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# Insights on plant responses to abiotic stressors: copper and drought tolerance in *Bixa orellana* and *Populus* × *canescens*

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*Doctor Scientiae*

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Thesis submitted to the Botany Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Luzimar Campos da Silva

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Assent:

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*À minha mãe, e às minhas irmãs por todo amor, apoio e inspiração.*
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## ABSTRACT

REZENDE, Franklin Patrocínio, D.Sc., Universidade Federal de Viçosa, June, 2025. **Insights on plant responses to abiotic stressors: copper and drought tolerance in *Bixa orellana* and *Populus* × *canescens***. Adviser: Luzimar Campos da Silva.

Human activities such as mining, industrial emissions have contributed to large soil contamination by heavy metals and increased frequency of extreme drought events. Investigating how plants respond to these abiotic stresses is an essential tool for the development of sustainable mitigation strategies. This study examines two contrasting environmental stresses, copper toxicity and water deficit, through experimental studies focused on the morphoanatomical, biochemical and physiological responses of plants. In the first study, *Bixa orellana* seedlings were cultivated in soil amended with copper at concentrations of 0, 100, 200 and 400 mg/kg. The aim was to assess their phytoextraction potential, as well as their structural and metabolic responses to excess copper. Copper was mainly retained in roots, with no significant translocation to shoots or reduction in biomass. Increases in pigment content were observed at higher concentrations, and anatomical analyses showed alterations such as xylem vessel deformation, trichome collapse, cell hyperplasia and collapse, and phenolics accumulation in leaves. These results indicate that *B. orellana* possesses strategies to tolerate copper toxicity, and it is not an accumulator species. The second study focused on drought response in genetically modified *Populus* × *canescens* lines overexpressing the Sc*WS* and Ma*FAR* genes, both involved in the wax biosynthesis pathway. Plants were grown under well-watered and drought conditions for 16 days, and physiological and growth parameters were evaluated. The transgenic lines displayed reduced gas exchange in both treatments but showed improved water-use efficiency under drought. Although they exhibited reduced height and stem diameter compared to the wild type, no differences were detected on dry biomass. These findings suggest that upregulation of wax biosynthesis may contribute to drought tolerance by limiting water loss, but trade-offs could occur in other parameters. Together, these findings provide valuable information on plant strategies under distinct types of abiotic stress and highlight both the potential and the limitations of using native species in phytoextraction and genetic engineering approaches for drought stress tolerance.



Keywords: abiotic stress; morphoanatomy; copper; drought

## RESUMO

REZENDE, Franklin Patrocínio, D.Sc., Universidade Federal de Viçosa, junho de 2025. **Perspectivas sobre as respostas de plantas a estresses abióticos: tolerância ao cobre e à seca em *Bixa orellana* e *Populus* × *canescens***. Orientadora: Luzimar Campos da Silva.

Atividades antropogênicas como mineração e emissões industriais têm contribuído significativamente para a contaminação de solos por metais pesados e para o aumento na frequência de eventos de seca extrema. Investigar como as plantas respondem a esses estresses abióticos é uma ferramenta essencial para o desenvolvimento de estratégias sustentáveis de mitigação. Este estudo avalia dois tipos de estresses ambientais, toxicidade por cobre e seca, por meio de experimentos focados nas respostas morfoanatômicas, bioquímicas e fisiológicas das plantas. No primeiro estudo, mudas de *Bixa orellana* foram cultivadas em solo contaminado por cobre nas concentrações de 0, 100, 200 e 400 mg/kg. O objetivo foi avaliar seu potencial fitoextrator, bem como as respostas estruturais e metabólicas ao excesso de cobre. O cobre foi retido principalmente nas raízes, sem translocação para a parte aérea ou redução na biomassa. Houve aumento no teor de pigmentos em concentrações mais elevadas, e as análises anatômicas revelaram alterações como deformação das paredes dos elementos de vaso do xilema, colapso de tricomas, hiperplasia e colapso de células, e acúmulo de fenólicos nas folhas. Esses resultados indicam que *B. orellana* possui estratégias eficientes na tolerância a toxicidade por cobre, embora não seja uma espécie acumuladora. O segundo estudo investigou a resposta à seca em linhagens geneticamente modificadas de *Populus* × *canescens*, superexpressando os genes Sc*WS* e Ma*FAR*, ambos envolvidos na via de biossíntese de cera. As plantas foram cultivadas em condições ideais de irrigação e de seca por 16 dias, e parâmetros fisiológicos e de crescimento foram avaliados. As linhagens transgênicas apresentaram trocas gasosas reduzidas em ambos os tratamentos, mas demonstraram maior eficiência no uso da água sob estresse hídrico. Apesar da redução na altura e no diâmetro do caule em comparação ao tipo selvagem, não foram observadas diferenças na biomassa seca. Esses resultados sugerem que a regulação positiva da biossíntese de cera pode contribuir para a tolerância à seca por limitação da perda de água, embora possam ocorrer trade-offs em parâmetros de crescimento. Em conjunto, os resultados fornecem informações valiosas sobre as estratégias adaptativas de plantas sob diferentes tipos de estresse abiótico e destacam o potencial e as limitações do uso de espécies nativas em fitorremediação e do uso de

engenharia genética para aumento da tolerância à seca.

Palavras-chave: estresse abiótico; morfoanatomia; cobre; seca

**SUMMARY**

## GENERAL INTRODUCTION

Environmental changes due to human activities and natural processes have become one of the most alarming problems of the modern world. Deforestation, industrial emissions, agricultural runoff, fossil fuel burning and land use change are some factors strongly associated with climate change, biodiversity loss and chemical pollution (Gabric 2023; Sigmund et al., 2023; Keck et al., 2025). Heavy metal pollution and climate change are some of the key drivers responsible for the changes in biological, chemical and physical environmental quality nowadays (Islam and Sandhi, 2022). The increase in the frequency of extreme drought events due to climate change and soil heavy metal contamination are considered some of the biggest global concerns due to their direct impact on crop production and food safety, being recognized as global-scale emergencies that require massive mitigation strategies (Mansoor et al., 2021; Sigmund et al., 2023).

One of the most used heavy metals in the world, copper is an essential element for all organisms, and it can cause severe environmental pollution due to several anthropogenic activities such as mining, industrial emissions, pesticide application, and wastewater management, entering air, water and soil systems (Poggere et al., 2023; Wang et al., 2025). Copper pollution has emerged as a significant concern because of its deleterious effects on humans and plants (Wang et al., 2025).

In general, excess copper can induce several toxicity symptoms in plants such as increased reactive oxygen species (ROS), necrosis, reduction in plant growth and biomass allocation, reduced root nutrient and water uptake, pigment content changes and reduction in gas exchange parameters (Shabbir et al., 2020; Kumar et al., 2021; Wang et al., 2025). Plants possess many different strategies to tolerate high levels of copper in soil and some species can accumulate substantial amounts of copper in their tissues. These species are classified as hyperaccumulators, defined as those able to accumulate more than 300 mg of copper per kilogram of dry shoot tissue, and are considered key elements for phytoremediation of contaminated soils (Reeves et al., 2018).

Climate change is another result of human activities' impact on the environment, and, in recent years, it has increased the frequency of extreme weather events such as heat waves and long drought periods (Sato et al., 2024). Drought stress has many effects in plants such as changes in the internal and external morphology of leaves, stems and roots and limitation of vital processes such as photosynthesis, respiration, and stomatal movement, which has direct

impact in physiological metabolism and plant growth (Yang et al., 2021). Thus, crop production is significantly impacted by long drought events, with losses ranging from 30-90% depending on the species, which is the main reason these phenomena had become a global concern (Dietz et al., 2021).

In primary growth, the aerial parts of land plants are covered by the cuticle, a lipophilic layer that plays a crucial role in the protection against intense UV light, bacteria, fungi, insects and especially reduces water loss due to its hydrophobic nature (Kong et al., 2020; Lewandowska et al., 2020). The cuticle has a dynamic structure and composition, and it can change in response to environmental parameters such as temperature, relative humidity and irradiation (Dimopoulos et al., 2020; Heredia et al., 2024). Many studies have investigated the biosynthetic pathways involved in the production and regulation of the cuticular components, but there is still a lot to be discovered. Since food security has become a major concern due to extreme drought events, the investigation and genetic engineering of stress-adaptive traits, such as cuticle biosynthesis, have emerged as scientific priorities to maintain crop productivity under climatic stress conditions (Shinwari et al., 2020; Wang et al., 2021; Sato et al., 2024).

The investigation of plant responses to different abiotic stress conditions such as heavy metal stress and drought stress can provide valuable information for developing strategies involving phytoremediation of contaminated soils and increasing crop productivity under the frequent drought events (Liu et al., 2022; Sharma et al., 2025).

Furthermore, in this study, we aim to answer the following questions:

- Is *Bixa orellana* L. “Piave vermelha” (Bixaceae) a copper hyperaccumulator species and does it present any morphoanatomical and physiological alterations in response to excess copper?
- Does the overexpression of Sc*WS* and Ma*FAR* genes improve *Populus canescens* drought tolerance?

In order to answer these questions, this thesis is divided into two chapters:

- Chapter 1: “Copper in *Bixa orellana* L. (Bixaceae): uptake, morphoanatomical and metabolic responses to different soil concentrations”, which will be submitted to the journal *Environmental Science and Pollution Research* (ISSN 1614-7499) after reviewing and incorporation of the board’s suggestions;

- Chapter 2: “Co-Expression of Jojoba Wax Ester Synthase (Sc*WS*) and *Marinobacter aquaeolei* Fatty Acyl-CoA Reductase (Ma*FAR*) genes in Gray Poplar: initial characterization of transgenic lines under drought stress”, which will also be submitted to the journal Environmental Science and Pollution Research (ISSN 1614-7499) after reviewing and incorporation of the board’s suggestions.

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# Copper in *Bixa orellana* L. (Bixaceae): uptake, morphoanatomical and metabolic responses to different soil concentrations

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## ABSTRACT

Heavy metal contamination in soils by anthropogenic activities poses risks to human health and to ecosystem balance. Understanding native plant species responses to such stress is a crucial point for tracing soil remediation strategies. We tested the hypothesis that *Bixa orellana* acts as a copper phytoextractor species while exhibiting morphoanatomical and physiological alterations that mitigate and adapt to excess copper stress. This study evaluated the phytoextraction potential and the morphoanatomical and metabolic responses of *Bixa orellana* seedlings to increasing copper concentrations in soil. Seedlings were grown in soils treated with 0, 100, 200 and 400 mg/kg of copper and assessed for its accumulation in roots and shoots, translocation factor, biomass production, pigment content, lipid peroxidation, antioxidative enzymes activity and structural alterations. Copper was mainly retained in roots, and no significant translocation or biomass reduction was observed. For metabolic parameters, only pigment levels increased significantly at higher doses. Anatomical and micromorphological analyses revealed xylem vessel wall deformation, trichome collapse, cell hyperplasia and collapse, and phenolic accumulation in leaves of copper treated plants. These results suggest that *B. orellana* is a tolerant non-accumulator species that possesses efficient strategies against copper toxicity.

**KEYWORDS:** *Bixa orellana*; Copper; Phytoextraction; Metabolic responses; Morphoanatomy; Micromorphology.

## INTRODUCTION

Heavy metal contamination is one of the main causes of soil pollution due to their toxicity and persistence in the environment (Angon et al., 2024). Anthropogenic activities such as mining, manufacturing, chemical industry waste disposal, fossil fuel consumption, waste disposal, irrigation, pesticide and fertilizer usage release many toxic metal and metalloids in the environment (Rehman et al., 2023). One of these contaminants, Copper ($Cu^{2+}$) is the third most

used metal in the world and the twenty-fifth most abundant element in the earth's crust (Karlin and Tyeklár, 2012). In nature, most part of it is found in igneous and volcanic rocks (Shabbier et al., 2020). Copper is widely exploited for its important application in wiring, electronics, car and construction industries (Henckens and Worrell, 2020; Müller et al., 2025).

Soil contamination by copper happens mainly due to mining activities, residues from agricultural and industrial activities and agrochemicals (Wu et al. 2010a; 2010b; Karimi et al., 2021; Marques et al., 2022; Neaman et al., 2024). According to Keller et al. (2017), more than 95% of the copper released into the environment is added to the soil and water bodies, reaching potentially toxic levels. High concentrations of copper in soil can pose a threat to the establishment of biological populations and human health (Radziemska et al., 2017; Shabbir et al., 2020; Kumar et al., 2021). Furthermore, the bioavailability and toxicity of this element may increase due to different characteristics of the substrate, such as changes in its oxide-reducing potential, low concentration of organic matter and pH destabilization (Alloway, 2012; Nirola et al., 2015).

In plants, copper is considered an essential micronutrient, being the main constituent of proteins such as plastocyanin and cytochrome oxidase; it is essential to cell wall integrity and acts in several physiological processes such as macromolecules metabolism, photosynthesis, respiration, in the antioxidative system and in symbiotic nitrogen fixation (Rehman et al., 2019; Kumar et al., 2021; Wang e t al., 2024). The satisfactory concentration of copper in plant tissues is 5 to 30 $mg/kg^{-1}$, and higher concentrations are considered toxic (Wuana and Okieimen, 2011). At high concentrations, copper can destabilize the integrity of the plasma membrane, reduce photosynthetic rates and alter enzymatic activity, leading to reduced plant growth and production (Shabbir et al., 2020; Alengebawy et al.; Mir et al., 2021).

