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D2.2. TOLERANCE TESTING OF SELECTED CROPS AT LAB/BENCH SCALE

pHYBi
Phytomanagement for a bio-based textile industry

PHYtomanagement as a sustainable feedstock source of lignocellulosic-based high-value BIo-based products for textile applications

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EXECUTIVE SUMMARY

This deliverable provides the first technical "tolerance filter" within WP2 by testing the capacity of selected high-biomass crops and genotypes to establish, grow and maintain functional performance under representative stress conditions of the pHYBi case-study soils (multi-element metal contamination, hydrocarbon contamination, and salinity). The work is organised by case study to ensure that the resulting evidence is directly traceable to operational decisions in subsequent WP2 tasks (optimisation with amendments and microorganisms, greenhouse and pilot-scale validation). In CS#1 (pollution by metals; France), fifteen somatic hybrid poplar cultivars were screened in Pierrelaye soil under semi-realistic greenhouse conditions. Establishment strongly differentiated hybrids: only ten lines survived and were therefore suitable for comparative analysis. A multi-criteria selection index integrating vigour, accumulation efficiency and stabilisation effects identified PHY_2025_8 as the most balanced candidate for upscaling, combining high biomass production and phenotypic stability with acceptable trace-element (TE) export. PHY_2025_1 remains a robust contingency option due to its high internal loading capacity and overall performance. In CS#3A (organic/mixed contamination; Spain), *Miscanthus × giganteus* and industrial hemp (*Cannabis sativa*) were tested across a TPH gradient. The Miscanthus material corresponded to the established commercially deployable genotype selected at proposal stage because of its availability for field implementation and low invasiveness risk, whereas the hemp material used in the tolerance assays resulted from a preliminary cultivar screening step based on germination and establishment performance. Miscanthus maintained good performance at low–medium contamination and showed a decline primarily at the highest TPH level, whereas hemp displayed a narrower tolerance threshold, with severe growth inhibition at high contamination that also limited some biochemical determinations. Physiological (SPAD, stomatal conductance) and biochemical indicators (protein, pigments, antioxidant enzymes) supported species-specific adjustment strategies under hydrocarbon stress. In CS#3B (organic/mixed contamination; Croatia), a complementary contamination scenario was assessed under Particula greenhouse constraints. The results provide preliminary evidence that the selected *Miscanthus × giganteus* material and the selected hemp cultivar were able to establish under the specific Croatian soil batches and greenhouse conditions tested. However, CS#3B should be interpreted as a limited complementary assay rather than as a definitive proof of broad crop tolerance across Croatian hydrocarbon-contaminated soils. In CS#4 (salinity; Spain), both crops were tested under increasing conductivity levels. Within the tested low-to-moderate salinity range, the selected *Miscanthus × giganteus* material showed comparatively higher stability, whereas the selected hemp cultivar, *C. sativa* cv. Futura 83, exhibited stronger growth penalties at the highest salinity level.

Overall, D2.2 provides a first operational tolerance filter and identifies crop × stress combinations that may be prioritised for WP2 optimisation and pilot validation within the limits of the tested plant material and experimental conditions. These results highlight where supportive strategies (amendments and microbial inoculants) are most likely to be impactful, but they should not be interpreted as a definitive genotype- or cultivar-level selection.

1. INTRODUCTION

The implementation of phytomanagement strategies in degraded or contaminated soils requires, prior to any agronomic optimisation or field validation, a basic yet decisive verification: the actual tolerance of candidate plant species under stress conditions representative of the target site. In pHYBi, this requirement is even more critical because phytomanagement is not conceived solely as an environmental intervention, but as an integrated approach in which the produced biomass must be sufficiently abundant and of adequate quality to feed lignocellulosic valorisation pathways and, ultimately, applications in the textile sector.

Within this framework, Deliverable D2.2 is conceived as the first technical filter within WP2 to identify robust crop × soil-condition combinations and to define an "operational envelope" (ranges of contamination/salinity and associated physicochemical conditions) in which selected species and genotypes maintain acceptable performance consistent with the project objectives. The deliverable is explicitly organised around case studies, so that subsequent interpretation and decision-making (optimisation with inoculants/amendments, modelling, pilot-scale validation) can be supported by comparable and traceable evidence.

The target soils of the project represent stress scenarios described in D1.2 & D1.3 (metal-contaminated soils, organically contaminated soils, and saline soils), in which plant responses depend not only on the primary contaminant, but also on its bioavailability, effective salinity, and modulating variables such as pH, organic matter content, or soil texture. Under these conditions, selection based solely on the literature or on "ideal" experimental trials tends to fail for two main reasons: (i) plant performance and survival may collapse as stress intensity increases or as the soil matrix changes; and (ii) even when plant growth is sustained, the distribution of contaminants within plant tissues (and therefore the resulting risk/biomass-use profile) may not be compatible with the intended valorisation chain.

For this reason, WP2 includes a specific task devoted to laboratory/bench-scale tolerance assays (T2.2), aimed at generating early decision-making data before investing in optimisation steps and pilot-scale activities. Accordingly, D2.2 is structured around four case studies covering the main stress types and crop groups addressed within the project:

- CS#1 (metals): screening of poplar hybrids in metal-contaminated soils.

For over a century (1890–1990s), the Pierrelaye plain (Val-d'Oise, France) was subjected to intensive irrigation with raw urban wastewater from Paris. While this practice fuelled local agriculture, specifically, market gardening activities, it left a "legacy" of complex contamination, including heavy metals (Pb, Cd, Cu, Zn, Hg), arsenic (As), and organic pollutants like PAHs and PCBs. The site is now transitioning into a 1,000-hectare forest designed for carbon sequestration and long-term ecological restoration.

The successful afforestation of such a degraded site requires highly resilient plant material capable of thriving under multi-element stress. To meet this challenge, innovative breeding strategies are being employed to develop non-GMO hybrid poplars specifically optimized for phytoremediation. Leveraging somatic hybridization via protoplast electrofusion, Phytowelt has combined the genomic strengths of four distinct species into resilient, fast-growing cultivars:

- ***Populus maximowiczii***: A key parental species for enhancing growth vigour, it is characterized by rapid vegetative development and substantial annual height increments.
- ***Populus nigra***: The European black poplar, valued for producing wood of high industrial quality and widely used in breeding programs to improve biomass density and stem form.
- ***Populus alba***: A highly resilient species that tolerates a broad range of soil conditions, including nutrient-poor substrates. Its isohydric behaviour and reflective leaf pubescence allow it to adjust physiologically to severe environmental stressors, such as intense heat and drought.
- ***Populus tremula***: Known for its broad ecological amplitude, the European aspen possesses inherent low-temperature tolerance and adapts well to disturbed or impoverished soils.

Unlike traditional breeding, this method allows for the creation of stable polyploid genotypes (3n and 4n) without genetic modification. These polyploid lines often exhibit superior traits (a phenomenon known as "triploid vigour") resulting in increased biomass, larger leaf areas, and enhanced photosynthetic capacity. Furthermore, the *in vitro* recovery process can harness somaclonal variation, potentially selecting for novel variants with spontaneous epigenetic resilience to environmental pollutants.

While internal field observations and nursery trials at Phytowelt facilities have shown that these hybrids can outperform standard controls (such as the *Max4* line) in terms of vegetative growth and vigour under non-contaminated conditions, their potential for contaminated site management is particularly promising:

- Stress Tolerance: The inclusion of *P. alba* genetics suggests high tolerance to the poor soil conditions and heavy metal loads found at Pierrelaye.
- Enhanced Biomass: Increased biomass production in triploid lines facilitates greater contaminant uptake and immobilization and physiological resilience.

By merging high-yield industrial traits with ecological hardiness, these somatic hybrids represent a data-driven solution for converting the Pierrelaye legacy into a productive, bio-based landscape.

➢ **CS#3A and CS#3B (organic contamination): tolerance assessment of Miscanthus and hemp in soils contaminated with TPH, PAHs, and co-contamination scenarios.**

Case studies CS#3A (Sargentes de la Lora, Spain) and CS#3B (Donja Gračenica, Croatia) address phytomanagement under organic and mixed contamination conditions linked to historical petroleum exploitation, refining, and transport activities. As described in D1.3, CS#3A corresponds to a former onshore oil extraction area in Sargentes de la Lora (Ayoluengo oil field), where soils are primarily affected by total petroleum hydrocarbons (TPHs) and also contain PAHs, with likely co-occurring metal contamination associated with crude oil residues. D1.3 reports very high contamination levels at this site, including maximum values of 18,665.62 mg kg^{-1} TPH and 44.67 mg kg^{-1} total PAHs, confirming the relevance of this site as a high-stress scenario for tolerance testing.

On the other hand, CS#3B represents an oil-affected area in the Sisak-Moslavina region (Croatia), where contamination is associated with historical leaks from refinery and pipeline infrastructure. According to D1.3, this site presents localised TPH hotspots (rather than diffuse contamination), with TPH concentrations in the sampled soils ranging from 12.48 to 594.02 mg kg^{-1}, and a broader context of mixed industrial pressure and low soil fertility. This makes CS#3B a complementary scenario to CS#3A: while CS#3A captures a severe hydrocarbon-contaminated hotspot, CS#3B provides a lower-intensity but heterogeneous contamination setting, more representative of many legacy oil-impacted agricultural landscapes. Together, both case studies allow D2.2 to evaluate crop responses across a realistic gradient of hydrocarbon stress and co-contamination conditions.

The tolerance assays in CS#3A and CS#3B focus on *Miscanthus × giganteus* and *Cannabis sativa* (hemp), which were already prioritised in D1.3 and D2.1 as high-biomass lignocellulosic crops with strong potential for phytomanagement on marginal and contaminated land. D1.3 explicitly identifies both species as the core crops for remediation planning in these organic-contamination case studies, in combination with plant growth-promoting microorganisms, mycorrhizae and soil amendments for later optimisation and pilot implementation. In parallel, D2.1 supports this crop choice by highlighting miscanthus and hemp as low-input, adaptable species suitable for marginal soils, with value not only for biomass production but also for ecological functions such as soil protection, carbon accumulation and phytoremediation, fully aligned with pHYBi's bio-based textile valorisation strategy.

The choice of plant material within these species was guided by the operational scope of pHYBi and by the requirement to work with commercially available, field-deployable material that would not raise relevant agronomic or environmental concerns, including invasiveness risk. For Miscanthus, *Miscanthus × giganteus* was selected because, at proposal stage, it represented the established commercial reference genotype for biomass production in Europe, while other Miscanthus genotypes were mainly available in breeding, research or pre-commercial contexts. This is consistent with previous European work on Miscanthus biomass production, which identified M. × giganteus as the commercially available genotype in Europe and highlighted the need for further breeding to broaden site-specific adaptation (OPTIMISC, 2016). The use of M. × giganteus is also consistent with the practical upscaling perspective of perennial biomass crops, where field deployment depends not only on biological performance, but also on availability of planting material, supply-chain readiness, establishment logistics and agronomic risk management (Hodgson et al., 2024).

Therefore, the results reported in D2.2 should be interpreted as a tolerance assessment of the commercially deployable reference Miscanthus genotype selected for operational implementation, not as a comprehensive screening of the full Miscanthus genetic pool. The project acknowledges that additional Miscanthus genotypes or novel hybrids may show improved performance under specific marginality constraints. Where suitable, safe and commercially relevant material becomes available within the project timeframe, additional genotype comparison may be considered in subsequent optimisation or validation activities.

Within D2.2, CS#3A and CS#3B therefore act as the experimental basis for defining the operational tolerance envelope of Miscanthus and hemp under TPH/PAH and co-contamination

stress. The results generated here will support three downstream decisions in WP2: (i) identification of the most robust crop × soil combinations to advance; (ii) selection of the stress ranges where amendment and microbial support is most relevant in T2.3; and (iii) preparation of evidence-based crop deployment strategies for the future greenhouse and in situ pilot validation stages.

- CS#4 (salinity): tolerance assessment of Miscanthus and hemp across salinity gradients.

CS#4 tests both crops under increasing electrical conductivity levels to delimit salinity thresholds affecting growth, physiology, and biomass allocation, and to identify the ranges in which supportive strategies (amendments/microorganisms) are most meaningful in subsequent WP2 steps.

In the context of this deliverable, CS#4 should be interpreted as a low-to-moderate salinity benchmark rather than as an extreme saline-soil scenario. The experimental gradient was designed around electrical conductivity values representative of the selected case-study soils, reaching approximately 3.5–3.7 dS m^{-1} in the highest treatment. These values approach, but generally remain below, the conventional 4 dS m^{-1} threshold often used to identify saline soil conditions, although direct comparison depends on the EC measurement method. This level is nevertheless agronomically relevant, since sensitive crops may already show growth penalties under moderate salt stress. At European scale, salinisation is recognised as a spatially uneven but relevant soil degradation threat: the ESDAC/JRC Saline and Sodic Soils Map identifies saline, sodic and potentially salt-affected areas across the EU, while RECARE estimates that salinisation affects around 3.8 million ha in Europe (ESDAC, 2008; RECARE, 2016).

This case-based structure is not merely narrative; it is a means to ensure that tolerance results are interpreted in relation to specific operational decisions within WP2. For example, the screening of poplar hybrids in CS#1 informs the selection of plant material that will subsequently be optimized and validated, whereas the results obtained for Miscanthus and hemp (CS#3 and CS#4) allow the identification of species × stress-level combinations for which the application of support strategies (microorganisms/amendments) is meaningful in subsequent stages.

Within the pHYBi framework, “tolerance” is not limited to survival or to a single indicator. Instead, it is considered as a set of functional responses that enable the maintenance of: biomass growth and partitioning (productive capacity under stress); physiological and biochemical stress signals (indicators of metabolic cost and resilience); and patterns of contaminant accumulation and translocation within plant tissues (with implications for safety, management, and valorisation).

Accordingly, D2.2 enables three practical decisions within WP2: (i) selection of candidate species/hybrids to advance to optimisation (T2.3) and pilot-scale validation (T2.5); (ii) definition of operational ranges (stress levels at which phytomanagement is viable under standard management versus those requiring intensive support or where it is not advisable); and (iii) prioritization of intervention strategies (for example, determining when optimisation should focus on enhancing growth, modulating contaminant bioavailability, or providing microbial support to improve resilience).

2. CASE STUDY SECTIONS (METHODS + RESULTS INTEGRATED PER CS)

2.1 CS#1. METALS: POPLAR HYBRID SCREENING

2.1.1. Materials and methods

2.1.1a Plant material:

Fifteen somatic hybrid poplar cultivars were provided by Phytowelt to UMLP for evaluation under site-relevant soil stress conditions. These hybrids were derived from the following specific parental lines, each selected for targeted physiological advantages:

- P10 (*P. nigra* × *P. maximowiczii*): A high-yielding commercial cross (e.g., commercial reference clone: "Max 4") that combines the rapid biomass accumulation of the Asian balsam poplar with the structural wood quality of the black poplar.
- P31 (*P. alba*): A white poplar line selected for its drought avoidance mechanisms, pathogen resistance, and low nutrient requirements, supporting stability under fluctuating water availability.
- P2 (*P. tremula*): An aspen line characterized by high vegetative resilience and adaptability to variable soil textures.

The hybrids integrate key traits from these species and have demonstrated enhanced early vegetative vigour under open-air nursery conditions, including increased stem height and leaf area relative to the Max4 parental line (Table 1).

Branches were collected in late winter (February 2025) while trees were still dormant. Poplar propagation material for hardwood cuttings is typically harvested from at least one-year-old wood during the dormant season, as this stage maximises rooting potential and reduces the risk of desiccation and disease during establishment. Branches were cut using clean, sharp tools to minimise tissue damage; cuttings were immediately stored at low temperature to maintain bud dormancy until preparation for planting (Figure 1a).

From these branches, single-node cuttings were prepared as internodal segments, each containing a single quiescent axillary bud. Explants were selected based on the presence of a visible bud primordium without evidence of shoot elongation or anthesis. Cuttings were prepared with an oblique basal cut to maintain morphological polarity and increase the surface area for moisture absorption (Figure 1b). During planting, cuttings were oriented vertically within the substrate; the axillary bud was positioned just above the soil surface to facilitate gas exchange and prevent pathogenic decay (basal rot). The sub-nodal stem tissue was firmly embedded to ensure maximum hydraulic contact with the medium, promoting the induction of adventitious roots.

