Samanta de Oliveira Pereira

Study of the Physiological, Molecular and Biochemical Mechanisms in *Populus alba* L. "Villafranca" clone under salinity stress

Universidade do Algarve

Faculdade de Ciências e Tecnologia

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Master in Biotechnology

Supervised by

Profº Dr. Luca Sebastiani – Institute of crop science, Scuola Superiore Sant'Anna

Profª Dr. Anabela Romano – Mediterranean Institute for Agriculture, Universidade do Algarve

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Declaração de autoria | Declaration of authorship of work

Study of the Physiological, Molecular and Biochemical Mechanisms in *Populus alba L.* "Villafranca" clone under salinity stress

Declaro ser a autora deste trabalho, que é original e inédito. Os autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

I declare to be the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and are included in the list of references.

Samanta de Oliveira Pereira

Acknowledgments

First and foremost, I would like to express my gratitude to God, my source of strength and motivation.

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Obrigada! Grazie! شكرًا! Thank you!

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List of abbreviations

CDS	Coding DNA sequence
DEPC H_2O	Diethylpyrocarbonate treated water
GPX	Glutathione peroxidase
GPX1	Glutathione peroxidase 1 gene
GSH	Glutathione
GSH1 / γ-ECS	Gamma-glutamylcysteine synthetase
GSH1	Gamma-glutamylcysteine synthetase gene
GST	Glutathione-s-transferase
GSTF1	Glutathione-s-transferases Phi 1 gene
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
ROS	Reactive oxygen species

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Abstract

The present work aimed to characterize the morphological, physiological, biochemical, and molecular responses of the *Populus alba* L. "Villafranca" clone to salt stress. To analyze the effects of salt stress, plants were treated for one month with different concentrations of sodium chloride (NaCl): 0 mM (control), 60 mM, 120 mM, and 180 mM NaCl.

Morphologically, no significant changes were observed in plant length. However, a decrease in the fresh weight of all organs was noted as salinity levels increased. Symptoms of chlorosis were observed in groups treated with 120 mM and 180 mM NaCl. Physiologically, decreases were observed in all analyzed parameters across all groups, with the 180 mM NaCl group being the most affected. Stomatal conductance (G_s) and net photosynthetic rate (P_n) in the 180 mM NaCl group decreased by 81.4 % and 69.2 %, respectively. Chlorophyll and flavonol content decreased by 40.4 % and 29.4 %, respectively, in the 180 mM NaCl group.

Biochemically, proline concentration and mineral element absorption in leaves and roots were quantified. The highest proline concentration was in the 120 mM NaCl treatment (43.2 ± 9.36 mg/g FW), while the 180 mM group had the lowest content (14.5 ± 7.47 mg/g FW). In basal leaves, the group treated with 60 mM NaCl showed the highest proline concentration (8.8 ± 0.68 mg/g FW). Sodium concentration increased in all organs, with the highest concentration in the 180 mM group. The potassium concentration was changed only in roots, where there was a decrease in the 120 and 180 mM groups. Unlike calcium, where there were changes only in the leaves and not in the roots. There was an increase in calcium concentration as salt stress increased, reaching its peak in the 180 mM NaCl group.

At the molecular level, the expression of three genes from the glutathione pathway was analyzed: *GSTF1*, *GPX1*, and *GSH1*. Only *GSTF1* expression changed under salt stress, increasing by 162.5 % in basal leaves and 179.6 % in roots. *GPX1* did not change during stress but was more abundant in leaves. *GSH1* was present in similar concentrations in both organs.

Keywords: *Populus alba*; NaCl stress; salt response; glutathione pathway

Resumo

Nos últimos anos, os solos salinos emergiram como um dos principais desafios enfrentados pela agricultura global, representando uma ameaça significativa à produtividade das plantas e à segurança alimentar global. O excesso de sais iônicos, o baixo potencial hídrico e o conteúdo reduzido de nutrientes em solo salino representam desafios significativos para o crescimento e desenvolvimento das plantas (Zheng *et al*., 2023). Embora a salinização do solo seja um processo natural, resultante de sais solúveis na precipitação e mecanismos acionados pelo vento, atividades humanas como a irrigação com água salina e o uso excessivo de fertilizantes aceleram esse processo (Li *et al*., 2014; Hassani *et al*., 2021). A atual magnitude global do problema é alarmante, a FAO (2023) informou que mais de 1100 milhões de hectares de solo são afetados pela salinidade. Além disso, espera-se que este problema aumente devido aos impactos contínuos das alterações climáticas (Kibria e Hoque, 2019).

Neste contexto, o presente trabalho teve como objetivo caracterizar as respostas morfológicas, fisiológicas, bioquímicas e moleculares de *Populus alba* L. "Villafranca" clone ao stress salino. *Populus* é uma espécie modelo no estudo de árvores e também é de grande importância ecológica e económica, e por isso seu estudo é de muita importância. Para analisar os efeitos do stress salino em *P. alba* "Villafranca" clone, as plantas foram tratadas por um mês com diferentes concentrações de cloreto de sódio (NaCl), 0 mM (controlo), 60 mM, 120 mM e 180 mM NaCl. Após o tratamento as plantas foram separadas em, folhas basais e apicais, caule basal e apical e raízes, para que fosse possível analisar não só o efeito em diferentes órgãos, mas também a nível de translocação.

Referente as medições morfológicas, não foram observadas mudanças significativas no comprimento das plantas. Entretanto, no peso fresco de todos os órgãos é possível observar uma diminuição conforme aumenta o grau de salinidade. Ainda foram notados sintomas de cloroses nos grupos tratados com 120 mM e 180 mM NaCl. Fisiologicamente, foram observadas diminuições em todos os parâmetros analisados, e em todos os grupos, e como esperado o grupo mais afetado foi o tratado com 180 mM NaCl. Os valores de condutância estomática (G_s) e taxa fotossintética líquida (P_n) do grupo tratado com 180 mM NaCl diminuíram em 81,4 e 69,2 %, respetivamente, em comparação com o primeiro dia de experimento. Relativamente ao teor de clorofila e flavonol a diminuição foi de 40,4 e 29,4 %, respetivamente, no grupo 180 mM NaCl.

Para analisar as mudanças a nível bioquímico, foi quantificado a concentração de prolina e a absorção de elementos minerais nas folhas e raízes. A concentração de prolina atingiu o maior nível no tratamento com 120 mM NaCl (43,2 ± 9,36 mg/g FW), enquanto o grupo tratado com 180 mM resultou no menor teor (14,6 ± 7,47 mg/g FW). Enquanto nas folhas basais, o grupo tratado com 60 mM NaCl é o que apresenta maior concentração de prolina (8,8 ± 0,68 mg/g FW). Nas raízes não foram observadas mudanças significativas. Quanto a absorção de minerais, foi analisada a concentração de sódio, potássio e cálcio. Relativamente ao sódio, houve um aumento em todos os tecidos, e como esperado o grupo com maior concentração foi o tratado com 180 mM. A concentração de potássio foi alterada apenas raízes, onde houve uma diminuição nos grupos 120 e 180 mM. Ao contrário do cálcio, onde houve alterações apenas nas folhas e não nas raízes. Houve um aumento na concentração de cálcio conforme aumentou o stress salino, atingindo seu pico no grupo 180 mM NaCl.

Relativamente aos mecanismos moleculares, foi analisada a expressão de três genes pertencentes a via da glutationa, glutationa s-transferase F1 (*GSTF1*), glutationa peroxidase 1 (*GPX1*) e gama-glutamilcisteína sintetase (*GSH1*). Esta análise foi realizada através de um RT-PCR, onde foram analisadas o as folhas basais e raízes do grupo controlo e 180 mM NaCl. Dos três genes, apenas o *GSTF1* sofreu alteração com o stress salino. Ao comparar o grupo 180 mM com o controlo a sua expressão aumentou em 162,5 % nas folhas e 179,6 % nas raízes, durante o stress. Confirmando assim ter a sua expressão regulada em condições de stress. O gene *GPX1* não sofreu alterações durante o stress, mas é possível notar a diferença na sua abundância através dos tecidos, este gene está muito mais presente em folhas do que nas raízes. Enquanto *GSH1* esta presente em concentrações semelhantes em ambos os tecidos.

Em conclusão, *Populus alba* L. "Villafranca" clone demonstrou uma série de adaptações ao estresse salino, destacando-se como uma espécie modelo valiosa para estudos de tolerância ao sal. Esses achados contribuem para uma melhor compreensão dos mecanismos subjacentes à tolerância ao sal em árvores, oferecendo insights que podem ser aplicados no desenvolvimento de práticas agrícolas mais eficientes e sustentáveis.

Palavras-chave: *Populus alba*; NaCl stress; resposta a salinidade; via da glutationa

1. Introduction

1.1. Soil salinization

Soil salinization is one of the main environmental problems that limit the growth and development of plants due to the excessive amounts of ionic salts, low water potential and low nutrient content (Zheng *et al.,* 2023). In the face of accelerating environmental changes, the widespread issue of soil salinization has become a focal point of concern, requiring urgent attention and comprehensive research to mitigate its far-reaching implications on agricultural productivity and ecological stability in the current era (Hassani *et al.,* 2021).

Soil salinization is characterized as the accumulation of soluble salts, such as potassium, magnesium, calcium, chlorides, sulphates, carbonates, bicarbonates, and sodium to the point that soil fertility is severely reduced (European Soil Data Centre - ESDAC, 2008). When it comes to the increase in the concentration of sodium it is referred to as sodicity. This problem has two main causes, which are primary and secondary salinization. Primary salinity results from the increase of salts over long periods of time through natural processes in the soil or groundwater. It is caused by two natural phenomena. The first stems from the breakdown of parent materials that harbor soluble salts due to weathering processes. These processes involve the gradual disintegration of rocks, liberating a variety of soluble salts, predominantly sodium, calcium, and magnesium chlorides, alongside sulfates and carbonates to a lesser degree. The second, is the deposition of oceanic salt transported inland by wind and subsequently deposited through rainfall. These "cyclic salts" predominantly comprise sodium chloride and are carried from the ocean by wind and deposited during precipitation events. In secondary or anthropogenic soil salinization, however, the main sources of salinization are human interventions, such as irrigation with saline water, seawater intrusion due to rising sea levels and excessive use of fertilizers (Li *et al.,* 2014; Hassani *et al.,* 2021). Furthermore, climate change also contributes to increased soil salinization. Climate change leads to an increase in temperatures and the frequency and magnitude of droughts in regions with wetter climates. All these processes make soil salinization a worldwide problem nowadays.

According to FAO (2023) over 1100 million hectares of soil are affected by salinity and sodicity (Fig. 1), resulting in a $ 27.3 billion loss per year and these numbers are expected to increase due to the evolution of climate change and human activities. It is estimated that by 2050 more than 50 % of the arable land in the world will be salinized (Kibria and Hoque, 2019). It is estimated that the world population will reach 9.7 billion

people by the year 2050 (Gu *et al.*, 2021), and with the increase in demand for food due to the global population explosion, it is necessary to develop saline areas for agriculture and to develop salt-tolerant plants. Therefore, it is extremely important to elucidate the mechanisms by which plants respond and adapt to saline stress.

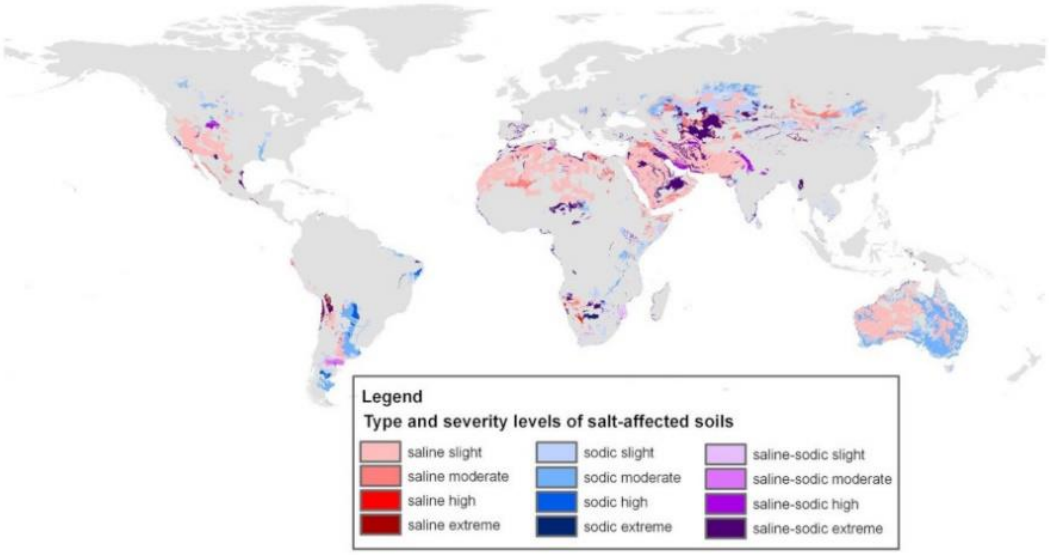

Figure 1. Global distribution of saline, sodic, and saline-sodic soils. Soil salinity level scale, pink being low salinity and red being extreme salinity. Following the soil sodicity scale, light blue is the lowest level and dark blue is the extreme level. And level of saline-sodic soil, with light purple being the lowest and dark purple being extreme (Wicke *et al.*, 2011).