Many plant species are capable of thriving in soils contaminated with high concentrations of different metals (Reeves et al., 2017). Acknowledging the adaptive strategies behind metal tolerance, as well as the dynamics of metal uptake and distribution within the plant body, represents the first step towards developing phytoremediation approaches. Phytoremediation is a technique that uses plant species to mitigate soil contamination by metals or other pollutants, either by removing them from the soil or by immobilizing, transforming, or volatilizing them (Awa and Hadibarata, 2020; Wang et al., 2020; Sharma et al., 2025).

In Brazil, some plant species occur naturally in areas contaminated by mining tailings and already accumulate significant amounts of copper in their tissues (Coelho et al., 2021). The

investigation parameters such as plant growth, antioxidant defense system activity, together with the analysis of structural damage, are crucial for the understanding of how different plant species respond to high concentrations of copper in the substrate (Silva et al. 2000; Le Lay, 2006; Sant'Anna-Santos et al. 2006; Sant'Anna-Santos e Azevedo 2007; Santana et al., 2014; Goswami and Das, 2016). The understanding of these responses contributes to the development of phytoremediation approaches using native species, valued for their environmental adaptability and ecosystem compatibility.

*Bixa orellana* L. is a pioneer plant species, native to the Atlantic Forest, which occurs in the Rio Doce Basin, a copper-contaminated region affected by mining tailings from the Fundão Dam (Lorenzi, 2002; Barbieri et al., 2011). *B. orellana* is the only species known to produce bixin, an apocarotenoid widely used in the dyeing industry. Due to its applications in natural and sustainable dyes, bixin represents a highly promising industrial compound, with a market value of approximately USD 210.7 million at the end of 2022 (Gonçalves et al., 2025). Besides its high commercial importance in the production of dyes, which are widely used in the food, pharmaceutical and textile industries, the species is extensively used in reforestation of degraded areas (Lorenzi, 2002; Barbieri et al., 2011; Demczuk Jr and Ribani, 2015). Thus, the investigation of morphoanatomical and metabolic responses of *B. orellana* growing under high concentrations of copper in the soil can contribute with valuable information on the use of this species in remediation and reforestation of copper contaminated soils. Due to its natural occurrence in copper-rich soils, we tested the hypothesis that *Bixa orellana* exhibits biological adjustments enabling it to thrive under high soil copper concentrations.

In this way, the aims of this study were (i) to quantify the copper contents in roots, shoots and soil of *B. orellana* seedlings growing under different copper concentrations in soil, to understand copper dynamics between soil and plant; (ii) to determine the biomass production, pigment content, lipid peroxidation and antioxidative enzymes activity to characterize possible metabolic responses associated with copper exposure; and (iii) to identify possible structural alterations that can be used as biomarkers of copper toxicity in leaves of *B. orellana*.

## MATERIAL AND METHODS

### Plant material and growing conditions

*Bixa orellana* L. "Piave-vermelho" (Bixaceae) seeds, a variety widely used for commercial purposes due to its high bixin content (Faria et al., 2019), were obtained at the Tissue Culture Laboratory, at the Federal University of Viçosa and grown in a greenhouse. The

seeds were germinated in Vida Verde Tropstrato® commercial substrate, in 250 ml plastic bags. After standardization, the 6-month-old seedlings were selected and transplanted into 3.5 L pots with the same substrate with different concentrations of copper (0, 100, 200 and 400 mg/kg), obtained by the addition of $CuSO_4$ solution. The substrate was incremented with copper one month prior to the start of the experiment and left at rest during this period. The seedlings were maintained for 30 days at rest, being irrigated 3 times a week with water.

**Determination of Cu concentrations in dry matter and exchangeable Cu in soil**

To determine Cu concentrations in plant material, the samples were dried in an oven at 68–72 °C for 72 hours. After drying, the samples were ground using a knife mill. The Cu content in roots and shoots was determined using the nitric-perchloric digestion method (Sarruge & Haag, 1974). Copper was quantified using an inductively coupled plasma optical emission spectrometer (ICP-OES; Perkin Elmer Optima 8300 DV model). The equipment was calibrated with multielement standard solutions prepared in the same matrix as the samples, across different concentration ranges.

For the determination of copper (Cu) availability, soil samples were dried in an oven at 68–72 °C for 72 hours. Cu was extracted using the Mehlich-1 solution (Mehlich, 1953) and quantified by atomic absorption spectrometry (AAS) with the aid of a calibration curve. All procedures followed standardized methods established by the Brazilian Agricultural Research Corporation (EMBRAPA, 1997).

The whole plant Cu concentration (mg $kg^{-1}$) was calculated as a dry biomass (DB) - weighted average of Cu concentrations in shoots and roots, using the formula:

$$\text{Whole plant Cu concentration (mg kg}^{-1}) = \frac{[\text{Cu}]_{\text{shoot}} \times \text{DB}_{\text{shoot}} + [\text{Cu}]_{\text{root}} \times \text{DB}_{\text{root}}}{\text{DB}_{\text{shoot}} + \text{DB}_{\text{root}}}$$

The translocation factor (TF), which indicates plant ability to transfer metal from roots to shoots, was calculated according to Mellem et al. (2012), using the formula:

$$\text{TF} = \frac{[\text{Cu}]_{\text{shoot}}}{[\text{Cu}]_{\text{root}}}$$

**Biomass production determination**

At the end of the experiment, shoots and roots of the plants were separated and dried in an oven until reaching a constant weight to determine the dry biomass.

**Determination of chlorophyll a, b and carotenoid contents**

The levels of chlorophyll a, b and carotenoids were quantified using the extractor dimethylsulfoxide (DMSO) (Wellburn, 1994). 5mm diameter leaf disks were obtained from leaves collected from the third node. The disks were immersed in 4ml of DMSO saturated with calcium carbonate. The extract absorbance values were obtained after 24 hours in a water bath at 65º using a Genesys 2 PC spectrophotometer (Thermo Spectronic, Rochester, USA). The stipulated values for absorbance of chlorophyll a, b and carotenoids were respectively, 663.2, 646.8 and 470 nm. The equations for the obtention of pigment content in µg/mg of dry weight were calculated according to the methods proposed by Wellburn (1994). Chlorophyll a/b ratio and total chlorophyll content were also calculated.

**Determination of lipid peroxidation**

At the end of the experiment, 200 mg of leaves from the third node were collected and stored at -80ºC. Samples were macerated in 80% ethanol (v/v) and centrifuged at 3,000 xg for 10 minutes. Subsequently, 1 mL of the diluted sample was added to a tube with 1 mL of solution (I) – 20% (w/v) trichloroacetic acid (TCA), and 1 mL to a tube containing solution (II) – 20% TCA with 0.65% thiobarbituric acid (TBA). After mixing, the tubes were incubated at 95ºC for 25 minutes. Once cooled, absorbance readings were performed using a spectrophotometer, and malondialdehyde (MDA) equivalents (TBARS) were calculated according to Hodges et al. (1999).

**Enzymatic assays**

In order to evaluate the activity of antioxidative system enzymes of *B. orellana*, the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (POX) were determined. The enzymatic extract was obtained using 0.3g of leaf blade, previously harvested and kept at -80 °C. Samples were homogenized in 10mL of 0.1M potassium phosphate buffer, pH 6.8, containing 0.1mM EDTA, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 12,000 xg for 20 minutes and the supernatant was used as an enzyme source, as described by Peixoto et al. (1999). The protein content was determined by the methodology proposed by Bradford (1976), with a calibration curve constructed using the Bradford protein assay and bovine serum albumin (BSA) as standard.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined using the reaction described by Giannopolitis and Ries (1977). 50 µL of the extract was added to 2.95ml of the reaction medium (50mM sodium phosphate buffer, pH 7.8; 13 mM methionine; 75 µM nitro

blue tetrazolium – NBT; 0,1 mM EDTA and 2μM riboflavin). The photoreduction of NBT was measured after 5 minutes of continuous exposure to fluorescent light in a spectrophotometer at 560 nm. The photochemical production of formazan blue was determined at 560 nm. 1 unit of SOD was considered the amount of enzyme capable of inhibiting the reduction of NBT by 50% (Beauchamp and Fridovich, 1971).

For determination of catalase (CAT, E.C. 1.11.1.6) activity, 0.1 ml of crude extract was added to 2.9 ml of the reaction medium constituted by the potassium phosphate buffer 50mM (ph 7) and $H_2O_2$ 40mM (Havir and Mchale, 1987). The activity was established through the consumption of $H_2O_2$ in the first minute of reaction, which is determined by the drop in absorbance at 240 nm. The molar extinction factor of $H_2O_2$ ($39.4mM^{-1}$ $cm^{-1}$) was used for calculation.

For the determination of peroxidase (POX, E.C. 1.11.1.7) activity, 0.1 mL of the crude enzyme extract was added to 4.9 mL of reaction medium consisting of 25 mM potassium phosphate buffer, pH 6.8, 20 mM pyrogallol and 20 mM hydrogen peroxide (Kar and Mishra, 1976). The production of purpurogallin was determined by the increase in absorbance during the first minute of reaction at 420 nm, at 25 °C. The enzyme activity was calculated using the molar extinction coefficient of 2.47 mM $L^{-1}$ cm-$^{1}$ (Chance and Maehley, 1955).

**Visual and structural characterization in light microscopy**

Fully expanded leaves from the third node were photographed weekly with a digital camera (Cyber-Shot DSC-W310, Sony Corporation, Japan) for the documentation of the visual symptoms.

In order to characterize changes in the leaf anatomy of *B. orellana*, at the end of the experiment, samples were collected from the central region of fully expanded leaf blades from the third node. The samples were fixed in 2.5% glutaraldehyde solution in 0.1 M sodium phosphate buffer (adapted from Karnovsky, 1965), followed by dehydration in ethyl series, and included in methacrylate (Historesin, Leica Instruments, Heidelberg, Germany). 5 μm cross-sections were obtained in a rotating automatic microtome (Model RM2155, Leica Microsystems Inc., Deerfield, USA) using glass razors. After the microtomy process, the samples were stained with toluidine blue (pH 4.0) (O'Brien & McCully, 1981). All samples were mounted in glass slides and the cover slips were glued with synthetic Permount resin. The slides were photographed in a photomicroscope (model AX70 TRF, Olympus Optical, Tokyo, Japan) coupled to an AxioCam camera (Carl Zeiss, Jena, Germany).

**Structural characterization in scanning electron microscopy (SEM)**

At the end of the experiment, leaf samples from the third node of *B. orellana* were collected and fixed in a 2.5% glutaraldehyde solution in 0.1M sodium phosphate buffer, dehydrated in an ethylic series and dried at critical point with $CO_2$ (equipment model CPD 030; Bal-Tec, Balzers, Liechtenstein). The samples were placed in stubs and covered with gold in a metallizer (Sputter Coater, Balzers, model FDU 010, Liechtenstein), observed and photographed in a scanning electron microscope (model Leo 1430VP, Zeiss, Cambridge, England).

**Statistical analysis**

The experimental design was done in randomized blocks. The treatments consist of four different concentrations of copper (0, 100, 200 and 400 mg/kg), with 5 samples for each treatment (n=5). Quantitative data was submitted to analysis of variance (ANOVA) and means were compared using Tukey's test at 5%.

**RESULTS**

**Determination of Cu concentrations in dry matter and exchangeable Cu in soil**

Copper (Cu) concentrations in roots, shoots and whole plant, as well as exchangeable Cu levels in soil were determined and represented in Table 1. No statistically significant differences were observed among treatments for translocation factor (TF) or for Cu concentrations in roots, shoots or whole plants. However, the 400mg/kg treatment exhibited significantly higher exchangeable Cu levels in soil compared to the other treatments.

**Table 1**: Copper (Cu) concentrations in shoots, roots, and whole plants; exchangeable Cu in soil; and translocation factor (TF) of *Bixa orellana* after 30 days of exposure to different concentrations of copper in soil. Values represent means ± SE. Statistically significant differences between treatments are indicated by * (Tukey's test, $p < 0.05$).

| Cu concentration (mg/kg) | Shoot Cu concentration (mg/kg) | Root Cu concentration (mg/kg) | Whole plant Cu concentration (mg/kg) | Exchangeable Cu in soil (mg/dm³) | Translocation Factor (TF) |
|---|---|---|---|---|---|
| 0 | 3.55 ± 0.42 | 7.17 ± 1.22 | 15.26 ± 1.40 | 1.22 ± 0.04 | 0.52 ± 0.10 |
| 100 | 3.83 ± 0.24 | 12.32 ± 3.34 | 17.68 ± 2.93 | 1.52 ± 0.13 | 0.40 ± 0.16 |
| 200 | 4.52 ± 0.45 | 7.95 ± 0.90 | 14.24 ± 0.59 | 2.35 ± 0.20 | 0.59 ± 0.09 |
| 400 | 4.97 ± 0.53 | 11.1 ± 1.62 | 17.23 ± 1.82 | 3.46 ± 0.84* | 0.45 ± 0.28 |

**Biomass production**

Dry biomass for roots and shoots was determined and represented in Figure 1. No statistically significant differences were observed among any of the treatments.

**Determination of chlorophyll a, b and carotenoid contents**

Chlorophyll A, B, total, A/B ratio and carotenoid contents were determined and represented in Figure 1. The 400mg/kg treatment exhibited significantly higher Chlorophyll B and Total Chlorophyll content compared to all the other treatments. As for the Chlorophyll A content, 400mg/kg treatment showed higher values when compared to the 0 and 100mg/kg treatments. No statistically significant differences were observed among treatments for Chlorophyll A/B ratio or carotenoid content.

