



Deliverable title	D4.1 Experimental plan design of cultivation trials
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Summary of Deliverable D4.1 – Experimental Plan for Cultivation Trials	To move from wild populations to sustainable farming, SEAFENNEL4MED designed an ambitious experimental plan for cultivation trials across the Mediterranean basin. Deliverable D4.1 describes the setup of demo fields in five locations—Italy, Croatia, Tunisia, and Türkiye—covering a range of climates from temperate coastal areas to semi-arid and desert zones. This diversity ensures that the plant's adaptability to different environmental challenges can be fully assessed. The trials followed a randomized block design with replications, guaranteeing scientific robustness. Seeds from carefully selected ecotypes (Mediterranean and Atlantic) were germinated in greenhouses, and seedlings with 3–4 true leaves were transplanted into the demo fields. Each site tested different treatments—such as bio-fertilization, biostimulation, irrigation regimes, and unfertilized controls—to evaluate how sustainable agronomic practices can enhance plant growth and resilience. In Italy, two sites (Camerano and Agugliano) hosted trials comparing Mediterranean and Atlantic ecotypes, with special focus on biostimulants based on nitrogen-fixing and phosphorus- or potassium-solubilizing bacteria. Croatia and Tunisia established trials with both local and Atlantic ecotypes, while Türkiye tested multiple native Aegean ecotypes alongside the Atlantic type, under both irrigated and biofertilized conditions. Detailed soil analyses at each site ensured that plant performance could be linked to environmental factors such as water availability, pH, organic matter, and salinity.

Versioning and Contribution History

Version	Date	Modified by	Modification reason
v1.0	20/11/2022	Branimir Ulrich	First version
V2.0	30/11/2022	Branimir Ulrich	Comments after peer reviewing process

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1 Experimental plan design of the cultivation trials

1.1 Italian sites

In Italy, two sites for demo field experiments were used during the project. Because in the first year the seeds did not germinate so we used another experimental site already in place with three-year-old plants in Camerano (AN) with mediterranean ecotype to carry out the research. The following year, however, we transplanted the seedlings and started the new experimental site in Agugliano (AN). On both experimental sites, we evaluated the application of THE SAME biostimulant on measured variables such as epigeal biomass, SPAD units in three phenological phases and in Agugliano also evaluated the agronomic performance of two different sea fennel ecotypes (Mediterranean and Atlantic)

1.1.1 Site 1

Site 1: AGUGLIANO

Soil properties in the 0-30 cm layer in the experimental plots in 2024 (n = 3) are shown below

Soil properties	Values
Sand (g kg ⁻¹)	65 ± 18
Silt (g kg ⁻¹)	504 ± 17
Clay (g kg ⁻¹)	431 ± 20
Volumetric soil water content (%):	
Field Capacity	35.2 ± 1.2
Permanent Wilting Point	18.0 ± 2.4
Total available water	26.0 ± 1.8

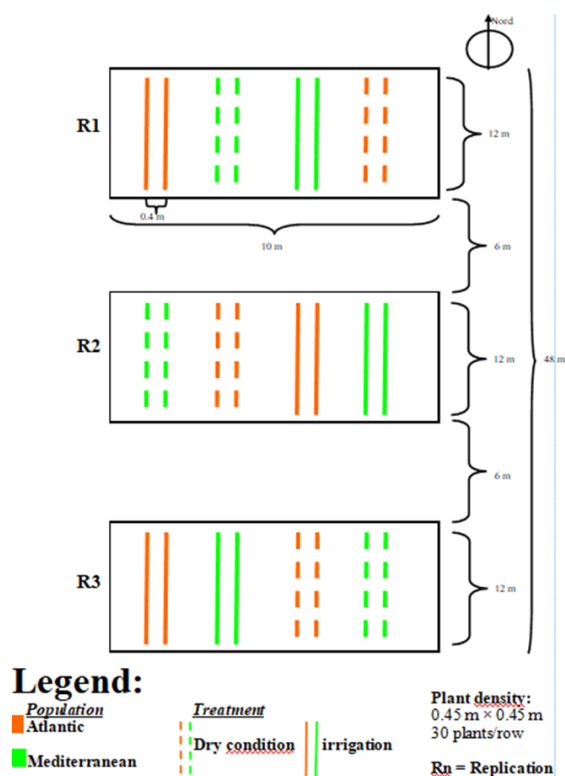
Values expressed in mean±standard deviation.



