BIOSYSMO

Deliverable D3.7

Report on genetically modified poplar lines with effective performances on metal uptake and endophyte colonization

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Executive Summary

Deliverable D3.7 – report on genetically modified poplar lines with effective performances on metal uptake and endophyte colonization is the seventh deliverable of BIOSYSMO WP3 - Development, optimization of biosystems.

D3.7 is the result of Task 3.3 activities - Genetic engineering of poplar for improved soil phytoremediation. Deliverable D3.7 is the continuation of the intermediate report D3.3. This document compiles data on relevant poplar genotypes with remediation capabilities, and the development of poplar-endophyte colonization protocols.

Deliverable D3.7 describes, firstly, the methodologies to perform metal tolerance assays and quantification of metal accumulation in poplar, the methodologies to perform RNA-seq studies of heavy metal resistant poplars to identify candidate genes related to phytoremediation capabilities, and the methodologies and protocols to inoculate poplar with endophytes.

Secondly, the report describes the results derived from T3.3 activities. The work developed in task T3.3 allowed the identification of two poplar tolerant genotypes for Zn and Cd excess. It also allowed the identification of a metal hypersensitive poplar genotype. Moreover, RNA-seq analysis performed in T3.3 allowed the identification of new candidate genes and the consequent genetic modifications in poplar to obtain new potentially metal resistant genetic engineered poplar genotypes. RNA-seq analysis also led us to investigate the biostimulant capacity of polyamines to alleviate heavy metal stress in poplar and its implications for enhancing poplar's metal resistance, tolerance, and accumulation performance.

Finally, because of Task 3.3 activities, we have developed several endophyte inoculation protocols (D3.5) for poplar relevant microbes isolated in the WP1 activities and that are being used for Task 4.2 activities (D4.2).

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Table of Abbreviations

Abbreviation	Definition
WT	Wild type
OX	Overexpressing
KO	Knock-out
LD	Long Day
DEG	Differential Expressed Gene
TE	Trace Element
AM	Arbuscular Mycorrhiza
EM	Ectomycorrhiza
GO	Gene Ontology
MS	Murashige and Skoog
UPM	Universidad Politécnica de Madrid
UMLP	Université Marie et Louis Pasteur

1 Introduction

Deliverable D3.7 describes the results of the work carried out by UPM and UMLP from WP3 Task 3.3, which focuses on the genetic engineering of poplar for improved soil phytoremediation. Our main objectives were, first, to develop poplar genotypes (UPM) that are tolerant to and can accumulate Zn and Cd, which are the main pollutants of sites 1 and 10, as identified in WP1 activities. Second, to develop endophyte inoculation protocols for poplar for Task 4.2 activities.

Poplar (*Populus* spp.) trees are well-suited for phytoremediation due to their fast growth rate and deep root systems. They can potentially absorb a significant amount of polluted water in water-rich areas but can still thrive in water-limited regions. Poplars are also considered a model tree species in forest genetics, genomics, and physiology due to their many advantages as a model system (Ciadamidaro et al., 2023). These include rapid growth, prolific sexual reproduction, ease of cloning, small and sequenced genomes, genomic resources, facile transgenesis, and tight coupling between physiological traits and biomass productivity (Cronk, 2005).

Poplar plantations have been successfully used for heavy metal phytoremediation of polluted lands unsuitable for agriculture (Giachetti and Sebastiani, 2006). Meta-analysis of the reported investigation indicates that poplar can accumulate significant amounts of Cd, Cr, Cu, Pb, and Zn in all plant parts. However, the accumulation of Ni is only moderate, and Mn accumulation is limited, which is dependent on soil pH and exposure time (Tőzsér et al, 2023). The mechanisms regulating plant-Zn and Cd interactions have been extensively studied in annual species with hyperaccumulating phenotypes (Unterbrunner et al, 2007; Takenaka et al, 2009; Zheleznova et al, 2017). However, these mechanisms are relatively unexplored in perennial species such as Poplar. The overexpression of poplar genes related to zinc accumulation from *Arabidopsis* into Poplar, such as HEAVY METAL TRANSPORTER (HMA4) and PHYTOCHELATIN (PCS1), nearly doubled the Zn accumulation in leaf tissues (Adams et al, 2011). This suggests that there may be a more significant pathway in perennials that should be investigated to engineer heavy metal hyperaccumulating poplars. It is also known that uptake and transport of both Zn and Cd in plants are strictly bound since Cd uses typical transporters implicated in Zn translocation such as HMA2 (Takahashi et al, 2012).

The phytohormone ethylene mediates heavy metal stress (Keunen et al, 2016; Thao et al, 2015). Recent work shows that poplar treated with zinc excess triggers a hypoxic response and activates a battery of ethylene-related pathways. It is hypothesized that a component of the hypoxic response pathway might play a role in Zn tolerance and increase Zn accumulation capacity (Laura Dalle Carbonare et al, 2019).

UPM has explored the Zn and Cd tolerance and accumulation capacity caused by poplar ethylene response transcription factors associated with hypoxic response. We engineered overexpression and knock-out plants to perform functional studies under Zn and Cd excess. We developed Zn and Cd tolerance assays of trees under a controlled environment. Tree physiology and metal accumulation were monitored, identifying genotypes that show high tolerance and accumulation capacity under Zn and Cd excess. Comparative transcriptomic analyses have been conducted to unravel the molecular framework to improve poplar heavy metal hyperaccumulating phenotype.

However, the success of phytoremediation strategies depends largely on the ability of plants to establish in stressed and degraded environments, as well as on their interactions with soil microorganisms. Microorganisms are ubiquitous across environments and play critical roles within ecosystems and they could create mutualistic associations with host organisms (Ciadamidaro et al.,

2022; Chalot and Puschenreiter, 2021). Salicaceae trees, particularly poplar, have been widely studied for their ability to create diverse microbial associations, including rhizospheric and endophytic interactions with microorganisms (Beckers et al., 2017; Ciadamidaro et al., 2023). In our recent studies, we underlined the capacities of ectomycorrhiza (EM) and arbuscular mycorrhiza (AM) inoculation on poplar cultivars “Skado”, growing at a multi-contaminated site, in enhancing biomass production (Ciadamidaro et al., 2017) and reducing trace element (TE) translocation in leaves (Phanthavongsa et al., 2017). In the past few years, special attention has been given to the use of inoculation of plants with microbial consortia, responsible for improving plant TE uptake and accumulation in phytoextraction techniques or limiting TE translocation from roots to shoots in phytostabilisation processes.

Within Task 3.3 activities, Zn and Cd tolerant and accumulative engineered (CRISPR-Cas9-mediated knock out plants and gene overexpressing plants) poplar lines have been described. Moreover, new genes have been selected, and consequent modifications have been performed in poplar to generate new Zn and Cd tolerant and accumulative poplar genotypes (UPM).

As a major innovation, the best-performing poplar genotypes have been bio-dynamized by selected fungal strains and microbial consortia (D3.5) and tested for their capacities to increase the export of metals to the aboveground tissues and to improve metal sequestration within the rhizosphere. Overall, this will lead to a bioaugmented, genetically engineered-based phytoremediation strategy that has never been applied to poplar (D4.2).

Selected poplar genotypes and microbial consortia are being used for the soil phytoremediation strategy that is part of Task 4.2 activities, some of which have already been reported in D4.2.

2 Materials and methods

2.1 Plant material and growth conditions

The hybrid poplar *Populus tremula* × *alba* INRA clone 717-1B4 was used as the plant model.

Poplar plantlets grown in vitro for 4–6 weeks were sectioned into 2–3 cm cuttings, each containing two axillary meristems. Cuttings were cultured in vitro for four weeks in 200 mL vessels containing 50 mL of Murashige and Skoog (MS) medium 1B (pH 5.7), solidified with 0.7% (w/v) plant agar and supplemented with 2% (w/v) sucrose, 0.5 mg/L indole-3-acetic acid (IAA), and 0.5 mg/L indole-3-butyric acid (IBA). Cultures were maintained at 22 °C under long-day photoperiod conditions (16 h light / 8 h dark), with a light intensity of 140 µmol m^{-2} s^{-1} and 70% relative humidity.

2.2 Metal tolerance assays

Four to six weeks old poplar *in vitro* plantlets are transferred to 1.7 L pots containing perlite and let them grow for 8 weeks in a growth chamber at 22 °C under long-day photoperiod conditions (16 h light / 8 h dark), with a light intensity of 140 µmol m^{-2} s^{-1} and 70% relative humidity until they completely acclimate to perlite culture and reach a full growth state. Plants are fertilized every two weeks with 0.5 g/L of NPK fertilizer with micro-nutrients 20-20-20 (Peters Professional).

$ZnCl_2$ anhydrous 99.9% (Fisher) and $CdCl_2$ anhydrous 99.9% (Fisher) salts were used for metal tolerance testing. Metal tolerance assays were conducted by treating the plants with 300 mL of 600 µM $ZnCl_2$ aqueous solution, 300 mL of 1200 µM $ZnCl_2$ aqueous solution, 300 mL of 300 µM of $CdCl_2$ aqueous solution or 300 mL of 500 µM of $CdCl_2$ aqueous solution, twice a week during the experiment. These concentrations were selected due to their best performance in the screening to determine the most effective concentration for the assays, already reported in D3.3. Control plants, on the other hand, were only treated with water. It's worth noting that the experiment ended once the wild-type poplar plants died due to metal toxicity. This range of concentrations was chosen based on previous similar studies from bibliography (Di Baccio et al, 2003; He et al, 2013; Di Baccio et al, 2014).

For *in vitro* assays, square plates containing 100 mL of Murashige and Skoog (MS) medium 1B (pH 5.7), solidified with 0.7% (w/v) plant agar and supplemented with 2% (w/v) sucrose is used for control conditions. The same medium supplemented with 600 µM $ZnCl_2$ aqueous solution is used for metal stress condition. The same medium supplemented with 1200 µM $ZnCl_2$ and 0.5 mM Spermidine is also used for metal tolerance *in vitro* assays.