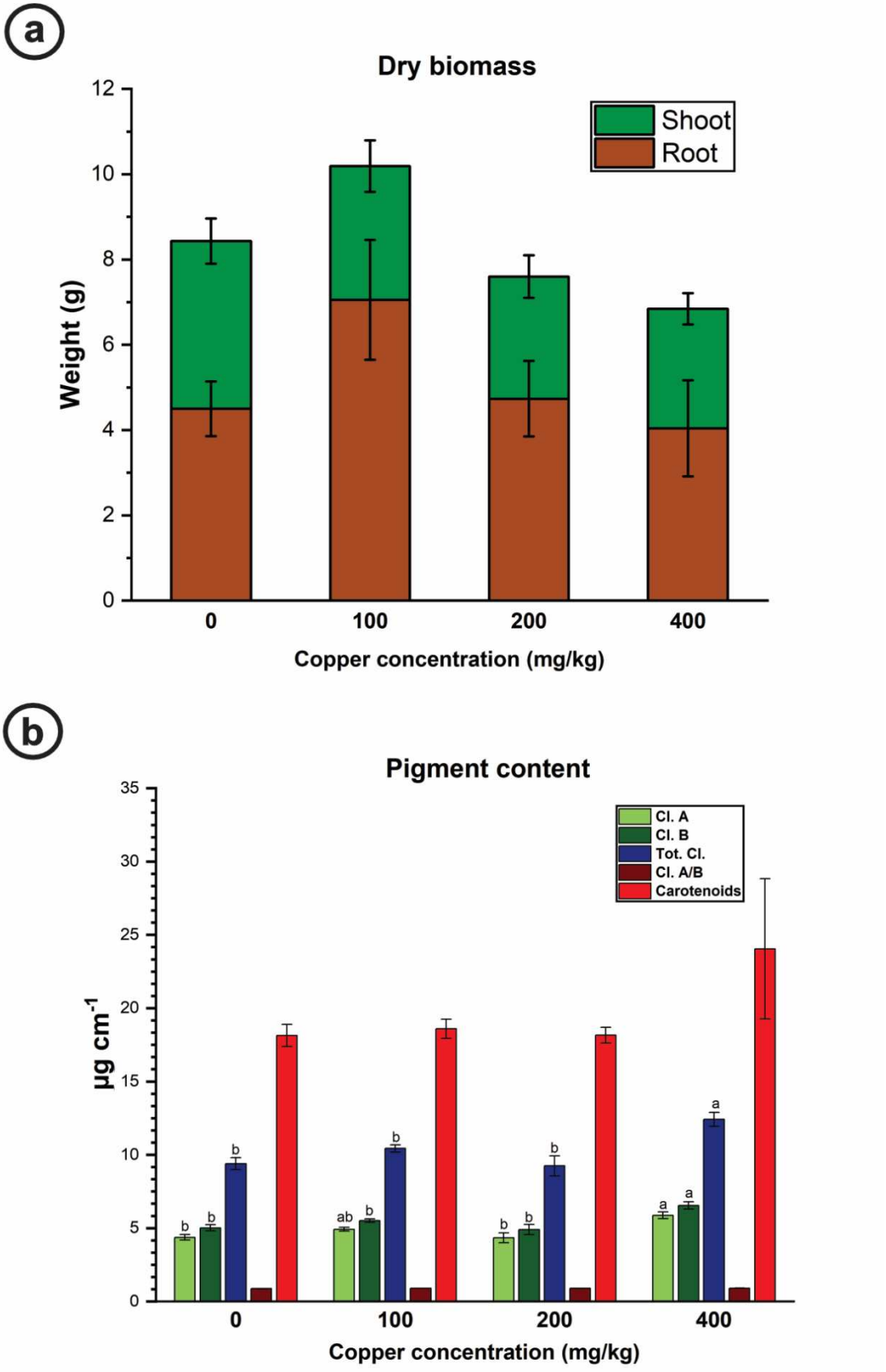
a
Dry biomass
Shoot
Root
Weight (g)
0
2
4
6
8
10
12
0
100
200
400
Copper concentration (mg/kg)
b
Pigment content
Cl. A
Cl. B
Tot. Cl.
Cl. A/B
Carotenoids
µg cm-1
0
5
10
15
20
25
30
35
b
b
b
ab
b
b
b
b
a
a
a
0
100
200
400
Copper concentration (mg/kg)

**Figure 1**: A – Root and shoot dry biomass (g) and B – Chlorophyll A, B, Total chlorophyll, A/B ratio and Carotenoid contents (µg $cm^{-1}$) of *Bixa orellana* after 30 days of exposure to different concentrations of copper in soil. Values represent means ± SE. Statistically significant differences between treatments are indicated by different letters (Tukey's test, $p < 0.05$).

**Determination of lipid peroxidation**

The lipid peroxidation was determined and represented in Figure 2. No statistical differences were observed among treatments.

**Enzymatic assays**

The superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) activities were measured and represented in Figure 2. No statistical differences were observed among treatments for any of the assays.

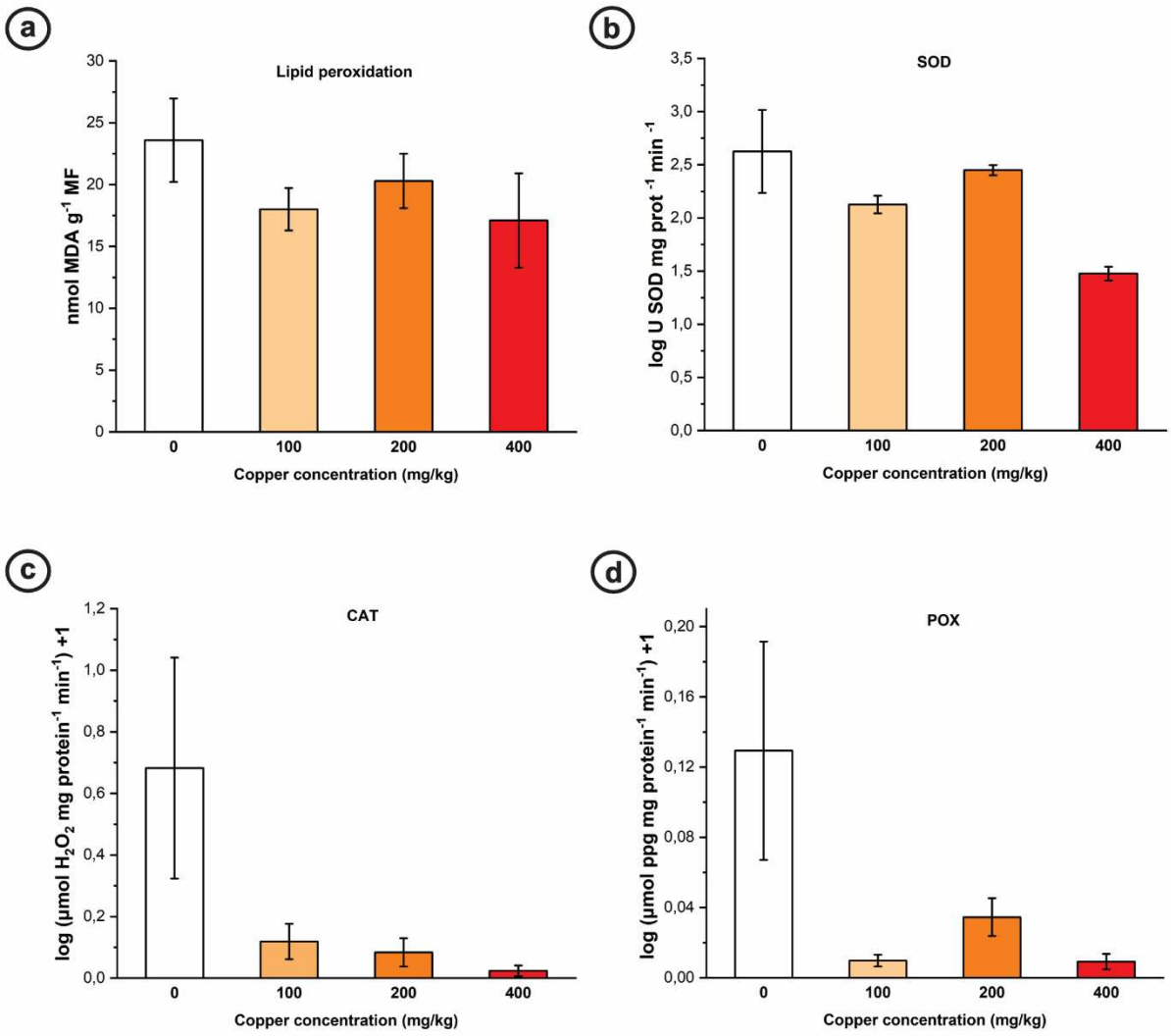

**Figure 2**: A – Lipid peroxidation; B – Superoxide dismutase (SOD) activity; C – Catalase (CAT) activity; and D – Peroxidase (POX) activity in *Bixa orellana* after 30 days of exposure to different concentrations of copper in soil. Valus represent means ± SE.

**Visual and structural characterization in light microscopy**

The visual characterization of leaves was done from 0 to 30 days of experiment and is represented in Figure 3. No apparent visual symptoms were observed in this period.


0 days
30 days
0 mg/kg
100 mg/kg
200 mg/kg
400 mg/kg
2 cm

**Figure 3**: Visual characterization of *Bixa orellana* leaves from 0 and after 30 days of exposure to different concentrations of copper in soil. Bar: 2cm.

*Bixa orellana* leaves are amphistomatic, and the epidermis is uniseriate and covered by a cuticle. Peltate trichomes are observed on both the adaxial and abaxial leaf surfaces (Fig. 4B). The mesophyll is dorsiventral, comprising 2–3 layers of palisade parenchyma and 3–4 layers of spongy parenchyma (Fig. 4A). Bixin-secreting laticifers are distributed within the spongy parenchyma (Fig. 4A), while crystal idioblasts are present throughout the subepidermal regions. The vascular system forms a closed arch with associated accessory vascular bundles (Fig. 5A; B). Crystal idioblasts and mucilage-secreting canals are scattered throughout the ground parenchyma (Fig. 5A).

On the leaf blade of 100mg/kg treatment plants, the peltate trichomes show a senescent aspect, with flaccid secreting-cells (Fig. 4C). Pectic protrusions can be observed in the epidermis (Fig. 4 D-E). In the middle vein, a layer of cells filled with phenolics can be observed surrounding the vascular system (Fig. 5C; E). In the xylem, secondary wall deformation can be observed in vessel elements (Fig. 5D).

On the leaf blade of 400mg/kg treatment plants, the trichomes also presented secretory cells with a flaccid appearance (Fig. 4F). As for the veins, damaged regions were observed in the epidermis and parenchyma, with cell disruption, cell hypertrophy and hyperplasia, phenolics accumulation and wound tissue formation close to the damaged area (Fig. 5F-H). Cell wall deformation was also observed in xylem vessel elements (Fig 5I). No anatomical changes were observed in leaves from the 200mg/kg treatment.

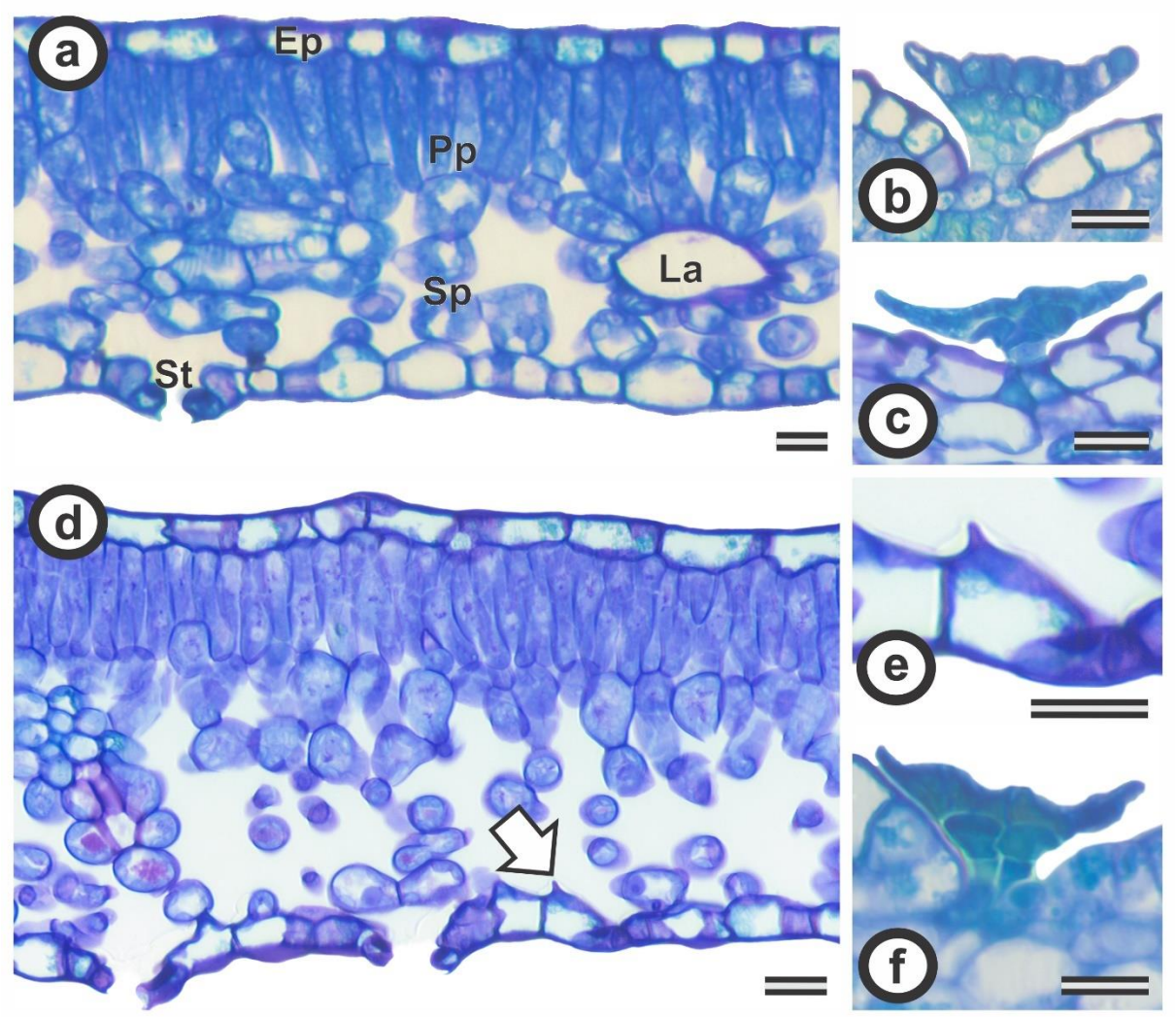


**Figure 4**: *B. orellana* leaf blade under light microscopy. 5µm cross-sections stained with Toluidine Blue. Leaves after 30 days under different copper concentrations. 0mg/kg (Control) (A-B); 100mg/kg (C-E) and 400mg/kg (F) treatments. A – Leaf blade; B – Peltate trichome; C; F – Peltate trichome with flaccid secreting cells; D-E – Pectic protrusion in the abaxial surface of the epidermis (arrow). Ep – Epidermis; Pp – Palisade parenchyma; Sp – Spongy parenchyma; La – Bixin-secreting laticifer; St – Stomata. Bars: 20 µm.