Table 1. Somatic hybrids used in the tolerance assay.

Hybrid ID	Parental Cross	Phenotype / Ploidy	Field Observations
PHY-2025-1	P10×P31	Mixoploid	Strong growth, larger leaves

PHY-2025-2	P10×P31	4n	Vigorous growth, elongated leaves
PHY-2025-3	P10×P31	2n	Standard growth, medium leaves
PHY-2025-4	P10×P31	4n	Rounder leaves, distinct pattern
PHY-2025-5	P10×P31	2n	Moderate growth, uniform leaves
PHY-2025-6	P10×P31	2n	Moderate growth
PHY-2025-7	P10×P31	2n	Standard growth
PHY-2025-8	P10×P31	2n	Standard growth
PHY-2025-9	P10×P31	3n	Faster leaf expansion, tougher leaves
PHY-2025-10	P10×P31	2n	Longer leaves
PHY-2025-11	P10×P2	2n	Vigorous growth, thin leaves
PHY-2025-12	P10×P2	2n	Standard growth
PHY-2025-13	P10×P2	2n	Moderate growth
PHY-2025-14	P10×P2	2n	Strong growth
PHY-2025-15	P10×P2	2n	Strong growth, thicker stems

Note: Phenotypic observations refer to early vegetative traits including stem height, leaf size, and general vigour. Ploidy levels were determined by flow cytometry at nursery stage.



Figure 1. Propagation material: (a, b) parent poplar branches and (c) standardized single-node cuttings used as experimental units.

b. Soil Material

The soil was sourced from a fallow plot within the Pierrelaye site (CS1-P3), which is designated for future pHYBi plantation establishment. A full physicochemical characterization of the plot is provided in Deliverable D1.2 (*"Initial Monitoring Campaign of Existing Phytomanagement Sites"*).

The area is currently under restricted land-use and managed through monitored natural attenuation under rain-fed conditions. Owing to more than a century of intensive inputs of exogenous organic and mineral matter, the topsoil exhibits features consistent with a Technosol. Total trace element (TE) concentrations were reported for the four LUCAS-like replicate sampling points within the plot (D1.2). Although moderate point-to-point variability was observed, no spatial gradient was detected, indicating that the material is sufficiently homogeneous for controlled pot experiments.

The soil is neutral to slightly alkaline (pH 7–8), with residual carbonate content (5–6 g kg^{-1}) and elevated organic matter (28–35 g kg^{-1}), resulting in a Cation Exchange Capacity (CEC) of approximately 6.3 cmol(+) kg^{-1}. Its coarse texture and low clay fraction further constrain TE mobility. Because these physicochemical conditions limit solubility and therefore the bioavailable fraction of many TEs, complementary extractions with 0.01 M $CaCl_2$ were carried out to quantify the plant-accessible pools. These values provide a more realistic basis for interpreting the responses of the poplar hybrids to the site's characteristic multi-element contamination pressure.

c. Pot Assay: Performance under Realistic Site Conditions

Ten replicates per hybrid were established, yielding a total of 150 experimental units. The assay was conducted using 1.0 L pots, each filled with soil equivalent to 1.0 kg dry weight of the previously described soil. To maintain the original contaminant profile and ensure realistic site conditions, the soil was used intact (undiluted); it was sieved (2 mm) only to remove large debris, with no sand or mineral amendments added. This ensured that the hybrids experienced the full chemical and physical complexity of the target site. Unplanted control pots were maintained in parallel under identical conditions.

The experiment was conducted over a 76-day period (June to August 2025) within an unheated exterior glass greenhouse in Montbéliard, France. This period was characterized by multiple heat wave events typical of the 2025 summer season. While the glass structure provided protection from wind and precipitation, it allowed for natural photoperiods and ambient temperature fluctuations, exposing the plants to significant thermal stress alongside the geochemical stress of the substrate. To compensate for high summer evapotranspiration while maintaining the geochemical integrity of the substrate, irrigation was performed twice weekly (or more frequently during peak heat events). Individual plastic trays were utilized under each pot to capture any temporary drainage; this leachate was subsequently reabsorbed by the soil via capillary action as it dried, ensuring a zero-leaching system.

Treatments were arranged in a randomized design within the greenhouse. Manual weed control was performed regularly, and the absence of pests was monitored throughout the duration of the assay. During the initial 4 weeks, sprouting success and shoot emergence were recorded. While non-sprouted cuttings were replaced once, those that failed to establish thereafter were retained in the final dataset to accurately calculate survival and sprouting rates. At the conclusion of the 76-day growth period, a comprehensive harvest was conducted to quantify:

- **Aerial Growth:** Overall shoot height and total leaf count.

- **Biomass Allocation:** Fresh and dry mass (dried at 60 °C to constant weight) for leaves, stems, and roots.
- **Root Development:** Total root length, determined after gentle washing of the root system.

Dried leaf and stem samples were subsequently used for trace element (TE) analysis. Plant tissues were finely ground and subjected to acid digestion using concentrated nitric acid (68%). The resulting digests were analysed for TE concentrations using inductively coupled plasma–atomic emission spectroscopy (ICP-AES; iCAP PRO DUO, Thermo Fisher Scientific). Elemental concentrations were expressed on a dry weight basis (µg g^{-1} DW). Phytoextraction potential per plant, representing the total TE accumulated in the aboveground shoot system (leaves + stems), was calculated as the sum of leaf and stem contributions:

$$\text{Phytoextraction Potential} = ((\text{Conc}_{leaf} \times \text{DW}_{leaf}) + (\text{Conc}_{stem} \times \text{DW}_{stem})) / 1000$$

where Conc_{leaf} and Conc_{stem} represent TE concentrations in leaves and stems (mg kg^{-1} DW), and DW_{leaf} and DW_{stem} correspond to the dry biomass of leaves and stems (g). The resulting value represents the total TE mass per shoot (µg per plant).

Following plant harvest, soil samples were collected from each pot to evaluate the impact of different hybrids on TE dynamics. In parallel, soil was collected from unplanted control pots maintained under identical greenhouse conditions to distinguish plant-mediated changes in TE bioavailability from background changes in soil chemistry occurring during the experimental period. Readily soluble TE fractions were estimated by extraction with 0.01 M $CaCl_2$: soil suspensions were shaken, centrifuged, and the supernatant analysed using ICP-AES, with results expressed in mg kg^{-1} DW.



Figure 2. Experimental pot assay setup in an unheated greenhouse.

2.1.2 Results

The most striking initial result concerns the differential survival rate among the tested hybrids. Although 15 hybrids were initially screened, only 10 established successfully and are included in

the analyses. Notably, almost the entire P10×P2 (Aspen) lineage (Hybrids 11, 12, 13, and 14) failed to establish or survive under the combined stress of the contaminated soil, with only PHY_2025_15 persisting. In addition, PHY_2025_5 (P10×P31) did not survive. These failures may be partly attributable to the extreme summer heatwaves of 2025 during the establishment phase and/or variability in the quality and physiological state of the initial cuttings.

Regarding statistical robustness, some hybrids are represented by a limited number of replicates. PHY_2025_9 (n = 2) and PHY_2025_4 (n = 3) therefore present a higher sensitivity to outliers, and their results should be interpreted cautiously. For completeness, phenotypic and trace element (TE) accumulation data in the shoot system are nevertheless reported for these hybrids, with this limitation in mind. Conversely, PHY_2025_1, PHY_2025_6, PHY_2025_7, and PHY_2025_8 maintained higher replicate numbers (n = 9), providing the most robust datasets for comparative analysis. Their higher survival and establishment rates may indicate greater tolerance or sprouting capacity under the tested soil and climatic conditions.

Following the initial establishment phase, the vegetative performance of the surviving hybrids was assessed after 76 days of growth in Pierrelaye soil. Table 2 summarizes the main phenotypic traits, including the number of surviving replicates (n, out of ten initial cuttings per hybrid), together with leaf number, plant height, shoot dry biomass, root length, and root dry biomass.

Table 2. Vegetative performance and biomass allocation of *Populus* hybrids (PHY_2025_x) after 76 days of growth in CS1 soil under controlled conditions. Values represent mean ± standard deviation of all surviving replicate plants (n as indicated). Highest values are highlighted in bold green and lowest values in light brown; when the lowest value corresponds to hybrid PHY_2025_9 (n = 2), the second lowest value is highlighted as well due to the low replicate number.

Hybrid	n	Number of leaves	Height (cm)	Shoot dry weight (g)	Root Length (cm)	Root dry weight (g)
PHY_2025_1	9	**14.67 ± 0.71**	26.22 ± 2.40	1.679 ± 0.358	29.61 ± 4.14	0.220 ± 0.076
PHY_2025_2	7	13.14 ± 1.57	22.86 ± 3.53	1.396 ± 0.401	28.86 ± 5.73	0.180 ± 0.054
PHY_2025_3	8	11.12 ± 1.46	19.06 ± 2.19	0.899 ± 0.186	23.94 ± 6.03	0.133 ± 0.045
PHY_2025_4	3	12.00 ± 1.00	22.40 ± 2.48	1.403 ± 0.334	25.00 ± 6.95	0.149 ± 0.046
PHY_2025_6	9	13.67 ± 1.50	22.72 ± 3.57	1.659 ± 0.472	24.17 ± 3.58	**0.289 ± 0.068**
PHY_2025_7	9	13.67 ± 3.04	27.22 ± 6.87	1.723 ± 0.773	31.78 ± 10.70	0.267 ± 0.091

PHY_2025_8	9	13.22 ± 1.48	**29.33 ± 1.46**	**1.975 ± 0.230**	**33.67 ± 6.69**	0.268 ± 0.042
PHY_2025_9	2	10.00 ± 2.83	9.00 ± 1.41	0.697 ± 0.236	22.50 ± 0.71	0.107 ± 0.005
PHY_2025_10	4	13.00 ± 3.16	21.50 ± 8.58	1.519 ± 1.049	21.25 ± 12.37	0.254 ± 0.197
PHY_2025_15	5	12.60 ± 1.34	25.50 ± 3.71	1.909 ± 0.776	29.70 ± 4.83	0.223 ± 0.084

Vegetative vigour differed notably among the surviving clones (Table 2). PHY_2025_8 and PHY_2025_15 achieved the highest shoot biomass (>1.9 g DW), suggesting greater tolerance to the semi-alkaline conditions and trace element (TE) profile of the Pierrelaye soil. However, TE accumulation in the shoot system (Table 3) did not scale linearly with biomass. For instance, PHY_2025_1 and PHY_2025_8 both exported relatively high zinc (Zn) (>180 µg per shoot), yet PHY_2025_1 did so with lower biomass, indicating higher tissue concentration and potentially more efficient phytoextraction, although growth limitation due to TE accumulation cannot be excluded.

PHY_2025_15 exhibited substantial TE export, but only about half of the cuttings successfully sprouted, which reduces the robustness of these data. In comparison, PHY_2025_1 exported considerable amounts of TEs despite having a lower shoot dry weight. These plants, however, were taller and produced more leaves. Since the average fresh weights of the two hybrids were similar (5.37 g vs. 5.74 g), differences in tissue water content do not explain the lower dry weight of PHY_2025_1. The discrepancy likely reflects structural differences, such as smaller or thinner leaves and stems. These observations highlight that high TE export can result from either greater biomass or higher tissue concentration, underscoring the relevance of reporting phytoextraction potential (i.e., total mass µg per shoot) rather than tissue accumulation capacity alone (µg g^{-1} DW). This integrated metric provides a more representative estimate of total TE removal and avoids bias where plants with high tissue concentrations but low biomass appear artificially efficient. For the project's objectives, a balance between biomass yield and TE accumulation is particularly important, as TE content in the shoot could affect downstream lignosolv extraction in WP3.

Among all hybrids, Cu and Zn were the most highly exported elements, with PHY_2025_8, PHY_2025_10, and PHY_2025_15 achieving the highest Cu accumulation, and PHY_2025_8 exhibiting the highest Zn export. Cd, Cr, and Ni showed moderate variation, whereas PHY_2025_9 exhibited minimal TE export, consistent with low survival and biomass. Overall, PHY_2025_8 represents the most robust combination of growth and TE export, while PHY_2025_1 and PHY_2025_15 illustrate trade-offs between biomass, tissue concentration, and survival, providing insights for hybrid selection in phytoextraction strategies. The extraction data reveal three distinct geochemical patterns within the hybrid population: (1) **Zn–Cd co-accumulation**, consistent with Cd uptake via divalent cation transporters due to its chemical similarity to Zn^{2+};

(2) **a Ni–Cr association**, likely reflecting similar soil geochemical controls on their availability rather than a specific biological transport mechanism; and

(3) **independent Cu translocation**, highly variable among clones and consistent with tight physiological regulation through intracellular chelation and chaperone systems rather than passive transpiration-driven transport.

Table 3. Phytoextraction potential of TE (Cd, Cr, Cu, Ni, Zn) in the shoot system of *Populus* hybrids (PHY_2025_x) after 76 days of growth in CS1 soil. Values represent the total TE mass per shoot (µg per plant), calculated as the sum of leaf and stem contributions for each plant. Data are expressed as mean ± standard deviation of replicate plants. Highest values are highlighted in bold green and lowest values in light brown; when the lowest value corresponds to hybrid PHY_2025_9 (n = 2), the second lowest value is highlighted as well due to the low replicate number.

Hybrid	Cd	Cr	Cu	Ni	Zn
PHY_2025_1	**5.88 ± 3.30**	**5.67 ± 2.38**	16.57 ± 4.05	**5.44 ± 2.38**	186.08 ± 39.59
PHY_2025_2	3.72 ± 1.02	4.59 ± 1.51	12.55 ± 3.38	3.57 ± 0.78	149.13 ± 43.50
PHY_2025_3	1.64 ± 0.43	3.81 ± 1.52	6.69 ± 1.22	2.30 ± 0.92	75.84 ± 16.96
PHY_2025_4	2.60 ± 0.96	2.85 ± 0.89	11.80 ± 1.50	2.44 ± 0.60	121.58 ± 30.72
PHY_2025_6	4.72 ± 1.70	3.11 ± 1.50	10.19 ± 2.27	2.75 ± 2.16	181.48 ± 53.63
PHY_2025_7	4.07 ± 1.83	2.56 ± 1.24	14.10 ± 5.07	2.16 ± 0.56	168.20 ± 70.91
PHY_2025_8	3.45 ± 0.49	2.87 ± 0.90	18.01 ± 4.07	2.55 ± 0.66	**193.25 ± 26.02**
PHY_2025_9	0.77 ± 0.33	5.09 ± 2.17	9.81 ± 3.64	2.89 ± 0.48	51.60 ± 22.82
PHY_2025_10	2.29 ± 1.68	2.80 ± 0.72	**22.68 ± 14.07**	3.17 ± 0.77	128.36 ± 77.84
PHY_2025_15	2.36 ± 1.04	2.74 ± 0.70	22.11 ± 6.58	3.08 ± 0.98	160.35 ± 55.99

Evaluation of soil bioavailability (Table 4) highlights the plant-mediated effect of the hybrids. Comparing post-harvest bioavailable fractions to unplanted reference soil, several hybrids significantly reduced soluble Zn and Cd. PHY_2025_1 and PHY_2025_2 reduced bioavailable Zn from 1492 to approximately 869–898 µg/g, indicating effective uptake or immobilization. By contrast, PHY_2025_3, PHY_2025_4, and PHY_2025_15 maintained or slightly increased bioavailable fractions, potentially due to root exudation of organic acids or concentration effects from higher evapotranspiration in pots with larger biomass. Hybrids with high shoot TE accumulation (e.g., PHY_2025_8 and PHY_2025_15) did not always correspond to the largest reductions in soil bioavailability, showing that total extraction depends on both biomass production and chemical interactions in the soil-plant system. Overall, these results indicate that hybrids differ not only in shoot accumulation capacity (Table 3) but also in their modulation of soil bioavailability, reflecting a combination of uptake efficiency, root exudation, and water flux effects. This variation is critical for selecting the best hybrids for a phytoextraction or phytostabilisation strategy.