1.2. Effects of salt stress in plants

Salinity is one of the main abiotic stresses that has significantly affected plant growth and productivity. Salt stress occurs due to excessive uptake of ions such as sodium (Na^+) and chloride (Cl^-). This leads to an imbalance in ions, creates osmotic and oxidative stress and negatively affects several aspects of plant growth and development, such as their morphology, physiology, biochemical processes, and gene expression. Ultimately, this damage can be severe enough to cause the death of the plant (Isayenkov and Maathuis, 2019).

One of the first effects of salt stress is the decrease of growth rate. The salt present in the soil inhibits plant growth reducing the plant's ability to absorb water leading to slower growth, which is the osmotic effect of salinity. It can also enter the transpiration stream and eventually damage the transpiring leaf cells, further reducing growth (Parihar *et al.*, 2015). Furthermore, when the plant is under environmental stress, there is an increase in the production of free radicals and other oxidative species. As a consequence, there will be a stimulation of carbon flow from the primary metabolic pathways, which are related to plant growth, to the secondary ones, inducing the synthesis of antioxidant products.

Leading the plant to prioritize responding to stress rather than growth (Borges and Amorim, 2020). All these effects give rise to a two-phase growth response to salinity (Fig. 2).

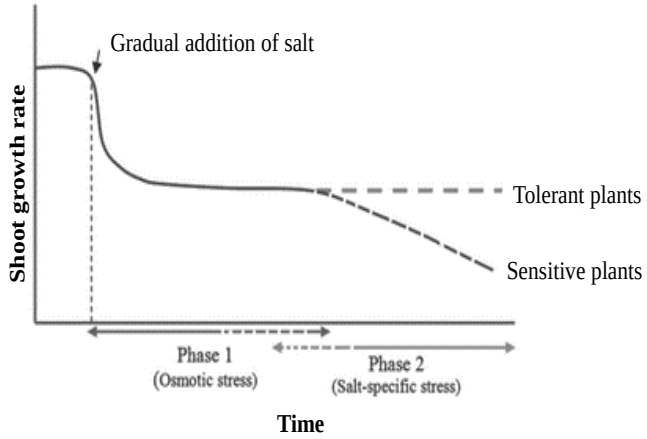

Figure 2. Schematic illustration of the growth response to salinity stress: Phase 1, rapid response to the increase in external osmotic pressure (the osmotic phase), and Phase 2, a slower response due to the accumulation of Na^+ in leave (salt-specific stress). Two-phase growth response differs the genotypes in salt sensitive and tolerant. The time scale is in days to months, depending in the salinity level and the salt tolerance ability (Parihar *et al.,* 2015).

The first phase of the growth response results from the effect of salt outside the plant. It is characterized by reduced growth of leaves and roots. However, at this stage, Na^+ and Cl^- do not accumulate in growing organs/tissues at concentrations that inhibit growth. For the meristematic tissues are largely sustained by the phloem, from which salt is effectively excluded. The second phase of the growth response results from the toxic effect of salt inside the plant. The plant accumulates salt mainly in older leaves, where continued transport to actively transpiring leaves gradually leads to excessively high concentrations of Na^+ and Cl^-, ultimately causing leaf death. This injury results from the salt concentration that exceeds the cells' ability to sequester salts within the vacuoles. Consequently, salts can quickly accumulate in the cytoplasm, impeding enzyme function (Munns, 2005; Parihar *et al*., 2015).

With excessive concentrations of Na^+ or Cl^- in cells and tissues, plant organs cannot function well, causing ionic and nutritional imbalance (Fig. 3). Heightened Na^+ levels typically coincide with reduced potassium (K^+) levels, and it may be critical for tolerance to maintain cytosolic K^+ levels at an acceptable level or to maintain homeostasis. Additionally, the toxic impact of Na^+ might stem from its competition with K^+ for enzymes reliant on potassium, suggesting that the cytoplasmic Na^+ to K^+ ratio could hold more significance than the absolute concentration of Na^+ itself. In addition to potassium,

soluble salts also compete with the absorption and metabolism of other essential nutrients such as phosphorus (P), nitrogen (N), magnesium (Mg^{2+}) and calcium (Ca^{2+}), causing nutritional disorders. The high concentration of Na^{+} causes a decrease in the uptake of K^{+} and Ca^{2+}, which reduces photosynthesis mainly by affecting stomatal conductance, while the high concentration of Cl^{-} reduces photosynthetic capacity due to non-stomatal effects and chlorophyll degradation. Decreased absorption of these nutrients can occur due to several factors. One of them is that with saline stress there is a reduction in root surface area, both in the density and length of root hairs, which are directly proportional to nutrient absorption (Parihar *et al*., 2015).

Along with its direct effects on plants, salinity often results in excessive production and accumulation of reactive oxygen species (ROS). ROS are highly reactive species that, in the absence of any defensive mechanism they can seriously disrupt the normal metabolism. At the cellular level, ROS production occurs due to incomplete or partial reduction of oxygen molecules. Therefore, ROS production is a general phenomenon because they are produced as a result of the oxidation–reduction reaction of several metabolic processes in multiple locations and compartments of plant cells, such as, chloroplasts, mitochondria, endoplasmic reticulum, cytosol, and peroxisome. ROS produced in plants are composed of both free radicals and non-radicals and the most common are hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), superoxide ($O^{2.-}$) and hydroxyl radicals ($^{\cdot}OH$). Increased ROS levels can interact with essential components of plant cells and cause serious oxidative damage to plants, such as DNA damage, lipid peroxidation, enzyme inactivation, protein oxidation, hormonal and nutritional imbalances, and even death (Kibria and Hoque, 2019).

Another process affected by salt stress is photosynthesis. Photosynthesis is one of the most important biochemical processes by which plants convert solar energy into chemical energy and grow. The reduction in photosynthetic rates in plants under salt stress is mainly due to the reduction in water potential - leading to stomatal closure - and chlorophyll content (Kibria and Hoque, 2019). The decrease in chlorophyll content in leaves is a phenomenon widely reported in the literature, as it is the first visible symptom of plants subjected to saline stress. This decrease may be the result of slow synthesis or rapid breakdown of chlorophyll. In addition to chlorophyll, other pigments can also be affected by salt stress, such as carotenoids and anthocyanins. Carotenoids help absorb light and protect photosynthetic complexes from damage caused by excessive light. Under salt stress, carotenoid synthesis can be negatively affected, which can compromise

the plant's ability to dissipate excess light energy and protect itself against oxidative damage. Anthocyanins, on the other hand, have antioxidant functions and can protect plants against damage caused by stress (Kibria and Hoque, 2019; Parihar *et al*., 2015).

Photosynthetic capacity is also affected by the deterioration of photosystem components, such as photosystem II (PS II). PS II plays a crucial role in photosynthesis as it is responsible for absorbing light, breaking down water and releasing oxygen, as well as initiating the electron transport chain. Thus, a decrease in PS II efficiency can affect the electron transport chain (ETC) and the assimilation rate of CO_2. However, there are other factors that can reduce photosynthetic rate under salt stress, such as dehydration of cell membranes that reduce their permeability to carbon dioxide, salt toxicity, increased senescence, changes in enzyme activity induced by changes in cytoplasmic structure, and negative feedback by reducing sink activity (Parihar *et al*., 2015).

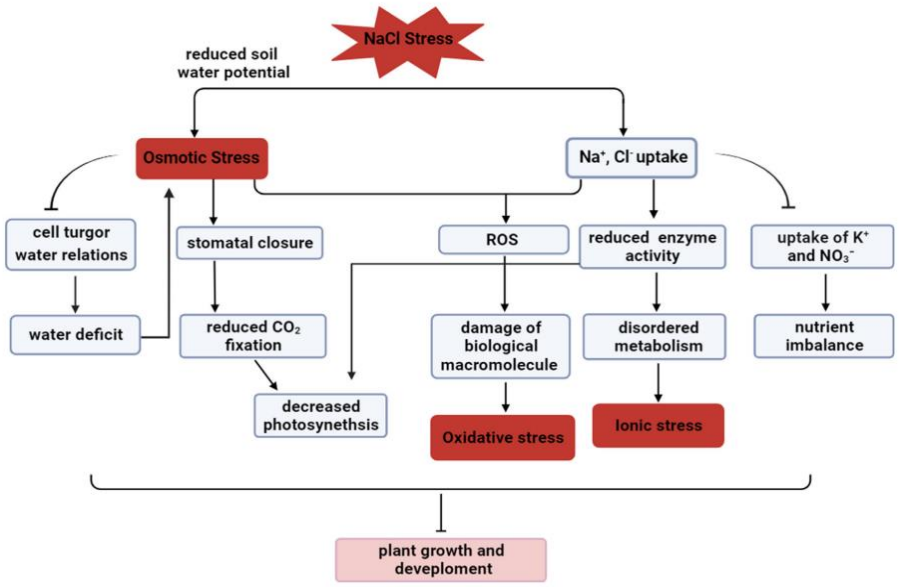

Figure 3. Scheme of adverse effects of salt stress imposed on plants. Salt stress in plants primarily results from water scarcity and elevated levels of intracellular Na^+ and Cl^- in saline conditions. Osmotic stress alters cell morphology and disrupts photosynthesis. The influx of Na^+ and Cl^- induces ionic stress, diminishing K-dependent enzyme activity, impeding nitrogen absorption, and leading to metabolic and nutrient imbalances. Osmotic and ionic stress also triggers reactive oxygen species (ROS) production, causing oxidative damage to organelles and hindering their functionality. Collectively, these adversities restrict normal plant growth and development (Zhou *et al*., 2023).

1.3. Plants response to salt stress

When exposed to salt stress, plants trigger many adaptive responses at the cellular, metabolic, molecular, morphological, and physiological stages (Fig. 4). High salinity causes osmotic stress in plants due to dehydration and as response plants could initiate osmotic adjustment to maintain turgor under salt stress. Osmotic adjustment is a process by which plants increase their water availability through the synthesis of compatible

solutions, known as osmolytes. Osmolytes mainly include organic substances and inorganic ions, proline, glycine betaine and soluble carbohydrates. Due to its strong hydration capacity, proline accumulation is an effective strategy to prevent dehydration and denaturation of proteins under osmotic stress in plants. Several studies have shown that proline treatment can alleviate the adverse effects of salinity on photosynthesis and seed yield (Wani *et al.,* 2019). Additionally, proline can function as an O_2 suppressant, thereby removing excess ROS produced under stress.

Another way for the plant to maintain homeostasis and ionic balance is through ion exclusion, wherein they regulate the uptake and transport of ions, particularly sodium (Na^+) and chloride (Cl^-), to prevent their accumulation in sensitive tissues. This involves selective ion uptake by roots and the exclusion of toxic ions from the transpiration stream, thereby safeguarding cellular ion homeostasis. Plants have developed sophisticated and effective mechanisms to maintain optimal Na^+ levels by removing Na^+ from the cytoplasm and compartmentalizing it in the vacuole using a variety of transport and channel proteins, symporters and antiporters. NHX, SOS, HKT and HAK are some of the transporters that play a crucial role in maintaining ionic homeostasis in plants. These transporters are responsible for the movement of ions, including sodium (Na^+) and chloride (Cl^-), across cell membranes, thus regulating the concentration of salts within various plant compartments (Zhou *et al*., 2023; Dzinyela *et al.,* 2023).

In response to the oxidative stress triggered by salinity-induced reactive oxygen species (ROS) accumulation, plants activate antioxidant defense systems. Salt stress induces enzymatic and nonenzymatic systems to mitigate ROS stress. Enzymatic scavengers include superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), glutathione peroxidase (GPX), and glutathione S-transferase (GST). SOD is the most effective scavenger of reactive oxygen species, and it forms the first line of defense against damage caused by ROS under environmental stress. SOD generates H_2O_2 from $O_2^{\cdot -}$, and H_2O_2 is further detoxified to H_2O by APX, CAT and GPX. In addition to enzymatic antioxidants, plants possess a variety of non-enzymatic antioxidants that scavenge ROS and mitigate oxidative stress. Nonenzymatic scavengers include ascorbic acid, alkaloids, carotenoids, flavonoids, GSH and phenolic compounds. These antioxidants function by donating electrons to neutralize ROS or by chelating metal ions that catalyze ROS production. The antioxidant defense system is one of the most important responses in plants, as it is not specific to salt stress, the system is activated in

response to a wide range of stress. In this way, it can be used as a biotechnological tool in plant improvement. The overexpression of components of the antioxidant system, such as glutathione, can confer resistance and tolerance to various abiotic stresses in plants.