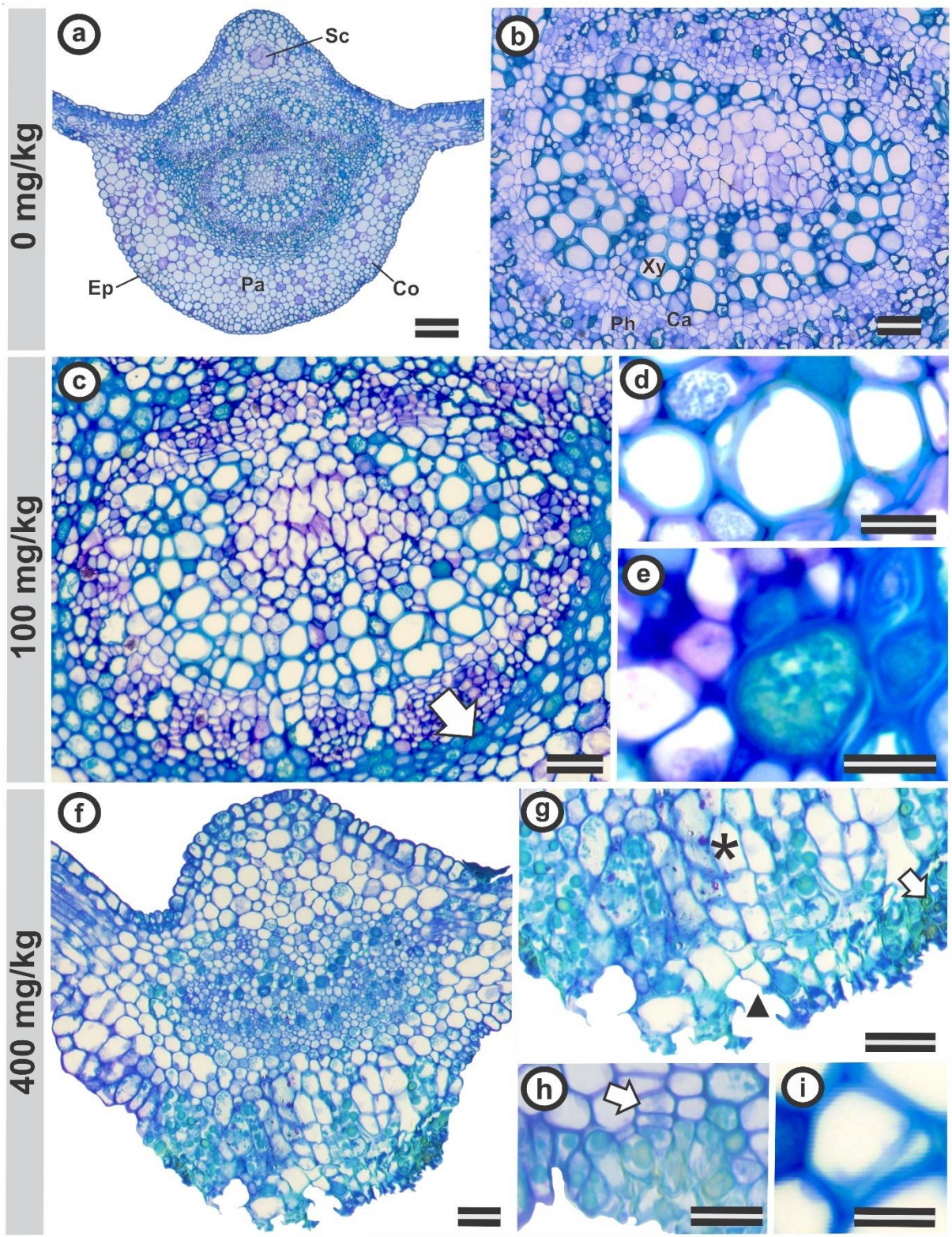


**Figure 5:** *B. orellana* veins under light microscopy. 5µm cross-sections stained with Toluidine Blue. Leaves after 30 days under different copper concentrations. 0mg/kg (Control) (A-B); 100mg/kg (C-E) and 400mg/kg (F-I) treatments. A – Middle vein general view; B – Vascular system; C – Vascular system surrounded by phenolic accumulating cells (arrow); D – Wall deformation in vessel element; E – Phenolic accumulating cell; F – Vein with damaged spot; G

– Damaged region in vein parenchyma and epidermis with phenolics accumulation (arrow), cell disruption (triangle) and cell hypertrophy and hyperplasia (asterisk); H – Cell hyperplasia with wound tissue formation (arrow); L – Wall deformation in vessel element. Ep – Epidermis; Pa – Parenchyma; Co – Collenchyma; Sc – Secretory canal; Xy – Xylem; Ph – Phloem; Ca – Cambium. Bars: A – 140; F, G – 50 μm; B, C – 40 μm; E, F, I, H – 20 μm.

**Structural characterization in scanning electron microscopy (SEM)**

*B. orellana* leaves characterized by juxtaposed cells in both surfaces of the epidermis. The stomata and trichomes are in the same level of the other epidermal cells and are present in both abaxial (Fig. 6A) and adaxial (Fig. 6B) surfaces. The adaxial surfaces presents ornamentations (Fig. 6B).

Epidermal grooves were observed in all treatments (Fig. 6D, E, F, H). At 100mg/kg, epidermal cell disruptions caused foliar tissue exposure and flaccid looking trichomes were observed (Fig. 6C). Fungal hyphae were observed around the damaged regions (Fig. 6C). At 400mg/kg, disruptions in the epidermal cells shape and cell rupture were detected (Fig. 6G).

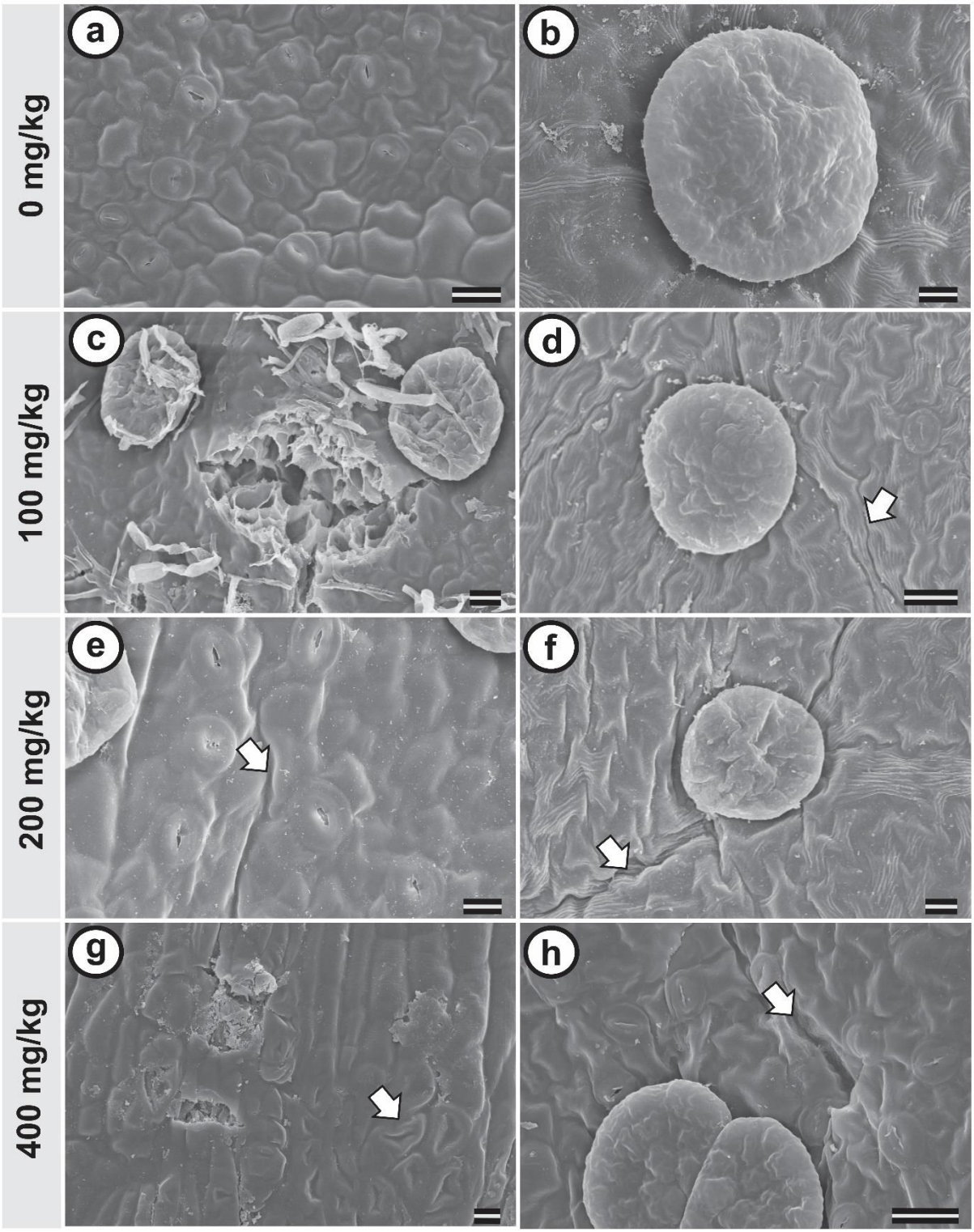


**Figure 6**: Leaf surface of *B. orellana* after 30 days exposure to different concentrations of copper in soil (Scanning electron microscopy). A – General view of the abaxial leaf surface with stomata; B – General view of the adaxial leaf surface with a peltate trichome; C – Epidermal cell disruption and exposure of the internal tissues, flaccid looking trichomes and fungal hyphae; D, E, F and H – Epidermal grooves (arrows); G – Epidermal cell shape alteration

(arrow). Abaxial surface – A, C, E, G, H; Adaxial surface – B, D, F. Bars: A, C, D, H – 20μm; B, E, F, G – 10μm.

**DISCUSSION**

In this study, the determination of copper content in roots, shoots, and soil provides crucial information on the uptake dynamics and internal distribution of the metal in *Bixa orellana*. Soil enrichment with copper to potentially toxic levels was successfully achieved, as its available fraction to plants ranged from 1.52 mg/dm³ in the lower treatment to 3.46 mg/dm³ in the highest. According to classification criteria proposed by Prezotti and Guarçoni (2013) regarding the available fraction, concentrations ranging from 0.8 – 1.8 mg/dm³ are considered moderate, with values exceeding 1.8 mg/dm³ being classified as high. However, even under high Cu availability, *B*. *orellana* accumulated relatively low amounts of the metal in roots and shoots. Even though root Cu concentrations were almost two times higher in 100 and 400 mg/kg treatments in comparison to 0, the concentrations still fall within the normal range of 5 – 30 $mg/kg^{-1}$ for non-accumulator plants (Wuana and Okieimen, 2011). As for shoots, the values were even below this range for control and high soil concentrations. This indicates a species-specific copper regulation for *B. orellana*, which can maintain relatively low concentrations in shoots while still functioning normally.

The internal regulation of copper uptake in root tissues involves a complex interplay of physical barriers, intracellular detoxification and transporter regulation. Copper can be adsorbed by the cell wall due to the negative charges of its constituents (pectin, cellulose) and by the endodermis, chelated in the cytosol and sequestered to vacuoles in less or non-toxic forms, and tightly regulated by xylem-loading transporters which control the root-to-shoot translocation (Ryan et al., 2013; Wairich et al., 2022; Zehra et al., 2023; Wang et al., 2024). Due to such a tight and integrated regulation system, for the majority of non-accumulator species, copper is likely to be retained in the root system (Nicolic et al., 2023). This tendency is confirmed in our study, since all treatments exhibited translocation factors (TF) below 1, which indicates that the biggest part of the absorbed metal was kept in roots and not transported to aerial organs.

