxidation of the organic matter was titrated with 0.5 M $FeSO_4$ until the final color was changed to green. The whole procedure was carried out on a sample containing everything except soil. At the end of the procedure, the amount of $FeSO_4$ that was consumed was recorded.

1.5 Analysis of particle size distribution

Firstly, 50 g of soil and 50 mL of dispersing solution were placed in beakers and left for 16 hours. The samples were stirred for 10 minutes in an electric mixer, transferred to 1 L volumetric cylinders and filled with water. This was followed by re-stirring with

a special rod, the measurement was conducted with Bouyoucos hydrometer and the temperature with a thermometer. After two hours of rest, the measurement was re-calculated. Finally, the percentage of clay, sand, and silt was estimated, by applying the findings to standard formulas

1.6 Calcium carbonate

A quantity of soil of about 10g (exactly recorded) was added in a conical flack. Then the dilute acid was placed in a beehive, without contact with the soil. The calciometer measured the CO_2 gas pressure forming as a result of the reaction. According to the values, the soil $CaC0_3$ percentage was found with special calculations.

1.7 Extraction of available trace elements with DTPA

The availability of trace elements in soil was determined, by adding 10 g of soil and 20 mL of diethylene triamine penta acetic acid (DTPA) solution in falcon vials. After shaking for 2 hours, filtration followed. The trace elements were detected by atomic absorption.

1.8 Total concentrations of trace elements (Aqua regia)

In digestion tubes 1 g of soil, 15 mL of concentrated HCl and 5 mL of concentrated HNO_3 were added. The digestion tubes were placed in a special digestion block in a gas extractor. The samples were left for 16 hours and the digestion was performed at 140 ˚C for 5 h. The samples were filtered with distilled H_2O into 100 mL volumetric flasks. This extract was measured in atomic absorption apparatus for detective trace elements.

1.9 K as exchangeable

A weight of 3 g of soil was weighed into falcon-type plastic bottles and 30 mL of CH_3COONH_4 (1 M, pH 7) was added. The samples were shaken for 1 hour, filtered and analyzed in a flame photometer.

1.10 Extractable N (in the form of NO3-N)

A weight of 2 g of soil was weighed into falcon-type plastic bottles and 20 mL of KCl 2 M was added. The samples were shaken for 1 hour, filtered and analyzed in a UV spectrophotometer at 210 and 270 nm.

1.11 Phosphorus extraction method

Phosphorus measurement is carried out in two steps a) extraction, b) development of blue color and measurement. Phosphorus can be extracted by various methods, but the method that has been used in the laboratory is the Olsen-P method. Specifically, in 50 mL plastic falcon bottles 1g of soil weighed and 20 mL of 0.5 M NaHCO3 were added. Shaking for 30 minutes and filtration with slow filter paper in plastic bottles and extraction. Then 5 mL extract and 2.5 mL of Reagent B (ammonium vanadomolybdate/ascorbic acid) were added in 25 mL volumetric flasks. The pH was adjusted to 7 by adding 8 mL 1 M NaOH and the volume was made up to the mark with distilled H_2O.The same procedure was used to develop color in solutions of known phosphorus concentration. Thus creating standard solutions with P concentrations of 0, 0.2, 0.4, 0.8 and 1 ppm (mg P/ L). Then the samples were left 30 minutes for color development and P measured (both the unknown and known samples) in a spectrophotometer at a wavelength of 882 nm.

2. Plant analyses

2.1 Samples preparation

Plants samples (aboveground and roots) were washed with distilled H_2O, placed in paper bags, and placed in an oven at 70 ˚C until totally dry. After that, the received dried biomass was grinded to a fine powder and stored until analysis. A weight of 0.5 g of powdered plant tissues was weighed in porcelain crucibles to be burned in an oven at 500 ˚C for 5 hours. The ash of plant tissues was extracted with 20 mL of HCl 20% and filtered into 50 mL volumetric flasks and made up to the mark with distilled H_2O (Plank, 1992).

2.2 Trace elements

The above extract was measured in atomic absorption apparatus for detective trace elements.

2.3 K measurement

In the same extract, potassium was measured in a flame photometer.

2.4 P measurement

The measure was performed as in the soil extract with the development of blue color (ammonium vanadomolybdate/ascorbic acid), and measured in a spectrophotometer at a wavelength of 882 nm.

2.5 Total nitrogen

Total nitrogen was measured by following the Kjeldahl method. In digestion tubes 1 g of dried biomass and 20 mL of concentrated H_2SO_4 were added. The digestion tubes were placed in a special digestion block in a gas extractor and digested at 420 ˚C. After the samples have been cooled down, the distillation took place in the automatic distillation system with 40% NaOH into a flask containing 4% H_3BO_3. After the end of distillation, the samples were titrated with 0.1103 N H_2SO_4.

Figures

Figure 1. pH meter

Figure 2. Electrical conductivity

Figure 3. Flame photometer

Figure 4. Spectrophotometer

Figure 5. Distillation system

Figure 6. Atomic Absorption

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Analyses performed by Ege University (EGE; Turkey)

Soil Sampling Protocol for Wild Plant Growth Areas

Soil sampling protocols can vary depending on the specific purpose and objectives of the sampling. When conducting soil sampling in wild plant growth areas, it's important to consider the unique characteristics of the ecosystem and the specific objectives of the study.