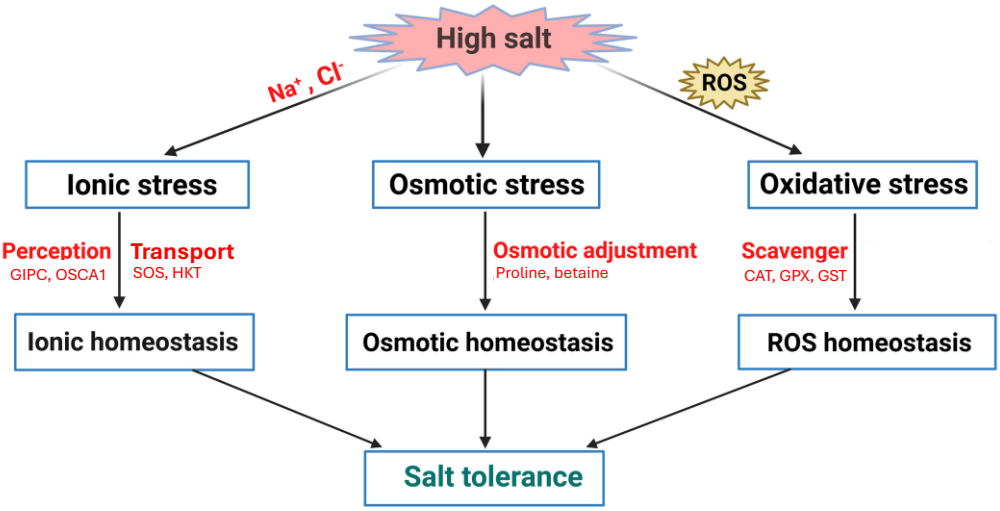

Figure 4. Illustration of plants response to salt stress. Salt stress primarily leads to osmotic stress, oxidative stress, and ionic stress. By detecting these stresses, plants trigger efficient signaling networks to gather substances for osmotic regulation and to sustain ionic and redox balance, resulting in increased salt tolerance in plants (adapted from Xiao and Zhou, 2023).

1.4. Glutathione

Glutathione (GSH; γ-glutamyl-cysteinyl-glycine) is an essential metabolite for plant survival, due to its role in the control of reactive oxygen species (ROS). Glutathione can be found in a free state or bound to other molecules. Free glutathione exists in two reversible states that help preserve redox homeostasis within the cell. Which is, oxidized form (GSSG) and reduced form (GSH). Under normal conditions, GSH is by far the most abundant of the two forms (Dorion *et al.*, 2021; Hameed *et al.*, 2014). In plants, a high level of glutathione is present in cell organelles especially in chloroplasts. This prevalence is attributed to the presence of the enzyme γ-ECS, a crucial component of the glutathione (GSH) pathway, which is localized in the chloroplasts. Consequently, chloroplasts are recognized as the primary site for GSH production in higher plants (Hasanuzzaman *et al.*, 2017).

Typically, the synthesis of GSH (Fig. 5) occurs in the chloroplast, but also in cytosol and mitochondria. The process involves a two-step reaction catalyzed by γ-glutamylcysteine synthetase (γ-ECS) encoded by the gene *GSH1* and glutathione synthase (GSHS) encoded by the gene *GSH2*. It begins with the uptake or synthesis of cysteine, a key amino acid containing a thiol group. The enzyme γ-glutamylcysteine synthetase catalyzes the combination of cysteine and glutamate, forming γ-glutamylcysteine (EC).

Subsequently, glutathione synthase adds glycine to produce the tripeptide glutathione. This synthesis process occurs at the expense of two molecules of ATP. It is also possible to export the γ-ECS from the chloroplast to the cytosol, where it serves as the precursor to GSH biosynthesis (Hasanuzzaman *et al.*, 2017; Reichheld *et al.*, 2009). After the biosynthesis of GSH in the cytosol, it can be efficiently reimported into the chloroplast or transported into mitochondria. After synthesis in the cytosol, glutathione (GSH) can be directly imported into various organelles or take alternate forms to fulfill metabolic needs. Nevertheless, under adverse or stressful conditions, the swift conversion of reduced GSH into oxidized glutathione or glutathione disulfide (GSSG) can occur through diverse biochemical reactions within different cellular compartments. The activities of glutathione reductase (GR) and glutathione peroxidase (GPX) maintain a balanced state of GSH/GSSG. Different enzyme activities are responsible for the GSH homeostasis and the GSH/GSSG ratio, which are also significantly affected by abiotic stress conditions (Noctor *et al.*, 2012; Hasanuzzaman *et al.*, 2017).

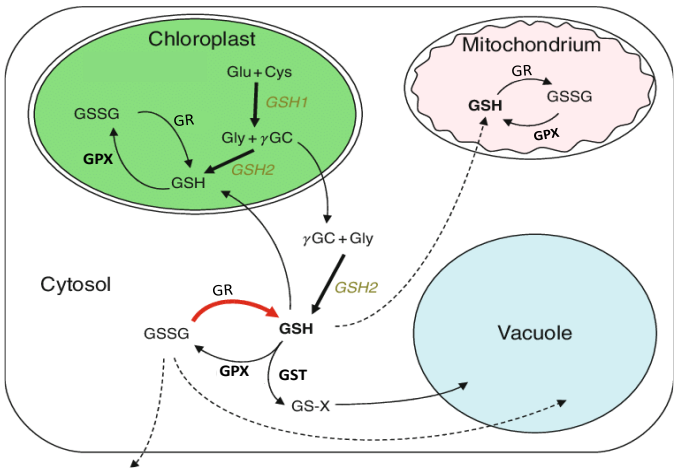

Figure 5. Biosynthesis and metabolism of glutathione. The first step occurs in the chloroplast or cytosol and involves the formation of γ-glutamylcysteine, a dipeptide, from glutamate and cysteine, catalyzed by the enzyme γ-glutamylcysteine synthetase (γ-ECS/GSH1). In the second step, the enzyme glutathione synthase (GSHS/GSH2) adds glycine to γ-glutamylcysteine, resulting in the final tripeptide, glutathione (GSH). The synthesized GSH can be utilized by cellular organelles like the plastids or mitochondria or in the cytosol. GSH participates in ROS scavenging and is converted into GSSG by the enzyme glutathione peroxidase (GPX). GSSG can be reconverted/recycled again into GSH by the activity of glutathione reductase (GR). Glutathione s-transferase (GST) catalyzed the conjugation of GSH to a generic xenobiotic (X), to facilities its degradation or excretion (adapted from Reichheld *et al.*, 2009).

Glutathione is widely known for its role in plant defense mechanisms. It is essential for plants under stress because it serves as a versatile molecule involved in antioxidant defense, detoxification, cellular protection, redox regulation, and various adaptive responses. The antioxidant function of GSH reduces oxidative stress, prevents lipid peroxidation, and protects the plasma membrane. GSH also participates in detoxification and as signaling in plant defense against biotic and abiotic stress. In addition to

glutathione (GSH), enzymes present in its pathway also play essential roles in plant responses to stress conditions. Among these enzymes, glutathione s-transferase (GST), γ-glutamylcysteine synthetase (GSH1) and glutathione peroxidase (GPX) stand out, all performing distinct but complementary functions in maintaining redox balance and protecting against environmental stress. Several studies have proven the protective role of these enzymes under stressful conditions. Increases in their activities during stress were observed and that gene expression is upregulated by stress, which confirms their potential as biotechnological tools to increase plant tolerance against abiotic and biotic stresses.

1.4.1. Glutathione s-transferase

Glutathione s-transferase (GST; EC 2.5.1.18) is a super family of multifunctional enzymes, consisting of three super families, cytosolic, mitochondrial, and microsomal, in plants they are mainly cytosolic (Csiszár *et al.*, 2014). Based in phylogenetic and genomic analysis, plants GST can be subdivided into 14 classes, namely tau (U), phi (F), theta (T), zeta (Z), lambda (L), γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bγ), dehydroascorbate reductase (DHAR), metaxin, tetrachloro-hydroquinone dehalogenase (TCHQD), Ure2p, and microsomal prostaglandin E synthase type 2 (mPGES-2). Recently, three new classes have been identified in plants, hemerythrin (GSTH), iota (GSTI) and glutathionyl-hydro-quinone reductases (GHRs) (Nianiou-Obeidat *et al.*, 2017). The two most widespread types in plants are Tau GST (GSTU) and Phi GST (GSTF) (Pégeot *et al.*, 2014). In *Populus*, a total of 81 GST genes have been identified in the annotated genome, including 8 GSTF genes (Gao *et al.*, 2022).

Plant GSTs play varied roles in plant development, stress tolerance, detoxification of xenobiotics and toxic lipid peroxides, reduction of dehydroascorbate, regulation of apoptosis and in biochemical reactions of secondary products such as flavonoid derivatives. Its main catalytic function is to conjugate the reduced form of glutathione (GSH; γ-glu-cys-gly) to endogenous and exogenous electrophilic molecules (Fig. 6) (Csiszár *et al.*, 2014). This conjugation reaction facilitates the excretion or degradation of these substances, promoting cellular detoxification. The Tau, Phi, and Theta classes also exhibit significant GSH-dependent hydroperoxidase (GPOX) activity toward fatty acid hydroperoxides, as well as GSH conjugation activity toward cytotoxic lipid peroxidation products such as alkenes and unsaturated aldehydes. Through these enzymatic functions, they play a direct role in increasing tolerance to abiotic stress by effectively reducing the production of reactive electrophilic species. Furthermore, it has been proposed that, in addition to the direct protective impact of GPOX activity, increased tolerance may result

from GPOX-mediated elevation of the oxidized form of glutathione (GSSG) concentration within cells, which then serves as a signaling mechanism to activate additional protective responses to stress (Csiszár *et al.*, 2014; Nianiou-Obeidat *et al.*, 2017).

Among all plant GSTs, GSTFs are one of the most used classes in tolerance studies, especially *GSTF1*. *GSTF1* expression can be induced by various environmental stresses, such as exposure to high concentrations of salt. This suggests that *GSTF1* plays an important role in plant response to stress conditions. Several studies have demonstrated that overexpression of the *GSTF1* gene confers even stronger resistance. In *Alopecurus myosaroides*, the *GSTF1*, which possesses a glutathione peroxidase activity, was found to be highly active in herbicide resistance (Cummins *et al.*, 2013). Gao *et al*. (2022), reported that *GSTF1* improves both biomass production and salt tolerance through ion homeostasis and ROS scavenging in transgenic poplar.

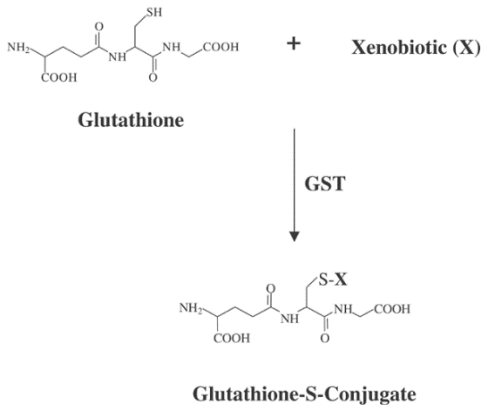

Figure 6. Glutathione s-transferase conjugation mechanism. Glutathione (GSH) conjugation to a xenobiotic (X) via glutathione s-transferase (GST) results in the formation of a glutathione-S-conjugate (Townsend and Tew, 2003).

1.4.2. Glutathione peroxidase

The Glutathione peroxidase (GPX; EC 1.11.1.9) is an important enzyme of the antioxidant system that belongs to a family of phylogenetically related oxidoreductases found in all living organisms. In different organisms, there are multiple isoforms or forms of GPX, in the poplar genome (*Populus trichocarpa*), for example, there are six complete GPX genes, namely *GPX1, GPX2, GPX3.1, GPX3.2, GPX4* and *GPX5* (Navrot *et al.*, 2006; Koh *et al.*, 2007). The number of GPX genes varies among plant species. It appears that plants with more complex genomes tend to have a higher count of *GPX* genes (Madhu *et al.*, 2023). GPX enzymes have a wide distribution in the plants, as they are present in chloroplasts, mitochondria, cytoplasm, extracellular and nuclear regions.

GPX enzymes play important roles in cellular protection and their functions include peroxide detoxification, protection against oxidative stress, regulation of the redox cycle and response to cellular signals. The main role of GPXs is to use glutathione and thioredoxin as reducing substrates to convert H_2O_2 or organic hydroperoxides into water or oxygen. Operating as thiol peroxidases, they transform reduced glutathione (GSH) into oxidized glutathione (GSSG), thereby reducing the accumulation of H_2O_2 (Fig. 7). This process plays a pivotal role in maintaining cell homeostasis and contributes significantly to the antioxidant response in plants (Madhu *et al.*, 2023; Wang *et al.*, 2023). Some studies have reported that in the detoxification of lipid hydroperoxides and other reactive molecules, GPX activity can be associated with GST enzymes (Bela *et al.*, 2015). Another activity of GPX is that to regulate cellular redox homeostasis, GPX maintains the level of thiol/disulfide or NADPH/$NADP^+$. Furthermore, this enzyme also plays a significant role in the growth and development of plants (Madhu *et al.*, 2023; Chen *et al.*, 2017).