Even though the values observed for copper accumulation in shoots of *B. orellana* were much lower than the thresholds for hyperaccumulator plants, they increased gradually in treatments up to 40% in the highest dose (400 mg/kg). The whole plant copper concentration increased by 15% at 100 mg/kg and 12,9% at 400 mg/kg, while it decreased by 6.7% at the 200

mg/kg dose. This suggests that copper toxicity in this species could be not solely dose-dependent but also occurs when internal detoxification mechanisms are saturated. At 100 mg/kg treatment, a relatively increase in Cu enrichment led to the higher enhancement in whole plant Cu uptake, which indicates that the detoxification system was not fully upregulated yet. In contrast, at 200 mg/kg, plants accumulated less copper than in control conditions, reflecting that this intermediate dose was able to induce an efficient response in detoxification strategies in the plants. However, at the 400 mg/kg dose, this system was probably saturated by the excess copper and plants were not able to stop copper translocation to shoots. Neutral or positive effects of copper in intermediate doses for plants are already well documented in the literature in other species (Tiecher et al., 2017; Trentin et al., 2022). The same pattern was also detected for anatomical damage in leaves, which were observed only at the two extreme doses.

The unaltered biomass production observed across the different copper doses in *Bixa orellana* align with results on copper toxicity in other tropical tree species such as *Cedrela fissilis* and *Myracrodruon urundeuva* (Asensio et al. 2019) as well as in herbaceous species like *Pelargonium graveolens* (Chrysargyris et al. 2021). Our findings suggest that, like these species, *B. orellana* probably possess efficient mechanisms to maintain biomass production even under potential metabolic and structural stress.

Excess copper is often associated with decreases in chlorophyll levels. However, some studies have shown also that under moderate stress conditions, chlorophyll content may actually increase a part of a compensatory physiological response (Chen et al., 2015; Li et al., 2023). This same pattern was observed in the present study, as 400 mg/kg treatment was able to induce an increase in Chlorophyll A, B and total chlorophyll in relation to the other treatments (except Cl. A at 100 mg/kg). This suggests that, in terms of photosynthetic apparatus, the highest dose of copper was still not able to impair the photosynthesis machinery or could even have induced a compensatory physiological response to mitigate the observed structural damage. Besides that, it is known that copper works as an activator for some enzymes in chlorophyll biosynthesis pathway (Sun et al., 2022), which in relatively moderate concentrations, could have enhanced the pigment production.

Reactive oxygen species (ROS) generated under excess copper can react with cell membrane lipids, leading to membrane disruption and cell content leakage (Giannakoula et al., 2021). However, despite localized damage, lipid peroxidation levels did not differ under the different treatments in this study. This can indicate that *B. orellana* possesses an efficient ROS-scavenging system. It is important to highlight that the lipid peroxidation assay is a systemic

analysis, which indicates that punctual tissue damage may not be significant for the final malondialdehyde content.

Antioxidant enzymes are key components of the plant defense system against oxidative stress, one of the earliest and most common responses to heavy metal exposure (Fujita and Hasanuzzaman, 2022; Mansoor et al., 2023). However, other non-enzymatic elements such as glutathione (GSH), ascorbic acid (ASA), proline, phenolic compounds and plant hormones play a major role in copper tolerance (Shabbir et al. 2020; Wang et al., 2024). In addition, *B. orellana* is a bixin-producing species, which is an apocarotenoid that besides possessing many other functions, is among the most effective biological quenchers of singlet oxygen ($^1O_2$) and scavengers of free radicals (Kovary et al., 2001; Vilar et al., 2014). Although the highest production occurs in seeds, all vegetative and reproductive organs of *B. orellana* possess bixin-secreting laticifers (Faria et al., 2019; Almeida et al., 2021). In our study, although none of the antioxidant enzymes (SOD, CAT and POX) exhibited any statistically different activity in comparison to the control, for catalase (CAT) and peroxidase (POX) even a decreasing pattern can be observed with increasing doses of copper. Such results suggest that in *B. orellana* some of the non-enzymatic antioxidants could be involved in scavenging the oxygen reactive species (ROS), probably bixin and phenolics, and there could be even a trade-off in this system, with a reduction in some antioxidant enzymes activity (CAT and POX) associated with an upregulation in the production of these other substances.

One of the major plant-responses to abiotic stress, including heavy metal and copper stress, is the production of phenolic compounds (Goncharuk and Kadoskina, 2023; Kumar et al., 2023). Excess copper induces key enzymes in the production of nonenzymatic compounds like flavonoids, coumarins and lignans, which can act as ion chelators and ROS scavengers in the plant body (Janczak-Pieniazek et. al., 2023; Kumar et al., 2023). The accumulation of phenolics in different organs of plants exposed to heavy metal stress is already well documented in the literature (Ambosini et al., 2015; Linggawati et al., 2022; Günthardt-Goerg et al., 2023). The same pattern was observed in the leaves of *B. orellana* under the 100 mg/kg treatment, with many cells surrounding the vascular system in the midrib. Since copper is translocated to shoots via xylem, in addition to its antioxidant effect, this layer of cells could work as a chelating barrier that reduces copper mobility to other leaf tissues.

Heavy metal movement in the plant body happens via symplastic and apoplastic pathways (Chao and Chao, 2024). As previously mentioned, cell wall components such as pectin and cellulose act as binding sites for heavy metals and chelators for these elements

(Shabbir et al., 2020; Chao and Chao, 2024). Cell wall remodeling by increasing the amounts of pectin and hemicellulose in response to heavy metals, including copper, is already reported in many studies for leaves and roots in different species (Wang et al., 2020; Guo et al, 2023; Yu et al., 2024; Wu et al., 2025). In our study, the 100 mg/kg treatment induced the formation of pectic protrusions in leaf epidermal cells, which can be characterized as defense mechanism against the increasing copper concentrations, as a way of limiting copper movement by binding it to cell walls.

Copper translocation from roots to shoots happens via xylem (Chen et al., 2022). Cu toxicity induces differential expression of enzymes related to the lignification patters in leaves of different species (Zhou et al., 2025). In *Citrus grandis* and *Citrus sinensis* leaves 9 and 4 out of 13 metabolites such as L-Phenylalanine, Cinnamic acid and Caffeic acid, which are associated with lignification, were downregulated in response to copper stress (Zhou et al., 2025). In tobacco plants, increasing copper doses induced a significant decrease in free phenolics content, which can be used as substrates for lignification (Tugbaeva et a.l., 2023). Modifications in enzyme activity as well as the substrates for the lignification process can causa an imbalance in the constituents of xylem vessels, affecting its rigidity and robustness (Parrotta et al., 2015). *B. orellana* leaves exhibited partial wall deformation in xylem vessels under the 100 and 400 mg/kg treatments, which suggests that copper may have induced certain disturbance in the activity of enzymes responsible for the lignification process, as well as the production of its substrates, affecting the integrity of xylem vessels.

One of the heavy metal detoxification strategies in plants is its chelation and deposition in vacuoles or trichomes (Nicolic et al., 2023). The role of glandular and non-glandular trichomes in sequestration of heavy metals is already well described for many different species (Gao et al., 2021; Li et al., 2022; Shukla et al. 2023). In tobacco plants, glandular trichomes were able to secrete excess Cd integrating it to calcium oxalate crystals (Zhang et al., 2021). In *Artemisia annua*, copper-treated plants exhibited reduced glandular trichome density and structural distortion, likely due to their role in detoxification through the sequestration or excretion of excess copper (Zehra et al., 2020). These findings align with the results obtained for *B. orellana* under 100 and 400 mg/kg treatments, in which the glandular trichomes exhibited secretory cells with flaccid appearance, probably due to the high secreting activity or senescence due to copper accumulation and toxicity. Future studies investigating the secretion process and chemical composition of *B. orellana* trichomes under different copper

concentrations in the soil would provide valuable information into the role of these structures in the species response to copper-induced stress.

Although lipid peroxidation levels did not differ among treatments at organ level, the presence of localized damaged regions suggest the presence of spatially restricted oxidative damage. Necrotic tissues are characterized by protoplast swelling and the breakdown of plasma membrane integrity (Sychta et al., 2021). This occurs mainly due to the increasing production of reactive oxygen species and harmful interactions at the cell level, and it is a common symptom of copper toxicity (Lange et al., 2017; Shabbir et al., 2020; Kumar et al., 2021; Liscakova et al., 2022). In the damaged region observed at the 400 mg/kg treatment in leaves of *B. orellana* some stress markers already reported for leaves of other tropical species under abiotic stresses can be detected, such as phenolics accumulation, cell hypertrophy and hyperplasia, wound healing tissue formation and cell disruption (Silva et al., 2005a,b; Andrade and Silva, 2016; Andrade et al., 2020; Rezende and Silva, 2024). The wound healing tissue is characterized by a newly differentiated cell barrier that prevents the lesion area from spreading to other parts of the tissue (Benatto Perino et al., 2025). Before dividing into this stacked layer of elongated cells, the precursor cells expand its size (hypertrophy) and number (hyperplasia) (Silva et al., 2005b). As previous mentioned, phenolics act as non-enzymatic antioxidants in cells under oxidative stress, and its presence suggests that this region was probably under severe stress, which culminated in cell disruption and death.

Visible symptoms such as chlorosis and necrosis are common in plants under copper stress (Shabbir et al., 2020; Kumar et al., 2021). However, even when not visible, micromorphological analysis can reveal many copper-induced alterations on leaf surface (Shang et al., 2024). In our study, even though no macroscopic symptoms were observed in leaves of *B. orellana*, 100 and 400 mg/kg treatments presented more severe alterations, while 200 mg/kg treatment exhibited subtler changes. Epidermal grooves were observed in all treatments, which could indicate mechanical weakening due to oxidative stress, leading to the observed constraints in the leaf surface. For 100 and 400 mg/kg the findings align with the observed anatomical damage, with plants exhibiting localized epidermal rupture with tissue exposure and cell shape alteration. These results confirm the presence of necrotic regions in 100 and 400 mg/kg treatments due to copper-induced oxidative stress. At the 200 mg/kg treatment, although the presence of epidermal grooves could indicate a mild stress level, plants were able to cope with it and prevent its progression to more severe damage. These findings

reinforce the prognostic value of anatomical and micromorphological analysis even in the absence of visual symptoms under abiotic stress.

## CONCLUSION

This study demonstrated that *Bixa orellana* exhibits limited copper accumulation, with most of it retained in roots and relatively low translocation to shoots, indicating a low phytoextraction potential. Although no changes were observed in biomass production, lipid peroxidation and in the antioxidative system enzymes, increased pigment content suggest that copper was capable of inducing metabolic responses in the species, which probably possesses other non-enzymatic antioxidative defense strategies. Micromorphological and anatomical changes in leaf tissues such as xylem vessels wall deformation, trichome deterioration, cell collapse, and phenolic accumulation were dose dependent and reinforce the prognostic value of morphoanatomical and micromophological analyses. Our findings suggest that *B. orellana* can be classified as a copper tolerant and non-accumulator species, being a reliable choice for reforestation of copper contaminated sites.

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# Co-Expression of Jojoba Wax Ester Synthase (Sc*WS*) and *Marinobacter aquaeolei* Fatty Acyl-CoA Reductase (Ma*FAR*) genes in Gray Poplar: initial characterization of transgenic lines under drought stress

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## ABSTRACT

Drought stress significantly affects plant productivity. Enhacing wax biosynthesis is a key strategy for enhancing drought tolerance in plants. This study provides a first characterization of *Populus canescens* overexpressing Sc*WS* and Ma*FAR* genes from the wax biosynthesis pathway. Transgenic lines and wild type were grown under well-watered and drought conditions for 15 days under greenhouse conditions. Plant height and diameter and gas exchange parameters were measured weekly; whole plant leaf area, fresh and dry biomass assessments were performed at the end of the experiment. The transgenic lines exhibited generally lower gas exchange parameters even under well-watered conditions; but better water-use efficiency under drought stress. They displayed lower height and diameter, but it did not reflect on final dry biomass. The results indicate a trade-off between greater water efficiency probably due to the enhanced wax biosynthesis, but loss in growth parameters such as height and diameter. Our study highlights wax biosynthesis enhancement as a viable mechanism for improving drought tolerance, but at the cost of reductions in growth parameters.

Keywords: Gray Poplar; Drought Stress; Sc*WS*; Ma*FAR*; Wax Biosynthesis

## INTRODUCTION

Climate change is defined as long-term critical shifts in weather patterns such as rainfall, temperature, wind and snow measurements (Bibi and Rahman, 2023; NASA, 2025). Such changes can be accentuated by natural processes but are mainly caused by global warming and greenhouse gases (GHG) emissions (Bibi and Rahman, 2023). Due to such factors, extreme weather events such as heat waves, heavy rainfalls and drought had become more frequent every year (Clarke et al., 2022). These changes have a significant impact in agriculture and many studies have shown that crop production has decreased over the years due to prolonged extreme weather events (Yang et al., 2024; Mirón et al., 2023).

