BeonNAT

Innovative value chains from tree & shrub species grown in marginal lands as a source of biomass for bio-based industries

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Protocol for intercropping tests establishment and follow up

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PROJECT INFORMATION

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Nº	Acronym	Participant organization name
1 (Coordinator)	CIEMAT	Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas
2	CESEFOR	CESEFOR
3	REC	Renewal Energy Consortium for Research and Demonstration
4	AIM	Instituto Tecnológico del Plástico
5	ATB	Leibniz Institute for Agricultural Engineering and Bioeconomy
6	BTU	Brandenburg University of Technology Cottbus-Senftenberg
7	USV	Universitatea Stefan el Mare, Suceava
8	IPB-CIMO	Centro de Investigação de Montanha / Instituto Politécnico de Bragança
9	CTA	Contáctica
10	IDS	IDOASIS 2002 S.L.
11	EJAR	El Jarpil
12	ENV	Envirohemp
13	NNFCC	The Bioeconomy Consultants NNFCC
14	TOLSA	TOLSA
15	MAVERICK	Laboratorios Maverick
16	PEFC	Asociación para la Certificación Española Forestal

DELIVERABLE DETAILS

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Task:	Task 2.2. Intercropping trials design and plantation of the selected species
Authors:	Steffi Schillem Werner Gerwin Dirk Freese Laura Bouriaud Ciprian Palaghianu Mihai-Leonard Duduman Irene Mediavilla Luis S. Esteban Marina Sanz Carlos Martín
Abstract:	The species selected in task 1.2 and multiplied/purchased in task 2.1 will be planted in selected marginal land in Spain by CIEMAT and EJAR, in Germany by BTU and in Romania by USV. Two (2) tests sites per country will be set up in marginal lands. Test fields will be prepared with a total of 12 plots per test based on that protocol.

1 Objectives

Field trials in three different European countries (Spain, Germany, Romania) will be established to test the benefit of intercropping/mixed-forest in marginal cultural land versus the natural growing/monoculture. This targets the investigation of plant interactions in intercropping systems, the ecological effects on marginal lands and the potential of native plant material. Furthermore, different harvesting and logistics systems will be tested in order to evaluate the whole value chain of the studied products. The provision of biomass for industry partners for the BeonNAT refinery will also be realized with these trials.

2 Field trial design

To investigate the performance of the species selected in Task 1.2 on marginal lands and to obtain wood-based feedstock for different industrial applications, field trials will be carried out in Spain by CIEMAT and EJAR, in Germany by BTU and in Romania by USV. In each country two test sites will be established on either agricultural or forest marginal land. On each site two species will be tested and compared between monoculture and intercropping.

2.1 Selected sites and species

2.1.1 Field trial sites

In Spain, two test sites will be established, one located in the north and the other in the south of Spain. The test site in Northern Spain will be on forest marginal land at Lubia (Soria). The field has a surface of about 1.5 ha and the soil is characterized by a pH of 6.6 with abundant stones, 56.7 % coarse soil texture fragments (> 2mm), and a soil depth of about 40 cm. The climate conditions are average annual precipitation of 493 mm and average annual temperature of 10.68 ºC. On this site *Juniperus communis* L. and *Ulmus pumila* L. will be planted. The second field trial will be carried out in the South of Spain on agricultural marginal land in Velefique (Almería). The test site is located at an altitude of 1,680 m above sea level. The field has about 1.5 ha and the soil is characterized by high stoniness and sandy loam texture. Annual precipitation is about 250 mm and average annual temperature about 15 ºC. *Rosmarinus officinialis* L. and *Cistus ladanifer* L. will be planted in this field trial

In Germany, both test sites are located in the east of Germany. One of the test sites will be established on marginal forest land close to Kromlau (northeast of Saxonia). The test site is about 1.5 ha in size and is characterized by a low pH of 3.7 and sandy substrate. On this site *Betula pendula* and *Cytisus scoparius* will be planted. The second field trial will be carried out on a reclamation site (agricultural land) in Welzow-South (south of Brandenburg). This nutrient poor, sandy site has pH values between 3.0 and 7.0 and the annual precipitation is about 560 mm. *Robinia pseudoacacia* and *Rubus fruticosus* are the species chosen for this field trial.

In Romania, both test sites are located in the northeast of Romania, in Suceava county. One of the test sites is located in the vicinity of Suceava city, in Moara (USV Campus 2). In this case the land has not been cultivated for more than 15 years. *Betula pendula* and *Carpinus betulus* will be installed in this site. The second test site will be established in the Siret river meadow, at Zamostea. *Robinia pseudoacacia* and *Populus nigra* were selected for this site. In this field trial, a nutrient-poor clay site was chosen, with significant wetness in the root zone for extended periods, affected by compaction.

2.1.2 Species

In Spain selected species will be multiplied from wild plants. In relation to *Ulmus pumila*, the cuttings were collected from CEDER plantations in May 2021 and the *Juniperus communis* cuttings in Barriomartín (Soria) and Fuentelcarro (Soria) between December 2020 and February 2021. The material for multiplying *Rosmarinus officinalis* was collected from Bonete (Albacete).

In Germany, due to very high costs and limited material for multiplying, plants will be purchased by local nurseries as 1-year-old saplings. However, to enable the investigation of the potential of native wild plants, two rows per species (1 in monoculture, 1 in intercropping) of propagated wild species will be established.

In Romania, since *Carpinus betulus* and *Betula pendula* are not economically valuable forest species, they are not produced in local nurseries. Instead, birch saplings will be produced from seeds in a local nursery, and hornbeam saplings will be harvested from natural regeneration. In the case of *Robinia pseudoacacia* and *Populus nigra*, due to the limited material for multiplication, saplings will also be purchased from local nurseries.

Due to the nature of the species selected, different plant distances within one row are recommended:

Table 1 Selected species for field trials in the respective countries

Species	Leave trait	specific distances between plant individuals		
		Spain	Germany	Romania
Betula pendula	Deciduous		1 m	1 m
Carpinus betulus	Deciduous			1 m
Cistus ladanifer	Evergreen	0.5 m		
Cytisus scoparius	Evergreen		1.5 m	
Juniperus communis	Evergreen	1 m		
Populus nigra	Deciduous			1 m
Robinia pseudoacacia	Deciduous		1 m	1 m
Rosmarinus officinalis	Evergreen	0.5 m		
Rubus fruticosus	Deciduous		1.5 m	
Ulmus pumila	Deciduous	1 m		

2.2 Monoculture and intercropping design

Ecological and economical restoration of marginal lands and avoiding competence between food agriculture and industrial forestry have been considered as main issues to select these lands for the cultivation of new and underutilized tree and shrub species in BeonNAT. To improve the fertility of soils and enhance the organic carbon stocks of marginal lands intercropping techniques are a suitable solution.

The term “intercropping” is usually associated with agriculture. As intercropping can increase crop productivity and the overall sustainability of systems, it is an important tool to facilitate sustainable intensification. In forestry, intercropping techniques result in so-called mixed-forest (two species cultivated in same line at same time) or in mixed-plantations (two species cultivated in different lines at same time). The concept of mixed-plantations will be tested in the present field trials.