2.2.1 Plant physiology monitoring

Plant's physiology parameters of stomata conductance and photosystem II activity were measured using a CIRAS-3 Portable Photosynthesis System (PP Systems). After the assay, the fresh weight of roots and stem was measured using a weighing scale.

2.2.2 Metal content in plant tissues determination

Metal concentrations in plant tissues were measured from the dried plant material after milling and mineralization. Total metal concentrations of leaves and roots were determined in wet-digested samples in HNO_3 and H_2O_2. After filtration to 1 µm was performed, metal concentrations were determined using the ICP-AES analysis (Radial ICAP 6500 Model, Thermo Fischer Scientific, Courtaboeuf, France) (Assad et al., 2017). All samples were analysed in triplicate using the certified reference materials Oriental Basma tobacco leaves (INCT-OBTL-5, LGC Promochem, Molsheim, France). Metal content determination is performed by UMLP.

2.3 RNA-seq studies

2.3.1 Sample preparation

For RNA-seq analysis, the plants were treated with a solution containing 600 µM $ZnCl_2$ once a week for four weeks before sample collection. The samples were collected by cutting the leaves and immediately freezing them in liquid nitrogen. RNA extraction was performed on frozen ground leaf material by adding cetyltrimethylammonium bromide (CTAB) extraction buffer with 2% β-mercaptoethanol at 65°C for 5 min, followed by two washes with chloroform. RNA was precipitated by adding 7.5 M $LiCl_2$: 50 mM EDTA and incubated overnight at 4°C. RNA was collected by centrifuging at 10,000 RPM for 20 min at 4ºC and then purified according to the manufacturer's instructions of NZY RNA purification kit (NZYTech, Lisboa, Portugal) and eluted in water. The RNA was then sent to Macrogen Inc. (Seoul, Republic of Korea) for Illumina TruSeq stranded mRNA library generation and Illumina 150bp PE sequencing.

2.3.2 Quality control

The raw reads of FASTQ files obtained after sequencing undergo quality assessment and filtering using the FastQC package. This step allows us to identify adapter content and low-quality reads. These issues are first fixed by trimming the Illumina adapter sequences using the trimmomatic package (Bolger, A. M et al., 2017). Second, the reads are also filtered based on their quality score using the leading, trailing, and sliding window parameters with standard values defined by the trimmomatic tool.

2.3.3 Alignment

To map our reads against a reference genome, the STAR (Spliced Transcripts Alignment to a Reference) package (Dobin, A. et al., 2013) is used. The genome FASTA and GTF files of our *Populus alba* reference genome can be acquired from the phytozome website (https://phytozome-next.jgi.doe.gov). *Populus alba* genome and the trimmed reads from the quality control step are used as input to the STAR aligner to map the reads to the genome. The alignment results are saved in BAM file format. As a final step, the generated BAM files are used as input to the featureCounts package (Liao et al, 2014) that counts mapped reads for each gene.

2.3.4 Gene expression analysis

To discover quantitative changes in expression levels, differential gene expression analysis is performed using the Bioconductor EdgeR package (Robinson et al, 2010). The obtained counts of the mapped reads per gene are used as an input to EdgeR. For further analysis and data mining, differentially expressed genes are filtered based on the adjusted p-value, where all the genes with an adjusted p-value higher than 0.01 are discarded.

2.3.5 Gene Ontology analysis

Gene ontology analysis was performed using the online tool g:Profiler g:GOSt (Kolberg L. et al., 2023).

2.4 Production of microbial inoculum and poplar inoculation protocol

Several inoculation methods were tested for poplar. Finally, root inoculation was selected for individual fungal inoculation for Task 4.2 activities (reported in D4.2). Direct spores' inoculation will be the chosen method for fungal consortia inoculation within Task 4.2 context.

2.4.1 *In vitro* inoculation

Poplar plantlets were grown *in vitro* for four weeks as described above. Plants were then placed in square plates containing 100 mL of Murashige and Skoog (MS) medium 1B (pH 5.7), solidified with 0.7% (w/v) plant agar and supplemented with 2% (w/v) sucrose; 100 mL of ½ Murashige and Skoog (MS) medium 1B (pH 5.7), solidified with 0.7% (w/v) plant agar and supplemented with 2% (w/v) sucrose and 100 mL Murashige and Skoog (MS) medium 1B (pH 5.7), solidified with 0.7% (w/v) plant agar.

Fungal inoculum was grown in Maltose Extract Agar media plates for two weeks at 25ºC in dark conditions. Plant inoculation was performed by coculture taking a 1 cm^2 agar plug from the fungal plates and transferred to the plates where plants were placed. Plants and fungi were grown for 10 days at 22 °C under long-day photoperiod conditions (16 h light / 8 h dark), with a light intensity of 140 µmol m^{-2} s^{-1} and 70% relative humidity.

Inoculated plants were then transferred to pots containing peat to evaluate inoculation performance in a growth chamber at 22 °C under long-day photoperiod conditions (16 h light / 8 h dark), with a light intensity of 140 µmol m^{-2} s^{-1} and 70% relative humidity.

This protocol was generated by UPM in the scope of Biosysmo.

2.4.2 Root inoculation

Fungal inoculum was grown on SDA medium (Sabouraud Dextrose Agar, Sigma) in the dark at 25°C for 15 days. The spores were harvested by scraping the mycelium in the Petri dish and then transferred into 10 mL of sterile H_2O containing 0.1% Tween80. Spores were then filter through 70 µM sterile filter and concentration was measured using a hematocytometer to adjust the solution to 1.10^8 spores/mL.

Poplar plantlets were grown *in vitro* for four weeks as described above. Root tips were cut and then dipped in 10 mL of the spore solution for 2h under gentle agitation in a 50 mL Falcon tube.

Inoculated plants were then transferred to site 10 soil and grown in a growth chamber at 22 °C under long-day photoperiod conditions (16 h light / 8 h dark), with a light intensity of 140 µmol m^{-2} s^{-1} and 70% relative humidity.

This protocol was generated by UMLP in the scope of Biosysmo, as described in D3.5.

2.4.3 Direct soil inoculation with spore solution

Poplar plantlets were grown *in vitro* for four weeks as described above and then planted into pots containing site 10 soil. Fungal inoculum was prepared as described in root inoculation section and then each pot was inoculated with the same volume of fungal solutions by pouring spore solutions on rhizospheric soil at the base of the plantlets, at a final spore concentration of 10^4 spores/g of soil.

This protocol was generated by UMLP in the scope of Biosysmo, as described in D3.1

2.4.4 Reisolation test

To prove the presence of the fungi in plant tissues after plant growth in pots, a reisolation test from root and leaves is performed. Start by harvesting leaf, stem or root tissues and thoroughly wash with tap water. Then surface-sterilize plant tissues in successive baths: 3 min in 1% sodium hypochlorite, 2 min in 70% EtOH, and 3 consecutive baths of sterile distilled water. Let dry under sterile conditions and plate 100 µL of the last rinse water on Sabouraud Dextrose Agar medium (SDA) to confirm that epiphytic microorganisms have been successfully eliminated or print the leaf on SDA. Cut small parts of plant tissues with a sterile scalpel and plate the tissues on SDA. Then incubate in the dark at 25°C until fungal growth.

3 Results

3.1 Searching for metal-tolerant poplar genotypes by metal tolerance assays

The selection of the most effective stress-inducing treatment of $ZnCl_2$ and $CdCl_2$ together with the results of $ZnCl_2$ tolerance assays in *GENE1* poplar genotypes were already reported in D3.3, however, they will be included here as well to provide better contextualization and support a more comprehensive understanding.

3.1.1 Metal-tolerant and accumulative *GENE1* poplar genotype identification

After choosing the most efficient concentration for metal tolerance experiments, we tested the effects of overexpressing and knocking out, by CRISPR-Cas9, *GENE1* in plants. *GENE1* is a transcription factor that responds to ethylene, a hormone induced by heavy metal stress. We expected that poplar genotypes with engineered *GENE1* have the potential to tolerate and accumulate metals successfully.

Wild type (WT) poplar was used as a genotype control in the metal tolerance experiment. Two independent knock-out (CRISPR-Cas9) lines and three independent overexpressing (OX) lines were also used. The treatment group of plants received a weekly dose of 300 mL of a 1200 µM $ZnCl_2$ aqueous

solution, while the control group received only water. As shown in Figure 1A-B, after eight weeks of treatment, the WT plant and *GENE1* knock-out lines #12 and #16 were completely dead, whereas *GENE1* overexpressing lines #3, #6, and #11 remained alive, as evidenced by comparison with the control conditions.

We went beyond visual phenotyping and decided to monitor some physiological parameters of plants. Among the various physiological parameters measured, stomata conductance data is shown (Fig. 1C). Under treatment conditions, neither WT nor *gene1* KO lines showed any stomata activity, as the plants were dead when phenotyping was performed. In contrast, *GENE1* OX lines showed stomata activity, indicating they were alive. The metal content in leaves was also measured using ICP-AES analysis. As shown in Figure 1D, the *GENE1* OX lines #6 and #11 significantly accumulate more Zn^{2+} in their leaves compared to the WT poplar, especially line #11, which is the one that was chosen for transcriptomic analysis.