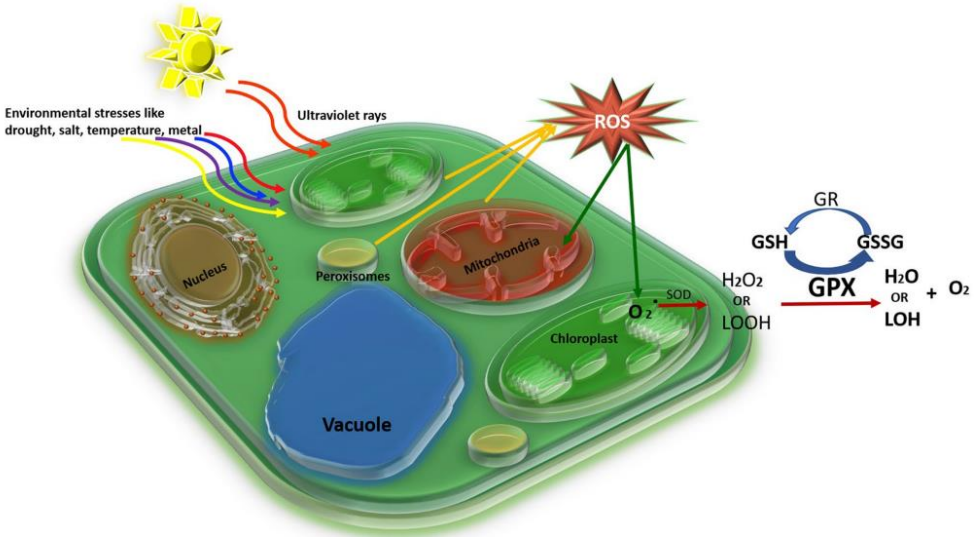

Figure 7. Glutathione peroxidase mechanism. Illustration of the production of reactive oxygen species (ROS) in different cell organelles under abiotic stress conditions and their scavenging by enzymatic antioxidant glutathione peroxidase (GPX). GPX transforms reduced glutathione (GSH) into oxidized glutathione (GSSG), to convert H_2O_2 or lipid hydroperoxides (LOOH) into water and oxygen (Madhu *et al.*, 2023).

Due to these functions, GPX has become a focal point in studies involving plants under stress conditions. The literature suggests that gene expression of GPXs is up regulated in response to environmental stresses, including salt stress, drought stress, temperature stress and biotic stress, in multiple plant species. Which strongly reinforces their protective role. For example, Cheng *et al*. (2009), reported that *GPX1* and GPX7 regulate cellular photooxidative tolerance and immune responses in *Arabidopsis thaliana*. In drought stress, Kang *et al*. (2004) and Anderson and Davis (2004) reported that several

GPX genes, including *GPX1-3*, were overexpressed, and were found to be useful against the stress in *Oryza sativa* and *Euphorbia esula* respectively. In the salt-tolerant plant production approach, *GPX* is an important gene family, as these genes play a vital role in providing salt tolerance. In Arabidopsis, overexpression of two *GPX* genes has been shown to confer high salt tolerance (Zhai *et al.,* 2013). These studies highlighted the fundamental role of GPXs as essential antioxidant enzymes in protecting plant cell machinery against environmental stresses. This underscores the significance of GPX as a crucial enzyme in the realm of crop breeding (Madhu *et al.,* 2023).

1.4.3. Gamma-glutamylcysteine synthetase

γ-glutamylcysteine synthetase (γ-ECS; GSH1; EC 6.3.2.2.), encoded by the *GSH1* gene, is a crucial enzyme in plants that plays a central role in the biosynthesis of GSH. Glutathione homeostasis is critically important for maintaining both intracellular redox balance and defense against oxidative or chemical stress. The enzyme GSH1 is responsible for catalyzing the first and rate-limiting step in GSH biosynthesis, linking glutamate and cysteine to form the intermediate compound γ-glutamylcysteine (Fig. 8). The first reaction has been the rate-limiting step in GSH biosynthesis as it is controlled via the feedback inhibition by GSH to avoid over-accumulation of GSH (Tang *et al.*, 2015). In addition to this mechanism, the GSH1 activity can also be controlled by the limited availability of cellular Cys, and the transcriptional and posttranscriptional regulation of the enzyme's expression under various physiological conditions. GSH1 is typically localized in the chloroplasts or cytoplasm of cells, where it carries out its function in glutathione biosynthesis (Samuilov *et al.,* 2016; Galant 2011).

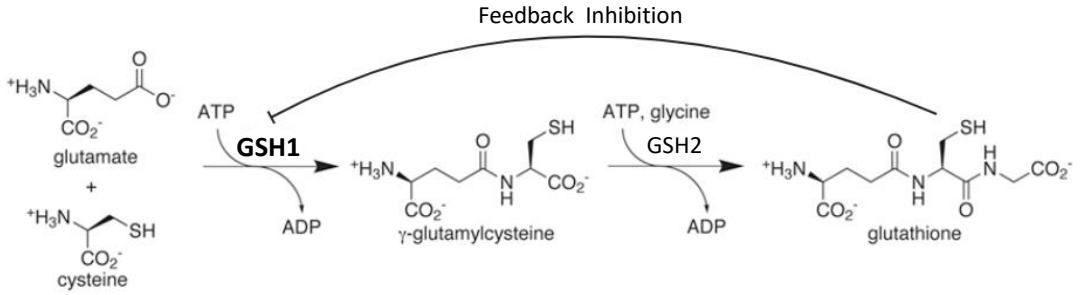

Figure 8. Glutathione Biosynthesis. Schematic diagram showing the two ATP-dependent steps synthesis of glutathione from the three constituent amino acids (glutamate, cysteine, and glycine). γ-glutamylcysteine synthetase (GSH1; γ-ECS) catalyzes the first step, the first step, the conjugation of glutamate and cysteine, forming the intermediate compound γ-glutamylcysteine. In the second step, glycine is added to γ-glutamylcysteine, by the enzyme glutathione synthase (GSH2; GSHS), forming the final product, glutathione. Feedback inhibition of GSH1 by the final product is also represented (adapted from Galant *et al.*, 2011).

Environmental and cellular conditions that cause oxidative stress increase glutathione levels in plants. Consequently, this increase in glutathione production may be caused by the overexpression of GSH1, the first enzyme in the glutathione pathway, in response to this stress. However, different stresses exert different effects on the genes that encode the enzymes responsible for glutathione production in plants (Noctor *et al.,* 2012). Several studies have reported an upregulation of the expression of GSH1 gene during different stress regimes. For example, transgenic poplar overexpressing the GSH1 showed better adaptation under mild drought exposure partially due to improved glutathione synthesis (Samuilov *et al.,* 2016). Ivanova *et al.* (2011), demonstrate that poplar overexpressing GSH1 were more tolerant to heavy metal stress than wild-type plants. Transgenic rice plants overexpressing *OsGSH1* in the cytosol showed strong tolerance to salt stress through maintenance of cellular homeostasis via an enhanced GSH redox state. Moreover, *OsGSH1* overexpression in transgenic plants showed increased total biomass and grain yield under paddy field conditions (Choe *et al.,* 2013).

GSH1 also plays a role in seedling development, hypocotyl elongation, and anthocyanin accumulation. GSH1 is crucial for maintaining redox homeostasis and signaling in plants, as it regulates the GSH/GSSG ratio, which is important for physiological functions and as an indicator of oxidative stress. Additionally, GSH1 is involved in the regulation of plant growth, development, and response to changing environments (Herschbach *et al.,* 2010). Biotechnological approaches using genes such as *GSH1* could provide insights for molecular breeding under environmental stress. Therefore, understanding the functions of GSH1 is extremely important for developing strategies to improve crop yield and increase plant resistance to saline stress and many other biotic and abiotic stresses.

1.5. *Populus alba* L.

Populus alba L. also known as white poplar belongs to the *Populus* genus, which includes around 35 tree species from the Salicaceae family (Chen and Polle, 2010). This tree is native to the riverine steppes and coastal forest communities of Central and Southern Europe (Fig. 9). It covers a wide geographic area stretching from North Africa to Poland and from the Iberian Peninsula to Western Siberia and Central Asia. It was introduced to the United States in the 18th century for its ornamental quality, and since then it has spread to all other continents, naturalizing in several regions, and becoming invasive in some countries (Caudullo and Rigo, 2016).

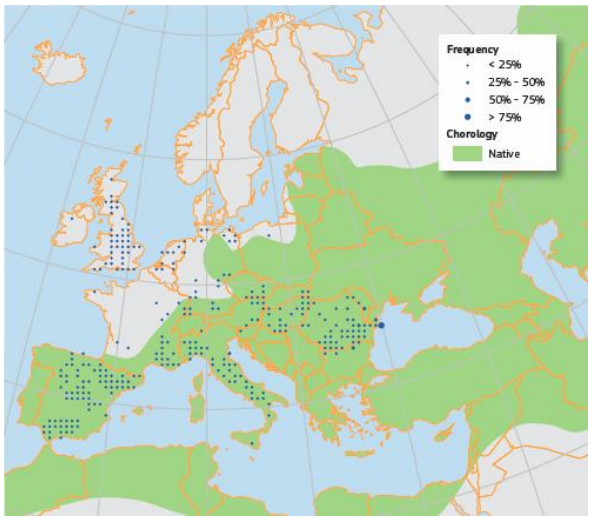

Figure 9. Distribution map of *Populus alba L.*. Frequency and native range of *P. alba* in Europe and north of Africa (Caudullo and Rigo, 2016).

P. alba (Fig. 10) is characterized by rapid growth, high biomass yield and adaptability to survive in different environmental conditions. Therefore, they are widely used to produce pulp, paper, wood, and biofuels, as well as for environmental protection such as agricultural shelterbelts and phytoremediation (Wang *et al.*, 2023). Their application for soil recovery is very important because they can prevent erosion through their deep root system and can also improve microclimatic conditions (Chen and Polle, 2010). *Populus* trees are also model species for biotechnology research due to the characteristics mentioned above and the availability of the complete genome sequence of *Populus trichocarpa* (Chen and Polle, 2010; Tuskan *et al*., 2006). For these reasons and their natural ability to tolerate stress, poplars have been the subject of several studies in response to biotic and abiotic stress, including saline stress.

A **B**

Figure 10. *Populus alba L.*, White Poplar. (A) Appearance of white poplar leaves (Arbolapp: https://www.arbolappcanarias.es/en/species/info/populus-alba/); (B) Representation of the *Populus alba* L. tree (Caudullo and Rigo, 2016).

Different species and clones of the genus *Populus* exhibit varying levels of sensitivity or tolerance to excess salt. For example, *Populus euphratica* is recognized as the most salt-tolerant species, while *Populus alba L.* displays a moderate level of salt tolerance, with differences observed between different clones and geographic origins. Genetic and molecular studies have also provided insights into the mechanisms underlying poplar salt tolerance. Many genes related to ion transport, osmotic adjustment and antioxidant defense have been identified and investigated for their contributions to increasing salt tolerance in poplars. Overall, the ability of poplars to resist salt stress is attributed to a combination of physiological, biochemical, and molecular mechanisms. In this way, improving these tolerance mechanisms could expand the usefulness of poplar trees for reforestation on degraded soils, agroforestry on marginal lands, and environmental conservation efforts. Sustainable wood bioproduction in areas unsuitable for traditional agriculture, such as salinized soils, could help respond to competing land use demands between food production and bioenergy production.

2. Objectives

2.1. General objectives

Given the increasing incidence of saline soils in agricultural areas around the world, it is extremely important to study and understand the impacts of these adverse conditions on plant development and survival. Poplar is an example of a tree that has been used in these studies in recent years. This is due to its known genetic structure, its role in mitigating climate change and its rapid growth, making it an excellent species for studying tolerance to saline stress.

The present work has analyzed the morphological, biochemical, and molecular changes in *Populus alba L.* "Villafranca" clone, caused by salinity with the aim of understanding its response in order to improve its tolerance to saline stress.

2.2. Specific objectives

- Evaluate morphological changes, in growth and appearance of plants.
- Evaluate physiological changes, in chlorophyll and flavonol content, leaf stomatal conductance and net photosynthetic rate.
- Analyze the effect of salt stress in the concentration of biochemical molecules, such as proline.
- Analyze the effect of salt stress on the uptake of mineral elements (Na, K and Ca).
- Assess the *GSTF1*, *GSH1* and *GPX1* genes expression as a response to salt stress.

3. Materials and Methods

3.1. Plant growth conditions and experimental design

Sixteen plants of *Populus alba L.*"Villafranca" clone were used in this experiment. Plantlets from *in vitro* culture were transferred from Magenta vessels containing WPM half-strength solid medium (Lloyd and McCown, 1981) to pots filled with perlite (Laterlite, Milano - Italy), closed in plexiglas boxes to maintain 100% humidity. The plants derived from *in vitro* culture were acclimatized *in vivo* for six weeks in a growth chamber under controlled environmental conditions (23:18 °C day:night temperature, 65–70 % relative humidity and 16 h photoperiod at photosynthetic photon flux density of 400 μmol m^{-2} s^{-1} supplied by fluorescent lights). During the acclimation process, the nutrient supply was gradually changed from WPM half-strength liquid medium to a modified Hoagland's solution (Table 1) at pH 6.2 (Arnon and Hoagland, 1940), while relative humidity was reduced from 100 % to 65–70 % (growth chamber parameter) in order to adapt the plants to the *in vivo* conditions, as described in Pierattini *et al*. (2016).

Table 1. The modified Hoagland's solution composition used during the acclimation process.