While the distances between plants depend on the growth behaviour of plants, the distances between rows are mainly based on the space harvest machines will need. Therefore, an important factor for the determination of plant row distances is the harvest interval linked with the final size of plants at harvest.

To realize the objectives of the field trials, on each test site three subtests with one plot each will be established, one plot per species (monoculture A and B) and one plot for intercropping both species (A+B). Each **plot** has a **length of 99 m** and the **distance between plots** or outside rows of the plots should be **at least 4 m**. Additionally, one **control area** has to be defined close to the test site having similar characteristics to represent

the status of the field trials before planting the chosen trees and shrubs. The size of the control area should be large enough for placing four 25 m² monitoring plots (see below in section 3) with some distance between them.

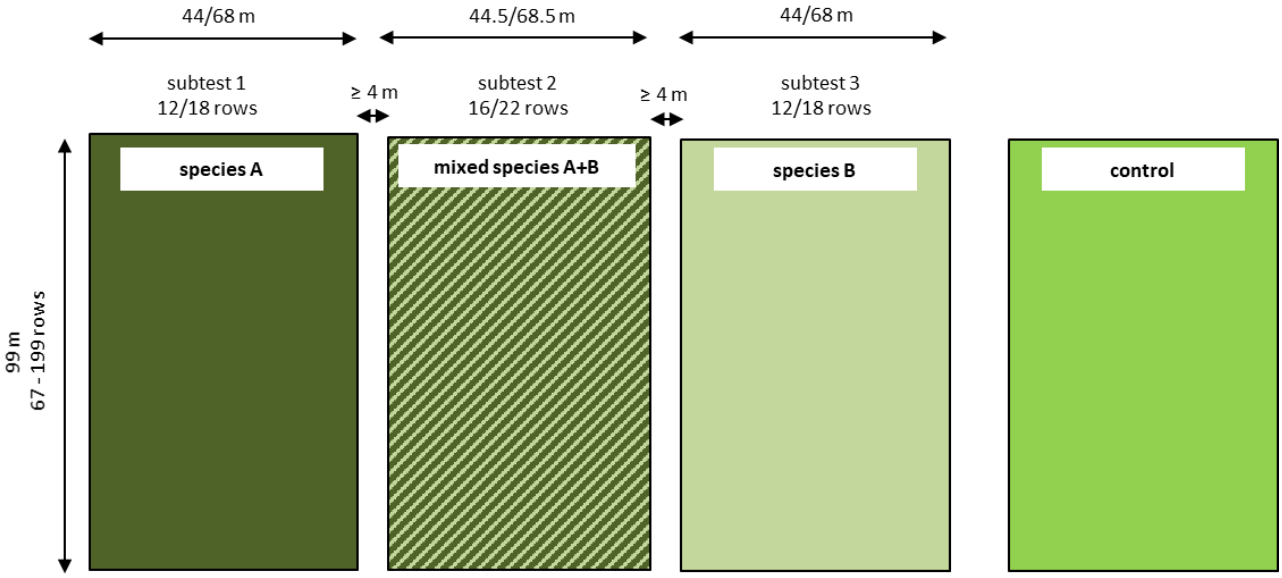


Figure 1 Field trial design

In Lubia (Soria) in Spain, the plots will be 68 m in width resulting in 18 rows for subtests 1 and 3. In Germany and Romania, the plots will be 44 m wide due to the size of the selected test sites. Therefore, 12 rows will be established for subtest 1 and 2. Based on the requirements of the intended harvest techniques the **distance between rows** will be **4 m** for subtest 1 and 3. However, in the field trial in Velefique (Almería) the distance between rows will be 2 m. If the dimensions of the plots cannot be realized on the sites selected, the total length of rows per species has to form the basis for the dimensions of the plots. For Spain, this would correspond to about 1.782 m (Lubia)/ 3.500 m (Velefique) and in Germany and Romania about 1.188 m for each species in subtest 1 and 3. This results in e.g. in Velefique in row numbers between 30 and 63. Plant and row distances have to be kept (Figure 1).

To investigate the effects of intercropping, subtest 2 will be divided into 2 **intercropping** subtests (IC). In IC 1 the **distances** between the rows will stay at **4 m** and **2 m** in Velefique that are necessary for the harvest techniques tested. In IC 2 the distances will be reduced to **1.5 m** between plant rows to enable plant interactions even in the first years of growing. IC 2 will consist of four rows in total to realize monitoring plots without site effects of other distances. The position within the field can be chosen individually depending on eventually inhomogeneous soil conditions. In Figure 2 a possible design with the dimensions of a plot in Germany or Romania is displayed. In the field trial in Velefique (Almeria) IC2 will not be established.