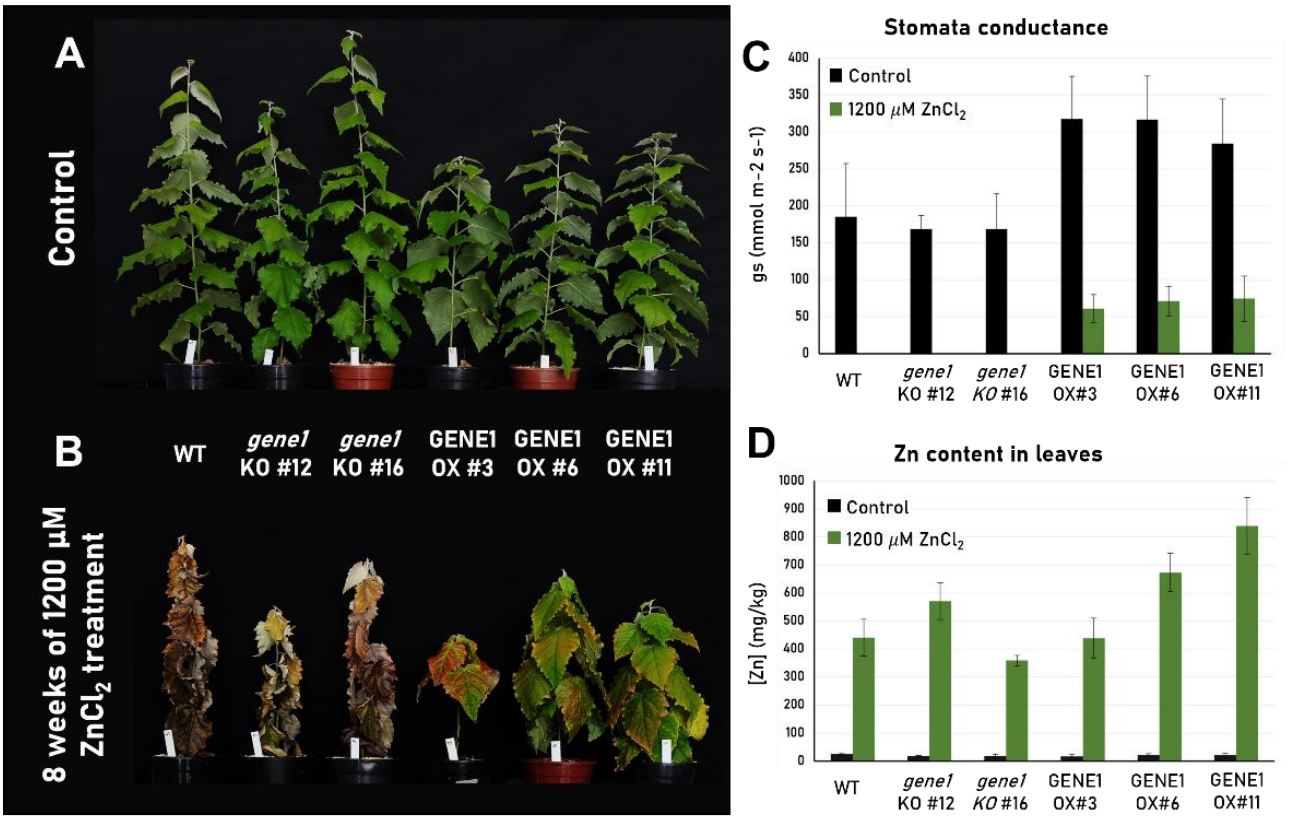

Figure 1. $ZnCl_2$ tolerance assay of *GENE1* poplar genotypes. Images of comparison between the different poplar genotypes after eight weeks of control conditions (A) and plants after eight weeks of weekly treatment with 300 mL of 1200 µM $ZnCl_2$ (B). Stomata conductance measured in mmol $m^{-2}s^{-1}$ (C) and metal content in leaves measured in mg/kg (D) of the different poplar genotypes after eight weeks in control conditions and after eight weeks of weekly treatment with 300 mL of 1200 µM $ZnCl_2$. N=6 for each genotype and treatment.

A similar experiment design was carried out to test *GENE1* function in response to $CdCl_2$ by exposing the WT, KO and OX plants to 300 mL of 300 µM $CdCl_2$ aqueous solution for five weeks. As shown in Figure 2A-B, after five weeks of treatment, the WT plant and *GENE1* knock-out lines #12 and #16 showed more sever tissue damage due to Cd toxicity respect to *GENE1* overexpressing lines #3, #6,

and #11, as evidenced by comparison with the control conditions. Unlike in *GENE1* $ZnCl_2$ toxicity test, *GENE1* genotype exposure to 300 µM $CdCl_2$ did not show a drastic phenotype of plant death in WT and KO mutants. However, an interesting development phenotype is appreciated in Figure 2C, that shows a close-up image of the shoot apex of the different genotypes. It can be appreciated that WT plants, *gene1* KO #12 and *gene1* KO #16 developed resistance buds in response to 300 µM $CdCl_2$ 10 weeks treatment, which is translated into a growth cessation, unlike *GENE1* OX #3, *GENE1* OX #6 and *GENE1* OX #11, where the resistance bud is not formed, indicating the plants are resisting to the toxic in the media and can continuing growing, although a slightly growth inhibition process is observed. After that, all poplar genotypes were forced to recovery, being only irrigated with water to remove Cd from the medium to alleviate the toxic conditions. After six weeks of recovery conditions (Fig. 2D), we saw that WT, *gene1* KO #12 and *gene1* KO #16 could not restore growth, since resistance buds were still formed, indicating the shoot apex meristem was dead. In contrast, *GENE1* OX #3, *GENE1* OX #6 and *GENE1* OX #11 could restore growth, reversing the growth inhibition process, which is appreciated in the new tissues' formation in these genotypes.

Given these results, we could associate *GENE1* OX genotype with a resistance and tolerance genotype to $CdCl_2$ excess in the media.

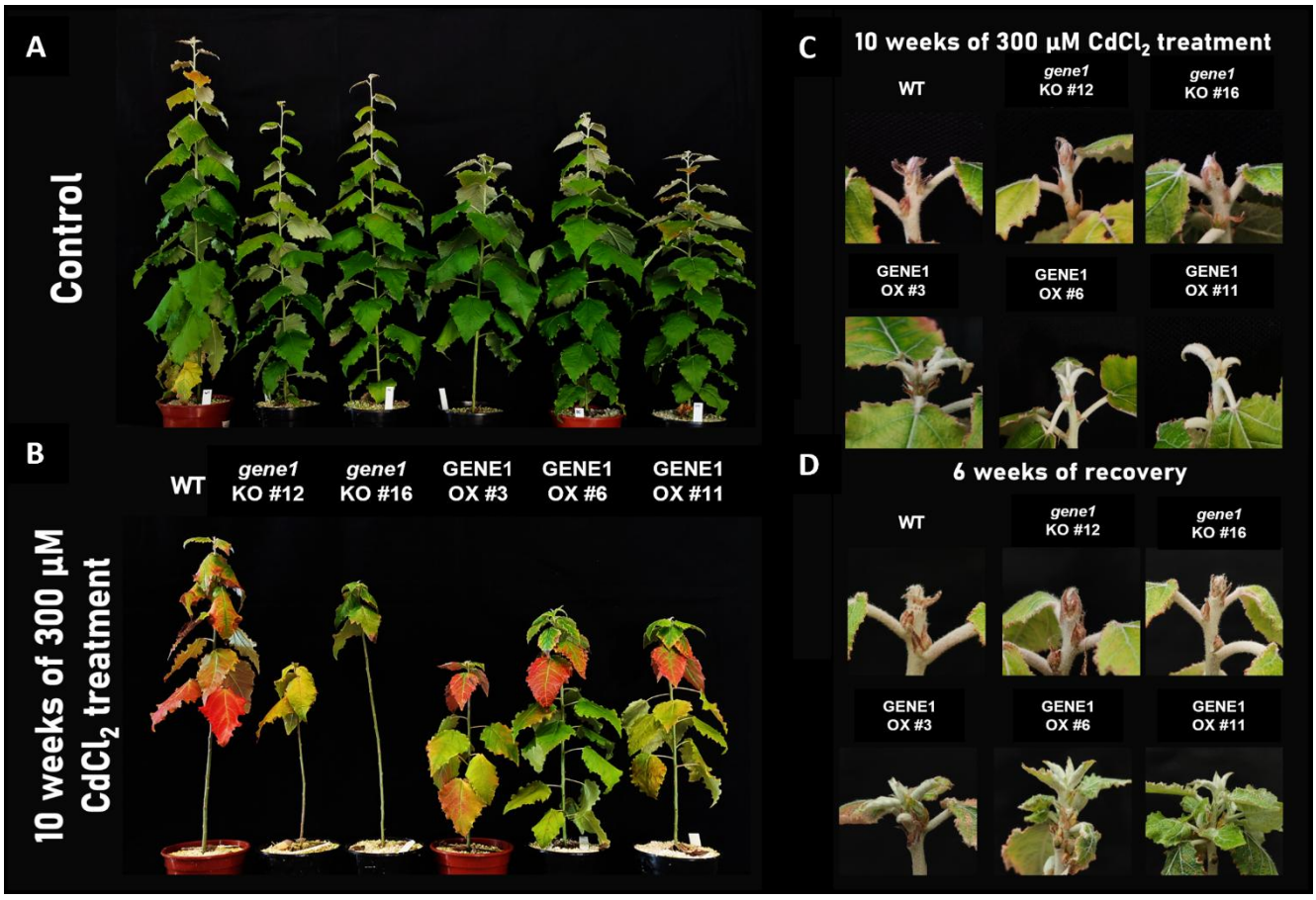

Figure 2. $CdCl_2$ tolerance assay and recovery of *GENE1* poplar genotypes. Images of comparison between the different poplar genotypes after ten weeks of control conditions (A) and plants after ten weeks of weekly treatment with 300 mL of 300 µM $CdCl_2$ (B). Zoomed images of shoot apex of the different genotypes after ten weeks of weekly treatment with 300 mL of 300 µM $CdCl_2$ (C). Zoomed images of shoot apex of the different genotypes after six weeks of recovery in water (D). N=6 for each genotype and treatment

To specifically evaluate resistance while avoiding the growth cessation–resistance trade-off observed with long-term treatment at 300 µM $CdCl_2$, we conducted a new assay using a higher concentration of 500 µM $CdCl_2$. As shown in Figure 3A-B, after five weeks of treatment, the WT plant and *gene1* knock-out lines #12 and #16 were several damaged, whereas *GENE1* overexpressing lines #3, #6, and #11 remained alive, as evidenced by comparison with the control conditions. Under treatment conditions, WT and *gene1* KO lines showed very low stomata activity due to several leaf damage. In contrast, *GENE1* OX lines showed acceptable levels of stomata activity, indicating a higher tolerance of these plants to the Cd in the media. The metal content in leaves was also measured using ICP-AES analysis. As shown in Figure 3D, the *GENE1* OX lines #6 and #11 can accumulate more Cd in their leaves (Fig. 3D) compared to the WT poplar.