Drought events are expected to increase in frequency, duration and intensity in future years (Rodell and Li, 2023). These events can lead to considerable disturbances in terrestrial ecosystems, with permanent effects on its structure and functionality over time (Müller and Bahn, 2022). In plants, drought is one of the major limiting elements for growth and biomass production, as it can inhibit essential processes such as photosynthesis, respiration and stomatal movement (Yang et al., 2021). Water scarcity conditions can induce many morphological, physiological, biochemical and molecular responses in plants (Hura et al., 2022; Seleiman et al., 2021). The development of strategies to improve plant performance facing such changes are of crucial importance (Yang et al., 2024).

Plants have developed many strategies to cope with biotic and abiotic stress. One key evolutionary innovation is the cuticle, which is an extracellular hydrophobic layer that covers all above-ground organs of land plants, acting as a primary barrier against environmental threats (Lewandowska et al., 2020; Xue et al., 2017). Besides restricting non-stomatal water loss, the cuticle also works as a protective layer against intense UV radiation, desiccation, pathogens and insects (Lewandowska et al., 2020; Wang et al., 2020; Xue et al., 2017). The cuticle is formed by a cutin polyester matrix and intra and epicuticular waxes, which are formed by a mixture of very-long-chain fatty acids (VLCs) and its derivatives such as aldehydes, alkanes, ketones, primary and secondary alcohols and wax esters (Lewandowska et al., 2020; Lee and Suh, 2015). Cuticular waxes composition can vary within species, plant organ, developmental stage and in response to changing environmental conditions (Grünhofer et al., 2022; Lewandowska et al., 2020; Kosma et al., 2010).

Wax biosynthesis occurs in epidermal cells, and it is a multi-step pathway involving numerous genes. It begins with the formation of VLCs, which are subsequently converted into

various wax components and transported to the outer surface (Lewandowska et l., 2020; Tafolla-Arellano et al. 2018). Two enzymes play key roles in this pathway: the Fatty acyl-CoA reductase3 (FAR) reduces the VLCs into primary alcohols that can be released to the outer layer; and the Wax ester synthase/acyl-CoA: diacylglycerol acyltransferase 1 (WSD1), which is a bifunctional enzyme that transforms primary alcohols or VLCs into wax esters that can also be released from cells (Domergue and Miklaszewska 2022; Lewandowska et al. 2020).

The overexpression of genes codifying for these two proteins combined has been widely used in genetic engineering to achieve a greater production of wax esters. The combination of the Jojoba (*Simmondsia chinensis*) wax ester synthase (Sc*WS*) and *Marinobacter aquaeolei* fatty acyl-CoA reductase (Ma*FAR*) in plants has shown a significant increase in the production of wax esters in seeds of *Arabidopsis thaliana* and *Camelina sativa* (Yu et al., 2018; Iven et al. 2016; Iven et al. 2013). However, little is known about how overexpression of these two proteins together affects the production of wax esters in leaves and how it affects plant drought stress tolerance.

Poplar (*Populus* spp.) is a widely studied crop that corresponds to 31.4 million hectares of world coverage. It has a high value for its woody biomass production, and it is considered a model organism for tree physiology and genomics (Rosso et al., 2023). It is well known that one of poplar's responses to drought stress is the enhanced wax biosynthesis (Rosso et al. 2023; Song et al. 2022; Meng et al., 2019). We tested the hypothesis that enhancing cuticular wax biosynthesis in *Populus canescens* through overexpression of Sc*WS* and Ma*FAR* genes improves drought tolerance by modulating physiological and growth responses. Therefore, the primary objective of this study was to characterize the responses of different *Populus canescens* transgenic lines, which overexpress both the Sc*WS* and Ma*FAR* genes, to drought stress conditions.

## MATERIAL AND METHODS

### Double mutant lines obtention

The plants were obtained from a collection of genetically modified lines overexpressing Sc*WS* and Ma*FAR* genes created at the Forest Botany and Tree Physiology Department from the Georg-August-Universität Göttingen, Göttingen, Germany, and were propagated *in vitro* through tissue culture using stem cuttings in sterile ½ MS medium (Murashige and Skoog, 1962) with a 16 h light/8 h dark photoperiod until the plantlets were well rooted.

## Greenhouse experiment and Plant Cultivation in Soil

Six- to eight-week-old plants from sterile culture were transplanted into 3 L pots with a soil and sand mixture (2 parts (*v/v*) N-type soil [Fruhstorfer Erde Type N, Hawite Gruppe GmBH, Vechta, Germany], 8 parts coarse sand (Ø 0.71–1.25 mm; Melo, Göttingen, Germany), and 2 parts fine sand [Ø 0.4–0.8 mm; Melo, Göttingen, Germany]) (Müller et al., 2013). To acclimate the plants to greenhouse conditions, each plant was initially covered with a small transparent beaker or plastic bag. After two weeks, the covers were gradually removed over the course of one additional week.

Post-acclimation, the plants were grown under temperatures ranging from 18°C to 27°C with an air humidity of 50% to 80%. Additional lighting, providing a photosynthetically active radiation (PAR) of 150 µmol photons $m^{-2}$ $s^{-1}$, was supplied by fluorescent lamps on a 16-hour photoperiod. The acclimated plants were grown for 10 weeks under greenhouse conditions. The double mutant lines used for the experiment (DFS3, DFS4 and DFS7) were selected based on relative gene expression assays. Wild type plants and empty vector control plants were also used, the latter serving as a baseline where the genetic transformation was performed without the introduction of the genes of interest, to ensure any observed effects were specifically due to the mutations.

**Table 1**: List of lines and number of plants used in this study.

| Selected lines | Construct | Name of the line |
|---|---|---|
| WT (*P. x canescens*) | (*Populus tremula* x *Populus alba*; INRA-717-1B4) | WT |
| *p*35S::*Sc*WS+*p*35S::*Ma*FAR | pK7WG+*p*35S::*Sc*WS+pB7WGF2.0+*p*35S::*Ma*FAR | DSF3 |
| *p*35S::*Sc*WS+*p*35S::*Ma*FAR | pK7WG+*p*35S::*Sc*WS+pB7WGF2.0+*p*35S::*Ma*FAR | DSF4 |
| *p*35S::*Sc*WS+*p*35S::*Ma*FAR | pK7WG+*p*35S::*Sc*WS+pB7WGF2.0+*p*35S::*Ma*FAR | DSF7 |
| Empty vector control | pB7WGF2.0+p35S | EV |

## Drought treatment

80 days after potting, plants were submitted to drought stress. For this experiment plants were divided into two groups and one group was subjected to drought withholding the irrigation. The control plants received 400 ml water daily to keep the soil moisture around 0.4 $m^3$ $m^{-3}$. The soil moisture of drought treated plants was kept to 0.1 $m^3$ $m^{-3}$; whenever it dropped below this threshold, plants were irrigated to restore soil moisture to 0.1 $m^3$ $m^{-3}$. Soil moisture

was measured using the HH2 device equipped with the ML2x sensor (Delta-T Devices Ltd., Cambridge, U.K.).

**Plant phenotype characterization**

At the end of the experiment, plants and leaves from all lines and treatments were photographed for phenotype documentation.

**Growth parameters and whole plant leaf area**

Stem diameter was measured at intervals once a week, approximately 1 cm above the soil surface, using a digital caliper (Tchibo GmbH, Hamburg, Germany). Stem height, from the base to the apex, was measured with a folding ruler at intervals of two to three days.

At the end of the experiment, three fresh leaves from the bottom, middle, and top of the plants were weighed and photographed using digital camera (Sony α6400, Tokyo, Japan). The digital images were uploaded in the software Image J (https://imagej.net/ImageJ) and used to determine the area per leaf. Whole plant leaf area was calculated as:

$$\frac{\text{Total leaves fresh weight (g) x leaf area of scanned leaves (cm}^2\text{)}}{\text{Fresh weight of scanned leaves (g)}}$$

**Gas exchange measurements**

For gas exchange measurements, leaves were numbered sequentially from the top of each plant. To maintain consistency in leaf position and age, measurements were taken from the same leaf position on each plant leaf number 7. Five biological replicates per measurement were included. Gas exchange measurements during the day were conducted between 08:30 am and 01:00 pm. Measurements were conducted using a multiphase Flash™ Fluorometer (LI-6800, LI-COR, Lincoln, USA). Following the attachment of the chamber to the leaf, an acclimation period of approximately 30 to 40 seconds was allowed. Subsequently, three replicate measurements were performed within a three-minute timeframe. The temperature set into 23-25 °C for day and 18°C at night, the humidity was 60-70% and the ambient $CO_2$ concentration was 400 µmol mol-¹. For the measurements, the light intensity was maintained at 800 µE $m^{-2}$ $s^{-1}$. Water use efficiency (WUE) was calculated using the following equation:

$$\text{WUE} = \frac{\text{A (Net Photosynthesis)}}{\text{E (Net Transpiration)}}$$

**Biomass assessments**

Plants were harvested at the end of the experiment. The stem was cut at the soil surface, the leaves were cut, and the roots were washed by rinsing them in tap water. All fractions were

immediately weighed to determine the total fresh biomass of each tissue. The fractions were dried for one week at 60°C and weighed to determine the total dry biomass of each tissue.

### Statistical analyses

We tested data normality and then used two-way analysis of variance (ANOVA) to test for significant effects among the lines at $p < 0.05$. The *post-hoc* Tukey test was applied to determine significant differences between means at $p < 0.05$. For all measurements and statistical analysis, the number of individual biological replicates was 4-5 replicates per line per treatment. Data are shown as mean value (± SE). The software Origin 2020 (OriginLab Corporation, Northampton, Massachusetts, USA) was used for statistical analysis.

## RESULTS

### Drought treatment

The experiment started with all plants under soil moisture of around 0.4 $m^3m^3$. Drought treated plants consumed the water from soil by day 10, when pretty much all lines reached the minimum of 0.1 $m^3m^3$, which was kept until the end of the experiment. Soil moisture of well-irrigated plant was kept at around 0.4 for the whole period. The variations of soil moisture for all lines and treatments over time are represented in Figure 1.

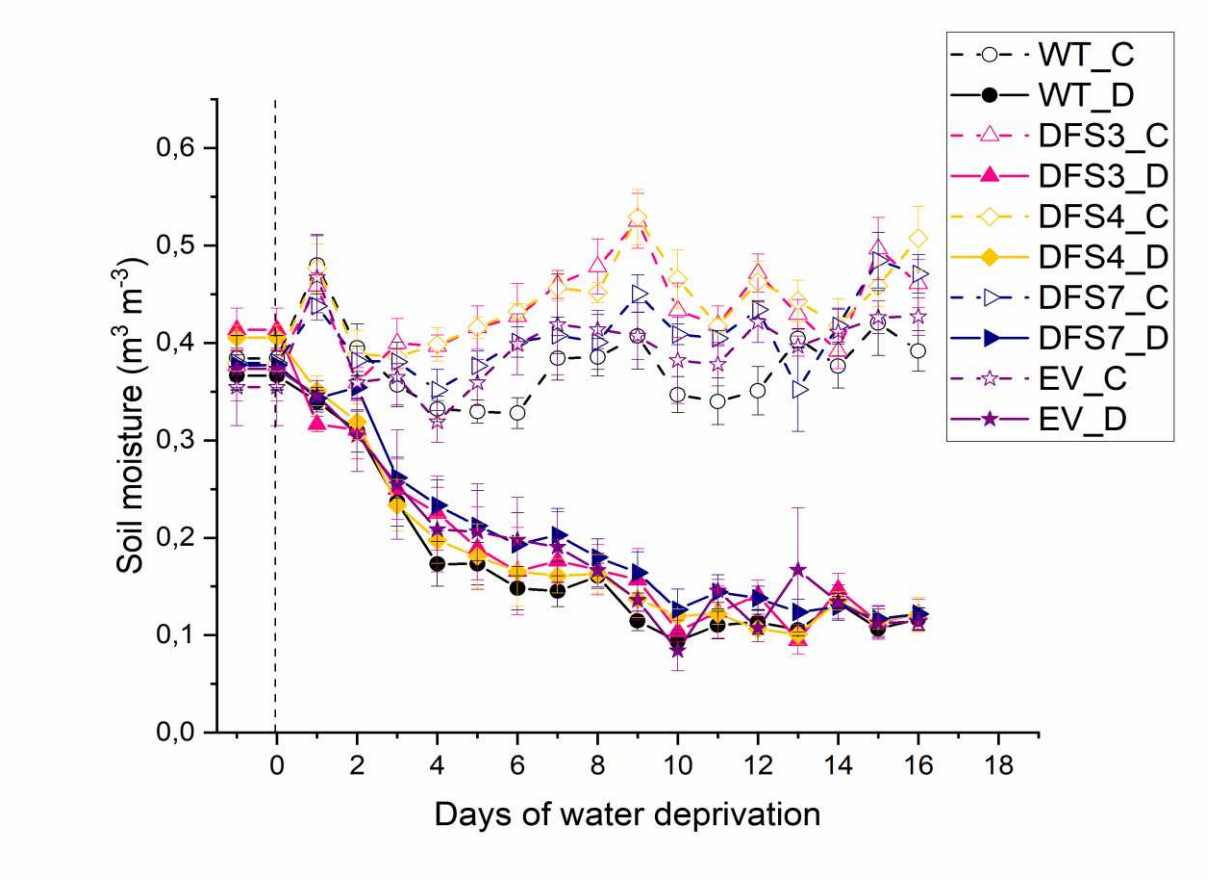


**Figure 1**: Soil moisture ($m^3m^{-3}$) of all lines and treatments over the experiment time. Data are the means (± SE). WT – Wild type; EV – Empty Vector control; DFS3, DFS4 and DFS7 – Double mutant lines. C – Control; D – Drought.