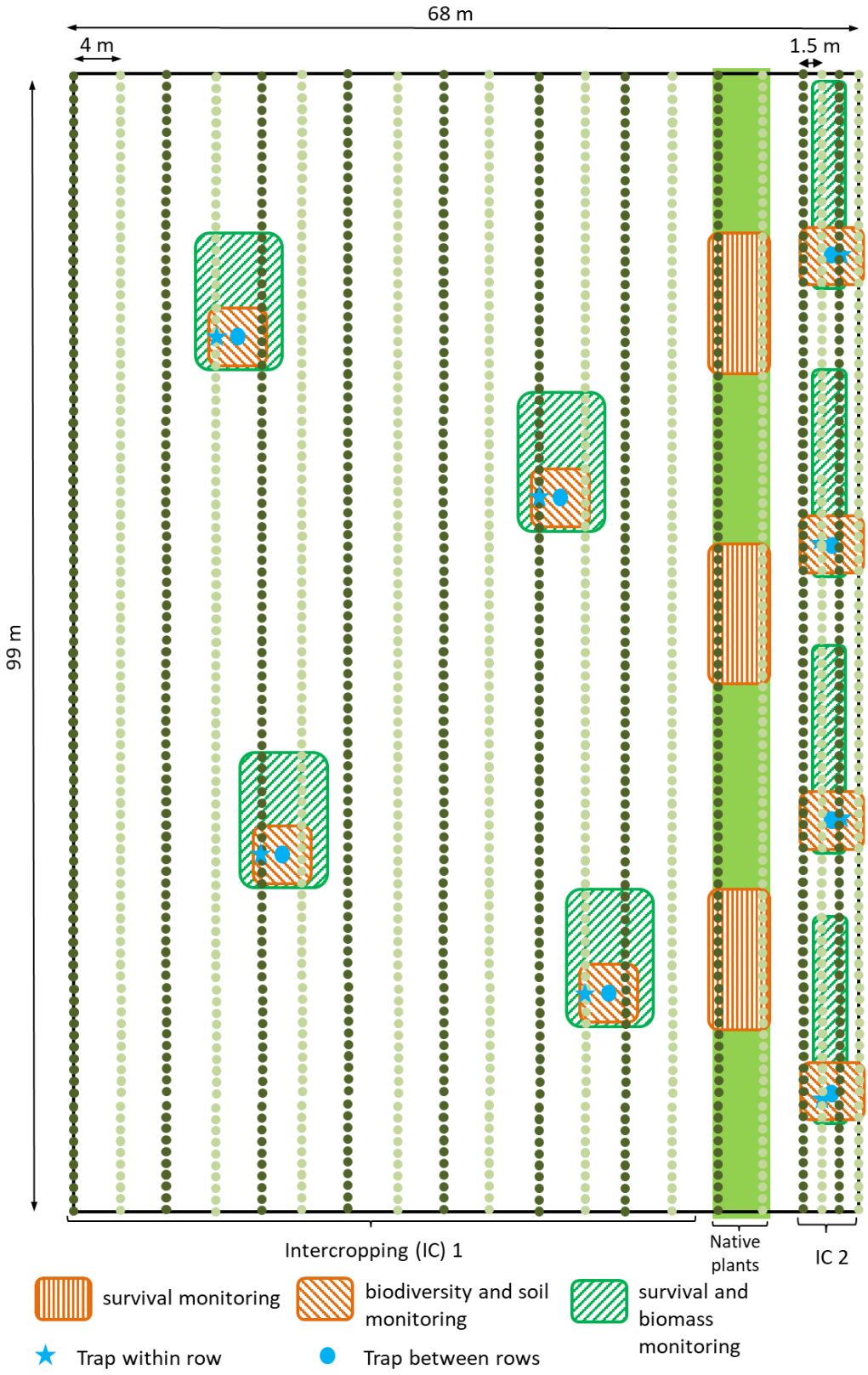


Figure 2 Design of the intercropping subtest with IC1 and IC 2 and the corresponding monitoring plots with one row per species (green area: one row per species with native plants only in Germany)

2.3 Fencing and soil preparation

Fencing is not mandatory. It depends on the conditions of the field trial location and on the conditions that are best practice in the country/region – e.g, in Spain where forest plantations always have a fence. If fencing is planned, it is mandatory to include the control area to not to investigate the effect of the fence but the effect of the cropping system.

Two fencing options can be realized:

1) Fencing of the whole field trial design including the control plots

2) fencing of parts of the field trial including all treatments and control to also investigate the effect of the fence

In Spain the option chosen for the plots located in Lubia (Soria) will be the number one, where fencing is for the whole field trial design including the control plots.

In Germany no fencing is planned. However, the Kromlau site is situated within a larger area which is already demarcated by fences.

In Romania, a large area of approximatively 30 hectares containing the test site from Moara (USV Campus 2) is already fenced, including the control area. In the second test site, at Zamostea, no fencing is planned.

Generally, **no preparation of field trials** (like fertilizing or mechanic soil preparation) is planned. But **if necessary** and common practice, soil preparation can be carried out; like in Spain where the compacted layers in the subsoil have to be broken (subsoiling in 70 – 80 cm depth) or in case of intensive growth of wild vegetation. Additionally, further management activities can become necessary. If the autumn and spring are too dry, watering will be necessary in Spain. And, if necessary, in Germany and Romania mechanical weed control at least once a year will have to be carried out whereas in Spain no weed control is planned.

3 Monitoring

During the field trials the biomass development of the planted shrub and tree species will be investigated. Furthermore, the impact of tree cultivation on the marginality of soils and biodiversity will be monitored by continuous soil sampling and observations. **This second element of the monitoring programme is part of WP 8 (Task 8.6 Biodiversity and soil quality assessments).**

The monitoring will be carried out at **4** different **monitoring plots for survival and biomass** development and additional **4 monitoring plots for soil and biodiversity** within each subtest and the control area (Figure 2, Figure 3). Each **biomass monitoring plot** will consist of at least **12 trees** per species – 6 trees in two rows for monocropping, 12 trees per row for intercropping. Within these plots the **monitoring plot for soil and biodiversity** will be located having a size of **5 x 5 m** (25 m²) containing two rows. The precise position of the monitoring plots will depend on the final dimensions of the subtest plots and should be randomized. Monitoring plots displayed in Figure 2 and Figure 3 show the approximate positions that should be realized by all partners.

For the interpretation of the biomass data, **weather data** of a nearby weather station should be collected including at least the monthly mean temperature and the monthly precipitation.