Compared ecotypes: *Crithmum maritimum* L ecotype Mediterranean, *Crithmum maritimum* L ecotype Atlantic coast
Seedbed preparation: sowing on February 2023, in greenhouse constantly at 20 °C, in polystyrene alveolate trays with peat, daily irrigation
Biostimulation: In greenhouse, a biostimulant is applied once a week (control: no irrigation), instead in the field, four applications of biostimulant for the growing season (2 L of biostimulant diluted in 10 L of water)
Plant Transplanting: About 800 seedlings of the Atlantic and Mediterranean ecotypes were transplanted in early spring 2024.



Compared Plant Density 45 x 45 cm
Plot dimension: 9.8 plant m⁻² corresponding 98,000 plants ha⁻¹
Field experiment dimension: 400 m²
Experimental design: Complete randomized blocks with 3 replications (R1, R2, R3), further divided into 3 plots based on different treatments (biostimulated, fertilized, Irrigation, Control) as previously reported by Zenobi et al. (2021, 2022)



The sea fennel plants in the experimental plots were biostimulated using the following mixture of bacterial strains (SAME BIOSTIMULANT as the Camerano site):

- free-nitrogen fixing bacteria: *Azotobacter chroococcum* DSM 2286, and *Azospirillum brasilense* DSMZ 1690 (mixed well together at a 1:1 (v/v) ratio);
- phosphorus-dissolving bacteria: *Priestia megaterium* DSMZ 339;
- potassium-solubilizing bacteria: *Niallia circulans* DSMZ 30598

The three microbial groups were inoculated at a 2:1:1 (v/v/v) ratio in load no lower than 10^8 cells/mL for each microbial group (details about preparation of the biofertilizer are reported in Section 1.1.1) and fertilized with seaweed-based compound with 5% nitrogen.

1.1.2 Site 2

Site 2. CAMERANO

Soil characterisation was performed before the *C. maritimum* L. growing season. According to the USDA classification, the soil detected is clayey loam, therefore with good water retention but subject to water stagnation.

Soil properties in the 0-30 cm layer in the experimental plots in 2023 (n = 3) are shown below:

Soil properties	Values
Sand (g kg ⁻¹)	125 ± 19
Silt (g kg ⁻¹)	445 ± 12

Clay (g kg ⁻¹)	430 ± 15
Volumetric soil water content (%):	
Field Capacity	44.3 ± 1.1
Permanent Wilting Point	18.4 ± 2.2
Total available water	26.2 ± 1.7

values expressed in mean±standard deviation.

Farm "Paccasassi del Conero" located in Camerano – Ancona – Italy. (43_530 N, 13_550 E).



Ecotypes: *Crithmum maritimum* L ecotype Mediterranean

Seedbed preparation: sowing on 26th January 2023, in greenhouse constantly at 20 °C, in polystyrene alveolate trays with peat, daily irrigation

Biostimulation: In greenhouse, a biostimulant applied once a week (control: no irrigation), instead in the field, four applications of biostimulant for the growing season (2 L of biostimulant diluted in 10 L of water)