**Plant phenotype characterization**

Under drought conditions, all lines, including wild type and empty vector plants, exhibited leaf desiccation, with many individuals undergoing leaf abscission. However, under well-irrigated conditions, the wild type and empty vector plants displayed a taller and more robust phenotype, characterized by well-developed, broader, and greener leaves. In contrast, the mutant lines demonstrated a stunted growth habit, with smaller plants bearing narrower, yellowish leaves. Plant phenotype is shown in Figures 2 and 3.

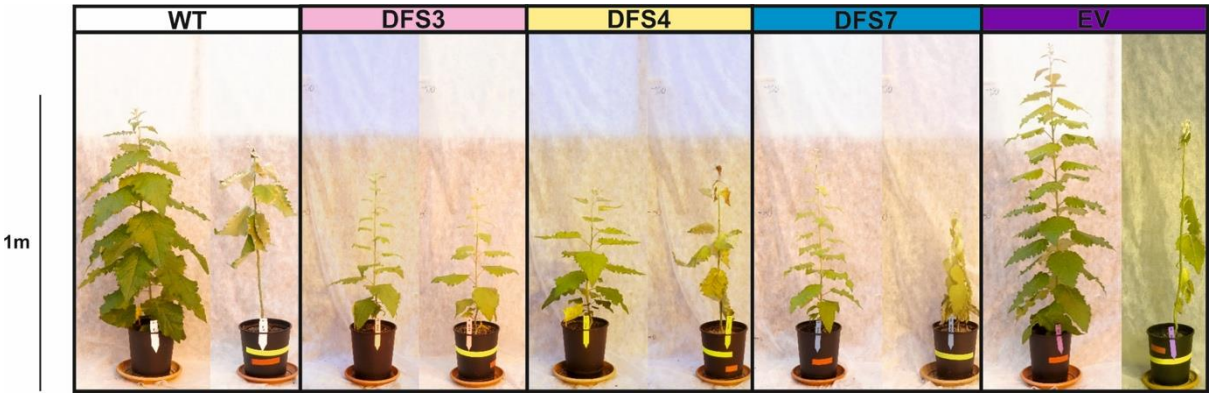


**Figure 2**: Plant phenotype before harvesting. Left – Well-irrigated plants; Right – Drought-treated plants. WT – Wild type; EV – Empty vector control; DFS3, DFS4 and DFS7 – Double mutant lines.

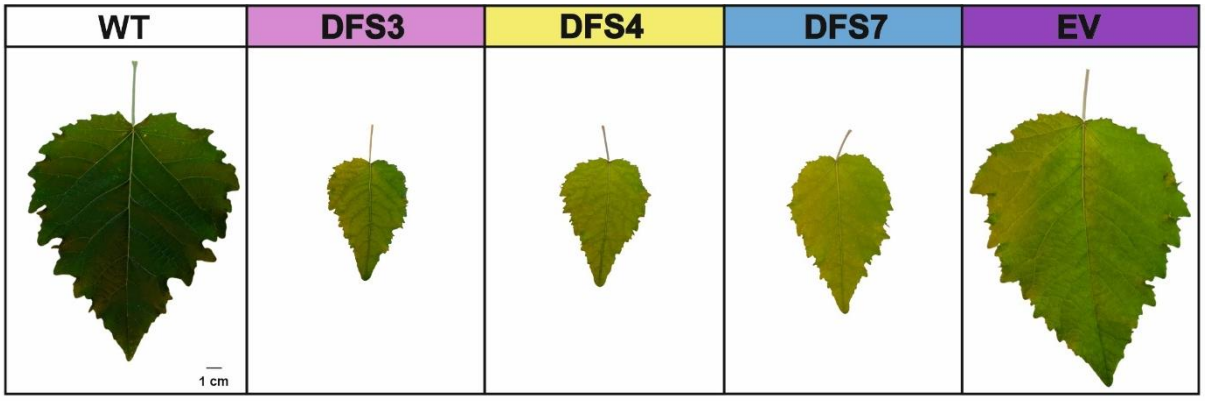


**Figure 3**: Well-irrigated plants – Fully developed leaves. WT – Wild type; EV – Empty vector control; DFS3, DFS4 and DFS7 – Double mutant lines.

**Growth parameters and whole plant leaf area**

In water-limited conditions, no significant differences were observed in height and diameters when comparing all lines. On the other hand, under well-irrigated conditions, wild type and empty vector control showed a greater increase in height and diameter over the time, as shown in Figure 4.

Among treatments, wild type, empty vector control, and DFS3 exhibited significantly larger whole plant leaf area in well-irrigated conditions compared to drought-treated plants. Within the lines, well-irrigated DFS4 plants displayed lower values in comparison to all other lines (except DFS7). The whole plant leaf area is shown in Figure 4a and b.

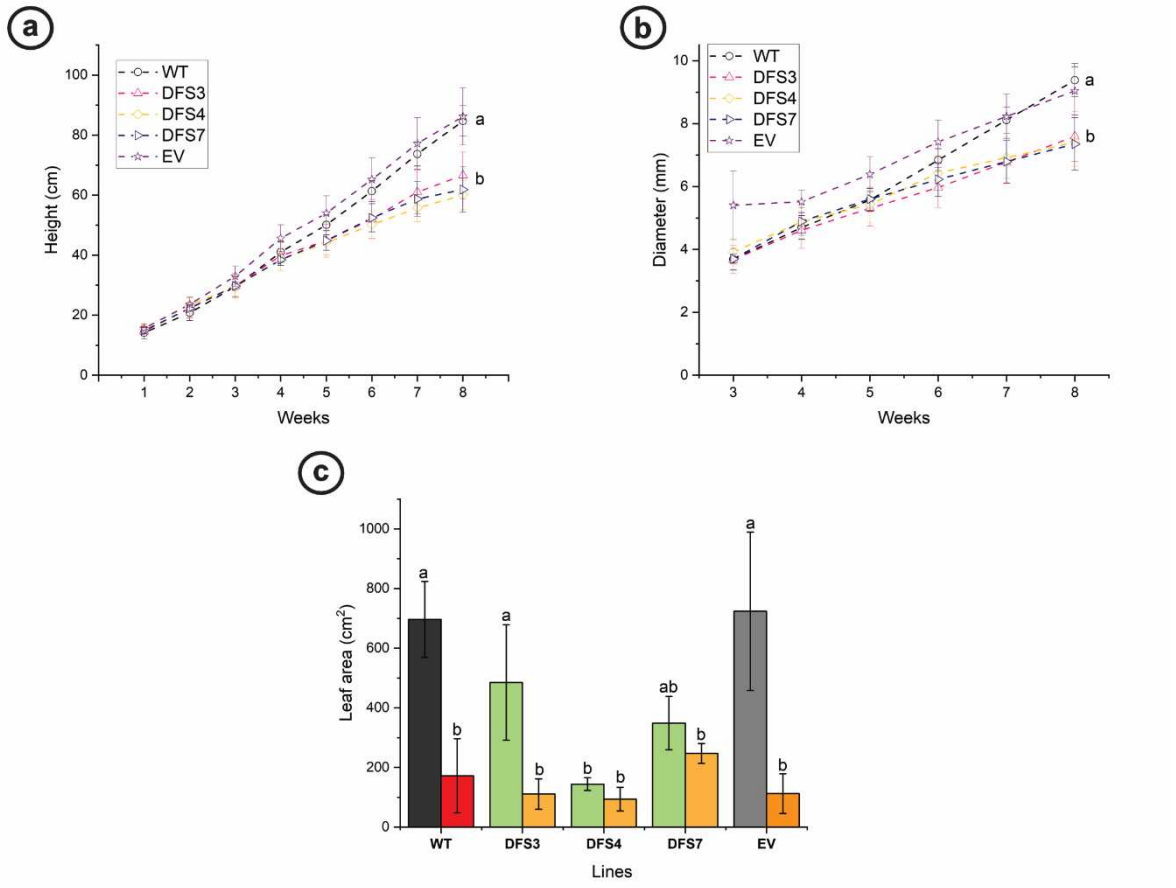


**Figure 4**: Height, Diameter and Whole plant leaf area measurements of *Populus canescens*. A – Height of well-irrigated plants from all lines over the weeks; B – Diameter of the stem of well-irrigated plants from all lines over the weeks; C – Whole plant leaf area of well-irrigated plants (left) and drought-treated plants (right) after 16 days of drought. Data are the means (± SE). Different letters indicate significant differences between lines and treatments (for A and B at the last measurement). WT: Wild type; EV: Empty vector control; DFS3, DFS4 and DFS7: Double mutant lines.

## Gas exchange measurements

### Stomatal conductance

No statistically significant differences were observed in stomatal conductance between well-irrigated and drought-treated plants across all double-mutant lines. However, in wild type plants and empty vector controls, well-irrigated conditions resulted in significantly higher

stomatal conductance compared to drought-treated plants and the double-mutant lines. Stomatal conductance for all lines is shown in Figure 5.

**Photosynthesis**

No significant differences were detected among treatments within any of the lines. However, well-irrigated wild type and empty vector plants exhibited statistically significant differences in comparison to all double-mutant lines. Photosynthesis for all lines is shown in Figure 5.

**Transpiration**

No significant differences were observed across treatments within any of the double-mutant lines. Well-irrigated wild type plants exhibited higher transpiration rates compared to the drought-treated plants. Additionally, wild type and empty vector control plants showed significantly greater transpiration rates under well-irrigated conditions compared to the double-mutant lines. Transpiration for all lines is shown in Figure 5.

**Water use efficiency (WUE)**

No significant differences were observed across treatments within any of the double-mutant lines. Drought treated wild type plants exhibited greater water use efficiency (WUE) than in control. Among lines, the double mutants showed greater water use efficiency under well-irrigated conditions when compared to wild type and empty vector, but under drought conditions, only in relation to the empty vector. The values for WUE are shown in Figure 5.

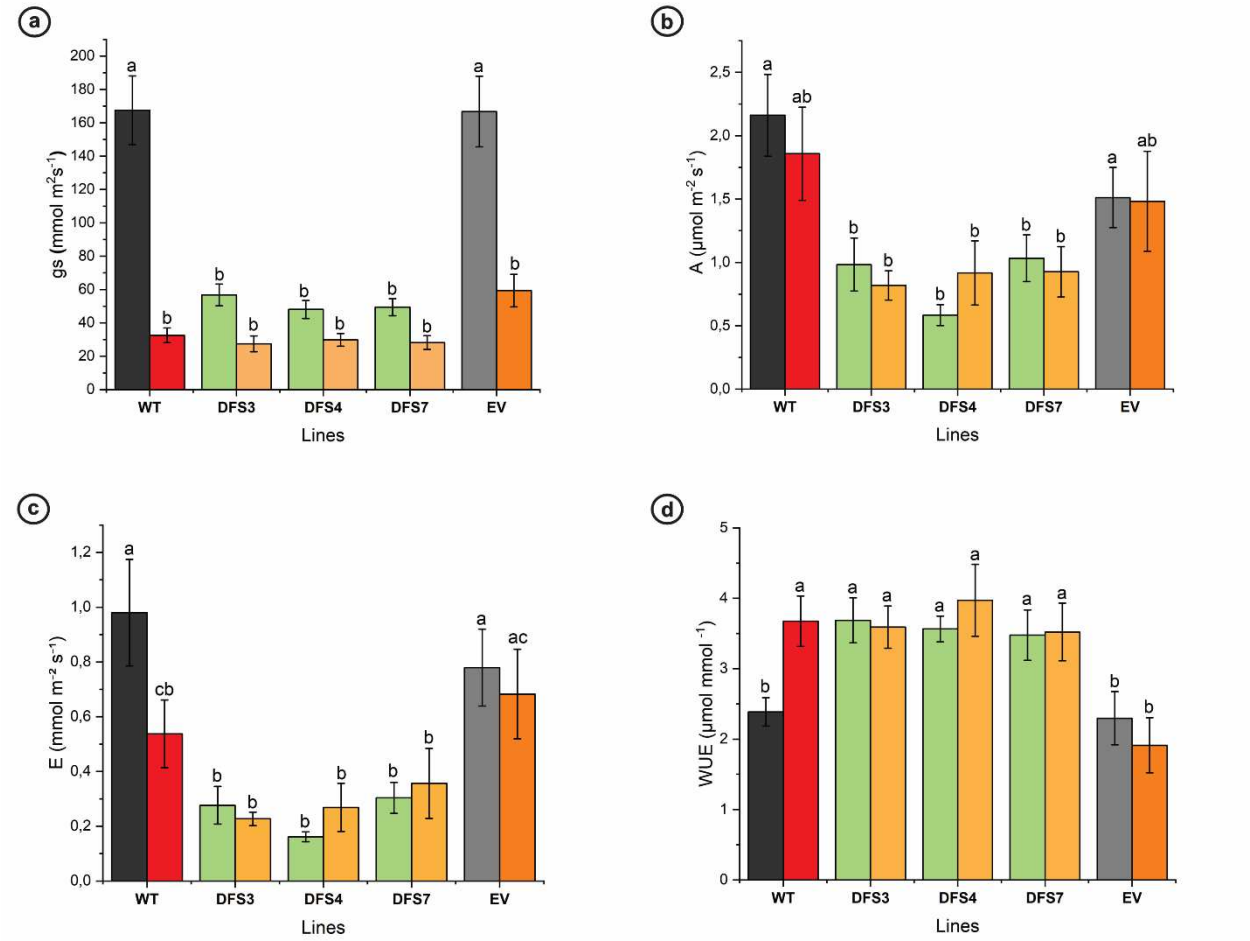


**Figure 5**: Gas exchange parameters of *Populus canescens* exposed to 16 days of drought. Stomata conductance (gs), Photosynthesis (A), Transpiration (E) and Water use efficiency (WUE) for well-irrigated plants (left) and drought-treated plants (right). A – Stomata conductance; B – Photosynthesis; C – Transpiration; D – Water use efficiency. WT – Wild type; EV – Empty vector control; DFS3, DFS4 and DFS7 – Double mutant lines. Data are the means (± SE). Different letters indicate significant differences between lines and treatments.