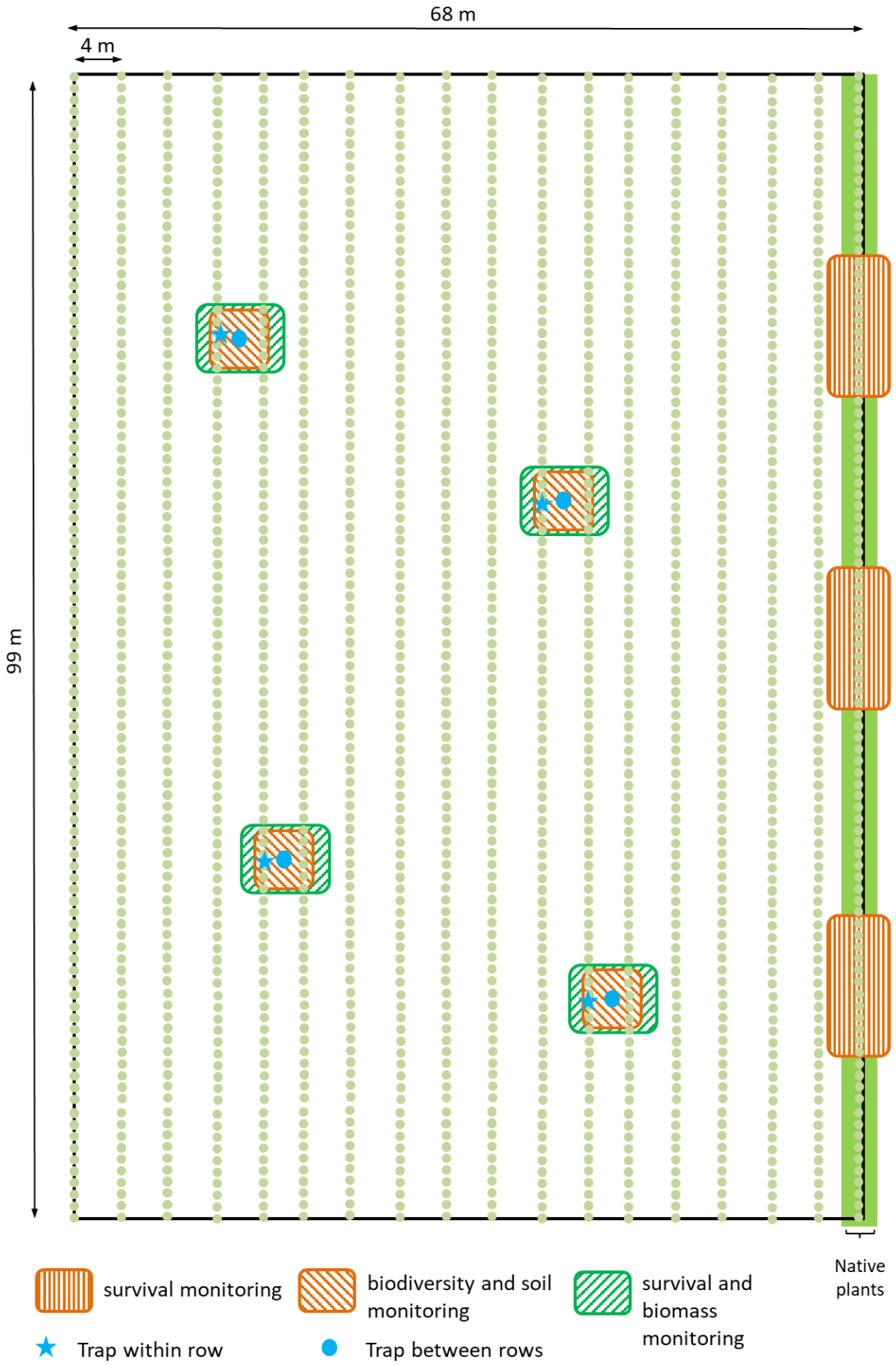


Figure 3 Subtest 1 or 3 with one species (A or B) with planting distance of 1 m within rows and different monitoring plots (green area: one row with native plants only in Germany)

The monitoring of biomass, soil and biodiversity will be carried out in regular periods of time:

Table 2 Overview about the BeonNAT monitoring program

Year	2021						2022						2023						2024						2025		
Season	Spring-Summer			Summer-Autumn			Spring-Summer			Summer-Autumn			Spring-Summer			Summer-Autumn			Spring-Summer			Summer-Autumn			Spring-Summer		
Month	4	5	6	8	9	10	4	5	6	8	9	10	4	5	6	8	9	10	4	5	6	8	9	10	4	5	6
WEEK																											
Plant survival		I-IV					I-II						I-II						I-II								
Biomass develop.						I-IV						I-IV						I-IV						I-IV			
Biomass yield												I-IV						I-IV						I-IV			
Roots																								I-IV			
Soil	I/3 – IV5						I/3 – IV/5						I/3 – IV/5						I/3 – IV/5						I/3-IV/5		
DNA in soil						I/10 – II/11																		I/10 – II/11			
TBI Instal-lation		I/5 – IV-6						I/5 – IV/6						I/5 – IV/6						I/5 – IV/6							
Plant biodiv.	I/4 – IV/7						I/4 – IV/7						I/4 – IV/7						I/4 – IV/7						I/4 – IV/6		
Install traps	III			III			III			III			III			III			III			III			III		III
Collect insects		I; III	I; end of II		I; end of II			I; III	I; end of II		I; end of II			I; III	I; end of II		I; end of II			I; III	I; end of II		I; end of II			I; III	I; end of II
Remove traps			end of II		end of II				end of II		end of II				end of II		end of II				end of II		end of II				end of II

The first investigations will be carried out immediately after planting.

Further details are described in chapters 3.1 to 3.3.

To guarantee an exact assignment all samples have to be named as follows:

Table 3 Code for naming samples

Country		Test site		Test plot/species		Number of monitoring plot	Number of tree
Spain	ES	Lubia	Lu	Betula pendula	BET	1	(1)
		Velefique	Ve	Carpinus betulus	CAR	2	(2)
Germany	DE	Kromlau	Kr	Cistus ladanifer	CIS	3	(3)
		Welzow-Süd	WS	Cytisus scoparius	CYT	4	(4)
Romania	RO	Moara	Mo	Juniperus communis	JUN		(5)
		Zamostea	Za	Populus nigra	POP		(6)
				Robinia pseudoacacia	ROB		(7)
				Rosmarinus officinalis	ROS		(8)
				Rubus fruticosus	RUB		(9)
				Ulmus pumila	ULP		(10)
							(11)
				for Intercropping e.g.	BET+CYT 1		(12)
					BET+CYT 2		
				Control	CO		

Example of a **sample code**: DE Kr BET+CYT 1 – 1; DE Kr BET- 1

3.1 Biomass monitoring

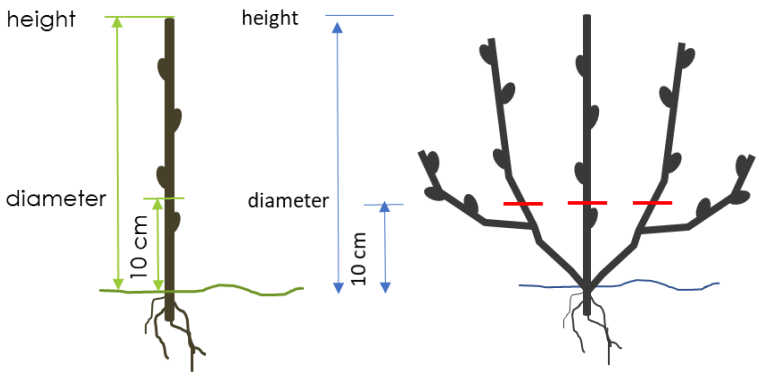
The biomass monitoring can be divided into three categories: survival of plants, biomass development and biomass of plants. These parameters will be investigated within the biomass monitoring plots containing 12 trees per species (see above). Each tree receives a number to enable the comparison between monitoring dates.

3.1.1 Survival of plants

The **survival of plants** will be investigated **once a year in spring** to evaluate the suitability of the plants for the chosen marginal field. Plants will be classified as “vital”, “poor vitality” and “dead”.

3.1.2 Biomass development

The **plant growth** will be evaluated within the monitoring plots at start of the field trial (planting) and afterwards once a year in autumn. The parameters shown in Figure 4 will be recorded as the basis for allometric functions to be established for all in BeonNAT cultivated tree and shrub species grown at marginal lands.



Parameter	Unit	Remarks/Formula
Stem diameter	mm	At 10 cm height from soil surface
Plant height	cm	From the soil surface to the highest tip of plant

Figure 4 Biomass monitoring: measuring plant growth parameters (trees and multi-stem shrubs)

For shrubs, the height and diameter of all first order shoots will be measured and number of shoots will be recorded (see Figure 4). For each individual shrub an average height and diameter is calculated based on these single measurements.

The collected data will be classified into 3 plant and site-specific growing classes (small, medium, large). The class boundaries have to be calculated individually for each plant species and year with equal class widths.

3.1.3 Surface biomass of plants

For establishing the needed allometric functions the total surface biomass needs to be measured by means of harvesting and weighing exemplary trees and shrubs from the test sites. For that reason, once a year in autumn 12 trees per species and subtest will be randomly harvested **outside the monitoring plots** at the end of the vegetation period to estimate the **biomass yield** [kg] after 1, 2 and 3 vegetation periods. Before harvesting **height and diameter (at 10 cm above ground) of each selected test plant must be measured and documented** for establishing allometric functions and for comparison with the plants of the monitoring plots. The selected plants should be representative for the site-specific distribution of height and diameter across the caclulated growing classes (see 3.1.2). At least half of the tree rows established at each subtest should be excluded from this yearly tree sampling, so that these rows are exclusively available for the final harvesting trials.