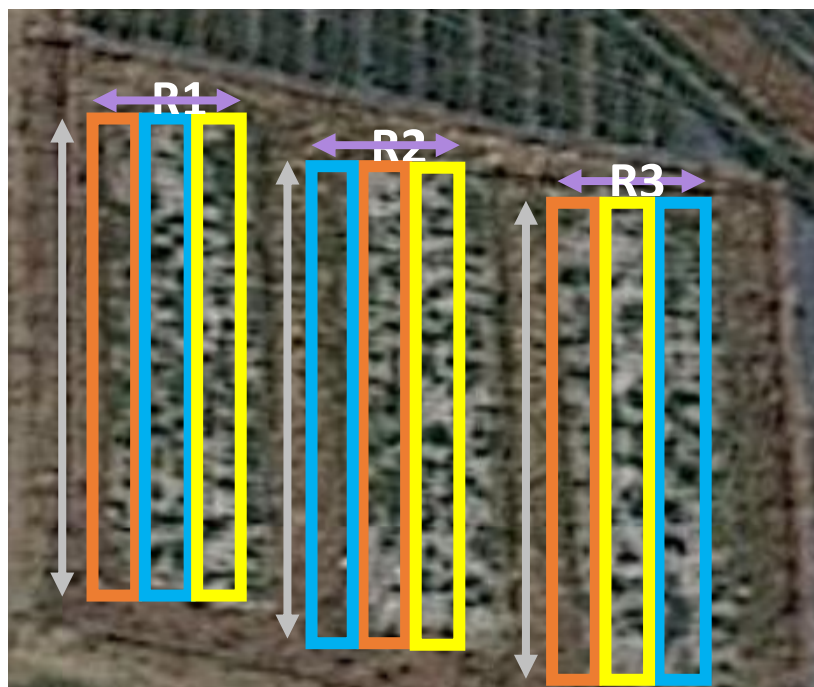
Plant Transplanting: In March 2023, the transplantation failed due to low seed germination; hence a demonstration plot consisting of 3-year-old sea fennel plants (Mediterranean ecotype) was used for bio-fertilization experiments in 2022-2023. These plants had previously been planted as part of the regional funded project "Bio-Veg-Conserve" (<http://www.biovegconserve.it/>).

Compared Plant Density 45 x 45 cm

Plot dimension: 9.8 plant m⁻² corresponding 98,000 plants ha⁻¹

Field experiment dimension: 400 m²

Experimental design: Complete randomized blocks with 3 replications (R1, R2, R3), further divided in 3 plots based on different treatments (biostimulated, Irrigation, Control) as previously reported by Zenobi et al. (2021, 2022)



Treatme : : : Control
 = 22.5 m = 5.4 m R =

The three year-old sea fennel plants in the experimental plots were biostimulated using the following mixture of bacterial strains:

- free-nitrogen fixing bacteria: *Azotobacter chroococcum* DSM 2286, and *Azospirillum brasilense* DSMZ 1690 (mixed well together at a 1:1 (v/v) ratio);
- phosphorus-dissolving bacteria: *Priestia megaterium* DSMZ 339;
- potassium-solubilizing bacteria: *Niallia circulans* DSMZ 30598

The three microbial groups were inoculated at a 2:1:1 (v/v/v) ratio in load no lower than 10^8 cells/mL for each microbial group (details about preparation of the biofertilizer are reported in Section 1.1.1)

1.2 Croatian site

Site: Institute for Adriatic Crops, Split (Croatia)



Experimental site in Split, Croatia

Soil characteristics

pH _{H2O}	pH _{KCl}	CaCO ₃	CaO	TOC	N	P available*	K available‡	Texture
		g kg ⁻¹			mg kg ⁻¹			
8.51	7.57	484	188	14.9	0.13	2.6	25.9	Silt loam

*available P was determined after extraction with (NH₄)₂SO₄ – Troug method

‡available K was determined after extraction with NH₄-acetate

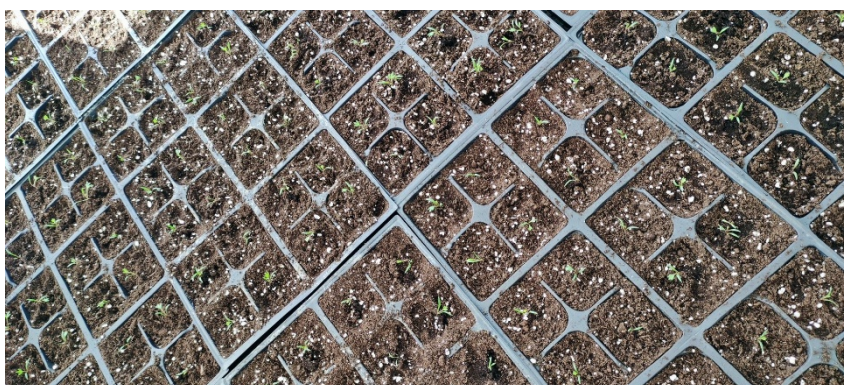
Compared ecotypes: *Crithmum maritimum* L ecotype Korčula, *Crithmum maritimum* L ecotype Atlantic coast

Seedbed preparation: sowing on 27th January 2023, in polystyrene plug trays filled with commercial peat (Brill 7, Austria), 3-4 seeds per plug was used, tray were put in heated germination tables inside unheated greenhouse, irrigation as needed.