Table 4. Bioavailable fraction of TE (Cd, Cr, Cu, Ni, Zn) in the soil after 76 days of growth of *Populus* hybrids (PHY_2025_x). Values represent 0.01 M $CaCl_2$-extractable concentrations, expressed as mean ± standard deviation of all replicate plants (n as indicated). Highest values are highlighted in bold green and lowest values in light brown; when the lowest value corresponds to hybrid PHY_2025_9 (n = 2), the second lowest value is highlighted as well due to the low replicate number.

Hybrid	n	Cd	Cr	Cu	Ni	Zn
PHY_2025_1	9	21.37 ± 5.16	12.05 ± 4.96	225.07 ± 36.95	54.46 ± 10.40	898.01 ± 109.84
PHY_2025_2	7	15.69 ± 6.58	13.31 ± 4.11	207.15 ± 60.33	47.00 ± 12.11	869.76 ± 283.05
PHY_2025_3	8	27.20 ± 7.93	**19.22 ± 14.08**	364.29 ± 108.94	67.97 ± 14.37	1582.63 ± 688.44
PHY_2025_4	4	27.56 ± 7.52	12.94 ± 1.84	**367.77 ± 22.25**	70.75 ± 7.99	1516.89 ± 562.63
PHY_2025_6	8	18.31 ± 2.67	11.80 ± 2.89	237.80 ± 25.35	55.81 ± 4.52	1034.00 ± 282.30
PHY_2025_7	9	21.98 ± 3.83	10.49 ± 3.04	257.48 ± 37.88	61.55 ± 7.73	1186.63 ± 232.55
PHY_2025_8	9	19.63 ± 4.91	10.39 ± 2.48	285.15 ± 26.26	62.24 ± 5.71	1021.22 ± 363.26
PHY_2025_10	4	19.95 ± 1.68	7.50 ± 0.62	203.41 ± 18.69	55.64 ± 4.57	1039.25 ± 112.89
PHY_2025_15	5	**31.70 ± 4.73**	11.98 ± 7.58	330.23 ± 43.30	**75.54 ± 5.68**	**1738.38 ± 386.58**
Reference Values	4	22.92 ± 6.98	6.38 ± 4.00	79.80 ± 14.96	60.84 ± 16.81	1492.90 ± 205.01

Final Hybrid Selection and Multi-Criteria Analysis

To identify the most suitable hybrid for the Pierrelaye site, a Multi-Criteria Selection Index (MCSI) was developed based on a three-step Z-score normalization approach.

- **Z-score normalization.**

For each parameter (e.g., shoot height, TE concentration), raw values were standardized using:

$$Z=(x-\mu)/\sigma$$

This transformation converts variables with different units (cm, g, µg) into a dimensionless scale, allowing direct comparison and integration.

- **Pillar aggregation.**

Standardized parameters were grouped into three functional pillars:

- **Vigour** – mean Z-score of shoot height and shoot dry biomass. Root dry weight was excluded to prioritize aerial biomass production and site coverage potential.
- **Accumulation Capacity (µg g^{-1})** – mean Z-score of tissue concentrations of Cd, Cr, Cu, Ni, and Zn. Using tissue concentration rather than total phytoextraction ensures that this pillar reflects uptake efficiency independently of plant size, since biomass effects are already captured by the Vigour pillar.
- **Stabilization** – mean Z-score of reductions in bioavailable soil TE concentrations relative to the unplanted reference soil.

Note: **Coefficient of Variation (CV) of Biomass (%)** is included to indicate the phenotypic stability of each hybrid, ensuring that the selected candidates provide reliable performance across replicates.

- **Final indexing.**

The Multi-Criteria Selection Index (MCSI) was calculated as the arithmetic mean of the three functional pillar scores. Consequently, an MCSI of 0 represents the cohort average; positive values indicate hybrids performing above the population mean, while negative scores reflect below-average performance.

To ensure statistical robustness and prevent outliers from skewing site-wide recommendations, PHY_2025_4 (n=3) and PHY_2025_9 (n=2) were excluded from the analysis due to insufficient replication. The resulting hierarchy (Table 5) highlights three distinct functional profiles among the surviving hybrids:

1. High-Vigour Biomass Producers: Optimized for rapid canopy closure and feedstock yield.
2. High-Efficiency Accumulators: Optimized for maximum internal trace element loading (µg g^{-1}).
3. Soil Stabilizers: Optimized for the reduction of soil bioavailability and leaching risk.

Table 5. Multi-Criteria Selection Index (MCSI) and pillar scores for *Populus* hybrid ranking. Scores were derived from Z-score normalization of mean values (n ≥ 4). CV Biomass is provided as a measure of phenotypic stability.

Hybrid ID	Vigour	Accumulation Capacity (µg g^{-1})	Stabilization	Final MCSI	CV Biomass (%)
PHY_2025_1	0.41	**1.13**	0.85	**0.80**	21.3
PHY_2025_8	**1.31**	-0.51	0.47	**0.42**	**11.6**
PHY_2025_2	-0.51	0.57	**0.94**	0.33	28.7
PHY_2025_6	-0.14	-0.06	0.43	0.07	28.4

PHY_2025_7	0.62	-0.43	-0.05	0.05	44.8
PHY_2025_10	-0.53	-0.08	0.41	-0.07	69.1
PHY_2025_15	0.64	-0.62	-1.77	-0.58	40.6
PHY_2025_3	-1.81	0.02	-1.29	-1.03	20.7

According to the MCSI, PHY_2025_1 achieved the highest overall MCSI score (0.80), reflecting strong internal loading capacity combined with good stabilization performance. Its high efficiency score indicates tissues with relatively high concentrations of the target TE while maintaining moderate vegetative vigour.

However, PHY_2025_8 represents the most balanced candidate for the pHYBi objectives. It clearly dominates the vigour pillar (Z = 1.31), which is critical for biomass production. Its lower efficiency score (-0.51) reflects a typical dilution effect, where rapid growth results in lower tissue concentrations. From a phytomanagement perspective, this trait can be advantageous because it allows high biomass production while limiting excessive TE concentrations in harvested tissues.

Importantly, PHY_2025_8 also exhibited the lowest biomass coefficient of variation (11.6%), indicating high phenotypic stability across replicates and therefore better reliability for large-scale deployment.

Upscaling, Operational Considerations and Contingency Strategy.

While PHY_2025_8 is selected as the reference hybrid for the field-scale pilot, operational constraints associated with large-scale vegetative propagation at the Phytowelt nurseries require a proactive strategy to mitigate risks related to plant material availability. Accordingly, PHY_2025_1 will also be deployed in parallel as a robust alternative, reducing reliance on a single genotype while maintaining performance objectives. Propagation capacity is further supported by optimized field management practices, including treatment of source plants and reduction of competition to enhance cutting production.

In addition, *in vitro* propagation via somatic embryogenesis will be initiated by Phytowelt, providing supplementary material for optimization trials, if needed, outside the optimal harvesting and storage window for vegetative cuttings. These plants will also be maintained as a strategic reserve and eventually to compare establishment performance with vegetative cuttings. As a contingency for *in situ* pilot validation, cutting size may be reduced (from 1 m to 25–30 cm) to increase the number of available propagules, and additional hybrids (e.g., PHY_2025_2 or PHY_2025_6) may be mobilized if required. Collectively, these measures ensure adequate material availability for downstream activities.

2.1.3 Deviations from the original work plan

Although the initial plan included transcriptomic and proteomic profiling of all 15 Populus hybrids in the CS1 soil, the absence of a suitable uncontaminated control prevents meaningful interpretation of differential expression across these lines. Consequently, the omics analyses have been refocused on a subset of priority hybrids, enabling a targeted evaluation of treatment effects; including microbial inoculants, mineral amendments, or biostimulants; within the same

contaminated soil matrix. This approach directly supports T2.3, focused on optimizing phytomanagement with the selected hybrid(s).

By examining treatment × hybrid interactions and comparing treated versus untreated conditions, the study can reveal how specific interventions modulate gene and protein expression under real-world multi-element stress. This ensures molecular-level comparability and allows interpretable contrasts to be reported in D2.3, providing actionable insights for phytomanagement optimisation without the confounding effects of hybrid genotype-only comparisons. Overall, this adjustment is fully aligned with WP2 objectives while maximizing the interpretability, impact, and usability of the resulting data.

2.2 CS#3A. ORGANIC / MIXED CONTAMINATION: MISCANTHUS & HEMP TOLERANCE

2.2.1 Materials and methods

a. Plant material

M. giganteus rhizomes were supplied by the partner PARTICULA and delivered to the facilities of the University of Burgos (UBU), where they were stored in darkness under humid conditions at 4 °C until use. *Miscanthus × giganteus* was selected as the Miscanthus reference material because the project required commercially available, field-deployable biomass material with low invasiveness risk. Although genotype-dependent responses are recognised as highly relevant under marginal conditions, this deliverable focuses on the established commercial reference genotype available for operational implementation within the project timeframe, rather than on a comprehensive Miscanthus breeding or genotype-screening programme.

For hemp, several certified industrial hemp cultivars were initially considered for the pHYBi trials. Before establishing the tolerance assays, a preliminary germination and establishment screening was performed using Futura 83, Eletta campana, and Futura 75. The cultivars Eletta campana and Futura 75 were discarded because their germination rate and/or establishment uniformity were below the minimum level required for robust replicated assays and for future field validation. This decision was taken to avoid confounding stress responses with poor initial seed performance. Based on this pre-screening, *C. sativa cv.* Futura 83 was selected for the tolerance assays because it showed the most suitable combination of germination capacity, seedling uniformity and practical availability. Seeds of *C. sativa* variety Futura 83 were purchased from EuroGrow and stored at 4 °C under the conditions provided upon receipt until use.

b. Soil material

Soils with varying levels of total petroleum hydrocarbons (TPHs) were collected from a petroleum exploitation site in Sargentes de la Lora (Burgos, Spain). Soil contamination at this site is dominated by TPHs, although polycyclic aromatic hydrocarbons (PAHs) and heavy metals are also present. These soils are fully described in Deliverable D1.3, “Characterisation of new candidate field sites”.

Due to the spatial heterogeneity of contamination across the site, three soils representing different contamination levels were selected (High ≈ 15,000 mg kg^{-1}; Medium ≈ 5,000 mg kg^{-1}; Low ≈ 1,000 mg kg^{-1}, dry-weight equivalent). In addition, a control soil with low contamination (≈ 100 mg kg^{-1}) was collected from a nearby area, exhibiting similar physicochemical properties to those of the contaminated soils. After collection, soils were homogenised and sieved (<2 mm) prior to pot preparation.

c. Tolerance assay

For the tolerance assays, pots measuring 24 × 24 × 26 cm were prepared with 8.5 kg of soil per pot amended with 5% sand and adjusted to a target water retention capacity (WRC) set at the start of the trial, and pots measuring 18 × 18 × 25 cm were prepared with 3.8 kg of soil per pot amended with 5% sand and adjusted to the corresponding target WRC, for *M. giganteus* and *C.*

sativa, respectively (70% WRC for *M. giganteus*; 30% WRC for *C. sativa*). The different target WRCs were used to ensure adequate aeration and avoid waterlogging under the contrasting pot volumes and crop requirements. Three pots were prepared for each contamination level (control, low, medium, and high) per species. Pots without plants prepared under the same conditions were also included to assess soil evolution throughout the experiment (Figure 3).



Figure 3: Representative diagram of the pot tolerance experiment design for each plant species.

Once the pots were prepared, sowing was carried out as follows: (i) *M. giganteus* rhizomes were washed with water to remove soil residues and impurities and then soaked in water for 2 h. Subsequently, one rhizome was planted per pot at a depth of approximately 6–8 cm, covered with soil without compaction, and the pots were covered with transparent film to maintain moisture. (ii) Hemp seeds were soaked in water for 2 h, then placed between two pieces of moist paper and allowed to germinate for one week in darkness at 25 °C. Germinated seeds were transplanted into 6 × 6 × 6 cm seedling trays prepared with the same soil used in the experimental pots, covered with transparent film, and incubated in growth chambers (Matek GROW 360) under controlled conditions (>70% relative humidity, day/night temperature regime of 23 ± 1 °C / 18 ± 1°C, and a 16 h/8 h day/night photoperiod) for one week. Subsequently, seedlings were transplanted from the trays into the experimental pots and covered with transparent film for two days to ensure successful establishment.

All pots were arranged in a randomized design and maintained under greenhouse conditions for the duration of the experiment (late May to late September, 4 months, for *M. giganteus*; and September to mid-November, 10 weeks, for *C. sativa*). During the experiment, plants were irrigated every two days, weed control was maintained, and the absence of pests was ensured. Irrigation was adjusted to maintain the target WRC as closely as possible throughout the trial.

For *M. giganteus*, images were taken every 30 days, and plant height, number of leaves, and the appearance of new rhizomes were recorded. For hemp, images were taken every 7 days, and plant height, main stem diameter, and number of leaves were measured. At the end of the experiment, in addition to the parameters described above, the fresh weight of aerial and root biomass was recorded for each plant. Both aerial and root tissues were collected and stored at −80 °C for further analysis. Additional aerial biomass was oven-dried at 60 °C and sent to CETIM for fibre quality assessment. Soil and rhizosphere samples were also collected.

d. Isolation of rhizosphere microorganisms

At the end of the tolerance experiments, epiphytic and endophytic microorganisms were isolated from plant roots. For the isolation of epiphytic microorganisms, root samples with adhering rhizosediment were first slightly washed and subsequently subjected to a more vigorous washing step in sterile saline solution to detach surface-associated microorganisms. The resulting suspensions were serially diluted, plated on Tryptic Soy Agar (TSA), and incubated at room temperature for 48 h. For endophytic microorganisms, root fragments were surface-sterilized using ethanol and sodium hypochlorite, followed by thorough rinsing with sterile distilled water. The sterilized roots were then macerated in sterile saline solution, serially diluted, and plated on TSA under the same incubation conditions. After incubation, bacterial colonies exhibiting distinct morphologies were selected and repeatedly subcultured to obtain pure isolates. Pure strains were cryopreserved in glycerol stocks and stored at −80 °C.

e. Soil analysis

The main soil physicochemical and biochemical properties (pH, electrical conductivity, total carbon and total nitrogen) were characterised prior to and at the end of the experiment to assess potential plant-induced changes in soil quality parameters (see D1.3 for baseline site characterisation). This characterisation aimed to determine whether the presence of Miscanthus and hemp influenced soil properties under the different experimental conditions.

At harvest, one soil sample was collected from each pot. These samples, taken for soil characterisation at the end of the experiment, were divided into two subsamples: one air-dried at 20-25 °C and sieved to 2 mm for the determination of physicochemical properties, and another stored at -20 °C for the assessment of biochemical properties. At this stage, the key parameter was the TPH concentration, determined at harvest to evaluate potential changes during the experiment (analytical method consistent with D1.3).

f. Plant physiological parameters

Throughout the experiment, plant growth and physiological performance were monitored using non-destructive measurements. Photographic records were taken to document plant development, and plant height, stem thickness, relative chlorophyll content, and stomatal

conductance were periodically assessed. All parameters were measured monthly in *M. giganteus* and weekly in *C. sativa*, as well as at the end of the experiment.