3.1.4 Subsurface biomass of plants

After the final biomass harvest in autumn 2024 the **rooting depth** [cm] and **rooting density** (number of roots per dm²) will be determined using a soil profile of 0.5 - 1 m depth (depending on depth of visible roots) along the tree row with a tree in the middle (see Figure 5 left and middle).

For estimating **root density**, a grid is used with a mesh width of 10 cm (Figure 5 middle). Practically, this can be easily made by means of a 1 x 1 m frame (e.g. made of wooden laths) with regularly stretched parallel wires or strings. A clear contrast between the colour of the wires or strings and the soil colour is needed (e.g. white). The number of fine roots (diameter < 2 mm) as well as of coarse roots (diameter > 2 mm) is counted for each square of the grid (number of roots/dm²).

For analysing carbon and nutrient allocation within the root system representative **root samples** will be taken. A metal frame with a volume of 1,000 cm³ is used to take in total nine subsamples from the soil profile (see

Figure 5 middle and right). The metal frame is open both at the bottom and the top so that it can be completely pressed (hammered) into the soil profile in the positions indicated in Figure 5 (middle). The soil sample with roots is stored in plastic bags for transport. In the laboratory, the samples are air dried, carefully loosened and sieved with a mesh wide of 2 mm. Coarse root particles that do not pass the sieve are separated from the mineral particles and weighed. For each species and country one mixed sample of coarse roots is sent to CIEMAT for further analysis (C, N, P, K) along with the biomass samples needed for Task 2.8.

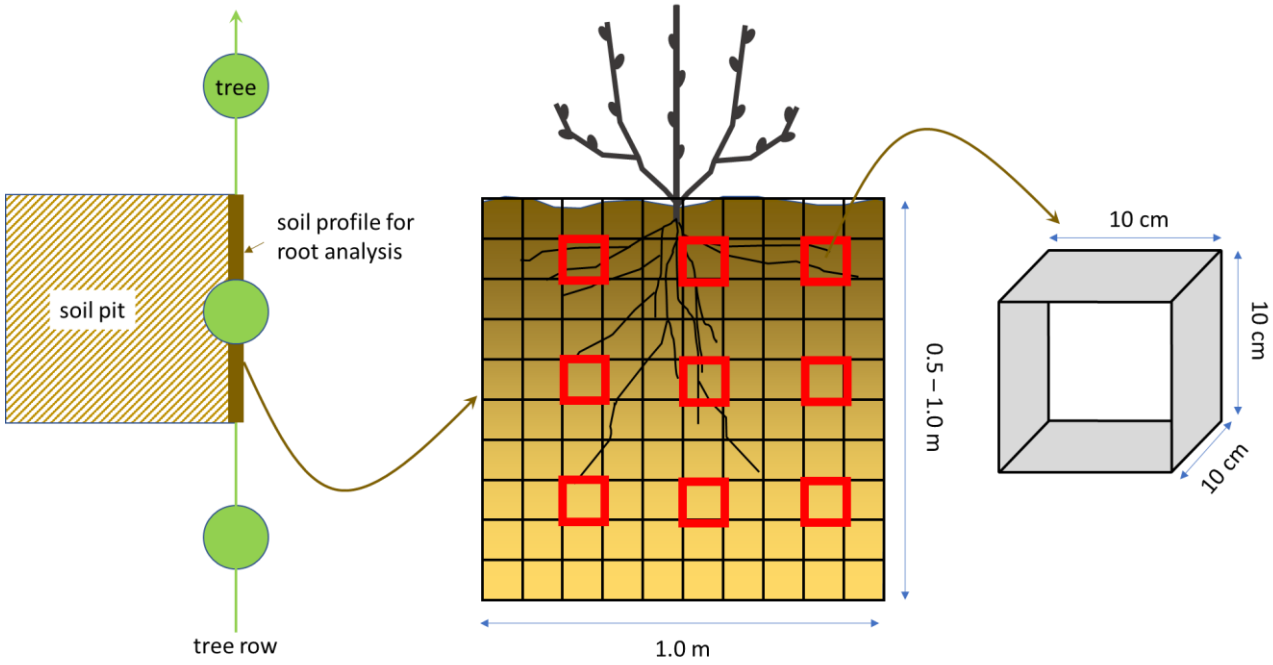


Figure 5 Root analysis: left: plan view of the soil profile; middle: soil profile with 10 x 10 cm grid for root density estimation; right: 1,000 cm³ sample cube for root sampling

This assessment and sampling procedure will be carried out for subtests 1 and 3 and in subtest 2 for both species each in IC1 – so four soil profiles in total.

For **leguminous plants** the presence and activity of root nodules will be determined. The nodules will be cut in halves and if the inner part is orange or red, the nodules are active.

3.2 Soil monitoring

For investigating potential effects of cultivating selected tree and shrub species on marginal lands in different European regions a joint monitoring program is implemented at each BeonNAT test field. An **initial soil characterization** is carried out before or immediately after planting the respective tree and shrub species. In the following years soil investigations are repeated **once a year in spring (March – May)**.

Several additional soil properties not mentioned here are part of the marginality assessment by means of the Soil Quality Rating Index (SQR) and data are collected within Task 2.3. They include, e.g., bulk density, soil texture, and stoniness (pedregosity). **These parameters are measured only once during soil quality assessment and not annually within the monitoring program.** They are not part of this protocol.

3.2.1 Soil organic matter and nutrients

3.2.1.1 Soil sampling

Soil samples are taken from the marked 25 m² monitoring plots (see above). Main objective is elucidating potential impacts of the cultivated tree species on soil properties. Therefore, soil samples are only taken **within the tree/shrub rows between the single plants** – not in the areas between the rows (Figure 6). If the trees have been planted by machines soil samples should be taken from the resulting furrows.