Polystyrene plug trays used for sowing of sea fennel seeds.

Seeds started to germinate 5 days after sowing and 45 days after plants were transplanted into PE trays with 24 places, one plant per pot were transplanted. Plants were irrigated every 2-3 days.



PE trays used for first transplantation of sea fennel seedlings.

Fertilization: Fertilizers or bio-stimulants were not applied at seedling stage.

Plant Transplanting: On the third week of May 2023, seedlings with four- five true leaves have been transplanted at IACKR site.

Compared Plant Density 40 × 60 cm

Field experiment dimension: The total area of the experimental field is 700 m².

Experimental design: Complete randomized blocks with 3 replications (R).

Experimental field was prepared with deep ploughing and motocultivator to obtain fine soil surface before transplanting. Before cultivation organic pelletized bovine and equine manure – NPK 2-2-2 (Fertildung, Fomet-IT) were applied at half of the experimental field at rate of 20 kg/100 m².



Motocultivator used for soil bed preparation.

Scheme of fertilized and unfertilized plots of experimental fields



Experimental plots at the Croatian demo field.

1.3 Tunisian site



Site: INRGREF experimental station in Nabeul-Tunisia (36.464325, 10.705955)

Compared ecotypes: *Crithmum maritimum* L. ecotype Tabarka (Tunisia), *Crithmum maritimum* L. ecotype Atlantic coast (France)

Seedbed preparation: seed were sown on December 2024, in germination plates at the rate of two-three seeds per well, regular irrigation, greenhouse (controlled conditions).

Fertilization: NPK fertilizer (20-20-20) was used

Plant Transplanting: In May, plants with three-four true leaves were transplanted in the site.

Compared Plant Density 45 x 45 cm

Plot dimension: 9.8 plant m⁻² corresponding 98,000 plants ha⁻¹

Field experiment dimension: 400 m²

Experimental design: Complete randomized blocks with 3 replications.



Sowing of sea fennel seeds on December 2023

Before planting, tillage was carried out by ploughing of depth between 35-40 cm and seed beds were prepared.

To assess the influence of soil composition and salinity on plant crop yield, 5 soil samples (0-30 cm depth) were taken from the experimental site to determine its physicochemical properties. In April 2024 all seedlings had only 1-2 true leaves. According to the protocol prepared by the international Consortium of Project partners, transplantation in the field was done when the seedlings had 3-4 true leaves (May 2024). Prior to transplantation, soil was prepared in order to have fine soil surface. Hand weeding was performed in order to control weed abundance and avoid competition with the crops.



Collection of soil samples for physicochemical analyses (Left picture), and transplantation of sea fennel in the field (May 2024) (Right picture)

Plant material was collected randomly in September 2024, January 2025 and April 2025.

Soil analysis

Soil analysis showed that the experimental station is installed on a sandy neutral soil with a pH level of 7. The soil analysis reveals a moderate level of organic matter at 2.167%, beneficial for enhancing soil structure, promoting microbial activity, and supporting overall soil health. The organic carbon content of 1.259% is consistent with the organic matter value, as carbon typically makes up about 58% of organic matter. This indicates a moderately fertile soil, capable of supporting healthy plant growth.

The soil pH is neutral at 7.0, which could optimize the availability of essential nutrients and encourage beneficial microbial activity. The electrical conductivity (EC) is measured at 300 $\mu\text{S}/\text{cm}$, which indicates low salinity.