**Biomass assessments**

For fresh biomass, significant differences were observed only between treatments in the wild type and empty vector groups, for leaves and roots. In contrast, no significant differences were detected among the transgenic lines under any treatment or organ. For dry biomass, no significant differences were observed among any organs of different lines or treatments. Fresh and dry biomass are shown in Figure 6.

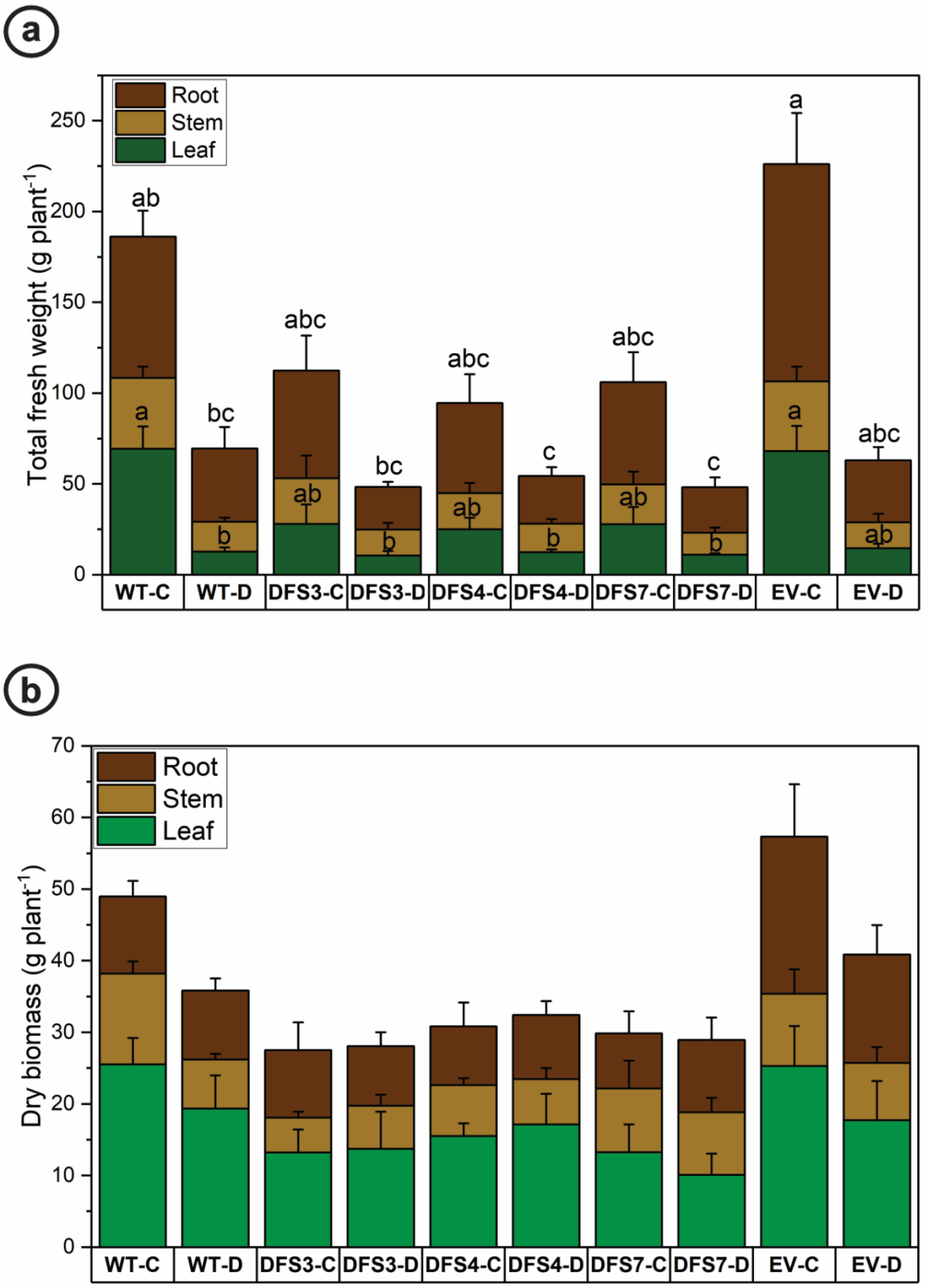


**Figure 6**: Total fresh and dry weight (g) per organ for well-irrigated plants (left) and drought-treated plants (right) for all lines of *Populus canescens* exposed to 16 days of drought. A –

Fresh weight; B – Dry weight. WT – Wild type; EV –Empty vector control; DFS3, DFS4, DFS7 – Double mutant lines. C: Control (well-irrigated conditions); D: drought. Data are the means (± SE). Different letters indicate significant differences between lines and treatments.

## DISCUSSION

This study demonstrates that the overexpression of Sc*WS* and Ma*FAR* genes, both involved in the wax biosynthesis pathway, alters significantly leaf morphology and plant growth in Poplar plants, evidencing metabolic trade-offs associated with cuticle enhancement. Genetic modification appears to confer some degree of stress resilience, but it also leads to trade-offs in some growth parameters. Smaller structures and reduced gas exchange rates suggest a conservative growth strategy that minimizes water loss and stress damage. In contrast, wild type and empty vector plants showed a more robust growth pattern, indicating that their unmodified genetic background allows for greater integration of growth and stress response, but being also more vulnerable to prolonged or severe stress in the long term.

Across all lines, drought-treated plants displayed typical water stress symptoms, including dehydration and substantial leaf shedding. Leaf color can be directly correlated to the amount of pigments and secondary metabolites present in the structure (Zhang et al. 2020; Fine et al. 2021). Wild type and empty vector plants appear to direct resource allocation to chlorophyll production resulting in greener leaves. Besides the modified wax production, mutant lines also probably produce secondary metabolites that help mitigate stress and also retain water. Overexpression of the wax biosynthesis pathway genes can be linked to a higher expression of secondary metabolites genes and its regulators, resulting in yellowish leaves (Zhou et al. 2018; Liu et al., 2023).

The overexpression of *Ma*FAR genes probably leads to a higher production of primary alcohols, an intermediate of the wax biosynthesis pathway. In yeast, the overproduction of fatty alcohols by the overexpression of FAR enzymes led to their secretion, which indicates that excess fatty alcohols may exert toxic effects on cellular homeostasis or require compartmentalization within specific organelles to prevent metabolic disturbances (Rowland and Domergue, 2012). The accumulation of such compounds can disrupt cell homeostasis by the interaction of fatty alcohols with the cell membrane, compromising its fluidity and cell integrity (Krishnan et al., 2020). In plants, such disruptions can affect critical physiological

processes, leading to stunted growth and development, characterized by reduced leaf size, decreased plant height, and smaller stem diameters, as observed in the mutant lines.

The wax biosynthesis pathway is known to be highly correlated to stomatal development, and it can affect ridge formation, number, shape and distribution of stomata (Tang et al., 2020; Wang and Cheng, 2024). The overexpression of *ScWS* in poplars altered stomata development, leading to the formation of closed or semi-closed stomata (Amirkhosravi et al., 2025). In *Arabidopsis*, the overexpression of genes of the wax biosynthesis pathway conferred greater drought tolerance to plants by reducing stomata number (Yang et al., 2011). In this experiment, the same probably happened to the mutant lines, once they showed much lower stomatal conductance under both conditions when compared to the wild type and empty vector. Around 90% of aerial water loss is due to transpiration (Seo and Park, 2011). Non-developed stomata and higher wax production can both be the reasons the double mutant lines presented lower transpiration rates even under well-watered conditions. As stomata are also the entry way for $CO_2$, one of the limiting substrates for photosynthesis, it is also expected that the mutant lines would also present lower photosynthesis rates when compared to the wild type and empty vector plants. The present study confirms this correlation between stomatal conductance, transpiration and photosynthesis rates.

Under drought conditions, wild type plants reduced water loss by decreasing transpiration while keeping stable photosynthetic rates. Consequently, they exhibited greater water use efficiency (WUE) compared to well-watered plants, highlighting their physiological plasticity in response to water-limiting conditions. Similar results were already documented for wild type poplar species under drought conditions, and the relatively high photosynthesis rates under drought even with lower stomatal conductance could be explained by an increase in rubisco efficiency, higher chlorophyll production or tighter stomatal regulation (Cirelli et al., 2015; Wang et al. 2015; Meng et al., 2019).

Under water-limiting conditions, the cuticle serves as the primary barrier regulating non-stomatal water loss in plants (Hasanuzzaman et al., 2023). In some cases, cuticular transpiration can account for up to 69% of total water loss in plants (David 2010; Yan and Castellarin, 2022). It is also known that the increase in wax production, modification in its composition and, specifically, enhanced wax esters production can increase cuticle impermeability, improving the water use efficiency (WUE) in different plant species (Abdullah et al. 2021; Wang et al., 2021; Grünhofer et al., 2024). In this study, all double mutant lines showed similar WUE under both conditions, and improved performance under well-watered conditions compared to the

wild type. This indicates that the modification in cuticular load or composition in the double mutants was able to successfully reduce cuticular transpiration and maintain essential metabolic processes under drought and well-watered conditions.

Leaf shedding is a key adaptive mechanism that minimizes water loss during drought stress, as it quickly reduces the total leaf surface area available for transpiration (Brodribb et al. 2020). Such avoidance strategy is very common in poplar species (Polle et al., 2019; Rosso et al., 2023). In response to drought, after reducing stomatal conductance, wild type plants started to lose water mainly via cuticular transpiration. As a way of reducing water loss, they shed their leaves, reducing the leaf area available for water loss. Most of the double mutant lines presented already smaller leaves even under well-watered conditions, which in addition to cuticle modification probably prevented plants from hydraulic failure during drought, maintaining their leaves for longer. Another reason for the reduction of leaf area in wild type plants is the reduction in relative water content, a commonly used stress marker, that triggers the increase of abscisic acid (ABA), which is the main responsible for the adjustment of the metabolism to drought (Bandurska., 2022). ABA is a key phytohormone in stress response, especially drought and, besides leaf shedding, it is also responsible for rearranging physiological and biochemical signal transduction cascades, biomolecules biosynthesis, seed germination, stomatal closure, root architecture modification and transcriptional and post-transcriptional regulation of stress-responsive gene expression (Ali et al., 2020; Muhammad Aslam et al., 2022). The probable lower ABA activation in the mutant lines could indicate their lower stress levels under drought conditions compared to the wild type.

As the wild type plants are more susceptible to water loss but have a highly plastic stress-response mechanism, a subtle but not significant reduction in dry biomass allocation is expected under drought stress. However, the mutant lines displayed very similar dry biomass values, with no difference between treatments. This, aligned with growth parameters such as height and diameter, could be indicators of different structural investments between the wild type and the mutant lines. Wild type plants probably invest in faster growth, with high rates of highly hydrated tissues such as parenchyma and collenchyma, which, for maintaining turgor, accumulate non-structural carbohydrates (NSCs), which do not account for final dry biomass. It is known that parenchyma is the tissue with the higher rates of NSCs and that such compounds play key roles in osmoregulation (Tomasella et al. 2019; Li et al., 2022; Ferraro et al., 2023). High water availability leads to its accumulation especially in leaves and roots, which are parenchyma-rich tissues, as it is shown by the fresh biomass rates. On the other hand, even

under well-watered conditions, the mutant lines are not able to retain as much water as the wild type plants, as evidenced by their fresh biomass values. This indicates that the mutant lines probably invest in a more “compact” growth, with waxy leaves, thicker cell walls and more robust tissues, which reflects in their smaller size and diameter. Anatomical analyses of different organs, micromorphology of the leaf surface and epicuticular wax composition analysis would be of great importance to the understanding of how the trade-offs occur in these lines.

## CONCLUSION

The transgenic lines exhibited enhanced drought tolerance by maintaining physiological function under water-limited conditions, despite generally lower gas exchange rates compared to wild-type plants, even under well-watered conditions. While these modifications were associated with reduced growth parameters and fresh biomass, they provided an advantage under water-limited conditions, confirming our hypothesis. These findings highlight the potential of cuticular and morphological modifications as a strategy for improving drought resilience in crops, although the trade-offs in growth and productivity must be carefully considered for practical applications.

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## FINAL CONCLUSIONS

Our findings confirm that *Bixa orellana* is not a copper hyperaccumulator species, since it did not accumulate high levels of the metal in any of its tissues even under relatively high concentrations of copper in soil. The species present and increase on pigment content under increasing copper concentrations in soil, and under the different copper treatments it exhibited morphoanatomical and micromorphological alterations that can be used as prognostic markers for impending copper stress. Our data suggest that *B. orellana* possess strategies that allows the species to survive and develop in copper contaminated soils.

For *Populus* × *canescens*, the overexpression of Sc*WS* and Ma*FAR*, genes involved in the wax biosynthesis pathway, was capable of enhancing drought tolerance in the species. However, in general, growth and gas exchange parameters were significantly compromised probably as a result of trade-offs.