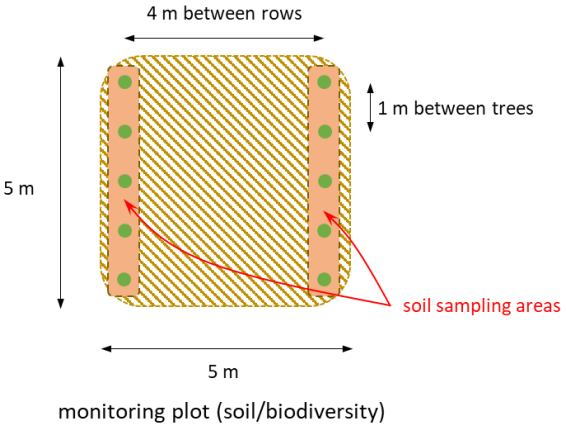


Figure 6 Left: Areas for soil sampling within the 25 m² monitoring plots (example for a mono-species subtest with 4 m distances between rows and 1 m planting distance); right: sampling between the tree individuals along the row of plants (here: *Betula pendula* with 1m distance between trees)

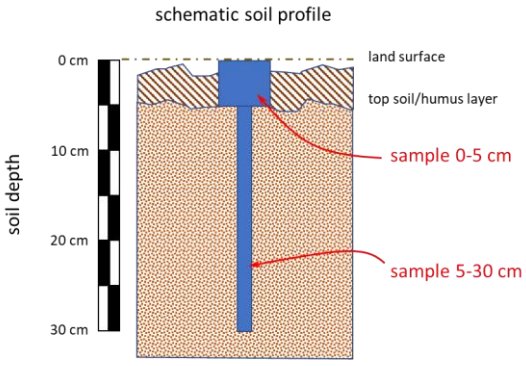


Figure 7 Sampling depths for the two sub-samples

Two sub-samples are taken from **0-5 cm and 5-30 cm below surface** as mixed samples (Figure 7). **Depth is measured from the level of land (not of the mineral soil) including possible humus layers** at the surface of the mineral soil. Fresh litter material or living plants (herbs) are excluded.

Soil material from the two depths is taken at in total **10 single sampling points** within the sampling areas of each monitoring plot (see Figure 6) and carefully **mixed for each depth and plot**. The single sampling points should be equally distributed along the tree rows of the monitoring plot for achieving a representative image of the plot. **This results in 2 soil samples per monitoring plot (0-5 cm and 5-30 cm).** Each sample should have a fresh mass of about 500 g. Excess material can be rejected. The exact sampling locations within the sampling areas should be slightly modified each year so that the effect of the disturbance caused by sampling can be avoided.

Sampling is carried out in two steps (see Figure 8): In a first step the humus/topsoil sample is taken from the upper 0-5 cm of the soil by means of suitable instruments (sampling rings or spatula/trowel) (Figure 8a). The sample from the second depth (5-30 cm) is taken by a push or hammer probe (Figure 8b). The remaining sampling hole should not be backfilled to avoid a second sampling on this place with wrong results.

Figure 8 Soil sampling devices: a) humus/top soil (0-5 cm) sample: spatula/trowel (above) or sampling ring (below); b) sub soil (5-30 cm) sample: push probe

At **intercropping subtests**, the above described procedure is applied accordingly. Within each monitoring plot **soil samples are taken from rows of both tree species and mixed to one soil sample per depth.** This procedure is carried out separately for IC1 and IC2 (see scheme of monitoring plots above). Accordingly, for each intercropping subtest field in total 16 soil samples (2 depths, 8 monitoring plots) have to be taken.

Soil sampling at the **control plots is only carried out in the beginning and in the end of the field trials** following the protocol above.

3.2.1.2 Laboratory analyses

After sampling soil samples are immediately dried **(air drying, maximum temperature 40°C)**. Dried samples are **sieved to a size of < 2 mm**. Particles with a larger diameter than 2 mm are rejected, only soil particles (including humus particles) < 2 mm are used for the laboratory analyses described below.

The following **parameters** will be determined **annually** to investigate the impact of the species on soil properties: total carbon (C_t in %), organic carbon (C_{org} in %), pH_{CaCl2}, total contents of N, P, K (in %) and plant available nutrients (P and K).

1. **$pH_{(CaCl2)}$**

   Soil pH is measured in 0.01 M $CaCl_2$ solution in a ratio of 1 : 2.5 (soil : solution).

2. **Total carbon (Ct) and nitrogen (Nt)**

   C_t and N_t are ideally simultaneously analyzed using an elemental analyzer system.

3. **Total organic carbon (Corg)**

Sub-samples (≥ 300 mg) are filled into ceramic crucibles and heated to at least 450°C in a muffle furnace (up to 8 h). The remaining total carbon content of the incinerated soil sample is then analyzed (see above). The difference between C_t of the air-dried sample and C_t of the incinerated sample is the total organic carbon content (C_{org}). C_{org} can be multiplied by 1.72 for calculating soil organic matter.

4. Total P and K content

P and K is analyzed after pressure digestion (HNO_3).

5. Plant available P and K

Nutrients are extracted using 0.01 M $CaCl_2$ solution. Eluates are prepared in a ratio of 1 : 2.5 (air-dried soil : $CaCl_2$ solution).

3.2.2 Soil biological activity

3.2.2.1 Tea bag index

In addition to monitoring soil organic matter and nutrients a standardized litter decay test can be **optionally** performed every year for estimating soil biological activity. The **Tea Bag Index (TBI)** is described by Keuskamp et al. (2013)[1] and is easy to perform. It is based on burying standardized types of tea bags (Lipton green tea and rooibos tea) in the topsoil of the test field. The mass loss due to the decay of the tea particles by soil fauna is measured and indices can be calculated.

- Always use fresh bags of Lipton Green tea (EAN 87 22700 05552 5) and Rooibos tea (EAN 87 22700 18843 8) as pairs.
- For each monitoring plots use 5 tea bags each. Tea bags should only be exposed within the soil sampling areas of each monitoring plot (see Figure 6) along the tree row (0.5–1 m distance from the row) with a distance of 15 cm from each other.
- Measure the initial, air-dry weight of the tea bag (.000 g) -including bag, cord and label-
- Mark the tea bags on the white side of the label with a permanent black marker.
- Bury the teabags in 8 cm-deep, separate holes while keeping the labels visible above the soil.
- Note the date of burial and geographical position of the site.
- Recover the tea bags after approximately 90 days.
- Remove adhered soil particles and dry in a stove for 48 h at 70 °C (not warmer!).
- Remove what is left of the label but leave the cord and weigh the bags (.000 g).
- Fill in the calculation sheet (available online, see below).

[1] Keuskamp et al. (2013): Tea Bag Index: a novel approach to collect uniform decomposition rates across ecosystems. Methods in Ecology and Evolution, doi: 10.1111/2041-210X.12097

Figure 9 Burying of tea bags with a soil corer

Exposition of tea bags in the soil of the test fields annually starts in May/June and should be finished after 3 months. The method description in detail and in different languages as well as a spreadsheet (Excel) for calculating the indices are available here:

Protocol: http://www.teatime4science.org/method/stepwise-protocol/

Calculation: http://www.teatime4science.org/publications/#Data (please chose the corresponding excel sheet for woven or non-woven bags depending on your tea bags)

The **tea** can be **order**ed here: http://www.teatime4science.org/method/availability-of-tea/

3.2.2.2 DNA extraction

This analysis will be performed for fungi and bacteria diversity and abundance in the soil. Soil sampling for DNA extraction will be carried out in **October or November** at each field trial sites in **2021** and **2024**.

On each of the 4 monitoring plots of each subtest (A, B, IC1, IC2 and Control) **one soil sample** will be taken. Each soil core (20 cm depth and 5 cm diameter) will be sampled with a metal probe. In these samplings, leaf litter, leaves and partially decomposed leaves will be excluded, whereas humus and mineral soil will be sampled together.