Plant height was determined as the distance from the substrate surface to the tip of the longest leaf using a measuring tape. Stem thickness in *C. sativa* plants was measured with a digital calliper. Relative chlorophyll content was assessed using a Chlorophyll Concentration Meter (CCM-200 plus, Opti-Sciences Inc., Hudson, USA), and stomatal conductance was measured with a leaf porometer (Medical and Scientific SDN, Bhd).

g. Total protein content

The soluble total protein content of leaves was determined by homogenizing 0.1–0.2 g of plant tissue in 50 mM sodium phosphate buffer containing 0.1 M NaH_2PO_4, 1 mM EDTA, 1mM Ascorbate (pH 7.5). After centrifugation and collection of the supernatant, protein concentration was determined using the Bradford method.

h. Photosynthetic pigments measurement

The photosynthetic pigments were extracted from the fresh leaves (0.1-0.3 g) using 80% acetone and fine sand. The samples were homogenized for 2 minutes and after centrifugation the supernatant was collected. The photosynthetic parameters were obtained following the protocol described by Lichtenthaler, 1987. The following formulas were used to obtain the quantification of chlorophyll and carotenoids:

$$Chlorophyll\ A\left(\frac{mg}{L}\right) = 12.25 * Abs663 - 2.79 * Abs646$$

$$Chlorophyll\ B\left(\frac{mg}{L}\right) = 21.5 * Abs646 - 5.1 * Abs663$$

$$Carotenoids\left(\frac{mg}{L}\right) = \frac{1000 * Abs470 - 1.82 * ChlA - 85.02 * ChlB}{198}$$

i. Antioxidant activities

The measurement of antioxidant activities was conducted following the protocol described by Khan et al. (2024), with minor modifications. Plant extracts were prepared using 0.1-0.3 g of leaf tissue homogenized in 1 mL of extraction buffer containing 0.1 M NaH_2PO_4, 1 mM EDTA, 1mM Ascorbate (pH 7.5), and 1% polyvinylpyrrolidone, under ice-cold conditions.

Catalase activity (CAT, EC 1.11.1.6) was determined by mixing 10 µL of plant extract with 185 µL of reaction buffer containing 0.1 mM NaH_2PO_4 and 1 mM EDTA (pH 7.5). For blank measurements, 10 µL of extraction buffer was used instead of plant extract. The absorbance was monitored at 240 nm for 3 min to confirm the stability of the reaction mixture. Subsequently, 5 µL of 2 M H_2O_2 was added to initiate the reaction, and the decrease in absorbance at 240 nm was recorded for 5 min. Catalase activity was calculated as mmol of H_2O_2 decomposed per minute per mg of protein, using an extinction coefficient (ε) of 39.4 $mM^{-1}\ cm^{-1}$.

Ascorbate peroxidase activity (APX, EC 1.11.1.11) was measured by combining 10 µL of plant extract with 185 µL of reaction buffer (50 mM potassium phosphate, 0.25 mM Ascorbic Acid, pH

7). Blank reactions were prepared using extraction buffer instead of plant extract. After verifying reaction mixture stability at 290 nm, 5 µL of substrate solution containing H_2O_2 0.2M was added to initiate the reaction, and changes in absorbance were monitored at 290 nm for 5 min. APX activity was expressed as µmol of oxidized ascorbate per minute per mg of protein, using an extinction coefficient (ε) of 2.8 mM^{-1} cm^{-1}.

2.2.2 Results

a. Soil properties

Soil pH values at the end of the experiment remained within a slightly alkaline range (8.33–8.90) across species, treatments, and plant presence conditions (Table 6), comparable to the initial values recorded prior to the start of the trial (8.38–8.80, see Deliverable 1.3). In both *M. giganteus* and *C. sativa*, the medium TPHs contamination level generally resulted in the highest pH values, particularly in pots with plants (8.85 ± 0.04 and 8.90 ± 0.01, respectively). The low contaminations levels tended to slightly reduce pH relative to the control, especially in pots without plants of *M. giganteus*. Differences between pots with and without plants were minimal, although in some cases plant presence was associated with marginally higher pH values under the medium and high contamination levels. Overall, the observed variations were small and remained within the range of the initial soil conditions, indicating no substantial shifts in substrate alkalinity during the experimental period.

In addition, electrical conductivity (EC) values at the end of the experiment ranged from 0.39 to 0.53 dS m^{-1} (Table 6), which is consistent with the low EC levels measured before the experiment (0.44–0.61 dS m^{-1}). In general, EC increased under the low and medium contamination levels compared to the control in both species. In *M. giganteus*, the highest EC was recorded in pots without plants under the low treatment (0.50 ± 0.04 dS m^{-1}), whereas in *C. sativa* the maximum EC was observed in pots with plants under the medium TPHs levels (0.53 ± 0.02 dS m^{-1}). Under the high contamination levels, EC values tended to stabilize or slightly decrease relative to the medium. Overall, TPHs contamination effects on EC were more pronounced than differences associated with plant presence; however, all values remained within the range of the initial soil conditions, supporting the absence of significant changes in substrate salinity throughout the study.

Table 6: Soil pH and electrical conductivity in pots with and without plants of *M. giganteus* and *C. sativa* under different TPH contamination levels.

Species	With / without plant	TPHs contamination level	pH	EC (dS m^{-1})
M. giganteus	Pots with plant	Control	8.59 ± 0.01	0.40 ± 0.02
		Low	8.46 ± 0.01	0.42 ± 0.01
		Medium	8.85 ± 0.04	0.44 ± 0.03

		High	8.76 ± 0.03	0.46 ± 0.02
	Pots without plant	Control	8.49 ± 0.01	0.39 ± 0.01
		Low	8.33 ± 0.02	0.50 ± 0.04
		Medium	8.70 ± 0.04	0.47 ± 0.02
		High	8.67 ± 0.02	0.45 ± 0.03
C. sativa	Pots with plant	Control	8.48 ± 0.02	0.44 ± 0.03
		Low	8.42 ± 0.02	0.48 ± 0.02
		Medium	8.90 ± 0.01	0.53 ± 0.02
		High	8.64 ± 0.01	0.47 ± 0.02
	Pots without plant	Control	8.54 ± 0.01	0.43 ± 0.01
		Low	8.54 ± 0.08	0.49 ± 0.02
		Medium	8.67 ± 0.08	0.45 ± 0.02
		High	8.76 ± 0.02	0.44 ± 0.02

Total carbon (TC) content prior to the initiation of the experiment ranged from 5.44 ± 0.50% to 10.26 ± 0.12% , while total nitrogen (TN) varied between 0.07 ± 0.01% and 0.20 ± 0.01%. At the end of the experiment, TC and TN values followed comparable distribution patterns across treatments in both *M. giganteus* and *C. sativa* (Table 7). In general, TC increased from the control to the low and medium contamination levels, reaching maximum values under the medium treatment, and subsequently declined at high contamination levels, particularly in pots without plants. Control treatments consistently exhibited the lowest TC values.

A similar trend was observed for TN, with the highest concentrations generally recorded under the low contamination levels and a marked decrease under high contamination, especially in the absence of plants. Despite these contamination-related differences, final TC and TN values remained within the range of the initial soil conditions, indicating that contamination level influenced the relative distribution of carbon and nitrogen among treatments but did not result in substantial deviations from baseline soil contents. Overall, contamination level exerted a stronger effect than plant presence, although pots with plants tended to maintain slightly higher TN concentrations under certain conditions.

Table 7: Total carbon (TC) and total nitrogen (TN) percentages in pots with and without plants of *M. giganteus* and *C. sativa* under different TPHs levels.

Species	With / without plant	TPHs contamination level	TC %	TN %
M. giganteus	Pots with plant	Control	2.96 ± 0.25	0.09 ± 0.00

		Low	6.36 ± 0.35	0.14 ± 0.00
		Medium	7.66 ± 0.56	0.10 ± 0.01
		High	7.44 ± 0.35	0.09 ± 0.01
	Pots without plant	Control	4.66± 0.39	0.09 ± 0.00
		Low	6.65 ± 0.20	0.16 ± 0.00
		Medium	7.87 ± 0.32	0.12 ± 0.00
		High	4.73 ± 0.15	0.07 ± 0.00
C. sativa	Pots with plant	Control	3.03 ± 0.31	0.12 ± 0.00
		Low	5.97 ± 0.35	0.16 ± 0.00
		Medium	7.59 ± 0.28	0.14 ± 0.00
		High	6.12 ± 0.41	0.08 ± 0.00
	Pots without plant	Control	4.08 ± 0.10	0.09 ± 0.00
		Low	6.55 ± 0.28	0.14 ± 0.00
		Medium	7.60 ±0.25	0.11 ± 0.00
		High	4.63 ± 0.39	0.07 ± 0.00

b. Effects of treatments on plant physiological parameters

At the end of the experiment with *M. giganteus*, we observed that plants grown in soils with low contamination levels exhibited a better phenotype (greener leaves, greater plant height, and more turgid leaves) compared to the other treatments (Figure4). Additionally, the more yellowish coloration of plants growing in highly contaminated soils was very evident.

On the other hand, at the end of the experiment with *C. sativa*, it was clearly observed that plants exposed to high levels of TPHs contamination were unable to grow in height and displayed a highly stressed phenotype, with senescing leaves and very thin stems. Additionally, noticeable leaf abscission occurred in these plants, with fallen leaves observed on the soil surface. In the other soil contamination levels, better development appeared in soils with low contamination compared to the medium contamination and control soils.

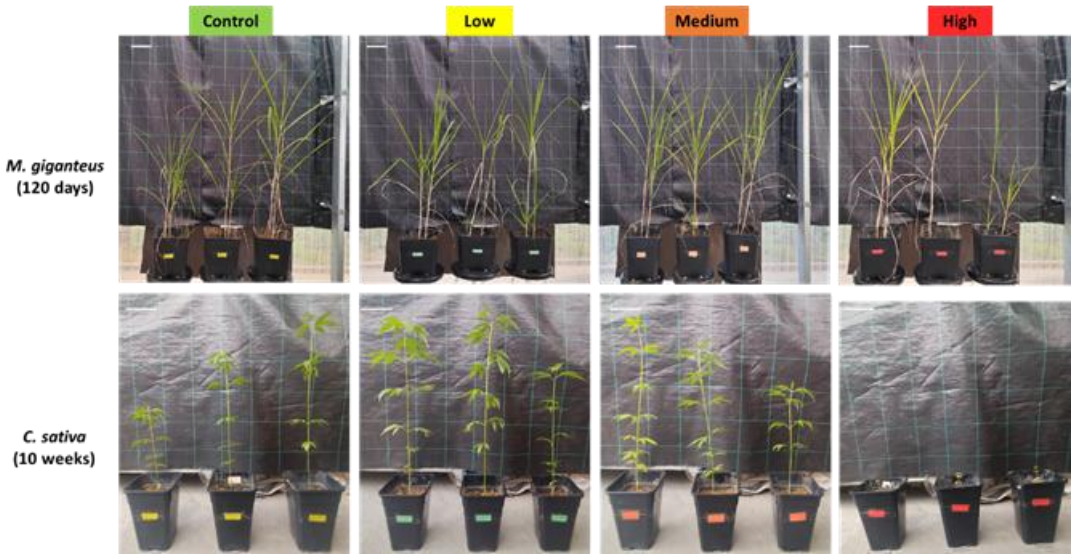

Figure 4: Growth of *M. giganteus* and *C. sativa* in TPH-contaminated soils. Images of *M. giganteus* after 120 days growing in TPH-contaminated soils and, *C. sativa* after 10 weeks growing in TPH-contaminated soils. Scale bars are indicated in each figure and correspond to 20 cm.

These results were corroborated by measuring other physiological parameters, such as plant height, number of leaves, and stem diameter throughout the experiments (Figure 5). In *M. giganteus*, plants exposed to low levels of TPHs showed greater height than the other treatments, although no clear differences were observed in the number of leaves (Figure 5A). In contrast, consistent with the observed phenotype of *C. sativa* plants, those exposed to high levels of TPHs exhibited much lower height compared to the other treatments, as well as reduced number of leaves and stem diameter (figure 5).

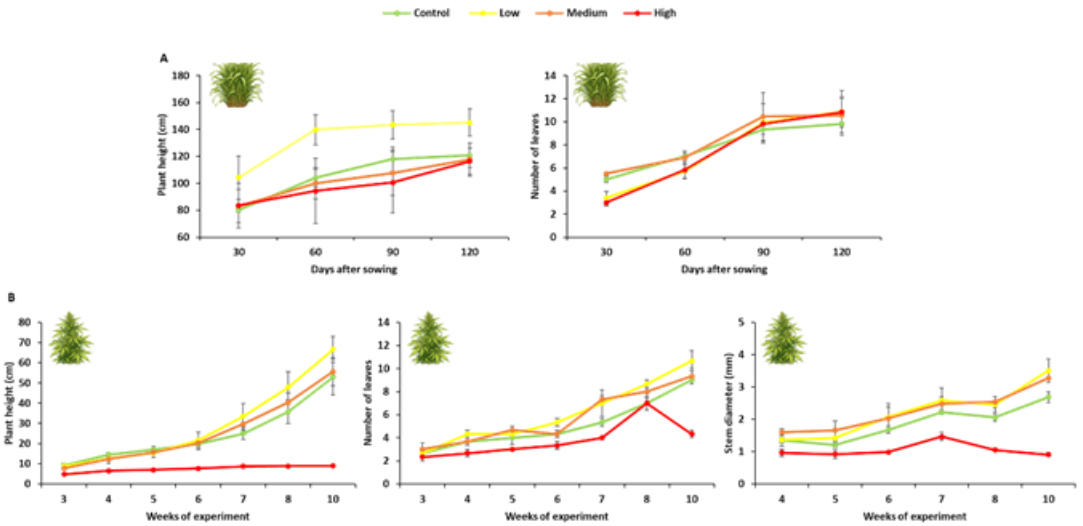

Figure 5: Growth dynamics of *M. giganteus* and *C. sativa* over the experimental assay with TPHs contaminated soils. A. *M. giganteus* growth throughout the experiment: plant height (cm) and number of leaves measured at 30, 60, 90 and 120 days of the experiment. B. *C. sativa* growth

throughout the experiment: plant height (cm), number of leaves and stem diameter (cm) measured at 4, 5, 6, 7, 8 and 10 weeks of the experiment. Data represent means ± 3 biological replicates.

Growth and physiological parameters responded differently to contamination levels in *M. giganteus* and *C. sativa*. In *M. giganteus*, plant height, number of leaves, and biomass (shoot and root weight) generally increased under low and especially medium contamination levels compared to the control, with the highest values typically observed at medium contamination. However, under the high treatment, most growth parameters declined markedly. Stomatal conductance and SPAD values followed a similar trend, increasing under low and medium levels and decreasing under high contamination (Table 8).

In *C. sativa*, a progressive reduction in plant height and shoot biomass was observed with increasing contamination, particularly under the high contamination levels. The number of leaves and buds also decreased at higher contamination levels. Stomatal conductance and SPAD values were highest under control or medium treatments and declined sharply under high contamination (Table 8). Stem diameter and root weight were reduced at high contamination levels in both species. Taken together, medium contamination appeared to stimulate growth in *M. giganteus*, whereas *C. sativa* showed greater sensitivity to increasing contamination, particularly at high levels.

Table 8: Growth, physiological, and biomass parameters of *M. giganteus* and *C. sativa* growing under different TPHs contamination levels. Data are means of three biological replicates ± S.E. n.m means not measured and n.d means not determinable.

M. giganteus								
	Height (cm)	Nº of leaves	Nº of buds	Stomatal conductance	SPAD	Stem diameter (mm)	Shoot weight (g)	Root weight
Control	120.67 ± 8.95	9.83 ± 1.01	8.67 ± 0.67	70.93 ± 9.08	12.86 ± 1.96	n.m	30.83 ± 3.27	92.70 ± 17.94
Low	145.17 ± 10.29	10.89 ± 1.84	15.00 ± 2.08	91.22 ± 8.60	16.71 ± 1.46	n.m	39.03 ± 0.58	101.78 ± 22.17
Medium	117.78 ± 12.39	10.56 ± 1.47	16.00 ± 3.00	106.34 ± 7.69	12.90 ± 1.63	n.m	36.70 ± 5.47	129.92 ± 25.27
High	116.33 ± 9.73	10.83 ± 1.30	5.33 ± 1.20	95.96 ± 24.89	7.90 ± 2.32	n.m	25.11 ± 6.59	75.81 ± 17.34
C. sativa								
	Height (cm)	Nº of leaves	Nº of buds	Stomatal conductance	SPAD	Stem diameter (mm)	Shoot weight (g)	Root weight
Control	53.00 ± 8.89	9.00 ± 0.00	n.m	419.44 ± 54.43	22.28 ± 1.46	2.69 ± 0.17	4.61 ± 0.72	1.30 ± 0.21
Low	66.67 ± 6.39	10.67 ± 0.88	n.m	401.26 ± 55.70	23.72 ± 1.45	3.51 ± 0.36	8.91 ± 2.03	2.61 ± 0.25
Medium	55.67 ± 6.96	9.33 ± 0.67	n.m	416.58 ± 57.78	16.14 ± 2.38	3.28 ± 0.04	6.22 ± 0.57	1.93 ± 0.10
High	9.00 ± 0.58	4.33 ± 0.33	n.m	n.d	n.d	0.91 ± 0.06	0.09 ± 0.01	0.13 ± 0.07

c. Rhizosphere microbial isolation

The isolation of microorganisms was performed on the rhizosphere part of the roots to obtain endophytic isolates and on the internal part of the roots to obtain epiphytic isolates. The variability of the isolates was higher for the epiphytic ones than for the endophytic (Table 9). The total number of microorganisms isolated from the rhizosphere varied depending on both plant species and the level of TPH contamination (Table 9). In *M. giganteus*, a progressive increase in the number of isolates was observed with increasing TPH concentrations, rising from 21 in the control treatment to 29, 28, and 32 in the low, medium, and high treatments, respectively, with the highest value recorded under the highest contamination level. In contrast, in *C. sativa*, the greatest number of microorganisms was detected in the control treatment (23), followed by a decrease under contaminated conditions, particularly at the medium level (16), and a slight recovery in the high treatment (19). These results indicate a differential response of the rhizospheric microbial community depending on plant species and the degree of TPH contamination.