Component	Concentration
$Ca(NO_3)_2 \cdot 4H_2O$	4.0 mM
$MgSO_4 \cdot 7H_2O$	2.1 mM
KNO_3	4.0 mM
$K_2HPO_4 \cdot 3H_2O$	1.0 mM
NH_4NO_3	1.5 mM
H_3BO_3	43.7 μM
$MnSO_4 \cdot H_2O$	20.1 μM
$CuSO_4 \cdot 5H_2O$	0.4 μM
$Na_2MoO_4 \cdot 2H_2O$	0.5 μM
$Zn(NO_3)_2 \cdot 6H_2O$	2.0 μM
Ferric tartrate	15.0 μM

After the acclimation period, plants were transferred into plastic pots of 15×15×20 cm (capacity of 3.6 litres), containing a mixture of 8–20 Ø mm expanded Agrileca clay (Laterlite, Milano - Italy) and sand (1:1, v/v). After one month of growth, plants reached an average height of 34.2 ± 7.8 cm and an average number of leaves of 18 ± 3, and the saline treatment was started. Plants were randomly divided in four groups (n = 4 for each group), control (CT) and treated plants (60, 120, and 180 mM NaCl). For four weeks, CT were supplemented with distillate water, while treated plants were supplemented with distillate water containing a concentration of 60, 120, and 180 mM NaCl. All plants were

watered three times per week, one time with the treatment and two times with a mixture of half Hoagland solution and half distillate water. All plants receive the same volume of solution (200 mL). The plants were maintained in a controlled environment throughout the experiment. The experiment was organized as showed in figure 11.

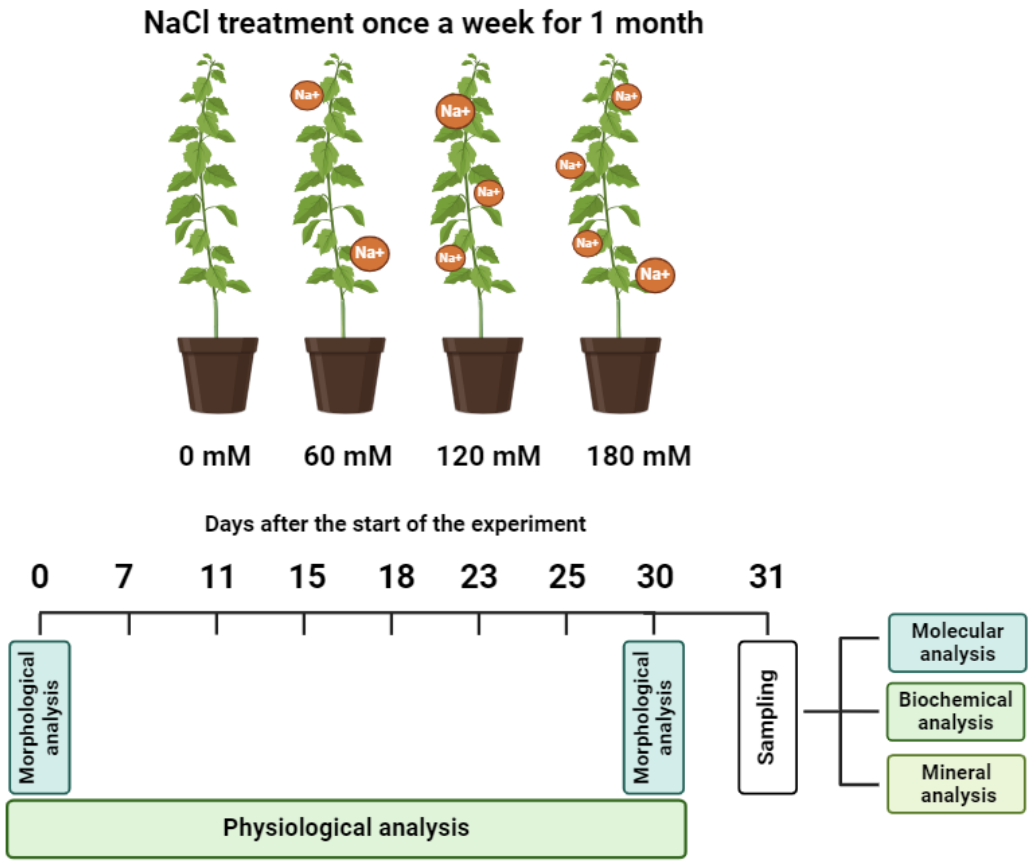

Figure 11. Schematic illustration of the experimental design. Sixteen plants of *Populus alba L.*"Villafranca" clone were used in this experiment. They were dived in to four groups (n=4), 0 (control), 60, 120, and 180 mM NaCl. The morphological measurements were performed at the beginning and the end of the experiment. The physiological analysis was performed two times per week for one month. After one month of experiment, the sampling was carried out. The collected samples were used to carry out molecular, biochemical, and mineral analysis.

3.2. Morphological and Physiological analysis

To analyze the effects of saline stress on the morphological appearance of *P. alba* "Villafranca" clone (12A), for each plant, the fresh weight (g) was measured of each organ of the plant separated (leaves, stem, and roots) at the end of the experiment. The height (cm) of each plant was measured on the first and last day of treatment.

To understand the effects of saline stress on physiological parameters such as chlorophyll, flavonol and photosynthesis rate, different non-destructive analyzes were carried out. Relative chlorophyll content in leaves was monitored two times per week, using a non-destructive measuring SPAD meter (SPAD-502 Plus, Konica Minolta, USA),

on three fully expanded leaves randomly selected on each plant. The chlorophyll meter takes instant readings (SPAD units) without destroying the plant tissue (Fig. 12B).

The leaf stomatal conductance (G_s, mmol m^{-2} s^{-1}) and net photosynthetic rate (P_n, µmol CO_2 m^{-2} s^{-1}) were measured for one leaf on each plant using a portable gas exchange system (CIRAS-2, PP Systems International, USA), operating at 100 ml min^{-1} flow rate, ambient temperature, 450 ± 10 ppm CO_2 and a photosynthetic flux density of 1000 µmol m^{-2} s^{-1}. Leaf was closed in the chamber and exposed to its controlled environment for 3-4 min before collecting the measures (Fig. 12C). Measurements were taken between 10:00 and 13:00.

Furthermore, flavonol content was measured for one leaf on each plant using a multiple wavelength pigment meter (MPM-100, Opti-Sciences, Inc., USA), two times per week (Fig. 12D). All analyzes were performed on days 0, 7, 11, 15, 18, 23, 25 and 30 after the start of treatments.

Figure 12. Non-destructive physiological measurements. (A) *Populus alba L.*"Villafranca" clone plants on time 0, placed in the growth chamber under controlled conditions. (B) SPAD (SPAD-502 Plus, Konica Minolta, USA) measurement on a fully expanded leaf. (C) Measurement of G_s and P_n using the portable gas exchange system, CIRAS-2 (PP Systems International, USA). **(D)** Flavonol measurements using the multiple wavelength pigment meter (MPM-100, Opti-Sciences, Inc., USA).

3.3. Plant sampling

The plants were sampled after 30 days from the start of the experiment. The plants were removed from the pots and leaves, stems and roots were sampled according to the

scheme in Figure 13. Leaves and stem were collected and separated in apical and basal division.

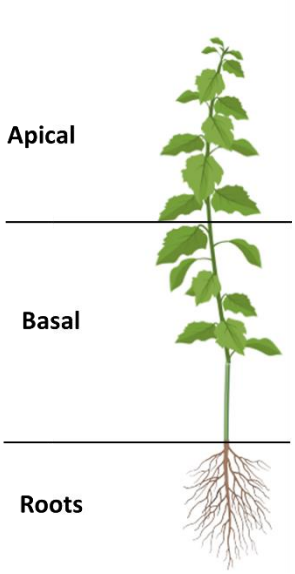

Figure 13. Schematic illustration of the plant's sections. Schematic representation of the sections in which the *P. alba* "Villafranca" clone was divided, after 30 days of NaCl treatment.

The collected roots were washed with distilled water to remove the soil residues (Fig. 14). All parts of the plants were then packed in aluminium foils. Half of the samples were dried in an oven and were later digested for mineral elements analysis, while the other half were frozen in liquid nitrogen for molecular and biochemical analysis.

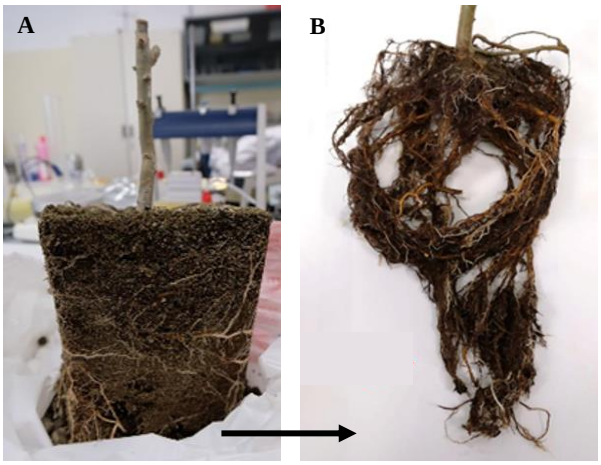

Figure 14. Example of sampling of the *P. alba* "Villafranca" clone after 30 days of treatment with NaCl. (A) Roots of *P. alba* "Villafranca" clone before washing. (B) Roots of *P. alba* "Villafranca" clone after washing.

3.4. Proline content quantification

Proline quantification analysis was performed in leaves and roots on four biological replicates. For the extraction, 0.5 g of frozen material was homogenized with 5 ml of a mix of 75 % methanol, 20 % miliQ H_2O and 5 % formic acid and incubated in dark at 4

ºC overnight. The samples were then sonicated in dark for 10 min on ice and then centrifuged at 4000 rpm for 15 min at 4 ºC. After that, the extracts were filtered through a membrane of 0.45 µm pore size. Analysis was carried out by liquid chromatography–mass spectrometry (LC-MS/MS) using a Sciex 5500 QTrap+ mass spectrometer (AB Sciex LLC, Framingham, MA, USA). The UHPLC chromatographic separation for quantification of proline was performed on a Biphenyl HPLC Column 100 x 2.1 mm, 2.6 µm. The elution was carried out using acetonitrile/water 15/85 containing 0.1% (v/v) formic acid as a mobile phase at flow rate 400 µL/min. Injection volume was 20 µL and column oven temperature was setup at 40 °C. MS/MS experiments were performed in Electrospray negative ion mode using nitrogen as collision gas, the operation source parameters are shown in table 2. Calibration curves for amino acids was done using Amino Acids Mix Solution standard (Supelco, Sigma) from 0.01 nmol/ml to 2.56 nmol/ml for proline.

Table 2. Liquid chromatography–mass spectrometry operation parameters for proline quantification.

Operation parameters	
Nebulizer gas (GS1)	60 (arbitrary units)
Turbo gas (GS2)	60 (arbitrary units)
Curtain gas (CUR)	20 (arbitrary units)
Temperature	500 ºC
Ionspray Voltage (IS)	-5400 V

3.5. Mineral elements analysis

Plant samples were dried at 70 °C until their weight remained constant. Then, 0.3 g of leaves and roots were ground and added to 8 mL of 65 % nitric acid, in 70 mL Teflon containers. The mixtures were digested using the COOLPEX Intelligent Microwave Reaction System (Yiyao Instrument Technology Development Co., Ltd., Shanghai, China). Digestion occurred in three steps, as shown in table 3. After digestion, the final samples were diluted in milliQ water, until reaching a volume of 30 mL.

Table 3. Microwave-assisted digestion protocol for plant samples.

Step	**Temperature (ºC)**	**Time (min)**	**Pressure (atm)**
1	120	2	15
2	150	4	20
3	180	4	25

Sodium (Na), potassium (K) and calcium (Ca) concentrations were determined using a Microwave Plasma-Atomic Emission Spectrometer (4210 MP-AES, Agilent Technologies,

Santa Clara, USA). Before reading, all samples were diluted in milliQ water according to the standard curve for each mineral analysed (App. 2). Calibration curves were obtained measuring a multi-element standard control in a 5 % HNO_3 (v/v) solution. Instrumental parameters (wavelength, nebulizer flow and viewing position) were optimized for each group of samples (App. 3 and 4). Results were expressed on a dry mass basis (mg/kg DW). The limits of detection (LOD) and limits of quantification (LOQ) for each mineral analysed were also calculated (Table 4).

Table 4. Limits of detection (LOD) and limits of quantification (LOQ) for each mineral analyzed.

Element	LOD (ppm)	LOQ (ppm)
Sodium (Na)	1.148	3.788
Potassium (K)	1.074	3.545
Calcium (Ca)	5.801	19.145

3.6. Bioinformatic analysis

To identify the sequences of the *GSTF1, GSH1* and *GPX1* genes in *P. alba*, a search was first carried out on the genome of *P. trichocarpa*, which is the reference genome. The search was carried out in the NCBI (https://www.ncbi.nlm.nih.gov/) and Phytozome (https://phytozome-next.jgi.doe.gov/) databases. Following, a BLAST of the collected sequences was performed to identify the most similar sequences in *P. alba*. For this purpose, some characteristics were considered, such as number of exons and 5'UTR and 3'UTR regions. After selecting the sequences of the respective genes in the *P. alba* genome, an alignment was carried out, using the Cluster Omega software, of the genomic, coding (CDS) and protein sequences of the three genes in the *P. trichocarpa* and *P. alba* to identify the difference between the sequences.