Soil samples will be sieved with a 2 - 3 mm mesh, stored at 4 °C for <24 h, freeze-dried and grouped to form and unique sample by subtest (A, B, IC1, IC2, Control) of about 400 cm^3. Ten soil samples (5 per location) will be stored at -20 °C and then will be sent to Madrid for DNA extraction packed with dry ice with the fastest transport available. This is about 4 L per country (2 L per site).

Then in Madrid, each of the composite soil samples will be grounded to fine powder using a pestle and mortar. The resulting fine powder will be used for DNA extraction.

3.3 Biodiversity assessment

The purpose of this component of the working protocol is to standardize the biodiversity assessment in test areas installed on marginal land, where the four tree and shrub species of interest for each participating country (Spain, Germany and Romania) will be cultivated.

Particular objective: Studying the biodiversity of marginal land used for bio-products development.

Biodiversity assessment period: April 2021 – June 2025 (April – September in 2021 before starting the cultivation; and in April – September 2022, 2023, 2024 in cultivated fields, respectively in April – June 2025, in the field after harvesting operations) (more details in Tab. 2).

The evaluation of the proposed crop's impact on biodiversity will be based on quantitative and qualitative analysis of plant and animal diversity in test areas versus control areas (one field area situated near the test area, which has similar characteristics in terms of biodiversity and those encountered in the test area before installing the crops). Thus, the principal components of the biodiversity that will be assessed are *Cormophyta* plants, insects (*Carabidae* Family), amphibians, reptiles, birds, and mammals. The principal units for biodiversity assessment are the monitoring plots (biodiversity monitoring plot), that were described in the first part of this chapter. In the monitoring plots the presence of *Cormophyta* plants, insects (*Carabidae* Family), amphibians, reptiles and micro mammals will be evaluated. The observations of birds and mammals will be made in all subtest areas.

In every subtest area biodiversity monitoring plots (BMP) will be installed, according to the protocol for intercropping tests (subchapter 2.2). In subtest areas 1 and 3, as well as in the control area, installed total 4 BMP will be established, and in subtest 2 (divided in two intercropping subtests IC1, IC2) further 8 BMP (4 BMP for every intercropping subtest) will be installed (Figure 2, Figure 3).

One BMP is represented by one square (5 x 5 m), arranged so as at least two rows of culture are included. In control area these four BMP will be installed randomly.

Cormophyta plant diversity will be evaluated through periodically direct observations (species identification and counting of individuals as well as estimation of cover degrees). Also, the *Carabidae* insects' evaluation will be made using the periodically catching method. Amphibians, reptiles (lizards) and micro mammals (mice, etc.) will be evaluated when they will be accidentally captured in the Barber pitfall traps used for insects and by direct observations of their presence or their traces imprinted on the ground or snow. Also, trap cameras could be installed at the same spot if their security can be ensured. Other mammals and birds' presence in the test areas will be carried through direct observations (visual and auditory assessment) or trap cameras' records. In addition to the trap camera, two fixed panoramic cameras could be installed. Installing a bird song recording devices is also recommended (Audiomoth - one device in every experimental plot).

3.3.1 Cormophyta plants

In every BMP (**5x5 m**) square, every **plant species** will be recorded. To evaluate the **density of the plants**, permanent square plots of **1 m²** are marked with a barber trap in their center. These permanent plots must be intersected by one of the culture rows and completely located within the 5 x 5 m BMP. All occurring plants will be annually counted per species.

In the field form (Annex 1 - left part), the identified species will be recorded. All specimens of identified plant species from the monitoring square will be counted and coverage ratio is estimated for each plant species following the **Braun-Blanquet** classes:

Coverage ratio [covered area by individuals of this species]	Exemplars/individuals of this species	Symbol
< 1 %	rare, only one individual	**r**
≤ 1 %	2-5 individuals	**+**
≤ 5 %	6-50 individuals	**1**
≤ 5 %	more than 50 individuals	**2**
5 – 25 %	any number	
26 – 50 %	any number	**3**
51 – 75 %	any number	**4**
76 – 100 %	any number	**5**

The information will be recorded in the right part of Annex 1. If there are plants that cannot be identified in the field, they will be collected and dried between paper sheets for proper identification. Also, photos of those individuals will be taken.

All the plant monitoring data will be recorded in the specific sampling form (Annex 1). It is recommended to take photos of the monitored areas at each field visit.

The plant information will be collected in the field between April and July every year (Tab. 2). The exact time for the vegetation survey depends on the regional climate and growing conditions.

3.3.2 Insects (*Carabidae* family)

In every BMP one **Barber trap** will be installed (Figure 10, Figure 2, Figure 3). This pitfall traps will be used for capturing *Carabidae* insects.

In the first year (2021), only one trap per monitoring plot will be installed (centered by BMP), after all plots will be identified and marked in the field. During the following years, the traps' position will be fixed, being placed approximatively **on a culture row** and, optionally, another one between lines for checking insects' behavior.

Another trap area will be installed in the 'control field', using a similar pattern as for the subtests (one trap (centered) in every BMP).

All the traps will be installed in the field in mid-April and mid-August (Tab. 2). The traps will be checked every two weeks, and the captured material will be collected. The traps will be removed in mid-June and mid – September.

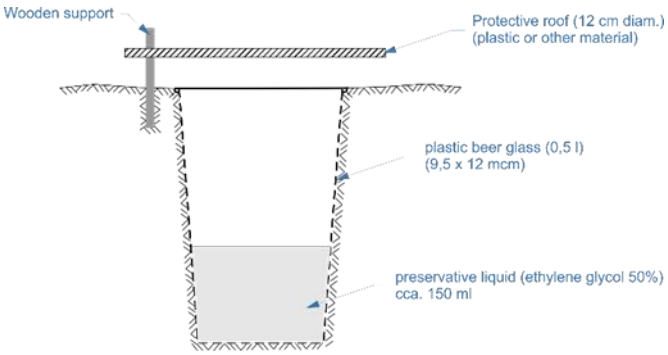


Figure 10 Barber trap installation

The traps will be made of plastic beer glasses (0.5 l), which will be buried in the soil up to their top edge. Each trap will contain about 150 ml of preservative liquid (ethylene glycol 50 %, or salted solution).

The trap will be protected on top by a dark plastic cover (preferably brownish, to mimic the color of the soil). This cover will be positioned on a wooden support placed at the height of 1-2 cm from the trap top edge.

Necessary materials:

- Plastic vials for storing the collected material (an approx. 100 ml volume)
- Permanent marker to write the code on the vials
- Inox colander to strain insects from the preservative liquid
- Reserve of ethylene glycol 50% or saline solution
- Liquid collection container (5 l) for used ethylene glycol
- Temporary liquid collection container (1 l)
- Reserve of plastic traps

Operations:

- Inspecting the integrity of the insects' trap
- Recording possible problems related to the integrity and function of the trap
- Recording the presence of micro mammals or reptiles accidentally captured in the trap
- Collecting the biological material using a colander and collecting the preservative liquid in a temporary container
- Inserting the biological material in plastic vials
- Marking the code and the collecting date on the vial
- Inspecting the preservative liquid collected (the possible traces of alteration are checked (smell of altered biological material, the presence of mucilage from snails/slugs, the changed color, etc.)
- If the preservative liquid is altered, the trap will be primed with fresh ethylene glycol, and the used fluid will be deposited in a container (5 l)
- Reinstalling the trap in the initial position.