Table 9: Number of total microorganisms isolated from the rhizosphere of the plants growing on TPHs contaminated soils.

	M. giganteus			*C. sativa*		
	Epiphytic	Endophytic	Total	Epiphytic	Endophytic	Total
Control	11	10	21	14	9	23
Low	13	16	29	12	9	21
Medium	16	12	28	7	9	16
High	22	10	32	10	9	19

d. Total soluble protein content in leaves

The total soluble protein content in leaves of *M. giganteus* and *C. sativa* after the tolerance assay in CS#3A soils contaminated with increasing levels of TPHs is shown in Table 10. In *M. giganteus*, plants grown in soils with low and medium TPH contamination exhibited an increase in leaf protein content compared to control plants, with the highest values observed under low TPH conditions. In contrast, plants exposed to high TPH levels showed protein contents close to those of control plants (Table10).

In *C. sativa*, leaf soluble protein levels were markedly higher than those measured in *M. giganteus* across all treatments. A slight increase in protein content was observed in plants grown in soils with low TPH levels relative to the control, whereas exposure to medium TPH contamination resulted in a reduction in protein levels. Protein content could not be determined in plants exposed to the highest TPH concentration due to the lack of sufficient plant material for analysis (Table10).

In general, these results indicate that moderate TPH contamination can differentially affect leaf protein accumulation depending on the plant species, suggesting species-specific physiological responses. Changes in total soluble protein content may reflect alterations in metabolic activity and stress-related responses associated with tolerance mechanisms operating under hydrocarbon-contaminated soil conditions.

Table 10: Total protein content (mg) of *M. giganteus* and *C. sativa* leaves after the CS#3A soil tolerance test. Data represent the total soluble protein content of three biological replicates, in mg ± S.E. n.d means not determinable.

	M. giganteus	*C. sativa*
Control	2.61 ± 0.16	14.32 ± 4.66
Low	3.76 ± 0.21	16.10 ± 0.88
Medium	3.02 ± 0.19	12.76 ± 1.47
High	2.78 ± 0.51	n. d

e. Photosynthetic pigment content

Changes in leaf photosynthetic pigment composition were evaluated in *M. giganteus* and *C. sativa* following the tolerance assay conducted in CS#3A soils with increasing TPH contamination (Table11). Chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoid contents are expressed as mean ± S.E. (µg g^{-1} fw).

In *M. giganteus*, low TPH contamination led to increased Chl a and Chl b contents compared to control plants, while medium TPH levels caused a decline in Chl a and carotenoids, with Chl b showing high variability; at high TPH concentrations, pigment levels generally resembled those of controls, albeit with greater variability.

C. sativa, however, consistently exhibited higher pigment concentrations than *M. giganteus* across all treatments. In this species, low TPH contamination markedly enhanced Chl a, Chl b, and carotenoid contents relative to controls, whereas medium TPH exposure reduced pigment levels compared to the low TPH treatment, though values remained above those of the control.

Table 11: Leaf photosynthetic pigment levels in *M. giganteus* and *C. sativa* were assessed after the tolerance assay in CS#3A soils. Chlorophyll a, chlorophyll b and total carotenoid contents are expressed as mean of three biological replicates ± S.E. (µg g^{-1} fw).

Species	TPHs contamination level	Chlorophyll a	Chlorophyll b	Carotenoids
M. giganteus	Control	154.62 ± 46.03	69.59 ± 34.63	60.24 ± 12.85
	Low	189.23 ± 30.94	86.64 ± 22.65	68.03 ± 7.40
	Medium	111.99 ± 31.72	77.50 ± 67.38	38.86 ± 3.35
	High	143.02 ± 58.61	67.97 ± 40.33	57.03 ± 19.36
C. sativa	Control	601.40 ± 59.49	285.10 ± 28.88	208.83 ± 19.10
	Low	960.44 ± 200.56	438.68 ± 83.15	275.48 ± 43.30
	Medium	783.49 ± 224.47	372.02 ± 105.99	258.29 ± 56.99

Overall, these results indicate that TPH contamination in CS#3A soils affects photosynthetic pigment accumulation in a species-dependent manner. The observed changes in chlorophylls and carotenoids suggest alterations in photosynthetic performance and photoprotective capacity under hydrocarbon-induced stress, supporting a differential tolerance and metabolic adjustment between *M. giganteus* and *C. sativa*.

f. Plant antioxidant activity responses

Antioxidant enzyme activities (Figure6) showed distinct responses to increasing TPHs contamination levels in both species. In *M. giganteus*, antioxidant enzymes exhibited enzyme-specific response patterns to contamination. Catalase (CAT) activity showed a moderate increase under low and high contamination levels, while remaining comparable to control values under medium contamination. This pattern suggests an early activation of hydrogen peroxide (H_2O_2) scavenging under mild stress, followed by a transient acclimation phase at intermediate contamination, and a renewed enzymatic stimulation under more severe oxidative pressure. In contrast, ascorbate peroxidase (APX) activity increased predominantly under medium contamination, whereas low and high treatments remained similar to the control. This response may indicate a differential regulation of H_2O_2 detoxification pathways, where APX plays a more prominent role under moderate stress conditions, possibly linked to fine-tuned redox homeostasis within chloroplasts and cytosolic compartments. Taken together, these results suggest a coordinated but enzyme-specific antioxidant adjustment in *M. giganteus*, reflecting a dynamic modulation of ROS-scavenging mechanisms depending on stress intensity.

In *C. sativa*, antioxidant enzyme responses differed markedly from those observed in *M. giganteus*, and no enzymatic activities could be determined under high contamination due to the severe stress-induced reduction in plant biomass. CAT activity increased under low contamination but declined under medium contamination relative to the control, suggesting an initial activation of H_2O_2 detoxification under mild stress followed by a possible impairment or downregulation of CAT activity as stress intensity increased. This decrease at medium contamination may reflect enzymatic inhibition, oxidative damage to proteins, or a shift toward alternative antioxidant pathways. In contrast, APX activity showed a progressive increasing trend with rising contamination levels (control < low < medium), indicating a gradual reinforcement of the ascorbate-dependent ROS-scavenging system. This pattern suggests that APX may play a more central role than CAT in sustaining redox balance in *C. sativa* under moderate stress conditions. The results point to a differential and stress-sensitive modulation of antioxidant defence in *C. sativa*, with limited tolerance to high contamination levels.

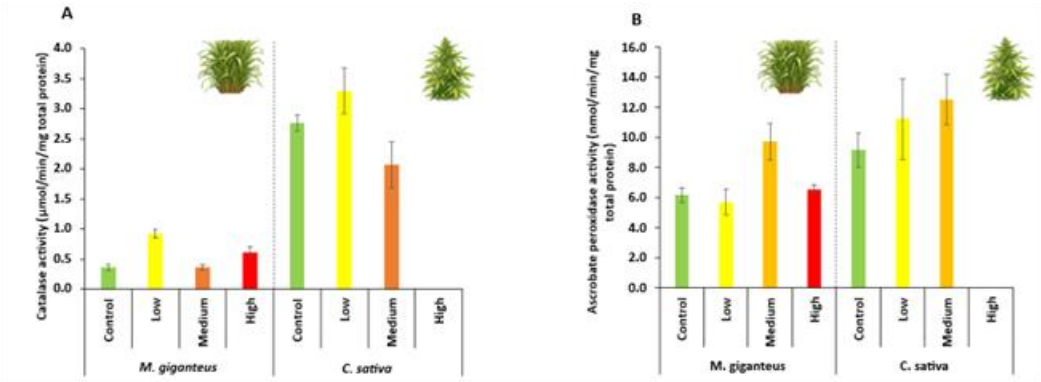

Figure 6: Antioxidant enzyme activities in *M. giganteus* and *C. sativa* exposed to increasing TPHs contamination levels. Bars represent mean values ± S.E. Colours indicate contamination levels.

2.3 CS#3B. ORGANIC / MIXED CONTAMINATION: MISCANTHUS & HEMP TOLERANCE

2.3.1. Materials and methods

a. Plant material

The plant material used in this study consisted of *Miscanthus × giganteus* rhizomes and seeds of *Cannabis sativa* (industrial hemp) belonging to the certified cultivar Futura 83. Rhizomes of *M. giganteus* were obtained from the proprietary propagation facilities of Particula Group, where planting stock was produced under controlled agronomic conditions. After harvest, the rhizomes were transported to the greenhouse facilities located in Rijeka, Croatia. To ensure their physiological viability prior to the start of the experiment, rhizomes were stored in complete darkness at 4 °C under high-humidity conditions. This storage regime minimizes metabolic activity, prevents premature sprouting, and maintains tissue moisture, all of which are essential for uniform establishment once planted.

Seeds of *C. sativa cv.* Futura 83 were acquired from the University of Burgos, an accredited supplier of certified hemp seed varieties. Upon delivery, seeds were kept at 4 °C, stored in airtight containers under low-humidity conditions to preserve germination quality and to prevent physiological ageing. Seeds were sown immediately before the beginning of the tolerance assays, ensuring high germination rates and consistent early growth among replicates.

The selection of *Miscanthus × giganteus* and *C. sativa cv.* Futura 83 followed the same operational rationale described for CS#3A.

b. Soil material

Soils used in the experiment were collected from historically contaminated sites belonging to the former INA oil refinery in Sisak, Croatia, and from sections of an oil pipeline crossing the same industrial zone. The selected sites represent complex contamination scenarios typical for petrochemical industry legacy locations. Total petroleum hydrocarbons (TPHs) constitute the predominant group of pollutants at these sites; however, polycyclic aromatic hydrocarbons (PAHs) and various heavy metals are also present due to decades of industrial activity, accidental spills, and diffuse leakage from oil infrastructure.

The physicochemical and contaminant profiles of these soils were previously characterized within the project and fully documented in Deliverable D1.3, “Characterisation of new candidate field sites.” These baseline assessments include pH, electrical conductivity, organic matter content, granulometry, and concentrations of hydrocarbons and trace metals. This prior characterization ensured that the selected soil batches covered a gradient of contamination levels (control, low, medium, high), allowing evaluation of plant tolerance under realistic environmental conditions.

c. Tolerance assay

To assess the tolerance of the two plant species to varying contamination levels, controlled greenhouse pot experiments were conducted. For *M. giganteus*, pots with dimensions of 22 × 20 × 17 cm (length × width × height) were used, each filled with 8 kg of soil. The soil was amended with 7% sand (w/w) to improve aeration and substrate structure. Soil moisture was adjusted to

70% of water retention capacity (WRC), a level suitable for optimal rhizome sprouting and early vegetative development of *M. giganteus*.

For *C. sativa*, a smaller pot size was used, measuring 18 × 18 × 15 cm, each filled with 3 kg of soil. Due to different water requirements and root system architecture, the soil for hemp was amended with 5% sand and maintained at 30% WRC, conditions known to support germination and early growth of industrial hemp while avoiding excessive moisture that may hinder root oxygenation.

For both species, four contamination scenarios were prepared: uncontaminated control soil and soils representing low, medium, and high contamination levels. Each scenario was replicated three times, resulting in a total of 12 pots per species. Plants were grown under greenhouse conditions with controlled temperature, regular irrigation, and standardized light exposure to limit environmental variability.



Figure 7. Pot preparation

d. Plant physiological parameters

Plant development and physiological performance were monitored throughout the experimental period using non-destructive measurement techniques. These techniques allow repeated assessment without harming the plants, enabling the evaluation of growth trends over time.

Photographic documentation was conducted regularly to visually track morphological progression, overall vigour, and any visible stress symptoms associated with contamination levels.

Plant height was measured using a measuring tape, with values recorded as the vertical distance from the soil surface to the tip of the longest leaf or shoot. For *M. giganteus*, height and other parameters were measured monthly due to its slower early growth and perennial habit. *C. sativa*, having a faster growth rate and shorter life cycle, was measured weekly to capture rapid physiological changes.

At the end of the trial, plants were harvested, and total biomass per pot was recorded. Biomass was measured as fresh mass, providing insights into tolerance and productivity under different contamination levels.



Figure 8. Growth trials in Particula Greenhouse

2.3.2. Results

a. Soil Physicochemical Properties

Soil pH measurements taken at the beginning and end of the experiment remained relatively stable. The pH ranged from 7.7 to 8.2 at the end of the experiment, consistent with initial values of 7.4 to 8.0 recorded during soil preparation. These data indicate that neither plant growth nor contamination levels resulted in substantial acidification or alkalinization of the soil substrate

during the experimental period. The slightly alkaline environment is typical of calcareous soils in the region and is known to influence the mobility of certain metals and organic pollutants.

Electrical conductivity (EC), which reflects the concentration of soluble salts in the soil, showed similarly stable behaviour. Initial EC values ranged from 0.2 to 0.3 dS m^{-1}, indicative of non-saline soils. By the end of the trial, EC values increased slightly to 0.3–0.4 dS m^{-1} but remained within the low-salinity range. Such small changes suggest minimal salt accumulation resulting from irrigation, plant activity, or contaminant degradation processes. Importantly, these EC values fall well below thresholds known to induce salinity stress in either *M. giganteus* or *C. sativa*.

b. Biomass Production

At harvest, both species produced biomass in the range of 0.02–0.04 kg per pot. This relatively low biomass yield can be attributed to several factors. First, both plants were grown in pots under controlled greenhouse conditions, which naturally restrict root expansion and overall plant size compared to field-grown individuals. Second, growth occurred in soils with varying levels of hydrocarbon and metal contamination, conditions that are known to suppress plant development by affecting nutrient uptake, root metabolism, and microbial interactions.

Nevertheless, the fact that biomass values remained within a comparable range across all treatments suggests that both *M. giganteus* and *C. sativa* demonstrated a degree of tolerance to the contamination levels tested. Although detailed statistical analysis is required to determine the significance of differences among treatments, the general trend indicates that neither species experienced catastrophic growth inhibition, even in highly contaminated soil.

c. Notes on Methodological Limitations

It is important to highlight that the Particula Group (a SME specialized in agricultural production) does not possess advanced laboratory equipment typically available in research and technology organizations (RTOs). Consequently, certain analytical procedures, especially those requiring specialized instrumentation (e.g., detailed hydrocarbon fractionation, metal speciation analysis, chlorophyll fluorescence measurements), could not be performed within this study.

As a result, the methodology employed may differ from protocols used at other sites participating in the broader project. However, the core aim of this case study—to assess general plant tolerance to mixed organic contamination under realistic growing conditions—was fully achieved. The data generated remain valuable for understanding plant–contaminant interactions and for supporting future field-scale applications

2.3.3. Interpretation and limitations of CS#3B

The CS#3B assay should be interpreted as a complementary, site-specific greenhouse test designed to assess whether the selected plant material could establish under Croatian oil-affected soil conditions, rather than as a full demonstration of broad tolerance to hydrocarbon contamination. Unlike CS#3A, where a clearer TPH gradient was used to evaluate dose-dependent responses, CS#3B represented a more heterogeneous and moderate contamination scenario, reflecting the variability of the sampled Croatian legacy oil-affected soils.