3.7. Polymerase chain reaction (PCR) analysis

To check the presence of *GSTF1, GSH1* and *GPX1* genes in the genome of *P. alba* "Villafranca" clone a PCR experiment was conducted (App. 5 and 6). Primers (Table 5) were designed in the coding sequence (CDS) for each gene, using NCBI blast primer (https://ncbi.nlm.nih.gov/tools/primer-blast). The characteristics were checked for product length, position, annealing temperatures, and GC content.

Table 5. List of forward (FW) and reverse (RV) primers, base pair number (bp) of amplified fragment and primer annealing temperature (T_{AN}), used to check the presence of each gene on *P. alba* "Villafranca" clone genome through PCR.

Gene	Primer	Fragment size (bp)	T_{AN} (ºC)
***GSTF1*- FW1**	CACCATTGTCCACGGCAGT	696	62
***GSTF1*-RV1**	CCAAGTAGTCCCCATTAGGCA		
***GSTF1*- FW2**	ATGGCAACTCCGGTGAC	843	60
***GSTF1*- RV2**	TCAAGCATTTTTCCTCATTTC		
***GSH1*-FW1**	AGAGCCTGGTGGTCAGTTTG	1784	66
***GSH1*-RV1**	GCCAGCACGATTGTTGTCAG		
***GSH1*-FW2**	CATGCCTAAAGTTGGCTCGC	1560	66
***GSH1*-RV2**	CTCCATCAGCACCCCTCATC		
***GPX1*-FW1**	ACCCTACAACAATGGCTCCC	1613	65
***GPX1*-RV1**	CAGGTTCTTGCCCACCAAAC		
***GPX1*-FW2**	ATGGCTTCCTTACCTTTCTCCTG	2198	64
***GPX1*-RV2**	TCATGCGGCGAGGAGCTT		

The fragments corresponding to the tree genes were amplified through PCR, using the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific.) reaction mix, using randomly selected samples of *P. alba* "Villafranca" clone. The PCR reaction components were meticulously assembled on ice and gently mixed. To determine the ideal annealing temperature, three different temperatures were employed for each set of primers (App. 7). A negative control (C-), composed of nuclease-free water, primers, and DNA polymerase, was included in the amplification process.

The PCR products were run in 1 % agarose gel, which was prepared with 1X TAE electrophoresis buffer. Additionally, 10 % of SYBR™ was added to the gel to allow visualization of the DNA with the UV transilluminator (Molecular Imager®, Gel Doc™, BIO-RAD). Depending on the expected size of the amplified fragment, 1Kb plus DNA ladders (GeneRuler™, Invitrogen) were used.

3.8. RNA extraction and cDNA synthesis

Total RNA extraction was performed according to Vennapusa *et al.* (2020) with minor modifications. Initially, 100 mg of the plant material was ground in liquid nitrogen and then further ground in 600 µl of extraction buffer (App. 8) containing 10 µl of 2-mercaptoethanol. Following, 84 µl of 3M KCl was added, and the mixture was incubated on ice for 15 minutes before centrifugation at 8000 rpm at 4°C for 5 minutes. The resulting suspension was transferred to a new tube, and an equal volume of 8M $LiCl_2$ was added, followed by overnight incubation at 4 °C. Subsequently, the mixture was centrifuged at 15000 rpm at 4 °C for 20 minutes, the supernatant was discarded, and 400 µl of DEPC

H_2O, 40 µl of 3 M NaOAC, and 450 µl of phenol:chloroform (1:1) were added and vortexed.

The solution was centrifugated at 15000 rpm at 4 °C for 5 minutes, the upper aqueous phase was transferred to a new tube, and an equal volume of chloroform was added. The mixture was centrifuged again at 15000 rpm at 4 °C for 5 minutes, and the upper aqueous phase was transferred to a new tube, to which 2.5 volumes of cold absolute ethanol and 10 µl of 3 M NaOAC were added. This mixture was incubated at -20 °C for 30 minutes and then centrifuged at 15000 rpm at 4°C for 10 minutes. Following centrifugation, the supernatant was discarded, and the pellet was resuspended in 30 µl of DEPC H_2O.

First-strand cDNA was synthesized from 500 ng of total RNA using the Maxima First strand cDNASynthesis kit (Thermo Fisher Scientific) with oligo (dT) 18 primer, following the manufacturer's instructions.

3.9. Gene expression analysis

Gene expression analysis was performed by qRT-PCR, using a CFX Connect real-time PCR System and iTaq Universal SYBR Green Supermix (BioRad, California, USA). Primers for gene-specific amplification were designed in the coding sequence (CDS) of each gene, using NCBI blast primer (https://ncbi.nlm.nih.gov/tools/primer-blast). The characteristics were checked for product length, position, annealing temperatures, and GC content (Table 6).

Table 6. List of forward (FW) and reverse (RV) primers, base pair number (bp) of amplified fragment and annealing temperature (T_{AN}) used in RT-qPCR for each gene studied.

Gene	Primer	Fragment size (bp)	T_{AN} (°C)
***GSTF1*-FW**	CGTAGATAAGTTGGCGAAAGTG	103	60
***GSTF1*-RV**	CAGGTGTGAAAGATCAGCAAAA		
***GSH1*-FW**	AGCCTGGTGGTCAGTTTGAG	237	60
***GSH1*-RV**	TATCTAGTCCCAGCGAGCCA		
***GPX1*-FW**	GGGAAGGATGTTGCTCTTAG	179	60
***GPX1*-RV**	GGTTCTTGCCCACCAAACTG		

To identify the most appropriate housekeeping genes for this work, three genes were tested, actin, tubulin-alpha 3 and elongation factor 1. In this work, the samples are heterogeneous, as different organs and treatments were used, therefore the most efficient housekeeping is the one with the lowest M value, which is the Elongation factor (Table 7).

Table 7. Tested housekeeping genes for *P. alba* "Villafranca" clone.

Target	M-value
Actin	0.6017
Tubulin-alpha 3	0.9051
Elongation factor 1	0.5484

Homogeneous sample: M<0.5
Heterogeneous sample: M:<1

3.10. Statistical analysis

The experiment was set up with four replicate plants (n = 4) for each treatment. All data reported are presented as mean ± standard deviation. Statistical analysis was carried out using GraphPad Prism version 8.0.1 software for Windows (GraphPad Software, San Diego, California USA). Statistical significance was tested by one-way ANOVA analysis with Tukey's Multiple Comparison Test ($P < 0.05$) and unpaired two-tailed t-test with Welch's correction ($P < 0.05$). A multivariate statistical technique, the Principal Component Analysis (PCA) was used to investigate and reveal the correlations between variables (mineral elements) within the measurements. Only PCA with eigenvalues > 0.7, explaining more than a single parameter alone, were extracted. For these principal components, the Varimax rotation was applied on the obtained factor. Statistical analysis was performed using GraphPad Prism version 10 software for Windows (GraphPad Software, San Diego, California USA).

4. Results

4.1. Morphological e Physiological measurements

After one month of NaCl treatments, noticeable phenotypic changes were observed in *Populus alba L.* “Villafranca” clone plants (Fig. 15). The most pronounced symptoms were observed in the group treated with the highest saline concentration (180 mM NaCl). Plants treated with 60 mM NaCl exhibited no significant differences and closely resembled the control group. However, at higher concentrations, such as 120 and 180 mM NaCl, distinct changes were observed, particularly on the basal leaves, which displayed clear signs of chlorosis (Fig. 15GH). These leaves are indicative that the impact of saline stress on leaf morphology is stronger on basal leaves than the apical leaves.

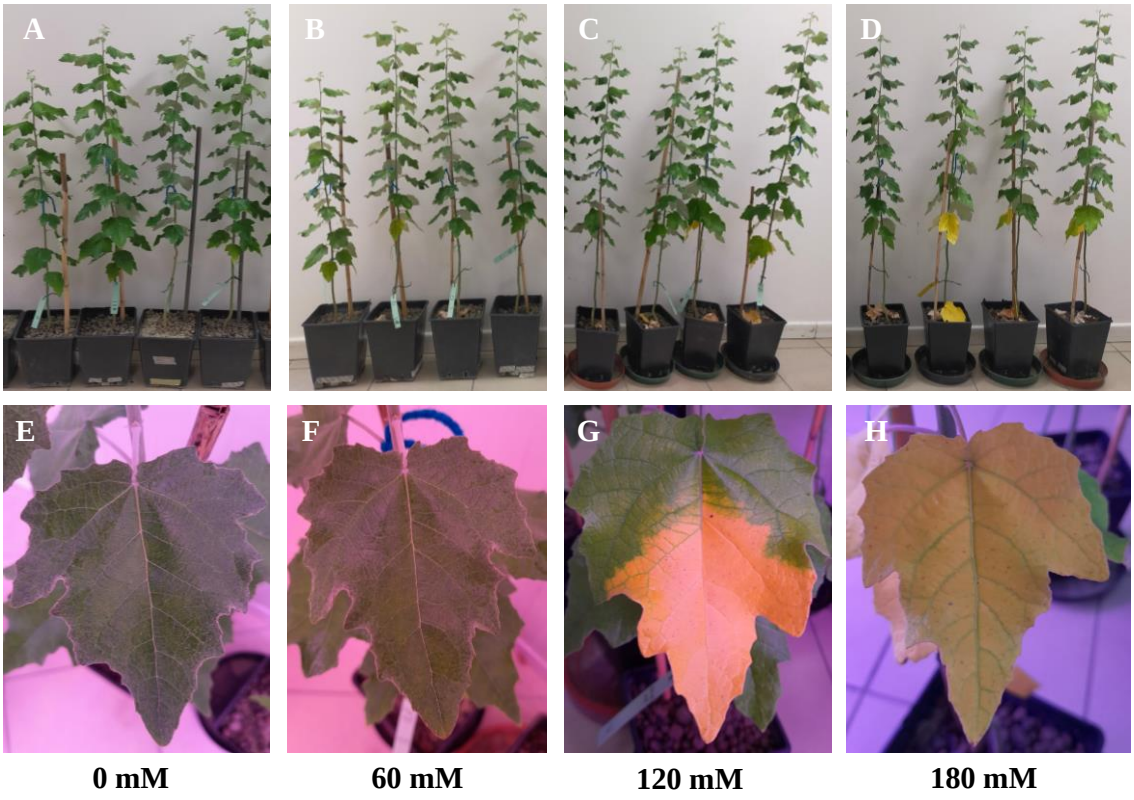

Figure 15. Phenotypic changes in *Populus alba L.* "Villafranca" clone plants (A, B, C and D) and their leaves (E, F, G and H) resulting from a one-month NaCl treatment at concentrations of 0, 60, 120 and 180 mM, respectively.

Regarding *P. alba* “Villafranca” clone growth, all plants were measured at the beginning and the end of the experiment, but no significant changes were observed between groups (App. 9). Plants were also weighed to analyze the effects of salinity on fresh weight. At the end of the treatment all plants were divided into leaves, stem and roots and weighed separately. Although all treated groups showed significant reductions in their weight, the group treated with 180 mM NaCl showed the most pronounced negative effects on the weight of all three organs (Fig. 16). When comparing the fresh

weight of leaves, the only group significantly different from the control is the one treated with 180 mM NaCl, with the control weighing 12.9 ± 1.72 g and the treated weighing 7.6 ± 0.58 g (mean ± standard deviation). The same is observed for the stem, the only group statistically different from the control (13.7 ± 2.27 g) was 180 mM NaCl (8.0 ± 1.54 g). In the roots, noticeable changes are evident in the groups treated with 120 mM NaCl (32.9 ± 1.78 g) and 180 mM NaCl (25.9 ± 1.79 g), both significantly differing from the control group (40.9 ± 4.00 g).

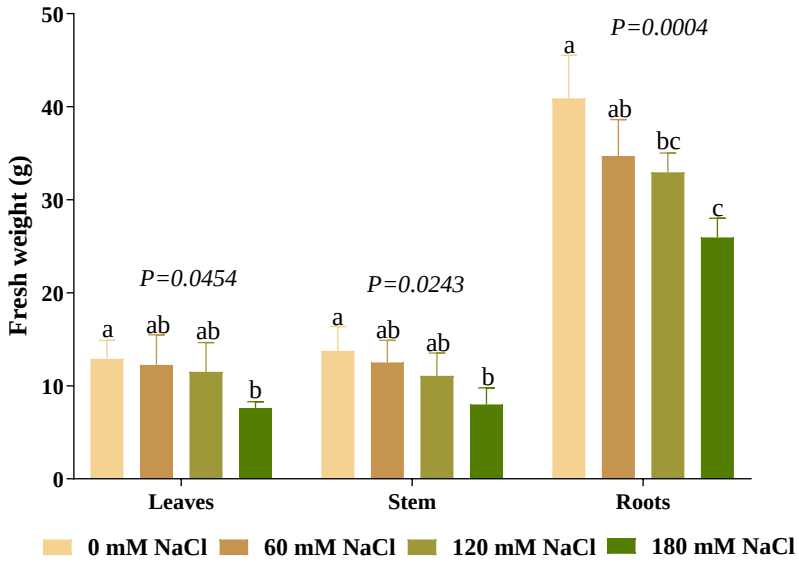

Figure 16. Fresh weight (g) of different organs of *Populus alba* L. "Villafranca" clone after one month under salt treatment (0, 60, 120 and 180 mM NaCl). The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test ($P < 0.05$).