Preserving the biological material: The biological material collected in traps in the field will be preserved in the laboratory by freezing until a further determination of species will be made.

Analyzing the biological material: Each partner is responsible for the analysis and identification of its own collected biological material.

All the partners will send the data regarding insect presence to USV for further analyses.

The field data about insect monitoring will be recorded in the specific sampling form (Annex 2).

3.3.3 Mammals, reptiles and birds

The main method used for monitoring this species is periodically **direct observations** in every subtest area. These observations will be made both on the target vertebrate groups' physical presence and on their tracks (footprints printed on the ground or snow, excrement, etc.).

Supplementary, the presence of some amphibians, reptiles and micro mammals will be observed using the insect traps. Thus, some reptiles, amphibians, and micro mammals will also be accidentally captured in Barber traps.

These observations can be supplemented by records from the photo traps for the monitored vertebrate groups and by Audiomoth devices used for bird sound recording. The use of these additional devices is not mandatory.

The photo traps can be installed in each subtest area, in a fixed spot, positioned in such a way as to capture the entire surface of test area. Moreover, two fixed panoramic cameras could be installed in two opposed corners of the field trial (test area). Supplementary, one of the Audiomoth devices will be installed in the same spot as the panoramic cameras.

In the first year (2021) the devices will be installed after plantation. The same location of the traps will be maintained during the experiment.

Photo traps will be optionally used for detecting birds, reptiles, amphibians and mammals that will enter the supervised area. The photo traps will be installed on a wooden pole at 25 cm above the ground, according to Figure 11. Moreover, the two panoramic photo traps (and Audiomoth devices) will also be installed on a wooden pole at 180 cm above the ground (Figure 12) for checking birds and mammals that won't cross the fence.

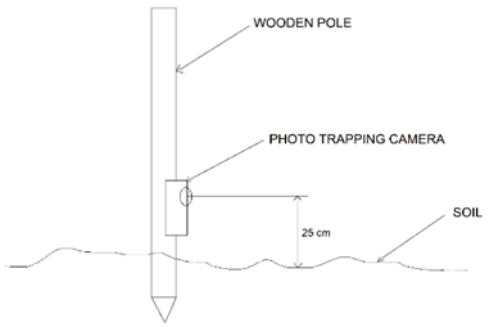


Figure 11 Photo trap installation

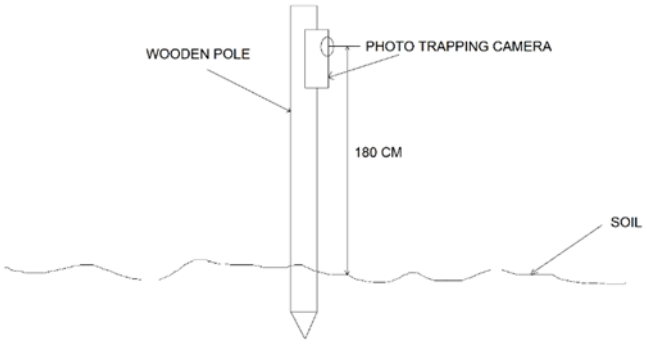


Figure 12 Panoramic photo trap installation

Monitoring calendar: During the growing season, the monitoring calendar will follow the same calendar used for insect monitoring.

The observations will continue during the winter season (using a two-week periodicity) and supplementary monitoring of birds or mammals' footprints in snow or ground.

Necessary materials:

- Plastic vials for storing the collected material (an approx. 1,000 ml volume)
- Permanent marker to write the code on the vials

Operations:

- Download pictures of trap cameras
- Download sound files of audiomoth
- Recording possible problems related to the integrity and function of the trap
- Establish a monitoring route
- Collect the material

Recording data: Every 14 days, the photos will be downloaded. The same route should be repeated every 14 days to assess the birds´, mammals´, and reptiles´ biodiversity. Operators should stroll, in the morning, after the sunrise. During the route, all species of mammals, reptiles and amphibians will be recorded, and signs of excrement, tracks, moults, etc. Operators will note the date, species, quantity, sighting point with respect to a fixed point that should be clearly defined. In case of doubts, collaborating experts should be consulted for the identification of the collected material.

The same route will be used for checking birds´ biodiversity. Operators should use binoculars and write down all the species that are observed or heard. The observations will be made each of the 14 days. The recorded data will include the species, date and hour, location (inside or outside the area).

All the field data about amphibians, reptiles, birds and mammals monitoring will be recorded in the specific sampling form (Annex 3).

Annex – sample forms

Annex 1. PLANTS DIVERSITY EVALUATION FORM

Country code:	ES or DE or RO
Name of the site:	
Test area number:	See table 3
Test plot ID:	See table 3
Monitoring point:	1, 2, 3, 4

Responsible person:	
Evaluation date:	

Plant species identified in square (5x5m)				
No	Species		No	Species

Exemplars per species counted in monitoring circle (1x1 m plots)								
No	Species	Count	Cover*		No	Species	Count	Cover*

Observations:

** plant cover: please use the Braun-Blanquet scale (see 3.3.1)*

Please attach photographs of the biodiversity areas

Country code:	ES or DE or RO
Name of the site:	
Test area number:	1 or 2

Annex 2. INSECT COLLECTING FIELD FORM

Responsible person:	
Collecting date:	

Test plot ID	Trap position	Trap No	Trap condition (answer with **y** (yes) or **n** (not))					Test plot ID	Trap position	Trap No	Trap condition (answer with **y** (yes) or **n** (not))				
			damaged	spilled content	has been replaced	presence of mammals, amhib. or rept.	other aspects				damaged	spilled content	has been replaced	presence of mammals, amhib. or rept.	other aspects
A	Along a culture row	1						**AB**	Along a culture row	1					
		2								2					
		3								3					
		4								4					
	Between culture rows (optional)	1'							Between culture rows (optional)	1'					
		2'								2'					
		3'								3'					
		4'								4'					
B	Along a culture row	1						**control**	Along a culture row	1					
		2								2					
		3								3					
		4								4					
	Between culture rows (optional)	1'							Between culture rows (optional)	1'					
		2'								2'					
		3'								3'					
		4'								4'					

Observations:

Annex 3. OBSERVATION FIELD FORM (AMPHIBIANS, REPTILES, BIRDS, MAMMALS)

Country code:	ES or DE or RO
Name of the site:	
Test area number:	1 or 2

Responsible person:	
Collecting date:	

Species	Identified after*	Locations (plot ID and number of biodiversity area)

Identified after: presence of individuals; flight; sound/song; footprint; excrements;

Please attach photographs of individuals, footprint, excrements, etc.