Define the objective: Clearly define the purpose of your soil sampling. Determine if you are conducting soil analysis for soil fertility, soil composition, nutrient availability, taxonomy, or any other specific application.

Identify sampling locations: Decide on the number and location of soil sampling points. Select representative sampling locations within the specific area. Consider factors such as soil type, topography, vegetation cover, plant diversity, topography, and any noticeable variations in the landscape. Pay attention to the distribution of stones and rocks when choosing the sampling spots. Choose areas that reflect the overall conditions of the ecosystem.

Prepare sampling tools: Gather the necessary equipment for soil sampling, including a soil auger, shovel, trowel, or soil probe. Make sure your tools are clean and free from any contaminants that could affect the soil samples. Depending on the vegetation density and site accessibility, you may need additional tools like pruning shears or a machete to clear the area for sampling. Additionally, you may need tools like a pickaxe or pry bar to remove stones obstructing the sampling process.

Sampling depth: Determine the appropriate sampling depth based on your objective, similar to a standard soil sampling protocol. Generally, a depth of 8-10 inches (20-25 cm) is sufficient for most purposes, unless there are specific requirements.
In wild plant growth areas, soil sampling depth can range from the surface litter layer to a deeper depth, depending on the specific research question. Consider factors such as root distribution and nutrient stratification when deciding on the depth.

Stone removal: Before collecting soil samples, remove stones or rocks that may hinder the sampling process or skew the results. You can use tools like a pickaxe or pry bar to

clear the area around the sampling location and ensure that you obtain a representative soil sample.

Sample collection: Once the area is prepared, follow these steps to collect soil samples:

a. Remove any surface litter or vegetation that may interfere with the sampling process. Clear a small area around the sampling location (Figure 1).

b. If there are large stones present, use a pry bar or pickaxe to create a hole or aperture in the soil to reach the desired sampling depth (Figure 2).

c. Use your sampling tool (e.g., auger, shovel) to collect a representative sample of soil from the desired depth, taking care to avoid excessive stone content. Take care to avoid contamination from nearby vegetation or debris.

d. Collect multiple sub-samples within a small area, as in a standard protocol, and mix them together in a clean bucket or sampling bag to create a composite sample.

e. Repeat the process at each designated sampling location, ensuring proper stone removal and representative sample collection.

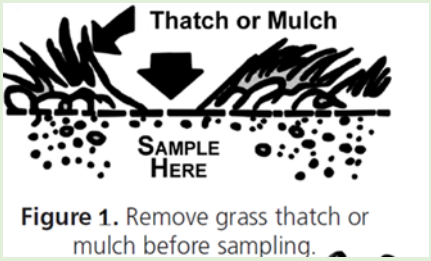

Figure 1. Remove grass thatch or mulch before sampling.

Figure 2. Soil sampling with a trowel.

(https://extension.uga.edu/)

Sample handling: Sample handling: Handle the soil samples carefully to maintain their integrity:

a. Place the composite soil samples in clean, labeled containers such as plastic bags or airtight jars.

b. Store the samples in a cool and dark location to minimize microbial activity and prevent moisture loss.

c. If there will be a delay before analysis, consider refrigerating or freezing the samples to preserve their quality.

Documentation: Record essential information about each sampling location, including the coordinates, plant species present, sampling depth, and any specific site characteristics. Proper documentation ensures traceability and assists in data interpretation.

Transport and analysis: Deliver the soil samples to a reputable laboratory for analysis. Record relevant information about each sampling location, package the samples securely. Follow the specific instructions provided by the laboratory for sample submission, including packaging, labeling, and required documentation.

Analyses performed by Dokuz Eylul University (DEU; Turkey)

1. **Soil Sampling Protocols**

    1. **Sampling from the nature and the field trials**

A clean shovel is used to collect soils from the top 10 cm of the ground during the samples from the nature, while top 20-25 cm was collected in the case of field trials. Composite sampling was applied by regarding the different parts of the land piece. Plant parts on the top of the soil and roots were removed and more than 1 kg of soil was collected and deposited into a plastic bag at each sampling site and/or trial lot. The bag was tagged with the sample number, date and time, location, the weather condition at the sampling date and any additional information, while sampling coordinates are also noted in saplings from the nature. The shovel was then cleaned by using a brush after each sampling and the first two scoop from each site were discarded. The samples were stored in an icebox until they were received by the laboratory. Soils that are subjected to the experimental analysis were dried at room temperature (20±2 °C) and the particles above 2 mm were removed by screening. The soil material was then homogenized in a large container, a representing sub-sample with an approximate mass of 200 g was generated by using quartering method in order to be used for the analysis. The remaining from the original dry sample was stored in the laboratory.

    2. **Sampling from pots**

The experimental plants were removed from the pot (having a volume of 8 L), if there was any, the pot content was emptied into a large container and homogenized by using shovel and gloved hands. In order to generate a sub-sample for laboratory analysis, the volume of the soil material was reduced by quartering until the desired sub-sample mass was reached. The sub-sample was dried at room temperature (20±2 °C) prior to the analysis and then sieved under 2 mm if it has larger particles.