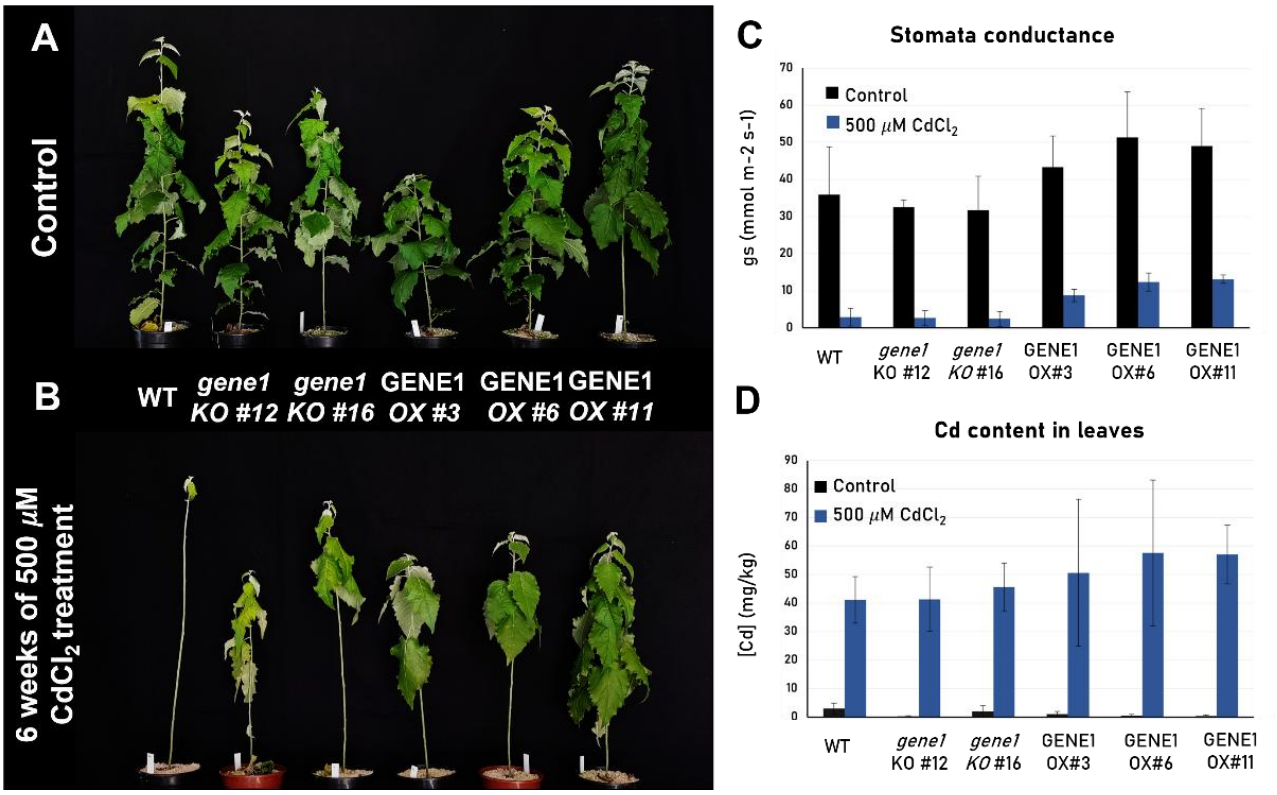

Figure 3. $CdCl_2$ tolerance assay of *GENE1* poplar genotypes. Images of comparison between the different poplar genotypes after six weeks of control conditions (A) and plants after six weeks of weekly treatment with 300 mL of 500 µM $CdCl_2$ (B). Stomata conductance measured in mmol $m^{-2}s^{-1}$ (C) and metal content in leaves measured in mg/kg (D) of the different poplar genotypes after eight weeks in control conditions and after eight weeks of weekly treatment with 300 mL of 500 µM $CdCl_2$. N=6 for each genotype and treatment.

Based on the results, we have found that *GENE1* OX can tolerate an excess of Zn and Cd and specifically accumulating it in leaves. This suggests that GENE1 OX plants could serve as a promising tool for phytoextraction applications.

WT plants, *gene1* KO #16 and *GENE1* OX #11 genotypes were chosen for subsequent transcriptomic analysis, which will be reported in the following sections, and for Task 4.2 activities, some of them already reported in D4.2.

3.1.2 Metal-tolerant and metal-hypersensitive *GENE2* poplar genotypes identification

We have also identified another metal tolerant poplar genotype, *GENE2* OX, and one metal hypersensitive poplar genotype, *gene2* KO. *GENE2* is a gene that encodes for a transcription factor related to the ethylene response pathway, therefore to the heavy metal stress signaling pathway.

To test *GENE2* function, we exposed WT poplar plants, two *GENE2* OX independent lines and two *gene2* KO independent lines to 300 mL of 500 µM $CdCl_2$ aqueous solution for five and six weeks. As shown in Figure 4A-B, the *gene2* KO lines #6 and #15 presented several leaf damage after five weeks of treatment, unlike the WT plant and *GENE2* OX lines #1 and #2, which maintained the plant vigour. One week later, after six weeks of treatment, WT plants started to die, *gene2* KO lines #6 and #15 were completely dead, but *GENE2* OX lines #1 and #2 remained alive (Fig. 4C).

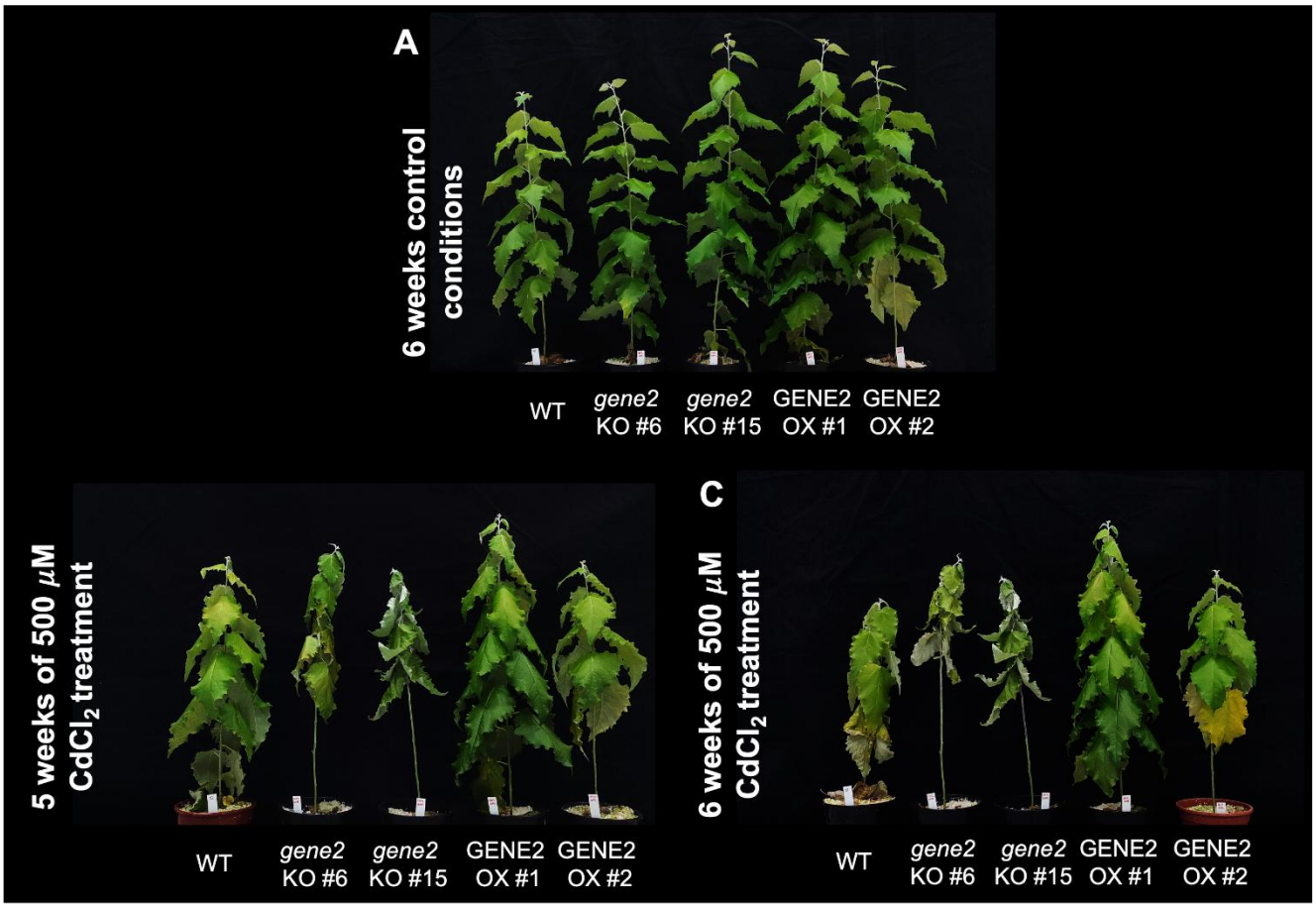

Figure 4. $CdCl_2$ tolerance assay of *GENE2* poplar genotypes. Image shows a visual phenotype comparison between WT, *gene1* KO #6, *gene2* KO #15, *GENE2* OX #1 and *GENE2* OX #2 poplar genotypes after six weeks of control conditions (A), after five weeks of weekly treatment with 300 mL of 500 µM $CdCl_2$ (B) and after six weeks of weekly treatment with 300 mL of 500 µM $CdCl_2$ (C) N=6 for each genotype and treatment.