Regarding macronutrients, the total nitrogen content is 2.14 g/kg, a moderate to high level that supports vigorous vegetative growth. The phosphorus content, at 0.93 g/kg, is also sufficient, contributing to healthy root development and reproductive processes like flowering and fruiting. Similarly, the potassium level of 1.13 g/kg is adequate to support various physiological processes.

Among the secondary nutrients, calcium is notably high at 8.17 g/kg, which is beneficial for plant structural integrity. The magnesium level, at 2.05 g/kg, is satisfactory, and Sodium is very low (<0.1 g/kg).

Finally, the chloride content of 73.67 mg/kg is within a normal range and should not pose a toxicity risk. The sulfate level, at 579.78 mg/kg, is relatively high, ensuring an adequate supply of sulfur for protein synthesis and enzymatic functions in plants.

Soil chemical analysis of experimental site

Variable	Value	Unit	Method
Organic Matter	2.167	% of dry matter	Calcination at 525°C
Organic carbon	1.259	% of dry matter	ISO 14235 (1998)
pH	7.0 (at20.1°C)		NF EN 12176 (1998)
Electric conductance	300	µS/cm	NF ISO 11265 (1995)
Total nitrogen	2.14	g/Kg of dry matter (DM)	NF ISO 11261 (1995)
Total phosphorus	0.93	g/Kg DM	ISO 11885 (2007)
Potassium	1.13	g/Kg DM	ISO 11885 (2007)
Sodium	<0.1	g/Kg DM	ISO 11885 (2007)
Calcium	8.17	g/Kg DM	ISO 11885 (2007)
Magnesium	2.05	g/Kg DM	ISO 11885 (2007)
Chloride	73.67	mg/Kg DM	ISO 10304-1 (2007)
Sulfate	579.78	mg/Kg DM	ISO 10304-1 (2007)

1.4 Turkish site

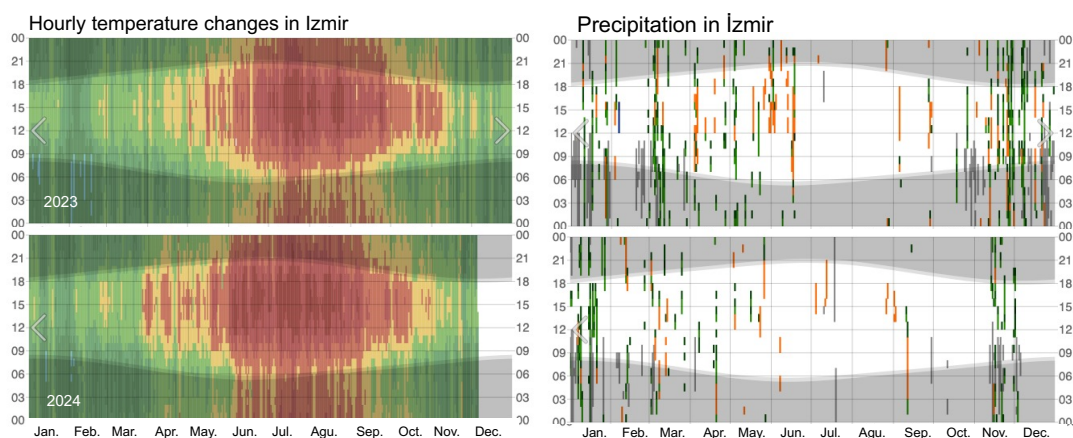
Site: Experimental field of Ege University, Faculty of Agriculture, Department of Field Crops, Bornova-Izmir/Turkey (38°27'06.0"N 27°13'31.9"E)

Soil samples (0–30 cm depth) were collected from the experimental site before transplanting. The samples were analyzed for various physicochemical properties, including soil texture, pH, organic matter content, electrical conductivity, total nitrogen (N), available phosphorus (P), exchangeable calcium (Ca), potassium (K), sodium (Na), iodine (I), magnesium (Mg), and major anions. Throughout the growing period, irrigation was carried out using a drip irrigation system. Field observations confirmed that sea fennel plants were unable to survive under rainfed conditions at the experimental site. Weed management was performed through mechanical removal, targeting *Sorghum halepense* L., the predominant weed species in the field. Hourly average temperature and total precipitation data for 2023 and 2024 at the experimental site (İzmir-Bornova) are presented in the Figure below. Mean temperatures in 2024 were recorded as 1.2°C (7.2%) higher than in 2023. Total precipitation in 2023 was 780 mm higher than long-term averages, while precipitation levels in 2024 were projected to be at least 20% lower by the end of the year.