Under these conditions, the observed establishment and biomass production indicate that *Miscanthus × giganteus* and *C. sativa cv.* Futura 83 were not completely inhibited by the tested Croatian soils. However, the available evidence is not sufficient to claim that both crops are broadly tolerant to all CS#3B contamination contexts. The results should therefore be read as preliminary evidence of establishment capacity under the specific soil batches, greenhouse conditions and crop material tested.

This limitation is relevant for WP2 decision-making. CS#3B does not provide a final crop recommendation by itself, but it helps identify which crop × soil combinations deserve further validation. The next step should be to test the selected material under better-defined field or semi-field conditions, ideally including replicated plots or larger containers, a clearer characterisation of spatial variability, and the integration of amendments and microbial inoculants. In this way, CS#3B will serve as a bridge between the initial lab/bench-scale tolerance filter and the future validation of a complete phytomanagement strategy.

Consequently, statements derived from CS#3B have been formulated cautiously in this deliverable. The results support the feasibility of continuing with the tested material in subsequent optimisation steps, but they do not constitute definitive proof of tolerance or a final crop/genotype recommendation for Croatian hydrocarbon-contaminated soils.

2.4 CS#4. SALINITY: MISCANTHUS & HEMP TOLERANCE

2.4.1 Materials and methods

All materials and methodologies used were the same as those described in Section 2.2.1, but using the soils described below. Soils with varying levels of electrical conductivity were collected from agricultural fields affected by saline intrusion associated with sodium sulphate mining activities. As in CS#3A, these soils are fully described in Deliverable D1.3, "Characterisation of new candidate field sites".

Four soil types were considered in this study: a control soil with similar physicochemical properties but normal electrical conductivity (≈ 0.4 dS m^{-1}); a low salinity soil with an electrical conductivity of approximately 0.8 dS m^{-1}, a medium salinity soil with an electrical conductivity of around 2 dS m^{-1}, and a high salinity treatment, which was artificially generated by amending the 0.8 dS m^{-1} mine-derived salts at a rate of 1.5% (w/w) to reach an electrical conductivity of approximately 3.5 dS m^{-1}.

This gradient was designed to represent a low-to-moderate salinity scenario relevant for agricultural soils affected by secondary salinisation, rather than an extreme saline-soil scenario. This distinction is important because soil salinity is spatially heterogeneous in Europe and depends strongly on climate, irrigation, groundwater dynamics, parent material and local industrial or mining activities. European-scale assessments identify saline, sodic and potentially salt-affected soils as a relevant but unevenly distributed degradation issue, particularly in Mediterranean and other vulnerable regions. Therefore, CS#4 should be interpreted as an operational test of crop response to increasing electrical conductivity within the range of the selected case-study conditions, not as a general assessment of crop performance across all European saline soils.

2.4.2 Results

a. Soil properties

Soil pH and electrical conductivity (EC) varied depending on the presence of plants, species, and salinity levels (Table 12). In general, the presence of plants tended to moderate changes in soil pH and EC compared to pots without plants.

For *M. giganteus*, soil pH in pots with plants showed a slight decrease under medium salinity but remaining relatively stable under low and high salinity. In contrast, pots without plants showed a slightly higher pH under control and high salinity, suggesting that plant uptake or rhizosphere activity may slightly acidify the soil under certain salinity conditions. EC increased with salinity for both pots with and without plants, but pots without plants exhibited higher EC values at low salinity and slightly lower EC at high salinity, indicating some plant-mediated reduction of soluble salts.

For *C. sativa*, soil pH in pots with plants decreased with increasing salinity, from 8.82 in the control to 7.99 under medium salinity, then slightly increased to 8.12 under high salinity. Pots without plants showed a more pronounced pH decline with salinity, reaching 6.97 at medium and 7.03 at

high salinity. EC increased with salinity in all cases, with higher EC in pots without plants compared to those with plants, suggesting that *C. sativa* can partially mitigate salt accumulation in the rhizosphere.

These results indicate that both species influence soil chemical properties, particularly under moderate and high salinity, with *C. sativa* showing a stronger effect in reducing pH decline and limiting EC increases compared to bare soil.

Table 12: Soil pH and electrical conductivity (EC) in pots with and without plants of *M. giganteus* and *C. sativa* under different salinity levels.

Species	With / without plant	Salinity levels	pH	EC (dS m^{-1})
M. giganteus	Pots with plant	Control	8.74 ± 0.02	0.41 ± 0.01
		Low	8.71 ± 0.03	1.10 ± 0.08
		Medium	8.15 ± 0.01	2.34 ± 0.01
		High	8.35 ± 0.03	3.66 ± 0.32
	Pots without plant	Control	8.82 ± 0.03	0.47 ± 0.03
		Low	8.82 ± 0.02	1.76 ± 0.12
		Medium	8.21 ± 0.02	2.44 ± 0.02
		High	8.49 ± 0.02	3.54 ± 0.42
C. sativa	Pots with plant	Control	8.82 ± 0.02	0.45 ± 0.02
		Low	8.55 ± 0.03	1.25 ± 0.03
		Medium	7.99 ± 0.05	2.42 ± 0.02
		High	8.12 ± 0.06	3.59 ± 0.12
	Pots without plant	Control	8.04± 0.06	0.61 ± 0.02
		Low	7.68 ± 0.04	1.54 ± 0.07
		Medium	6.97 ±0.03	2.52 ± 0.05
		High	7.03 ± 0.03	3.12 ± 0.25

Additionally, TC and TN in soils were influenced by the presence of plants, species, and salinity levels (Table 13). In the case of *M. giganteus*, TC in pots with plants increased with salinity, from 3.68% in the control to 4.88% under medium salinity, then slightly decreased under high salinity (4.68%). Pots without plants showed a similar trend but with consistently lower TC values, indicating that plant presence contributed to higher carbon retention in the soil. TN followed a comparable pattern, with values increasing from 0.16% in control pots to 0.22% at medium salinity in planted pots, whereas unplanted pots exhibited smaller increases (0.15–0.18%),

suggesting that *M. giganteus* may enhance nitrogen accumulation under moderate salinity conditions.

For *C. sativa*, TC in pots with plants increased markedly from 2.29% in the control to 4.76% at medium salinity, then slightly decreased under high salinity (4.49%). In pots without plants, TC also increased with salinity but remained lower than in planted pots, particularly at medium and high salinity levels. TN showed a similar trend, with higher percentages in planted pots (0.15–0.18%) than in unplanted pots (0.14–0.18%), indicating that *C. sativa* similarly contributes to the maintenance of soil nitrogen under salinity stress.

As a result, both species enhanced TC and TN under increasing salinity, with the effect being more pronounced under medium salinity and in pots with plants. This suggests that the presence of the plants can partially mitigate the depletion of soil carbon and nitrogen associated with saline conditions.

Table 13: Total carbon (TC) and total nitrogen (TN) percentages in pots with and without plants of *M. giganteus* and *C. sativa* under different salinity levels.

Specie	With / without plant	Salinity levels	TC %	TN %
M. giganteus	Pots with plant	Control	3.68 ± 0.15	0.16 ± 0.02
		Low	4.26 ± 0.10	0.18 ± 0.00
		Medium	4.88 ± 0.03	0.22 ± 0.00
		High	4.68 ± 0.07	0.18 ± 0.00
	Pots without plant	Control	3.57± 0.06	0.15 ± 0.00
		Low	3.98 ± 0.08	0.15 ± 0.01
		Medium	4.29 ± 0.17	0.18 ± 0.01
		High	4.02 ± 0.09	0.17 ± 0.00
C. sativa	Pots with plant	Control	2.29 ± 0.15	0.13 ± 0.00
		Low	3.99 ± 0.06	0.15 ± 0.00
		Medium	4.76 ± 0.02	0.18 ± 0.00
		High	4.49 ± 0.05	0.15 ± 0.00
	Pots without plant	Control	3.61± 0.02	0.14 ± 0.00
		Low	3.91 ± 0.04	0.14 ± 0.00
		Medium	4.42 ±0.15	0.18 ± 0.01
		High	4.27 ± 0.08	15 ± 0.00

b. Effects of treatments on plant physiological parameters

At the end of the experiment with *M. giganteus*, we observed that plants grown in soils with low salinity levels exhibited a better phenotype (greener leaves, greater plant height, and more turgid leaves) compared to the other treatments (Figure 9). Additionally, the more yellowish coloration of plants growing in high salinity levels was very evident. Moreover, the plants grown in medium salinity levels presented uneven results among the biological replicates due to the death of two biological replicates at day 60, and the emergence of two new plants few days later.

On the other hand, at the end of the experiment with *C. sativa*, it was clearly observed that plants exposed to high levels of saline concentration presented a less developed phenotype (less height, small leaves and thin stems) (Figure 9). In control and low salinity levels, plants presented a developed phenotype, nevertheless, leaves were starting to present a yellowish colour at the end of the experience. On the other hand, plants grown under medium saline levels presented better development, leaves presented advanced growth and bright green colour, and small inflorescences were beginning to bloom (Figure 9).

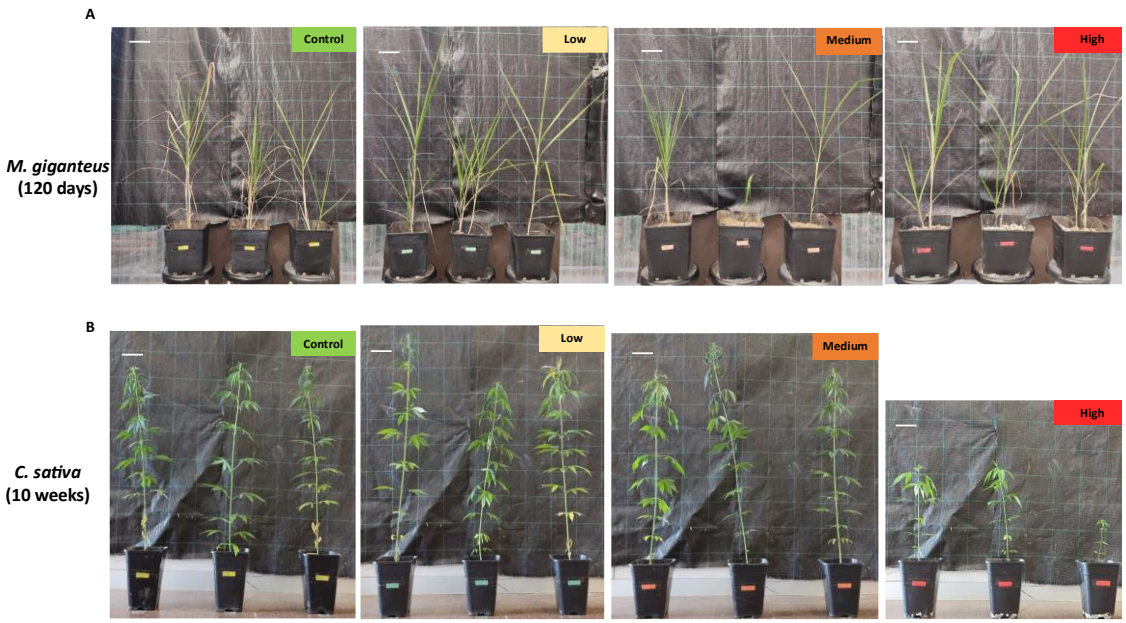

Figure 9: Growth of *M. giganteus* and *C. sativa* in Saline soils. Images of *M. giganteus* after 120 days growing in Saline-contaminated soils and, *C. sativa* after 10 weeks growing in saline-contaminated soils. Scale bars are indicated in each figure and correspond to 20cm.

These results were corroborated by measuring other physiological parameters, such as plant height, number of leaves, and stem diameter throughout the experiments (Figure 1010). In *M. giganteus*, control soil plants showed greater height than the other salinity levels, although no clear differences were observed in the number of leaves at the end of the experiment (Figure 1010A). In contrast, *C. sativa* plants exhibited more contrast between the high salinity level soils and the other treatments, showing less height, number of leaves and stem diameter. For the

other concentrations of salt on soils, these parameters exhibited similar results at the end of the experiment (Figure 10B).

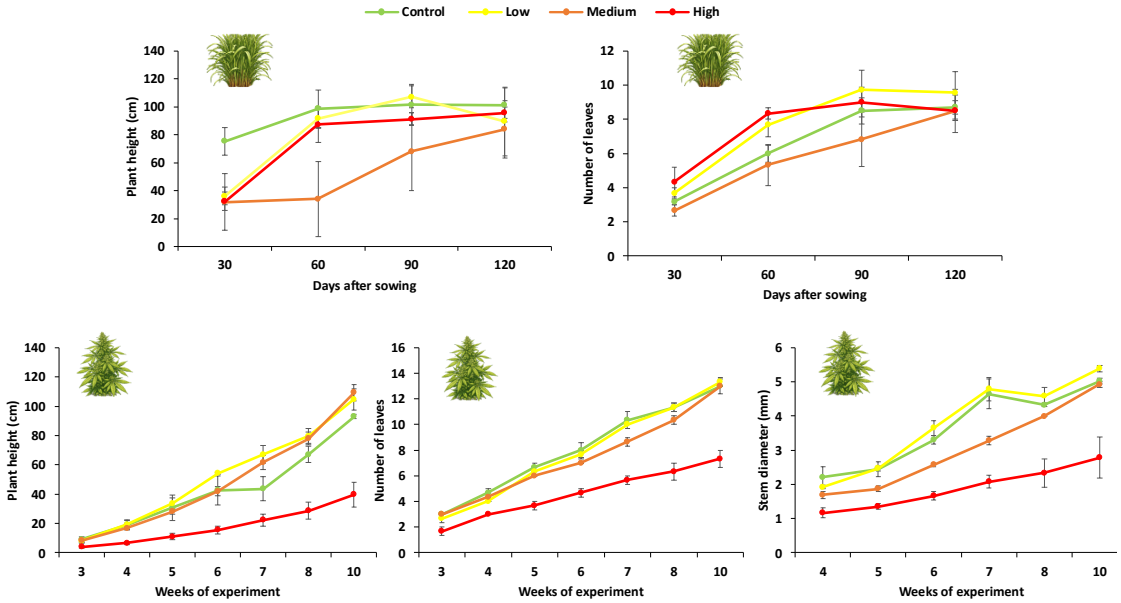

Figure 10: Growth dynamics of *M. giganteus* and *C. sativa* over the experimental assay with Saline soils. A. *M. giganteus* growth throughout the experiment: plant height (cm) and number of leaves measured at 30, 60, 90 and 120 days of the experiment. B. *C. sativa* growth throughout the experiment: plant height (cm), number of leaves and stem diameter (cm) measured at 4, 5, 6, 7, 8 and 10 weeks of the experiment. Data represent means ± 3 biological replicates.

Growth and physiological traits showed different responses to salinity levels in *M. giganteus* and *C. sativa*. In *M. giganteus* exhibit a descending tendency in higher salinity concentrations for the number of leaves (Table 14). For plant height, number of buds and stomatal conductance the tendency repeats for low, medium and high salinity concentrations, but the control salt concentration omits this pattern, probably due to the differences on the soil physicochemical parameters. SPAD values increase under low and medium concentrations and increase sharply under high saline concentration. Shoot and root weight follow the same tendency; under medium concentration the values are lower than for the high and low ones (Table 14).

In *C. sativa*, a reduction in height, number of leaves, SPAD values, stem diameter, shoot and root weight were observed under high salinity levels (Table 14). The stomatal conductance values were lower under low and medium levels of salinity. Given the circumstances, medium salinity levels seem to restrain growth in *M. giganteus*, on the other hand, *C. sativa* demonstrated greater susceptibility to increasing concentration, particularly under high salinity levels.

Table 14: Growth, physiological, and biomass parameters of *M. giganteus* and *C. sativa* growing under different Saline levels. Data are means of three biological replicates ± S.E. n.m means not measured.