Loss of *gene2* function in poplar triggers a hypersensitive response to elevated Cd levels in the medium. In contrast, *GENE2* overexpression enhances poplar tolerance and resistance to Cd exposure. Collectively, these findings underscore the pivotal role of *GENE2* in mediating responses to Cd stress and highlight its potential for developing Cd-tolerant poplar varieties.

We also tested *GENE2* function in response to Zn by exposing WT plants and *gene2* KO #6 genotype to 300 mL of 600 µM $ZnCl_2$ aqueous solution for eight weeks. As shown in Figure 5, WT poplar plants after eight weeks of $ZnCl_2$ treatment exhibit slight signs of metal toxicity, resulting in live plants with lower vigour and biomass compared to WT in control conditions. On the other hand, *gene2 KO* #6 plants after eight weeks of $ZnCl_2$ treatment show severe signs of metal toxicity, translated into dead plants with lower vigour and biomass and loss of leaves compared to *gene2* KO #6 plants after eight weeks of control conditions. Comparing WT plants and *gene2* KO #6 plants after eight weeks of $ZnCl_2$ treatment, *gene2* KO #6 plants shows deeper toxicity symptoms compared to WT plants in the same conditions.

Figure 5. $ZnCl_2$ tolerance assay of *gene2* KO #6 poplar genotype. Image shows a visual phenotype comparison between WT poplar in control conditions, WT poplar treated with 600 µM $ZnCl_2$, *gene2* KO #6 in control conditions and *gene2* KO #6 poplar plants treated with 600 µM $ZnCl_2$. Treatments were performed weekly for eight weeks. N=6 for each genotype and treatment.

These results suggest that loss of function of *GENE2* in poplar triggers a hypersensitive response to a high excess of Zn in the medium, making these plants more susceptible to the stress caused by the heavy metal.

We also tested the tolerance capacity of *GENE2* OX #1 poplar genotype to Zn by exposing WT and *GENE2* OX #1 plants to 600 µM $ZnCl_2$ in *in vitro* conditions. Poplar plantlets were grown *in vitro* as described in Materials and Methods (Section 2.2). After four weeks, poplar plantlets were transferred to square plates in control and in 600 µM $ZnCl_2$ treatment conditions. Results after 10 days of treatment are shown in Figure 6.

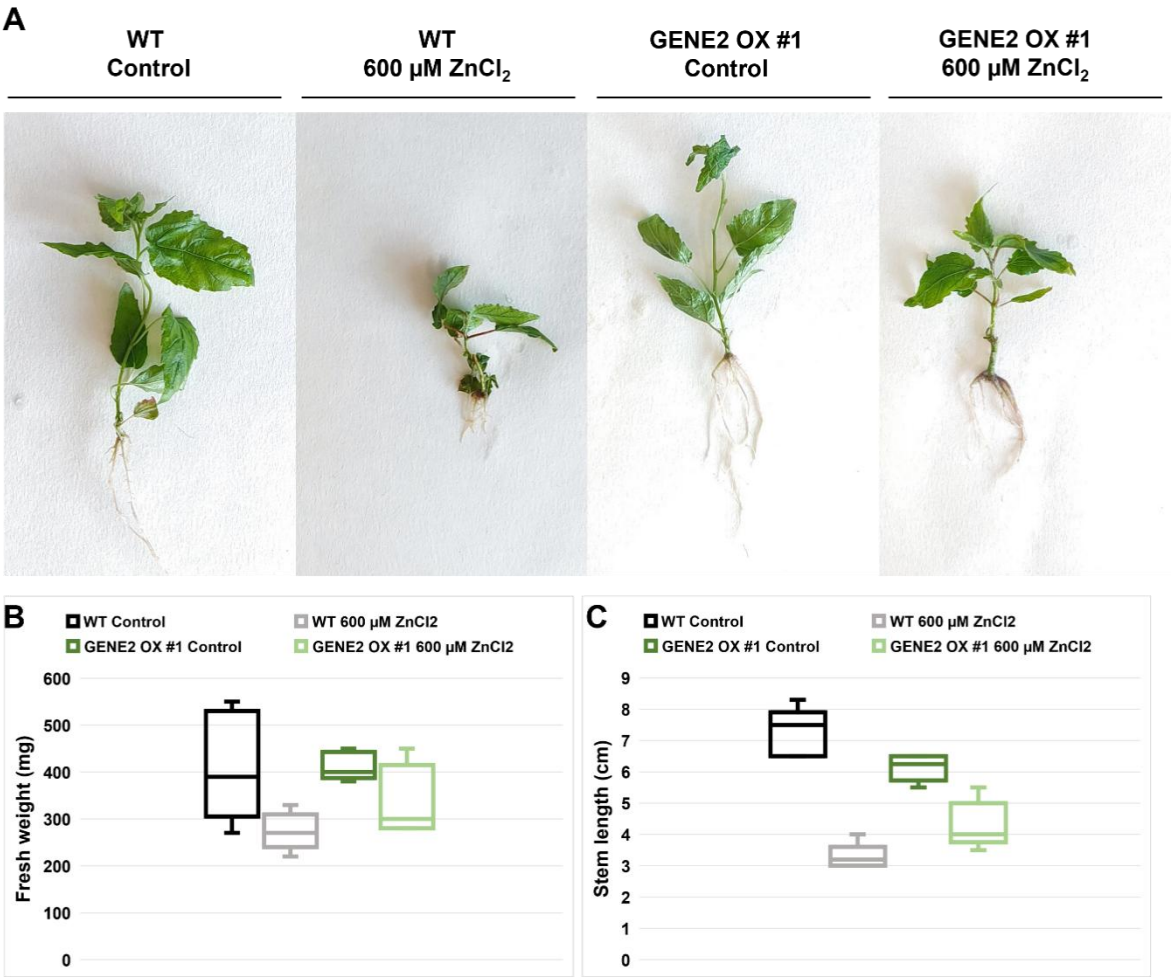

Figure 5. $ZnCl_2$ *in vitro* tolerance assay of *GENE2* OX #1 poplar genotype. Image shows a visual phenotype comparison after 10 days growing *in vitro* between WT poplar in control conditions, WT poplar treated with 600 µM $ZnCl_2$, *GENE2* OX #1 in control conditions and *GENE2* OX #1 poplar plants treated with 600 µM $ZnCl_2$ (A). N=6 for each genotype and treatment. Fresh weight expressed in mg (B) and stem length expressed in cm (C) of the different genotypes and conditions. N=6 for each genotype and treatment.

Similar plant vigour can be observed in WT and *GENE2* OX #1 in control conditions. In contrast, a lower biomass is appreciated in WT than in *GENE2* OX #1 under 600 µM $ZnCl_2$ treatment conditions, suggesting a better tolerance of *GENE2* OX #1 genotype to the stress caused by the metal stress (Fig. 6A). Less differences are also appreciated in fresh weight (Fig. 6B) and stem length (Fig. 6C) in *GENE1* OX #1 under control conditions compared to *GENE1* OX #1 under Zn treatment conditions than in WT under control conditions compared to WT under Zn treatment conditions. Also, stem length and fresh weight are higher in *GENE2* OX #1 compared to WT under Zn treatment conditions.

These results suggest that overexpressing *GENE2* in poplar leads to a higher tolerance to the high excess of Zn in the medium, making these plants more resistant to the stress caused by the heavy metal.

Putting all these results together, we can say that we've identified a second Zn and Cd tolerant and resistant genotype: *GENE2* OX. We have also identified a first genotype that is hypersensitive to stress caused by high concentrations of Zn and Cd in the medium: *gene2* KO.

3.2 Transcriptomic analysis of *GENE1* poplar genotypes

Transcriptomic analysis of *GENE1* poplar genotypes in response to high Zn excess was already reported in D3.3. Thus, we will report directly the new transcriptomic analysis of *GENE1* poplar genotype in response to high Cd excess.

3.2.1 RNA-seq of *GENE1* genotypes in response to $CdCl_2$

To further analyse and exploit our promising phytoextractor and hyperaccumulator *GENE1* poplar genotype, we performed an RNA-seq experiment. With this approach, we aim to better understand the molecular mechanism implicated in the capacity of *GENE1* poplar genotype to tolerate an excess of Cd and accumulate this element. Also, we aim to use RNA-seq analysis to find new and better genetic targets for further genetic modifications that can optimize tolerance and accumulation of metals in poplar.

The experimental design for the RNA-seq studies was similar to the one used for $CdCl_2$ tolerance assays (Fig. 3). We used WT, *GENE1* OX #11 and *GENE1* KO #16 lines based on their performance in the tolerance assay. For each treatment condition (control and 500 µM $CdCl_2$), six plants per genotype were used, resulting in a total of 36 plants for the experiment.

After four weeks of weekly treatment with 300 mL of 500 µM $CdCl_2$ in the treated plant group, plants developed a visual metal stress phenotype, so we collected the leaf samples. We collected 3 replicates of every treatment to ensure robust RNA-seq results. Each replicate consisted of one leaf pooled from two plants grown under the same conditions. In total, we obtained three independent samples for each genotype—WT, *GENE1* OX #11, and *gene1* KO #16—under both control and 500 µM $CdCl_2$ treatments.

RNA extraction was performed as described in the methods (Section 2.3). RNA samples were sent to Macrogen Inc. (Seoul, Republic of Korea) for Illumina TruSeq stranded mRNA library generation and Illumina 150bp PE sequencing.

Sequencing, quality control and alignment were performed as described in methods. In Table 1 we show the first raw data from aligning the sequences to *Populus alba* reference genome (*Populus tremula* x *Populus alba* HAP2 v5.1, https://phytozome-next.jgi.doe.gov).