Soil

pH	7.88
Org.(%)	1.99
Salinity (%)	0.09
CaCO ₃ (%)	14.2
Structure	Clay-loamy
Sand (%)	42
Silt (%)	21
Clay (%)	37
N (%)	0.05
P (ppm)	6.75
K (ppm)	506
Fe (ppm)	0.18
Cu (ppm)	1.10
Mg (ppm)	367
Zn (ppm)	1.95
Na (ppm)	73
Mn (ppm)	1.17

Physicochemical properties of the soil sampled from the experimental site in Izmir. The samples were collected before the sea fennel seedlings transplanted to the demo field trail (2023).



Hourly average temperature and total precipitation during 2023 and 2024 at the experimental site (İzmir-Bornova).

Compared ecotypes: *Crithmum maritimum* L ecotype AC-1 (Aegean Coast 1), *Crithmum maritimum* L ecotype AC-2 (Aegean Coast 2) and *Crithmum maritimum* L ecotype ATL (Atlantic Coast).

Seedbed preparation: As part of work package 3, fresh sea fennel samples and seeds were collected from four different locations in the Turkish flora and stored under suitable conditions in work package 4. In addition, sea fennel seeds of the French Ecotype were provided by Dr. Christian Magne, our project partner from France. The seeds of French Ecotype "Atlantis" were received later than scheduled, on March 18. Immediately upon receipt of the seeds, on March 20, Atlantis was planted as a control genotype along with seeds collected from the four different locations of the Turkish flora. These seeds were planted in plastic seedbed trays filled with sterilized

organic pits, with approximately 2,000 seeds planted from each ecotype, for a total of 8,000 seeds. The vials were irrigated with a sprinkler every day at 9:00-10:00 AM.



Planting of sea fennel seeds to the plastic vials filled with organic pit in 20th of March (left picture). Irrigation by a sprinkler head fixed garden pipe (right picture).

The first seeds germinated on April 11th 2023 and 57-66% of the seedlings had 1-2 true leaves by April 25th (Fig 2). According to the protocol prepared by the international consortium, the seedlings were transplanted into demo field after the appearance of 2-4 true leaves (May 2023).



Germinated seedlings in vials filled with organic peats. The pictures were taken on 25th April 2023.

Biostimulation: A bio-stimulant was applied to the seedlings at the beginning of the seeding process in the greenhouse. Half of the plots in the field trial will be treated with bio-fertilizer, while the other half will not receive any fertilizer application.

Plant Transplanting: The seedlings are scheduled to be transplanted to the field between May 8 and May 12. The field has already been prepared, soil samples have been collected, plots have been arranged, and the irrigation system has been installed.

Compared Plant Density: The experimental layout consisted of rows spaced 45 cm apart, with plants within each row spaced 22.5 cm apart (9.8 plants/m²).

Plot dimension: Each plot has a size of 1.8 x 5.0 meters, and there are a total of 36 plots.

Field experiment dimension: The total area of the experimental field is 390 m², of which 324 m² was used for planting.

Experimental design: The study incorporated two main treatment factors: (1) ecotype (ATL, CAN, and URL) and (2) fertilization (bio-fertilizer application versus control with no fertilization). A split-plot experimental design based on a randomized complete block (RCB) design with three replications was implemented. A biostimulant was applied to the seedlings at the early growth stage in the greenhouse. In the field trial, half of the plots received bio-fertilizer, while the other half remained untreated. The bio-fertilizer treatment consisted of a commercial organic fertilizer (Organomineral®) containing 12% N, 12% P₂O₅, and 12% K₂O, applied in comparison with a no-fertilization control.



*Transplanting of the sea fennel (*Crithmum maritimum* L.) seedlings to the field between 8-12 May 2023.*