M. giganteus								
	Height (cm)	Nº of leaves	Nº of buds	Stomatal conductance	SPAD	Stem diameter (mm)	Shoot weight (g)	Root weight
Control	106.61 ± 17.23	8.72 ± 0.72	8.67 ± 1.45	92.74 ± 11.80	15.78 ± 1.53	n.m	26.39 ± 2.93	55.77 ± 4.64
Low	89.61 ± 24.56	9.56 ± 1.24	10.67 ± 1.86	106.90 ± 7.36	17.98 ± 3.48	n.m	27.98 ± 2.81	68.05 ± 6.70
Medium	84.00 ± 20.60	8.50 ± 1.26	3.33 ± 2.03	103.48 ± 16.88	17.50 ± 1.00	n.m	11.02 ± 4.66	20.33 ± 9.20
High	95.83 ± 4.10	8.50 ± 0.58	9.50 ± 2.04	132.68 ± 14.10	20.31 ± 1.24	n.m	25.31 ± 1.03	60.37 ± 1.65
C. sativa								
	Height (cm)	Nº of leaves	Nº of buds	Stomatal conductance	SPAD	Stem diameter (mm)	Shoot weight (g)	Root weight
Control	93.00 ± 1.53	13.00 ± 0.58	n.m	164.18 ± 34.17	32.27 ± 4.95	5.02 ± 0.09	24.56 ± 0.57	16.25 ± 4.34
Low	104.67 ± 7.45	13.33 ± 0.33	n.m	124.89 ± 38.08	23.78 ± 6.13	5.39 ± 0.10	27.97 ± 0.98	17.79 ± 0.21
Medium	109.33 ± 5.61	13.00 ± 0.00	n.m	121.16 ± 13.80	36.06 ± 3.22	4.92 ± 0.08	26.24 ± 0.97	13.04 ± 1.16
High	39.67 ± 8.41	7.33 ± 0.67	n.m	161.50 ± 48.28	19.68 ± 2.18	2.78 ± 0.59	4.98 ± 2.10	1.60 ± 0.59

c. Rhizosphere microbial isolation

The total number of microorganisms isolated from the rhizosphere varied depending on both plant species and saline levels of the soils (Table 15). In *M. giganteus*, low saline concentration presented the greatest number of microorganisms (39), whereas control and high concentrations exhibited similar total microorganisms, 33 and 34, while medium saline levels presented lower number of microorganisms (30). On the other hand, in *C. sativa* control salinity levels presented higher number of microbial isolates (14), nevertheless, saline levels do not seem to condition the number of total microorganisms, since low medium and high saline levels present 12,13 and 12 total isolates. These results indicate a differential response of the rhizospheric microbial

community depending on plant species, for *M. giganteus* present a larger number of total microorganisms than *C. sativa*. Overall, both plant species presented more epiphytic isolates than endophytic isolates.

Table 15: Number of total microorganisms isolated from the rhizosphere of the plants growing in saline soils.

	M. giganteus			*C. sativa*		
	Epiphytic	Endophytic	Total	Epiphytic	Endophytic	Total
Control	22	12	34	8	6	14
Low	20	19	39	8	4	12
Medium	17	13	30	8	5	13
High	19	14	33	8	4	12

d. Total soluble protein content in leaves

Analysis of leaf total soluble protein content following the tolerance experiment with CS#4 site soils showed that (Table 26) in *M. giganteus*, plants grown under medium and high salinity conditions exhibited a marked increase in leaf protein content compared to control plants, with the highest values recorded at high salinity. In contrast, protein levels in plants exposed to low salinity were similar to those observed in control conditions.

Moreover, in *C. sativa*, leaf soluble protein content was consistently higher than in *M. giganteus* across all treatments. While protein levels under low salinity were comparable to control plants, a clear increase was observed under medium and high salinity conditions, reaching maximum values at medium salinity.

The increase in total soluble protein content observed under moderate to high salinity suggests the activation of stress-related metabolic adjustments in both species. These changes may be associated with osmotic adjustment, enhanced synthesis of stress-responsive proteins, or alterations in nitrogen metabolism, supporting a potential role of protein accumulation in salinity tolerance mechanisms.

Table 26: Total protein content of *M. giganteus* and *C. sativa* leaves after the CS#4 soil tolerance test. Data represent the total soluble protein content of three biological replicates, in mg ± S.E

	Control	Low	Medium	High
M. giganteus	4.14 ± 1.12	3.98 ± 0.29	8.03 ± 0.78	8.62 ± 0.73
C. sativa	10.50 ± 1.86	10.30 ± 1.08	15.61 ± 0.38	15.13 ± 1.21

e. Photosynthetic pigment content

Leaf photosynthetic pigment contents were differentially affected by the treatments in *M. giganteus* and *C. sativa* grown in salinity soils (Table 37). In *M. giganteus*, chlorophyll a, chlorophyll b, and total carotenoid levels showed a progressive increase with increasing treatment intensity, reaching the highest values under the high salinity levels, compared to the control. A similar trend was observed in *C. sativa*, although pigment accumulation was more variable. While chlorophyll and carotenoid contents under the low salinity levels were comparable to control levels, a marked increase was detected under medium and high salinity (Table 17). Overall, both species exhibited enhanced pigment accumulation at higher treatment levels, with a more pronounced relative increase in *C. sativa* under medium and high conditions. This likely represents an antioxidant and photosynthetic protective response to the stress induced by high salinity, which was more pronounced in *C. sativa*.

Table 37: Leaf photosynthetic pigment levels in *M. giganteus* and *C. sativa* were assessed after the tolerance assay in CS#4 soils. Chlorophyll a, chlorophyll b and total carotenoid contents are expressed as mean of three biological replicates ± S.E. (µg g^{-1} fw).

Species	Salinity levels	Chlorophyll a	Chlorophyll b	Carotenoids
M. giganteus	Control	277.77 ± 85.44	120.46 ± 34.80	88.46 ± 23.40
	Low	318.02 ± 102.99	137.33 ± 42.24	95.23 ± 31.28
	Medium	383.28 ± 143.97	162.98 ± 59.16	106.34 ± 35.64
	High	407.15 ± 192.41	179.54 ± 81.69	119.47 ± 47.24
C. sativa	Control	347.73 ± 10.17	247.04 ± 30.61	151.08 ± 16.08
	Low	339.49 ± 18.16	254.70 ± 16.30	140.28 ± 12.57
	Medium	550.40 ± 303.32	351.74 ± 135.80	176.54 ± 70.81
	High	570.24 ± 152.62	342.97 ± 85.03	188.98 ± 31.96

f. Plant antioxidant activity responses

Antioxidant enzyme activities (Figure 11) revealed distinct and species-specific responses to increasing salinity levels. In *M. giganteus*, the antioxidant system displayed enzyme-dependent response patterns. CAT activity showed only slight variations across treatments, remaining relatively stable from control to high salinity levels (Figure 11A). Although minor fluctuations were observed, particularly a tendency toward lower values under medium salinity, the overall differences were limited, indicating that CAT activity in *M. giganteus* was not strongly stimulated by increasing salinity. This stability suggests either an inherently efficient basal detoxification capacity or a limited induction of CAT in response to saline stress. In contrast, APX activity in *M. giganteus* showed greater responsiveness to salinity. APX activity increased under low salinity compared to the control and remained relatively elevated under medium and high salinity

conditions (Figure 11B). This pattern suggests that APX may play a more prominent role than CAT in modulating ROS homeostasis in *M. giganteus* under saline conditions, potentially compensating for the limited variation observed in CAT activity.

In *C. sativa*, antioxidant enzyme responses differed markedly from those observed in *M. giganteus*. Overall, CAT activity was substantially higher in *C. sativa* than in *M. giganteus* (Figure 11A). Moreover, CAT activity increased progressively with rising salinity levels, reaching the highest values under high salinity. This trend indicates a strong activation of H_2O_2-scavenging mechanisms in response to increasing stress intensity, reflecting a marked sensitivity of this species to saline conditions and a need for enhanced oxidative protection. Similarly, APX activity in *C. sativa* showed a clear increasing trend with salinity (low < medium < high), with maximum activity observed under high salinity (Figure 11B). The coordinated upregulation of both CAT and APX suggests an intensified activation of the antioxidant defence system to counteract elevated ROS production. However, the pronounced induction of these enzymes under high salinity may also indicate that *C. sativa* experiences substantial oxidative stress under these conditions, consistent with a comparatively lower tolerance to elevated contamination levels. In general, the data highlight contrasting antioxidant strategies between the two species, with *M. giganteus* exhibiting more stable enzymatic profile and *C. sativa* showing a strong stress-responsive activation of its antioxidant machinery.

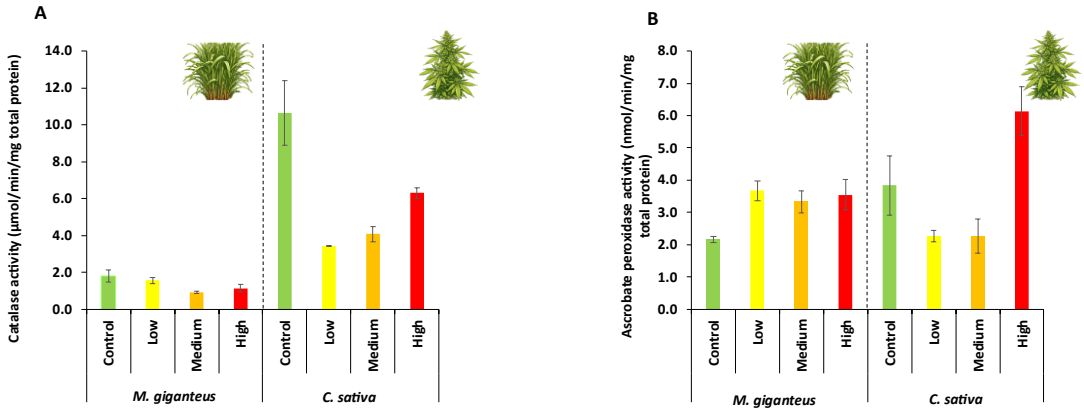

Figure 11: Antioxidant enzyme activities in *M. giganteus* and *C. sativa* exposed to increasing saline levels. Bars represent mean values ± S.E of three biological replicates. Colours indicate salinity levels.

3. DISCUSSION AND CROSS-CASE COMPARATIVE ASSESSMENT

The results obtained in CS#1 confirm that hybrid selection is a decisive step for phytomanagement in metal-contaminated soils, because plant performance was not determined by a single trait but by the interaction between establishment capacity, vegetative vigour, trace-element accumulation, and the ability to influence the bioavailable fraction of metals in soil. The strong differences observed among hybrids, especially during the establishment phase, indicate that tolerance under realistic site conditions cannot be inferred only from nursery performance or parental background. This agrees with recent evidence showing that poplar hybrids with different genetic backgrounds can display large differences in growth, stress resistance, and metal accumulation when exposed to multi-metal contamination, highlighting the importance of genotype-specific screening before upscaling (Li et al., 2024; Kovačević et al., 2025).

A second relevant outcome is that higher biomass production did not always coincide with the highest trace-element concentrations in plant tissues. In this sense, the comparison between hybrids such as PHY_2025_1 and PHY_2025_8 suggests that phytoextraction potential should be interpreted as the combined result of biomass production and tissue concentration, rather than either parameter alone. This is particularly important in phytomanagement systems where the harvested biomass is intended for downstream valorisation, because a highly productive hybrid with more moderate tissue concentrations may represent a more balanced option than a smaller but more concentrated accumulator. Similar biomass–accumulation trade-offs have been described in poplar-based phytoremediation studies, where increased metal uptake does not necessarily translate into greater overall remediation efficiency when biomass production is penalised (Komárek et al., 2008).

The post-harvest soil data further show that hybrids differed not only in their shoot accumulation patterns but also in their apparent influence on the soluble or readily extractable metal pool. This is relevant because, under field conditions, phytomanagement success depends not only on the removal of metals in harvestable biomass, but also on the progressive control of bioavailability and mobility in the soil matrix. The use of 0.01 M $CaCl_2$ extraction is therefore useful in this context, as it provides an operational estimate of the readily available fraction of inorganic pollutants in soil (Houba et al., 2000). From this perspective, the observed variation among hybrids suggests that some plant materials may be more suitable for a phytoextraction-oriented strategy, whereas others may contribute more clearly to risk reduction through partial stabilisation of the bioavailable fraction.

Taken together, the CS#1 results support the selection of PHY_2025_8 as the most balanced candidate for the next WP2 steps, because it combined strong vigour, good biomass production, acceptable trace-element export, and high phenotypic stability across replicates. At the same time, PHY_2025_1 remains highly relevant as a complementary option due to its stronger accumulation profile. This dual outcome is consistent with the general view that poplars are suitable candidates for phytoremediation, but that their performance is strongly clone-dependent and metal-specific (Li et al., 2024; Kovačević et al., 2025). Therefore, the results of CS#1 provide a robust basis for moving from broad screening to targeted optimisation, where the

selected hybrid(s) can be tested together with amendments, microbial inoculants, or biostimulants under the same contaminated soil conditions.

In CS#3A, the TPH gradient triggered clearly divergent responses between species: in *M. giganteus*, plant height, leaf number and biomass (shoot and root) increased under low and, especially, medium contamination, whereas under the high level most parameters declined markedly; in *C. sativa*, height and shoot biomass decreased progressively as contamination increased, with a strongly stressed phenotype at the high level (senescence, thin stems and leaf abscission). Still, these patterns should be interpreted cautiously because plant growth and physiological traits can be "co-determined" by soil properties that vary among treatments (fertility, N availability, pH, etc.) (Xia et al., 2024). Indeed, soils remained within a narrow alkaline range (pH 8.33–8.90), and the highest pH was consistently recorded in the "medium" treatment (e.g., 8.85 ± 0.04 in *M. giganteus* pots with plants and 8.90 ± 0.01 in *C. sativa* pots with plants), whereas the "low" treatment tended to slightly reduce pH (more acid, more favourable for plant growth). In Miscanthus, the non-linear pattern suggests a "working window" in which moderate stress is not only tolerated but may coincide with efficient physiological functioning before toxic effects dominate and growth and production decline. This reading aligns with independent evidence for hormesis-like responses in M. × giganteus exposed to hydrocarbons, where reinforcement of electron-transport-related processes has been described at lower concentrations (Burdová et al., 2023). At the same time, part of the biomass "peak" under medium TPH could be amplified if that soil also provided better nutritional conditions or a more favourable pH (Xia et al., 2024). In this case, the "medium" treatment coincides with higher pH, supporting the idea that part of the observed performance may be influenced by the soil context in addition to TPH.

Mechanistically, variables such as SPAD, stomatal conductance and biomass production are highly sensitive to nutritional status. Therefore, if N availability or mineralisation differed among soils along the gradient, which could help explain some of the improvements observed in Miscanthus at low–medium levels (Lee et al., 2017), beyond the strictly toxicological component of TPH. This nuance matters because it shifts the focus from "intrinsic tolerance" toward "tolerance conditioned by the edaphic environment". Here, there is a consistent signal in total TN: for both crops, TN was highest in "low" (e.g., *M. giganteus* 0.14–0.16% and *C. sativa* 0.14–0.16%, depending on whether baseline ("with plant") or end-point ("without plant") soil is considered) and declined in "high" to ~0.07–0.09%, with the lowest values typically observed in the end-point (post-harvest) soil.

In hemp, the results point to a narrower tolerance threshold: growth and biomass deterioration intensifies as contamination increases, and at the high level the loss of plant material limited some determinations. This is consistent with recent literature reporting strong biomass reductions in Cannabis grown in soils contaminated with organic pollutants (Gabriele et al., 2023). In addition, heterogeneity in N among treatments could modulate chlorophyll/vigour and partially "mask" contaminant effects at low–medium levels (Yang et al., 2021). In the present dataset, the "high" treatment combines clear plant deterioration with a decline in TN from baseline to the end-point soil (e.g., *C. sativa* baseline: 0.08%; end-point: 0.07%) and a reduction in TC relative to "medium" (e.g., TC in *C. sativa* decreases from 7.59% → 6.12% in baseline soils

across medium → high, and from 7.60% → 4.63% in end-point soils across medium → high), reinforcing that the C and N context accompanies the loss of performance at the most severe level.