Non-destructive methods were employed two times per week to investigate the physiological responses of *Populus alba L.* "Villafranca" clone plants to salt stress. The salt effects on photosynthetic parameters, such as stomatal conductance (G_s, mmol m^{-2} s^{-1}) and net photosynthetic rate (P_n, µmol CO_2 m^{-2} s^{-1}) appeared at 11 days after the start of the treatment. A decrease in these parameters is observed as the salt concentration increases and the duration of the treatment extends. At the end of treatment, the G_s values decreased by 32.5 %, 60.5 %, 72.0 %, 81.4 % for 0, 60, 120 and 180 mM NaCl, respectively, compared to the first day of experiment (Fig. 17B). A similar decrease was observed in the P_n of 17.8 %, 55.1 %, 53.8 % and 69.2 % for groups 0, 60, 120 and 180 mM NaCl respectively (Fig. 17A).

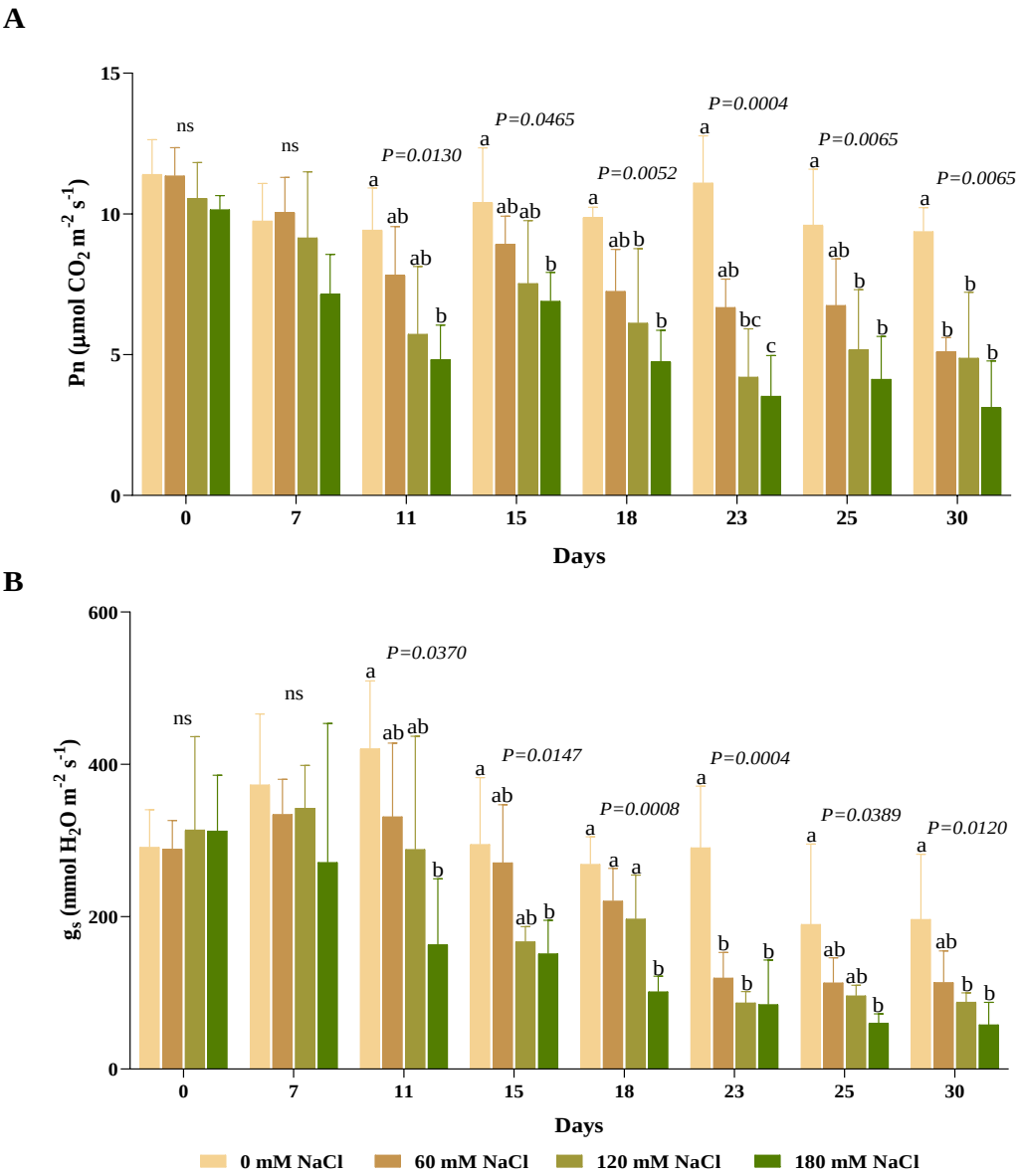

Figure 17. Foliar gas exchange parameters estimated to ambient CO_2 in *Populus alba L.* "Villafranca" clone during one month of salt treatment (0, 60, 120 and 180 mM NaCl). All analyzes were performed on days 0, 7, 11, 15, 18, 23, 25 and 30 after the start of treatments. (A) Stomatal conductance to water vapor (G_s, mmol m^{-2} s^{-1}). (B) net photosynthetic rate (P_n, µmol CO_2 m^{-2} s^{-1}). The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test ($P < 0.05$).

The chlorophyll and flavonols content were also analyzed by non-destructive measurements, twice a week, during one month of NaCl treatment. The chlorophyll content of the leaves was measured with the SPAD meter, which showed that the treatments did not significantly affect the chlorophyll content ($a + b$) until the 25th day of treatment (Fig. 18A). After the treatment period, we observed changes in chlorophyll content among the different experimental groups. In the control group, there was a slight increase of 4.4 % in chlorophyll content compared to the initial measurement. However,

in treated groups, was observed reductions in chlorophyll content. Specifically, the chlorophyll content decreased by 30.2 %, 32.5 %, and 40.4 % in the 60 mM, 120 mM, and 180 mM NaCl groups, respectively. Regarding flavonol content, changes were observed in the first week of treatment, on the 7th day. At the end of the treatment period, a consistent trend of decreasing values was observed in all experimental groups (Fig. 18 B). The control group had a modest decrease of 3.8 %, while the groups treated with 60, 120, and 180 mM NaCl decreased by 8.7 %, 25.9 %, and 29.4 %, respectively.

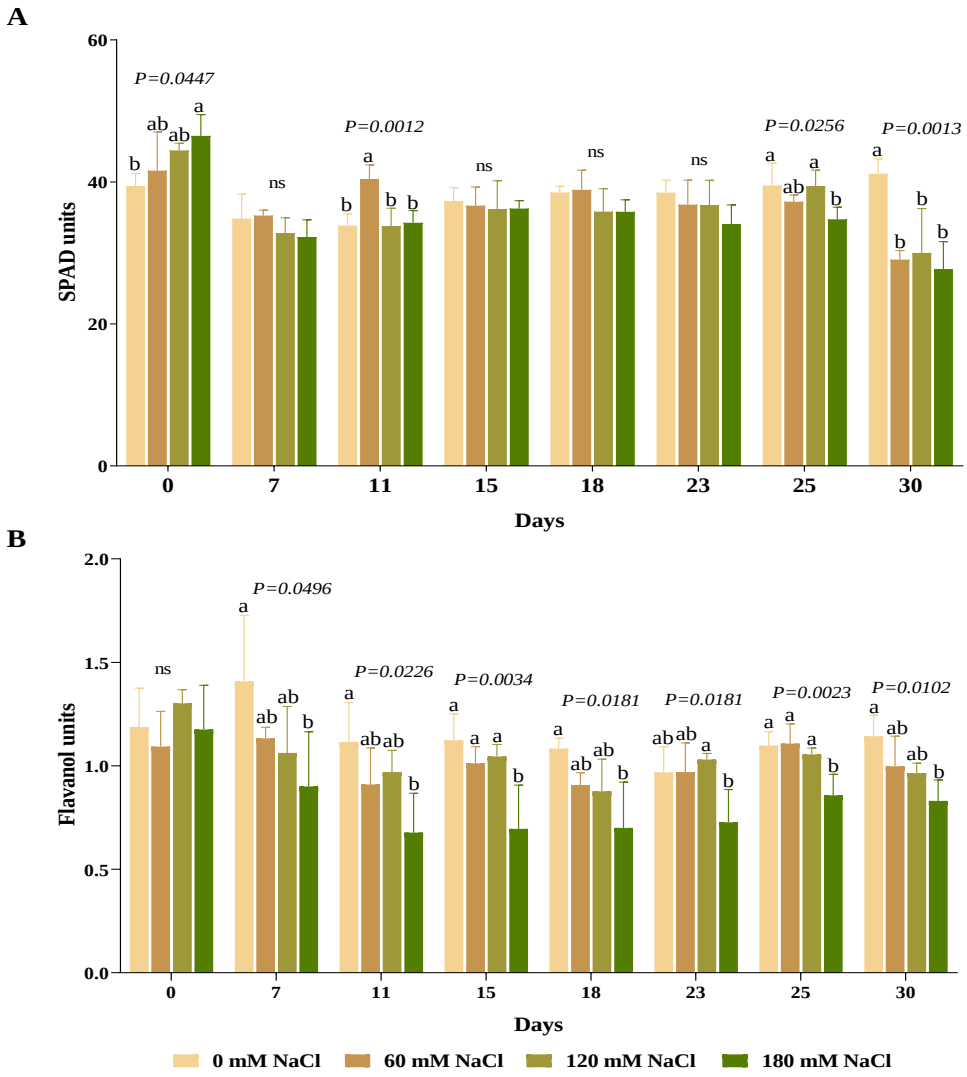

Figure 18. Non-destructive physiological analysis in *Populus alba L* .“Villafranca” clone during one month of salt treatment (0, 60, 120 and 180 mM NaCl). All analyzes were performed on days 0, 7, 11, 15, 18, 23, 25 and 30 after the start of treatments. (A) Flavanol content. (B) SPAD readings. The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test ($P < 0.05$). ns, non-significant.

4.2. Proline content

The proline content in the apical leaves and roots appears to follow a similar curve, with the proline content increasing proportionally with increasing NaCl, but with a

decrease in the group treated with 180 mM NaCl (Fig. 19). In apical leaves the treatment with 120 mM NaCl resulted in the highest concentration of proline (43.2 ± 9.36 mg/g FW), while the group treated with 180 mM NaCl resulted in the lowest content (14.6 ± 7.47 mg/g FW). In the basal leaves, the group treated with 60 mM NaCl is the one with the highest proline content (8.8 ± 0.68 mg/g FW), while the other groups are not statistically different.

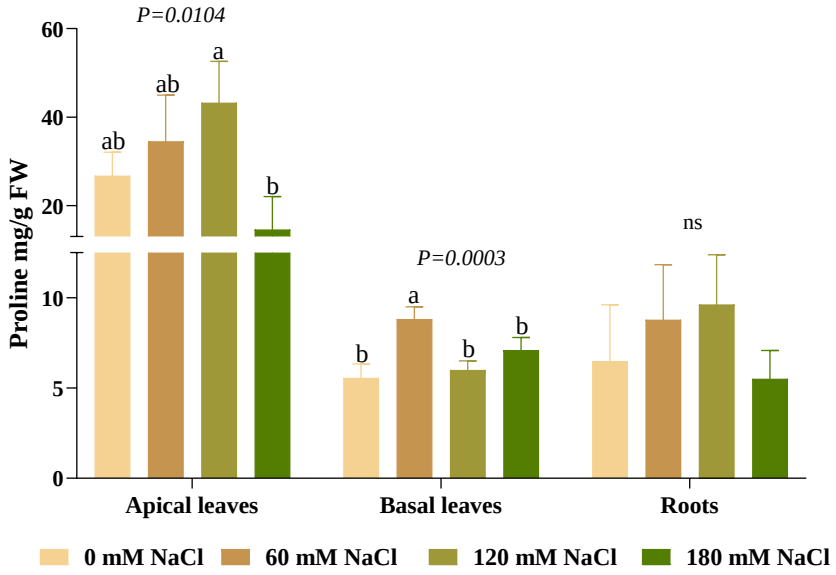

Figure 19. Proline content in *Populus alba L.* "Villafranca" clone organs after one month of NaCl treatment. The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test ($P < 0.05$). ns, non-significant.

4.3. Mineral elements content

As expected, the analysis of ion concentrations revealed that there was an increase in the concentration of sodium (Na) in all organs and especially in the plants in the group treated with the highest concentration (180 mM NaCl; Fig. 20). In the apical and basal leaves, the only group that showed significantly higher values than the control was the group treated with 180 mM NaCl. In the apical leaves the average Na concentration was 8.7 ± 2.28 mg/kg DW and 30.4 ± 1.73 mg/kg DW for the control and 180 mM NaCl respectively. While for the basal leaves the Na concentration was 23.7 ± 4.09 mg/kg DW for the control and 47.8 ± 9.75 mg/kg DW for the group treated with 180 mM NaCl. The roots are the organ with the highest concentration of sodium and all groups treated with NaCl present higher results than the control. The sodium concentration in the roots were 1396.9 ± 425.67, 9573.2 ± 1479.99, 11951.5 ± 1980.02 and 14412.9 ± 2757.01 mg/kg DW in the groups 0, 60, 120 and 180 mM NaCl respectively.