Table 1. Mapped data stats from RNA-seq analysis. Sample column refers to individual type of genotype, treatment and number of replicates used for sequencing. Processed reads refer to the number of cleaned reads after trimming. Mapped reads column refers to the total number of reads mapped to reference genome. The unmapped reads column refers to the total number of reads that failed to align to the reference genome.

Sample	Processed reads	Mapped reads	Unmapped reads
WT Control 1	45,242,380	41,421,343 (91.55%)	3,821,037 (8.45%)
WT Control 2	49,595,192	45,337,078 (91.41%)	4,258,114 (8.59%)
WT Control 3	50,962,008	46,736,694 (91.71%)	4,225,314 (8.29%)
WT $CdCl_2$ 1	46,487,986	42,374,271 (91.15%)	4,113,715 (8.85%)
WT $CdCl_2$ 2	41,698,970	38,154,862 (91.5%)	3,544,108 (8.5%)
WT $CdCl_2$ 3	42,640,506	39,044,380 (91.57%)	3,596,126 (8.43%)
GENE1 OX #11 Control 1	44,430,044	40,949,387 (922.17%)	3,480,657 (7.83%)
GENE1 OX #11 Control 2	42,628,436	39,126,374 (91.78%)	3,502,062 (8.22%)
GENE1 OX #11 Control 3	50,217,758	46,080,256 (91.76%)	4,137,502 (8.24%)
GENE1 OX #11 $CdCl_2$ 1	44,101,720	40,360,769 (91.52%)	3,740,951)8.48%)
GENE1 OX #11 $CdCl_2$ 2	45,868,156	41,892,516 (91.33%)	3,975,640 (8.67%)
GENE1 OX #11 $CdCl_2$ 3	40,828,282	37,163,157 (91.02%)	3,665,125 (8.98%)
gene1 KO #16 Control 1	40,834,962	37,308,017 (91.36%)	3,526,945 (8.64%)
gene1 KO #16 Control 2	41,998,230	38,569,198 (91.84%)	3,429,032 (8.16%)
gene1 KO #16 Control 3	43,730,812	39,771,305 (90.95%)	3,959,507 (9.05%)
gene1 KO #16 $CdCl_2$ 1	45,984,972	41,664,139 (90.6%)	4,320,833 (9.4%)
gene1 KO #16 $CdCl_2$ 2	38,356,030	35,097,860 (91.51%)	3,258,170 (8.49%)
gene1 KO #16 $CdCl_2$ 3	43,914,276	39,512,905 (89.98%)	4,401,371 (10.02%)

With the previous data, we performed gene expression analysis using EdgeR, as described in the Materials and Methods section, to identify candidate genes for further genetic modifications in poplar. The first step was to obtain lists of differentially expressed genes (DEGs) between the control and Zn conditions of every genotype. Thereby, we generated 3 different DEGs lists:

- WT Control / WT $CdCl_2$
- *GENE1* OX #11 Control / *GENE1* OX #11 $CdCl_2$
- *gene1* KO #16 Control / *gene1* KO #16 $CdCl_2$

Once we had these lists of DEGs, we generated a Venn Diagram (Figure 7) to see common and non-common genes between these lists.

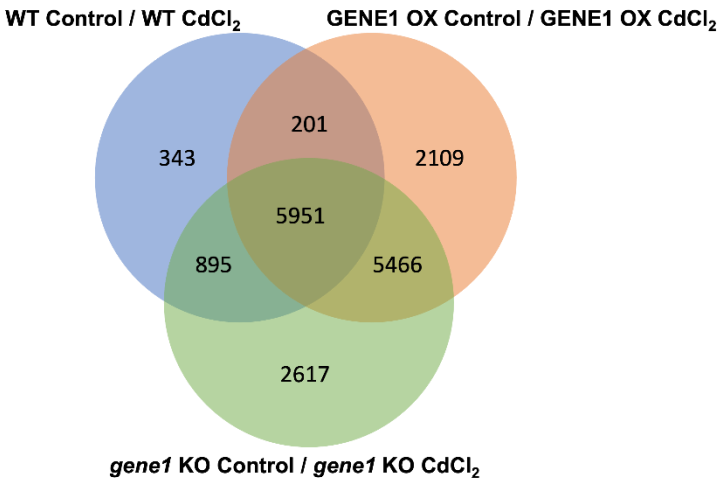

Figure 6. Venn Diagram of RNA-seq results. The diagram shows the intersections between the DEGs lists. On one hand common DEGs between genotypes and on the other hand unique DEGs of every genotype.

This result, together with RNA-seq analysis of *GENE1* genotypes in response to $ZnCl_2$ and further analysis of the function of the genes have given us a considerable amount of biological information to understand the tolerance and accumulative behavior of *GENE1* OX #11 poplar genotype and have led us to select candidate genes for new genetic modifications and to propose a new approach based on biostimulants to improve poplar's phytoremediation capacities.

3.2.2 Transcriptomic functional analysis of *GENE1*

To functionally analyse transcriptomic data from *GENE1* RNA-seq in response to $ZnCl_2$ and $CdCl_2$ from a biological perspective, we decided to analyse the differentially expressed genes (DEG) between WT control and WT Zn, WT control and WT Cd, WT Zn and *GENE1* OX #11 Zn, and WT Cd and *GENE1* OX #11 Cd. These results are shown in Venn diagrams format in Figure 8. We discarded the *gene1* KO #16 genotype for this analysis due to the absence of a clear phenotype different from WT (Figure 1).

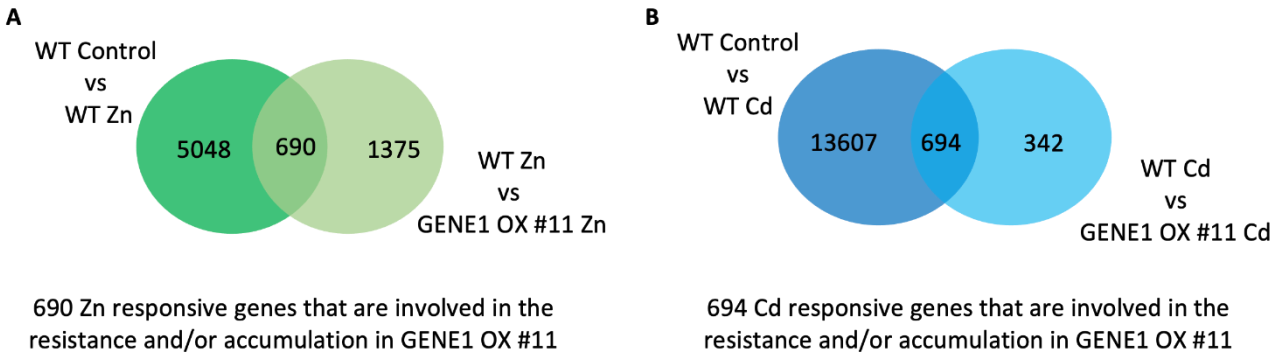

Figure 7. Venn Diagram of RNA-seq functional analysis. The diagrams show the intersections between DEG of WT control vs WT Zn and DEG of WT Zn vs *GENE1* OX #11 Zn (A) and DEG of WT control vs WT Cd and DEG of WT Cd vs *GENE1* OX #11 Cd.

DEG between WT control and WT Zn showed 5738 Zn responsive genes in WT poplar. DEG between WT Zn and *GENE1* OX #11 Zn showed 2065 genes that could be related to the Zn tolerance, resistance or accumulation in our *GENE1* OX #11 genotype. By crossing these two lists, we obtained 690 common genes. These genes are Zn responsive genes that are involved in the resistance and/or accumulation of Zn in *GENE1* OX #11 poplar genotype (Fig. 8A).

DEG between WT control and WT Cd showed 14301 Cd responsive genes in WT poplar. DEG between WT Cd and *GENE1* OX Cd showed 1036 genes that could be related to the Cd tolerance, resistance, or accumulation in our *GENE1* OX genotype. By crossing these two lists, we obtained 694 common genes. These genes are Cd responsive genes that are involved in the resistance and/or accumulation of Cd in *GENE1* OX #11 poplar genotype (Fig. 8B).

We also performed a Gene Ontology (GO) Enrichment Analysis from these 690 and 694 DEG to elucidate the main biological and molecular processes these genes are involved in. Top 30 GO terms are represented in Dot Plot graphs (Fig. 9) for Zn dataset (Fig. 9A) and Cd dataset (Fig. 9B).

For the Zn dataset GO Enrichment Analysis, we highlight important processes such as metal ion binding, oxidoreductase activity, anion transport, DNA-templated transcription, response to auxin, and polyamine oxidase activity. For the Cd dataset GO Enrichment Analysis, we highlight important processes such as ion binding, protein modification process, chlorophyll binding, carbohydrate metabolic process, and polyamine oxidase activity.