The physiological–biochemical profile reinforces the notion of contrasting strategies: in Miscanthus, the antioxidant response appears more modulated and enzyme-specific, whereas in hemp it intensifies up to medium levels, reflecting a growing metabolic effort that does not translate into sustained performance as stress escalates. At this point, nutrition (especially N) can act as a confounding factor because N is known to support photosynthetic functioning and antioxidant activity under stress, altering the magnitude of enzymatic responses (Tian et al., 2022; Wang et al., 2023). Supporting this interpretation, total TN does not remain constant along the CS#3A gradient: it tends to be higher in "low" and lower in "high", so part of the physiological variation could be "superimposed" on changes in total N in the substrate. This is particularly relevant when comparing baseline versus end-point soils, because TN is consistently lower at the end of the experiment in the most stressed conditions.

From a phytomanagement perspective, a practical takeaway is that Miscanthus deployment appears more robust in low–medium ranges (where performance and functionality are maintained), whereas hemp would require less severe conditions or additional support to avoid viability losses at high levels. This aligns with assessments of Miscanthus as a favourable crop for contaminated-land management due to its stabilisation capacity and its contribution to processes linked to the dissipation of persistent organic contaminants (Nsanganwimana et al., 2014). Consistently, the best-performing treatments are not associated with elevated salinity (EC remained low, 0.39–0.53 dS m^{-1}), but rather with relatively subtle shifts in the edaphic environment (pH and the distribution of TC/TN) on which TPH acts.

The rhizosphere comparison (more isolates in Miscanthus as contamination increases, versus a decline under contamination in hemp) further supports stronger operational robustness in Miscanthus: it appears to sustain functional microbial communities under TPH pressure, opening opportunities for biostimulation/bioaugmentation strategies under more demanding scenarios (Nsanganwimana et al., 2014). In hemp, the opposite trend suggests it may be more critical to "shield" the system from the start (inoculation/carriers/amendments) to avoid early functional losses.

CS#3B provides a complementary, field-relevant organic contamination scenario characterised by heterogeneous hydrocarbon pressure and realistic soil constraints. Within this setting, the successful establishment of both *Miscanthus × giganteus* and *Cannabis sativa* supports their use as operational crops for phytomanagement where the primary objective is to maintain vegetative function and generate usable biomass under stress. This outcome aligns with recent evidence showing that *Miscanthus × giganteus* can sustain stress tolerance and phytomanagement capacity in diesel-contaminated soils, while hemp has demonstrated applicability for the remediation of soils impacted by PAHs alongside co-contaminants in controlled trials (Burdová et al., 2023; Gabriele et al., 2023). In practical terms, CS#3B strengthens cross-site confidence that crop establishment is achievable under moderate-to-variable hydrocarbon scenarios, enabling the subsequent WP2 optimisation step to focus on supportive strategies (amendments and

microbial consortia) that improve growth consistency and resilience across spatially variable conditions.

In CS#4, the salinity gradient translated into clear soil changes and plant–soil responses: the final "effective" EC in the end-point soils can differ from the theoretical target of the high treatment. This requires discussing stress based on measured values (final EC) rather than on applied dose alone. Moreover, growth and physiological responses can be as strongly conditioned by soil chemistry (pH, N availability, cation balance) as by EC itself, especially when responses are not strictly monotonic (Xia et al., 2024). This is evident here: the baseline EC reported for the "high" treatment is ~3.59–3.66 dS m^{-1} (depending on species), and in "low" EC increased from baseline to end-point soil (e.g., *M. giganteus* 1.10 → 1.76 dS m^{-1}; *C. sativa* 1.25 → 1.54 dS m^{-1}), consistent with salt accumulation/concentration processes during the assay.

pH is an obvious candidate for such modulation: small changes can alter nutrient availability and, consequently, SPAD, soluble proteins and yield. Thus, if pH differed among soils or evolved differently by treatment, it could explain part of the variability in biomass and physiology even without large EC differences (Xia et al., 2024). Under salinity, this is particularly relevant because osmotic/ionic stress can co-occur with secondary nutritional constraints. In CS#4, pH shows species-dependent responses: in baseline *M. giganteus* soils pH drops in "medium" (8.74 → 8.15) and partially recovers in "high" (8.35), whereas in end-point *C. sativa* soils the drop is stronger (8.04 in control to 6.97 in "medium"), suggesting a scenario where rhizosphere processes and time-dependent soil chemistry jointly shape final conditions.

Phenotypically, Miscanthus appeared healthier at low salinity and showed more evident chlorosis/yellowing at high salinity; responses at medium salinity were more irregular (loss of some individuals and subsequent regrowth), pointing to sensitivity to micro-variations in the soil environment. In hemp, the strongest contrast occurred at high salinity (reduced height, smaller leaves and thinner stems), while medium salinity showed relatively more favourable development, including early flowering signs. This "non-linear" behaviour is consistent with scenarios where salinity stress overlaps with differences in water availability and nutrient status among soils.

To contextualise stress severity, it is useful to note that Miscanthus has been reported to respond differently to moderate versus severe salinity: moderate salinity typically causes limited biomass losses, whereas severe salinity can induce pronounced yield reductions (Stavridou et al., 2020). When effective final EC falls within a moderate range, biomass can remain measurable even as clear stress signals appear (chlorosis, growth changes, biochemical adjustments).

Growth and production data reflect these tendencies: in Miscanthus a monotonic relationship between salinity level and biomass is not always observed, pointing to interactions among osmotic stress, water availability and possible nutrient differences across soils; in hemp, the pattern is more consistent, with clear reductions in height, SPAD, stem diameter and biomass at the high treatment. In both crops, increased soluble proteins at medium–high salinity is compatible with osmotic/metabolic adjustment, but it may also be favoured by higher N availability if fertility differs among soils (Tian et al., 2022). In CS#4 there is indeed a parallel signal in TC/TN: both crops increase TC and TN toward "medium" in baseline soils (e.g., *M. giganteus*

TC 3.68 → 4.88% and TN 0.16 → 0.22%; *C. sativa* TC 2.29 → 4.76% and TN 0.13 → 0.18%), followed by a slight decline in "high", suggesting that soil C/N status tracks the non-linear behaviour around "medium".

The oxidative dimension reveals contrasting strategies: Miscanthus shows a more stable response, whereas hemp displays a stronger and progressive activation of antioxidant defences as salinity increases. This matches evidence in hemp where salinity tolerance is associated with lower oxidative damage and higher antioxidant enzyme activities in roots (Dixit et al., 2022). At the molecular level, the relevance of APX in hemp defence mechanisms under abiotic stresses has also been highlighted, helping interpret the importance of APX within the biochemical panel (Liang et al., 2024).

Because nutrition can strongly modify stress responses, a plausible explanation for part of the variability in CS#4 is the N × salinity interaction: N has been described as alleviating growth inhibition under salinity and improving antioxidant activity and osmoregulation-related traits (Tian et al., 2022). It has also been suggested that moderate N application can improve photosynthesis, antioxidant capacity and osmoregulation under salinity, while excessive N may reduce the benefit (Wang et al., 2023). Within this framing, differences between baseline and end-point TC/TN can be discussed as evidence of how soil C and N pools evolved over the experiment under each salinity level (rather than as a simple "with vs without plant" comparison across separate pots).

Across case studies, D2.2 supports the interpretation that tolerance is stressor- and context-dependent, and should be interpreted as an operational envelope combining establishment/survival, biomass production, physiological stability, and, where relevant, contaminant partitioning. In CS#1 (metals), performance was largely genotype-driven, confirming that poplar hybrid choice is a primary determinant of feasibility under multi-metal stress (Li et al., 2024). For CS#3A and CS#3B (hydrocarbons/mixed contamination), the results indicate contrasting responses between the tested *Miscanthus × giganteus* material and the selected hemp cultivar, but the strength of the evidence differs between both case studies. CS#3A provides the clearest basis for interpreting crop response along a hydrocarbon gradient, whereas CS#3B should be considered a complementary establishment assay under heterogeneous Croatian soil conditions. Therefore, the apparent higher functional tolerance of Miscanthus compared with hemp should be interpreted as a result observed for the selected plant material and tested conditions, not as a definitive ranking of all Miscanthus genotypes or hemp cultivars. In CS#4 (salinity), both crops responded along a clear gradient, reinforcing the need to match crop selection and management strategies to the specific marginality constraints of each target site.

This distinction is important for the next WP2 steps. The current evidence is sufficient to guide targeted optimisation trials, especially those combining the selected crop material with amendments and microbial support, but it is not sufficient to close the genotype/cultivar-selection question for Miscanthus and hemp. Where suitable commercial and field-relevant material is available, additional genotype or cultivar comparison should be considered in subsequent optimisation or validation activities. In parallel, the conclusions from CS#3B should

be used mainly to define follow-up validation needs, rather than to claim that crop tolerance has already been fully demonstrated.

4. CONCLUSIONS AND RECOMMENDATIONS FOR WP2 NEXT STEPS

4.1. CONCLUSIONS FROM THE TOLERANCE ASSAYS

The tolerance assays reported in D2.2 confirm that robust, case-specific crop selection is essential before advancing to optimisation and pilot deployment. Across the stressors assessed in this deliverable, tolerance should be understood as a functional response integrating biomass production, physiological performance and, where relevant, contaminant partitioning, since these factors jointly determine the feasibility of phytomanagement and the suitability of the resulting biomass for subsequent valorisation pathways. The results presented here should therefore be interpreted as a first operational tolerance filter to support subsequent WP2 activities, rather than as the final design of the complete phytomanagement systems.

In CS#1, the screening of poplar hybrids under the conditions of the Pierrelaye soil clearly differentiated plant material in terms of establishment, growth and trace-element-related performance. Several hybrids showed satisfactory adaptation, but PHY_2025_8 emerged as the most balanced candidate for further upscaling due to its strong vigour, good biomass production and stable performance across replicates. PHY_2025_1 should also be retained as a relevant secondary option because of its strong accumulation capacity and overall suitability under the tested conditions. These results provide a solid basis for the next WP2 stages, particularly for nursery multiplication and for optimisation trials evaluating microbial inoculants, amendments or biostimulants in the selected material.

Under hydrocarbon stress in CS#3A and CS#3B, the results indicate that the tested *Miscanthus × giganteus* material showed a broader operational tolerance envelope than the selected hemp cultivar, *C. sativa cv.* Futura 83, under the specific experimental conditions assessed in D2.2. In CS#3A, where a clearer TPH gradient was evaluated, *Miscanthus × giganteus* maintained acceptable performance over a wider range of contamination levels, whereas *C. sativa cv.* Futura 83 showed greater sensitivity to increasing contamination, particularly at the highest stress level, where plant growth and biomass production were more strongly affected. In CS#3B, the evidence should be interpreted more cautiously: the results indicate that both crops were able to establish under the tested Croatian soil batches, but they do not provide definitive proof of broad tolerance across Croatian hydrocarbon-contaminated soils.

Therefore, these findings support *Miscanthus × giganteus* as the priority crop material among those tested for further optimisation under hydrocarbon-affected conditions, while *C. sativa cv.* Futura 83 may remain a candidate under low to moderate contamination where establishment is not compromised. However, this conclusion applies only to the selected plant material tested in D2.2 and should not be interpreted as a definitive ranking of all Miscanthus genotypes or hemp cultivars. Where suitable commercially available and field-relevant material can be accessed, additional genotype or cultivar comparisons should be considered to strengthen the final phytomanagement strategy.

In CS#4, the salinity trial revealed a clear response gradient in both crops, with negative effects becoming more pronounced as salinity increased. However, these results should be interpreted within the tested low-to-moderate salinity range and according to the measured electrical conductivity of the soils, rather than as a general statement on species-level salinity tolerance. As in the hydrocarbon case studies, the tested *Miscanthus × giganteus* material showed a comparatively more stable response than the selected hemp cultivar, *C. sativa cv.* Futura 83, under the specific salinity gradient assessed in D2.2. *Cannabis sativa* cv. Futura 83 was more strongly penalised under the highest salinity conditions, particularly in terms of growth and biomass-related traits.

These findings support *Miscanthus × giganteus* as the priority crop material among those tested for subsequent optimisation under the CS#4 salinity scenario, while *C. sativa cv.* Futura 83 should be considered only for low-to-moderate salinity conditions or where supportive strategies can improve establishment and reduce stress impacts. This conclusion applies only to the selected plant material and experimental conditions tested in D2.2, and should not be interpreted as a definitive ranking of all Miscanthus genotypes or hemp cultivars under salinity.

Taken together, the results of D2.2 provide an evidence-based first prioritisation of crop × stress combinations for subsequent optimisation and validation within pHYBi. The deliverable confirms that crop performance under realistic soil constraints is highly dependent on the specific stress context, and that early tolerance testing is essential for identifying the most promising plant materials and guiding the design of tailored phytomanagement strategies in the following project tasks.

4.2. RECOMMENDATIONS AND WP2 NEXT STEPS

The next WP2 steps will move from tolerance screening to integrated phytomanagement design, combining selected crop material with soil amendments, microbial inoculants and case-specific management practices before and during field validation. The aim is not only to confirm that the selected crops can establish under stress conditions, but also to define complete crop × soil × treatment packages that can support phytoremediation, phytostabilisation and/or stress-resilience improvement depending on the objective of each case study.

Field implementation is already being prepared in parallel with the ongoing optimisation tasks. In Croatia, field experiments with Miscanthus have already started in order to complete a full crop cycle under local conditions and to generate site-specific evidence on establishment, persistence and biomass production. In Spain, the candidate plots in Burgos have already been selected, but full implementation will be launched once the optimisation strategy is sufficiently defined, with field establishment planned for the next suitable spring window. This sequencing is necessary to avoid transferring immature treatments to the field before the most promising combinations of crop material, soil amendments and microbial inoculants have been identified.

The field strategy will therefore follow an adaptive approach. The first field trials will use the crop × stress combinations prioritised in D2.2 and will incorporate the optimisation outputs generated in the subsequent WP2 tasks. If additional optimisation requirements emerge after the first field season, they will be implemented either by adjusting the management of the existing plots or by establishing additional treatment plots, depending on crop performance, plot availability and the nature of the intervention required. This will allow the project to maintain the planned field-validation timeline while preserving enough flexibility to integrate new evidence from greenhouse, microbial and amendment trials.

Microbial inoculants and soil treatments will be selected and applied according to the specific phytomanagement objective of each case study. In metal-contaminated soils, the main focus will be to support plant establishment and biomass production while modulating trace-element mobility, uptake and/or stabilisation depending on the selected poplar hybrid and site conditions. In hydrocarbon-affected soils, microbial and amendment strategies will primarily aim to enhance rhizosphere functioning, contaminant dissipation and crop resilience under organic or mixed contamination. In saline soils, supportive strategies will focus on improving establishment, root performance and physiological resilience under moderate salt stress, potentially in combination with water and salinity management. Therefore, microorganisms will not be applied as a generic treatment, but as part of case-specific phytomanagement packages targeting phytoremediation, phytostabilisation and/or stress-resilience improvement depending on the constraints and objectives of each site.

For CS#1, the immediate recommendation is to continue with the multiplication and optimisation of the most promising poplar hybrids, particularly PHY_2025_8 and PHY_2025_1, while testing whether amendments, microbial inoculants or biostimulants can further improve establishment, biomass production and trace-element management. For CS#3A and CS#3B, the next step is to optimise *Miscanthus × giganteus* as the main crop material under hydrocarbon-affected conditions, while retaining *C. sativa cv.* Futura 83 only for low to moderate contamination scenarios where establishment is not compromised. In CS#3B, additional validation is particularly important because the current results provide preliminary evidence of establishment under the tested Croatian soil batches, but not definitive proof of broad tolerance across all Croatian hydrocarbon-contaminated contexts. For CS#4, the next step is to validate *Miscanthus × giganteus* under field or semi-field salinity conditions and to test whether amendments, microbial support and water/salinity management can improve establishment and biomass production; hemp should only be considered where salinity remains low to moderate or where supportive treatments clearly reduce stress impacts.

Overall, D2.2 provides the starting point for a staged WP2 pathway: first, identifying crop material able to tolerate the target constraints; second, optimising this material with amendments, microorganisms and management practices; and third, validating the resulting phytomanagement packages under field or semi-field conditions. This staged and adaptive approach ensures that the project can progress towards complete phytomanagement systems while incorporating the optimisation results that are still ongoing.

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