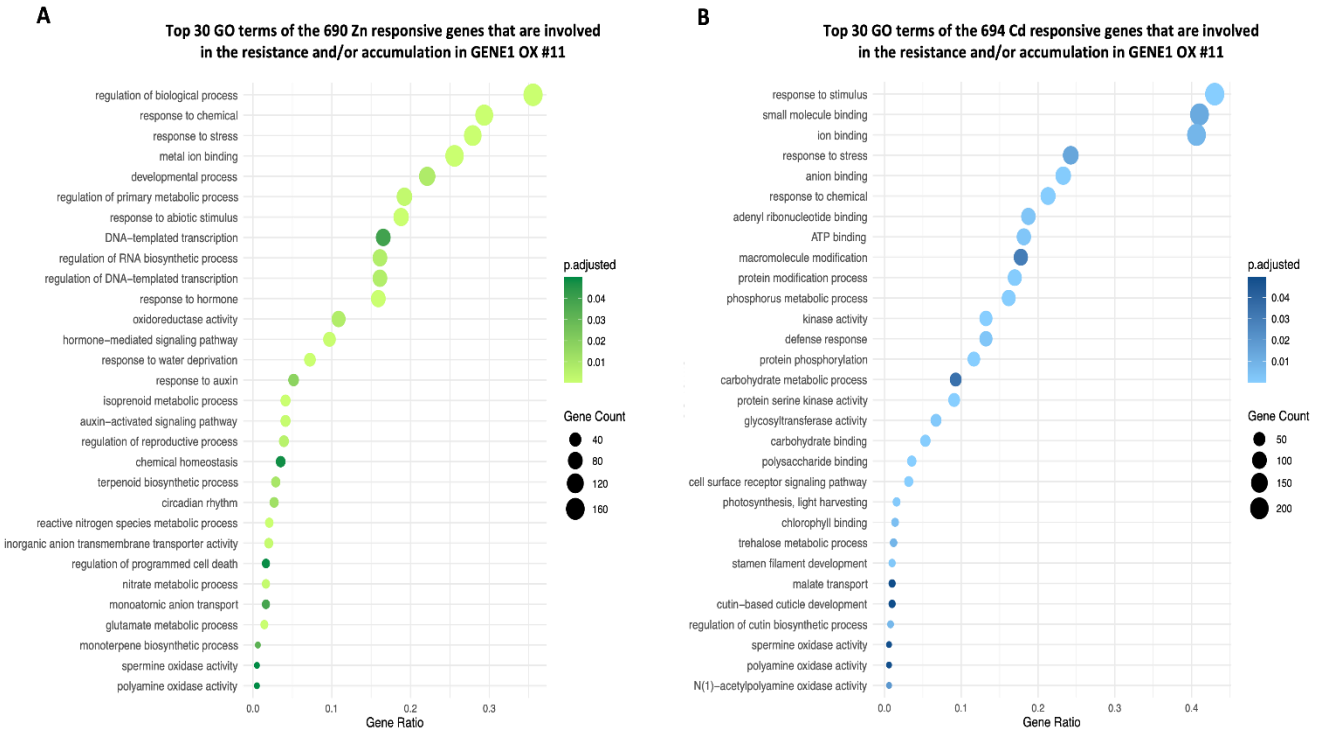

Figure 8. Top 30 GO terms of the DEG obtained from RNA-seq functional analysis. Dot Plot representing the GO terms from 690 Zn responsive genes that are involved in the resistance and/or accumulation of Zn in *GENE1* OX #11 poplar genotype (A) and from 694 Cd responsive genes that are involved in the resistance and/or accumulation of Zn in *GENE1* OX #11 poplar genotype (B). Gene Ratio represents the proportion of genes that are present in a GO term category with respect to the total number of genes. Gene Count represents the exact number of genes for a GO term category. Finally, p.adjusted represent the p.value < 0.05 of each GO term category within the dataset.

To investigate the common response to both metals (Figure 10), we identified the overlap between the 690 DEGs from the Zn analysis and the 694 DEGs from the Cd dataset. This intersection yielded 81 DEGs associated with Zn and Cd response in poplar, as well as with tolerance, resistance, and metal accumulation in the *GENE1* OX #11 genotype (Figure 10A). GO enrichment analysis of these 81 genes revealed 18 significantly enriched GO terms related to biological and molecular processes. Notably, the polyamine metabolic process emerged as the most prominently represented category among these DEGs.

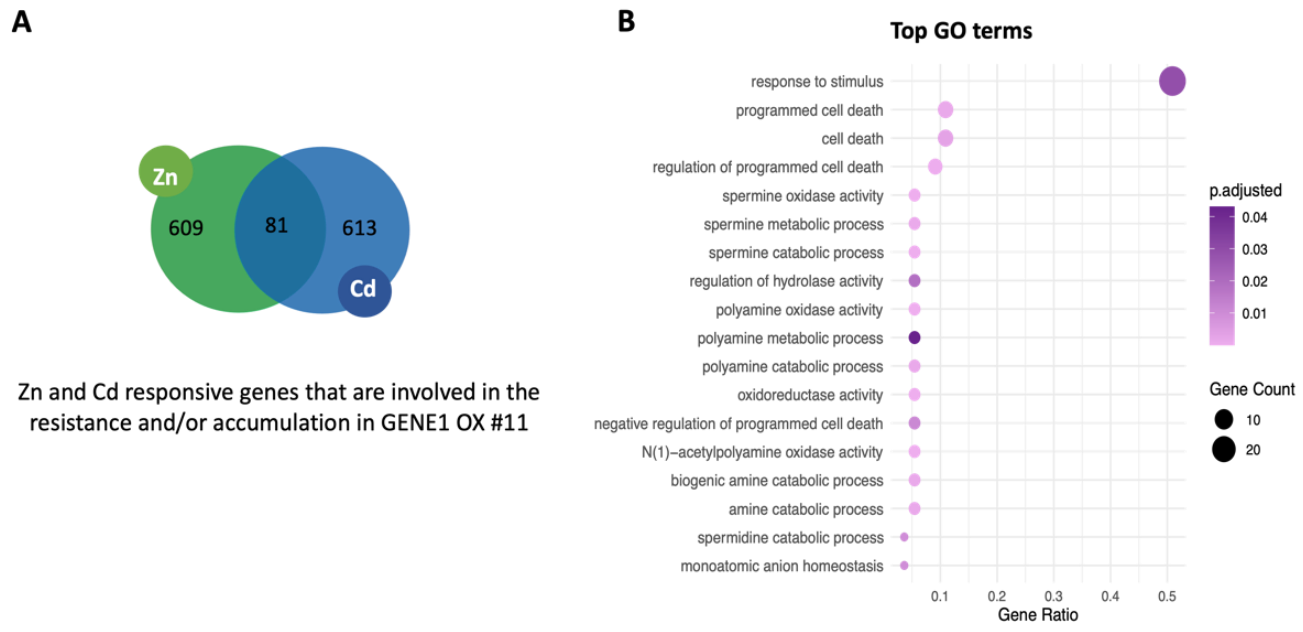

Figure 9. Analysis of the common DEG between Zn and Cd datasets. Venn Diagram of the Zn responsive genes that are involved in the resistance and/or accumulation of Zn in *GENE1* OX #11 poplar genotype and the Zn responsive genes that are involved in the resistance and/or accumulation of Cd in *GENE1* OX #11 poplar genotype (A). GO. Dot Plot representing the GO terms from 81 Zn and Cd responsive genes that are involved in the resistance and/or accumulation of Zn and Cd in *GENE1* OX #11 poplar genotype.

Based on all these results, two main strategies were implemented. First, seven different candidate genes have been selected to carry out genetic engineering in poplar with the goal of generating new transgenic lines exhibiting enhanced resistance, tolerance, and metal accumulation. Second, the biostimulant capacity of the polyamine spermidine is evaluated to alleviate heavy metal stress in poplar and to assess its impact on improving poplar's resistance, tolerance, and accumulation performances.

3.2.3 Generation of new transgenic lines

We have generated four new loss of function (knock-out) mutant lines by CRISPR-Cas9 technology, and three new overexpressing lines, based on their expression patterns in *GENE1* OX #11 line. These genes are implicated in auxin signalling, transcription regulation, metal transport and redox metabolism, based on previously highlighted GO terms from RNA-seq functional analysis.

Figure 11 shows the currently steps of poplar regeneration process to obtain the new genetic engineered lines. Three of these lines are currently in the shoot generation from callus state (Fig. 11A). The other four remaining lines are already being propagated for genotyping to identify the generated gene edition (Fig. 11B).

Figure 10. Currently state of new genetic engineered poplar lines regeneration process. Picture of shoot generation from callus (A) and picture of poplar explants amplification (B).

3.2.4 Polyamines

Based on the analysis of the common DEG between Zn and Cd datasets (Figure 10), we also decided to further investigate the function of the polyamine spermidine and its biostimulant capacity to alleviate heavy metal stress in poplar. The preliminary results are shown in Figure 12.

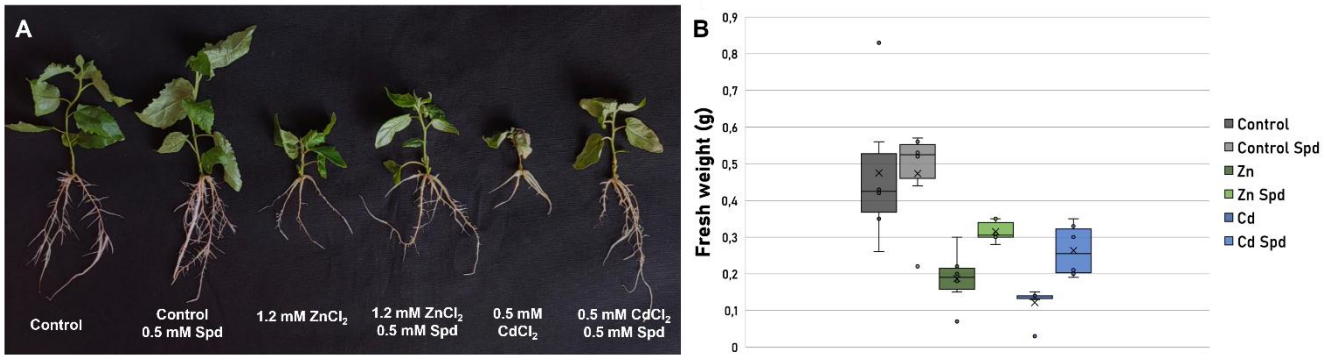

Figure 11. Metal tolerance and spermidine (Spd) *in vitro* assay in WT poplar. Picture of *in vitro* poplar plants after 10 days after the different treatments (A) and biomass quantification of the plants in terms of fresh weight measures of these plants, expressed in g (B). N=6 for each treatment.

We have performed an *in vitro* assay where we exposed WT poplar plants to 1.2 mM $ZnCl_2$ and 0.5 mM $CdCl_2$, adding 0.5 mM of spermidine to the medium to evaluate its potential (Fig. 12A). Through this, after ten days we observed that, on one hand, spermidine does not cause changes in terms of biomass in plants growing under control conditions, in fresh weight terms (Fig. 12B). In contrast, spermidine can increase the resistance and tolerance of plants to high concentrations of Zn and Cd. This can be observed by a higher biomass in plants treated with the metal in combination with spermidine, compared to those treated with the metal alone.

Therefore, we conclude that the polyamines exhibit a biostimulant effect under metal stress conditions.

3.3 Selection of inoculation methods for Task 4.2 activities

3.3.1 *In vitro* inoculation of poplar

For inoculating poplars with UFC003 and UBF009 fungal strains in *in vitro* conditions, UPM tested plant-fungi coculture in three different plant medium conditions, described in detail in the Materials and Methods (Section 2.4.2.).

Figure 13 shows the results after ten days of coculture between WT poplar and UFC003 and UFC009, separately, in MS1B media with sucrose (suc), MS1B media without sucrose and half MS1B without sucrose (Fig. 13A) and the reisolation test after four weeks of plants growing in peat containing pots (Fig. 13B-C). An optimal fungal growth in roots can be appreciated when sucrose is present in the media for both strains. UFC009 can also grow around plant roots in the other two conditions, although less fungal growth is appreciated when sucrose is not present in the media. For UFC003, slight growth is produced around poplar roots when sucrose is not present in the plant media (Fig. 13A).

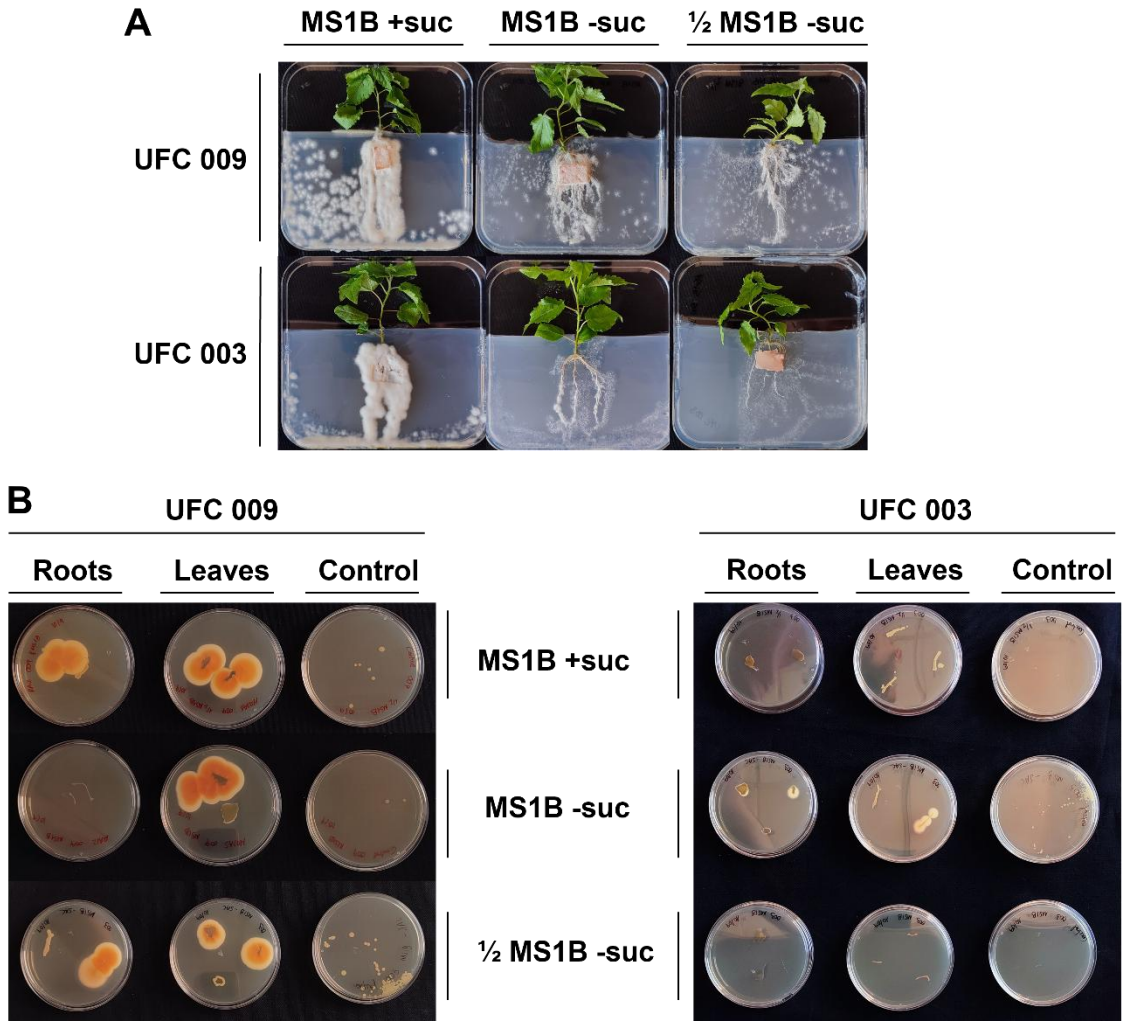

Figure 12. *In vitro* inoculation of WT poplar with UFC003 and UFC009 fungal strains and reisolation test. Poplar-UFC009 and UFC003 coculture after 10 days in the different *in vitro* plant media (A). Fungal growth in SDA plates from reisolation test from roots, leaves and controls of UFC009 and UFC003 after coculture in the three different *in vitro* media.

These plants were then transferred to peat containing pots and let them grow for four weeks. After that, plant leaves and roots were collected to perform a reisolation test in SDA plates to evaluate the endophyte colonization of both fungal strains in the plants in the long term (Fig. 13B). UFC009 was reisolated from the roots and leaves of inoculated plants under three different conditions, except in roots collected from MS1B without sucrose coculture medium. Control plates are prepared by spreading 100 µL of wash sterile water. Some rhizosphere bacterial colonies can still be appreciated in controls.

Several conclusions can be obtained from these experiments. It is possible to inoculate poplars using an *in vitro* approach and successfully maintain the fungi colonization after growing in pots for four weeks. Root inoculation of *in vitro* poplars depends more on the biology of the fungi being inoculated than on the coculture media composition. Successful colonization of poplar tissues by UFC009 fungal strains is possible independently of the coculture media composition, but UFC003 can only colonize poplar tissues when a complete nutrient profile and no sucrose is added to the coculture medium.

This inoculation method was ultimately discarded in favor of root inoculation and direct soil spore inoculation for WP4 activities (D4.2), as these two methods eliminate the coculture required by the *in vitro* inoculation, which lasts 10 days.

3.3.2 Root inoculation of poplar

Root inoculation is the method used by UPM to inoculate poplars with UFC003 and UFC009 in mesocosm experiments with site 10 soil. Results of these experiments are reported in D4.2.

3.3.3 Direct soil spores' inoculation of poplar

Soil spores' inoculation is the method used by UPM to inoculate poplar with fungal consortia from UMLP in mesocosm experiments with soil from site 10. Experiments are currently ongoing. They will be reported in the upcoming Deliverable D4.4.

4 Conclusions

As a result of Task 3.3 activities:

- Three different inoculation methods have been optimized for fungal colonization of poplar roots. These inoculation methods are being used in Task 4.2 activities (D4.2, and upcoming D4.4).
- One Zn and Cd resistant, tolerant, and accumulative poplar line (*GENE*1 OX #11) has been selected.
- *GENE*1 OX #11 line is being used for Task 4.2 activities to test fungal strains from UMLP and microbial consortia from UMLP, UBU and JSI in site 10 soil.
- Another Zn and Cd resistant, tolerant, and potentially accumulative poplar line has been identified (*GENE2* OX).
- *GENE2* OX line can be used for Task 4.2 activities on site 10 soil.
- One Zn and Cd hypersensitive poplar line has been identified (*gene2* KO).
- We have functionally studied the transcriptomic response of *GENE1* OX poplar genotype to Zn and Cd to understand its resistance, tolerance, and accumulation capacities.
- We selected seven novel candidate genes and generated new potentially Zn and Cd resistant and accumulative transgenic poplar lines.
- We show evidence of spermidine's biostimulant capacity to alleviate heavy metal stress in poplar and its implications in enhancing poplar's metal resistance and tolerance.

5 Future perspectives

The successful completion of our work carried under Task 3.3 context has not only provided significant insights into the genetic and molecular mechanisms of heavy metal tolerance in poplar but has also opened several promising avenues for future investigation. The findings from this work serve as a solid foundation for further exploration into phytoremediation applications.

The identification of the GENE2 OX and *gene2* KO poplar genotypes, with their contrasting resistance and hypersensitivity to Zn and Cd, presents a particularly compelling opportunity. Transcriptomic analysis of these genotypes under heavy metal stress is a key next step. By comparing the gene expression profiles of these two lines, we can gain a deeper understanding of the molecular pathways and genetic networks that underpin Zn and Cd resistance in plants. This knowledge will be crucial for developing strategies for future genetic improvements in poplar, ultimately enhancing its effectiveness for phytoremediation.

Building on the promising results of spermidine as a biostimulant, additional research is warranted to fully characterize its capacity to alleviate heavy metal stress. Further studies could explore the optimal application methods and concentrations of spermidine to maximize its effects on metal resistance, tolerance, and accumulation in poplar.

Finally, the seven novel candidate genes identified in our study and the transgenic poplar lines created represent a rich resource for future research. The characterization of their response to high Zn and Cd exposure is a logical next step to validate their potential roles in heavy metal tolerance. These lines could be used for metal tolerance experiments or on-site trials to evaluate their performance under real-world conditions. These future studies will provide greater knowledge of the fundamental mechanisms of heavy metal resistance in plants, paving the way for more effective biotechnological solutions.

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