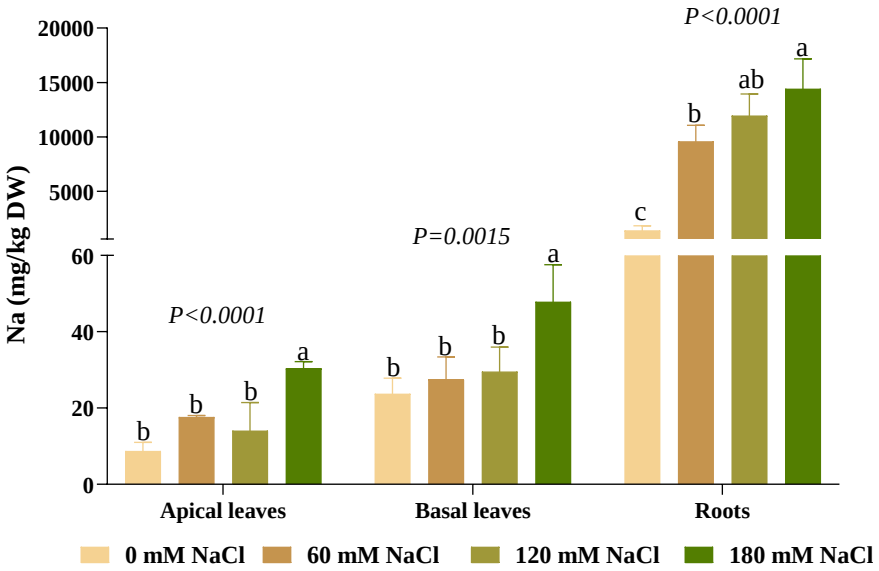

Figure 20. Sodium (Na) concentration in *Populus alba L.* "Villafranca" clone organs after one month of NaCl treatment. The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test (P < 0.05).

Regarding potassium concentration (Fig. 21), there was no significant difference in the leaves between the groups. However, in the roots it is possible to notice the difference between the control and the two highest concentrations (120 and 180 mM NaCl). The potassium concentration in the roots of the control group was 11140.7 ± 1455.68 mg/kg DW, while in the groups treated with 120 and 180 mM NaCl they were 6233.6 ± 438.87 and 8026.9 ± 2014.6 mg/kg DW, respectively.

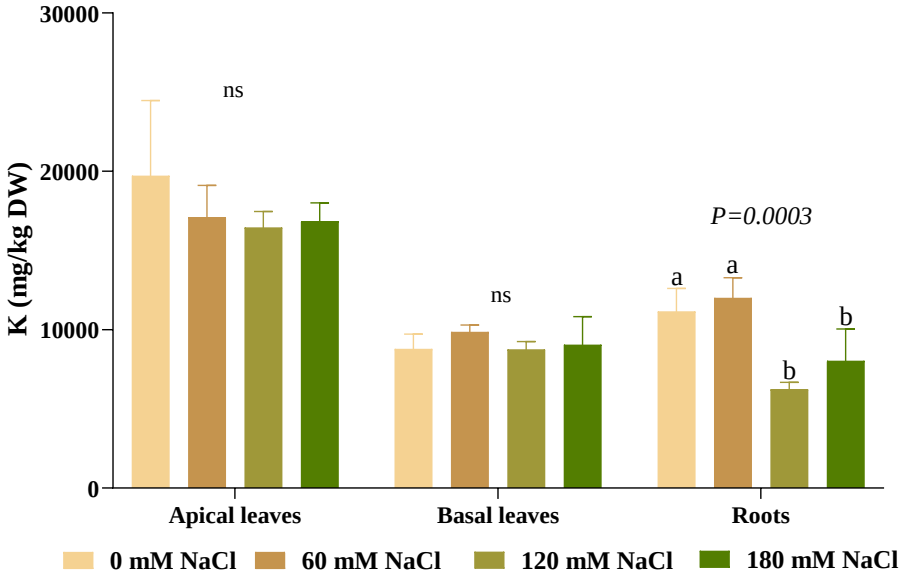

Figure 21. Potassium (K) concentration in *Populus alba L.* "Villafranca" clone organs after one month of NaCl treatment. The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test (P < 0.05). ns, non-significant.

In the calcium content, it is possible to observe changes only in the leaves and not in the roots (Fig. 22). In the apical leaves there was an increase in calcium concentration about 66% in all groups treated with NaCl compared to the control group. In the basal leaves the increase was more prominent. The control group had a content of 9372 ± 717.40 mg/kg DW, while the remaining groups had a content of 12699 ± 1683.50, 15570 ± 522.80 and 16388 ± 1519.60 mg/kg DW (60, 120 and 180 mM NaCl respectively).

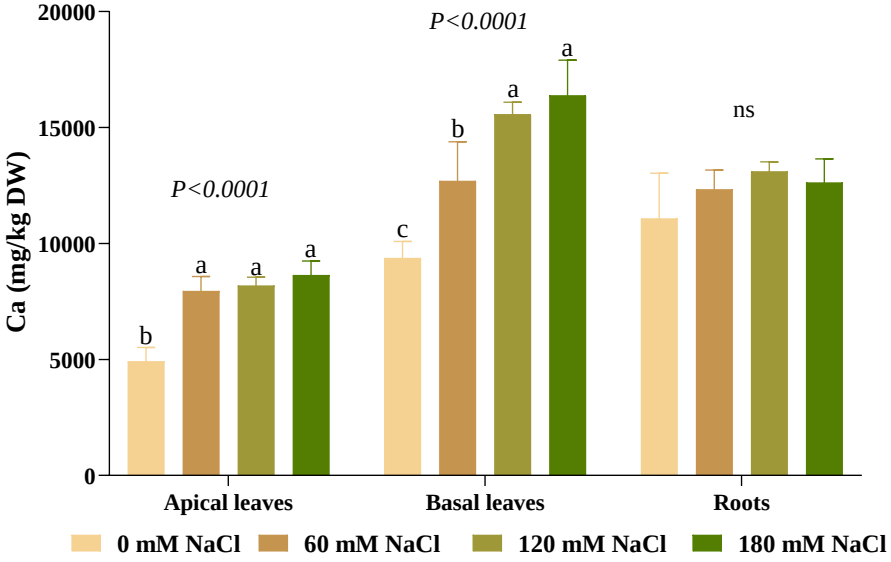

Figure 22. Calcium (Ca) concentration in *Populus alba L.* "Villafranca" clone organs after one month of NaCl treatment. The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test (P < 0.05). ns, non-significant.

PCA was performed (Fig. 22 A) in order to elucidate the correlation between mineral elements and the different parts and treatments of *P. alba*. The highest eigenvalues were achieved for two main components which explained a 92.4 % in total of the variability. With this result, it is possible to observe that the apical leaves and roots of the control group form a cluster with potassium, basal leaves form another cluster with calcium, while the treated roots form a group with sodium.

The linear correlation (Fig. 22 B) between the elements also clearly shows that the concentration of sodium has a negative correlation with potassium and a positive correlation with calcium. As the concentration of sodium increases, there is an increase in the concentration of calcium and a decrease in potassium.

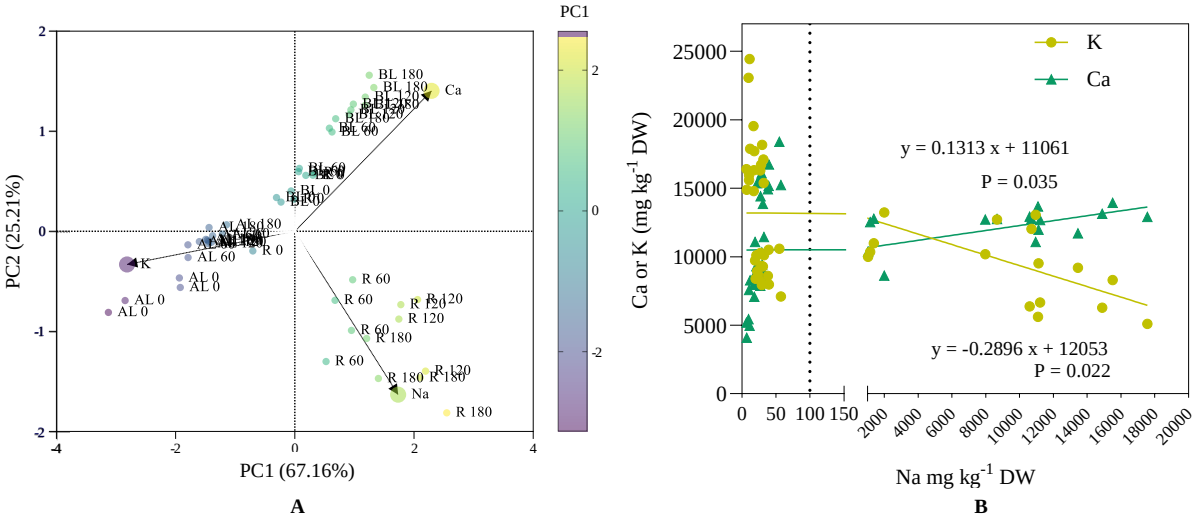

Figure 23. Correlation between sodium, potassium and calcium in *Populus alba L.*"Villafranca" clone after one month of NaCl treatment. (A) PCA scores plot of mineral elements parameters data *of Populus alba L.*"Villafranca" clone indicating clustering of basal leaves samples and Ca, apical leaves and K and treated roots with Na. R, roots. AL, apical leaves. BL, basal leaves. 0, 60, 120 and 180 mM NaCl. (B) Linear correlation between sodium, potassium and calcium in different part of plants of *Populus alba L.* "Villafranca" clone.

4.4. Bioinformatic analysis

To investigate the molecular mechanisms involved in the expression of glutathione S-transferase (*GSTF1*), γ-glutamylcysteine synthetase (*GSH1*), and glutathione peroxidase (*GPX1*) in *P. alba* "Villafranca" clone as a response to salt stress the target genes were identified by bioinformatic techniques. To identify the sequences of *GSTF1*, *GSH1* and *GPX1* within the genome of *Populus alba L.*, the corresponding sequences were initially identified from the reference genome of *P. trichocarpa*. Sequences were selected from the NCBI and Phytozome databases. To identify the sequences within the genome of *P. alba*, a BLAST search was conducted using the selected sequences from *P. trichocarpa* as queries. The sequences selected at NCBI for *P. alba* L. were XM_035063639.1 (*GSTF1*), XM_035038773.1 (*GSH1*) and XM_035060439.1 (*GPX1*) (App. 1). These sequences were selected considering some characteristics such as number of exons and 5'UTR and 3'UTR regions (Table 8).

Table 8. Location, Chr, exon number and gene ID information of target genes in *P. trichocarpa* and *P. alba.*

Name	Gene ID	Gene name	Location	Chr	Exon
GSTF1					
P. trichocarpa	LOC7496893	Glutathione s-transferase F11	NC_037286.2 (921674..922881)	2	3
P. alba	LOC118052639	Glutathione s-transferase F11-like	NW_023271512.1 (5003..6208)	Unplaced Scaffold	3
GSH1					
P. trichocarpa	LOC7472768	Glutamate--cysteine ligase, chloroplastic	NC_037285.2 (8421738..8429079)	1	16
P. alba	LOC118033691	Glutamate--cysteine ligase, chloroplastic-like	NW_023272025.1 (755799..763145)	Unplaced Scaffold	15
GPX1					
P. trichocarpa	LOC7468695	Phospholipid hydroperoxide glutathione peroxidase 1, chloroplastic	NC_037290.2 (26095899..26098509)	6	6
P. alba	LOC118050184	Probable phospholipid hydroperoxide glutathione peroxidase	NW_023271433.1 (225794..228406)	Unplaced Scaffold	6

The coding sequence (CDS) of the *GSTF1* gene in *P. alba* was 648 bp encoding 215 amino acids and exhibits a 95.5 % identity match with the corresponding sequence in *P. trichocarpa*. Both sequences have the same number of exons, highlighting their close evolutionary relationship. On the other hand, the *GSH1* sequence in *P. alba* does not have the same number of exons as *P. trichocarpa*, however they have a percentage of identity of 97.7 %. Its CDS was 1581 bp encoding 526 amino acids and 15 exons. Concerning the *GPX1* gene, its coding sequence spans 699 base pairs, translating into 232 amino acids. The *P. alba* sequence shares an identical number of exons to that of *P. trichocarpa*. The remarkable similarity is further highlighted by a percentage identity of 97.7 % between the sequences of *P. alba* and *P. trichocarpa*, indicating a high level of genetic resemblance. The high similarity in the protein sequences of these two species is a strong indication of a significant biological relationship, whether due to common ancestry or evolutionary conservation of the function of these enzymes (Fig. 24).

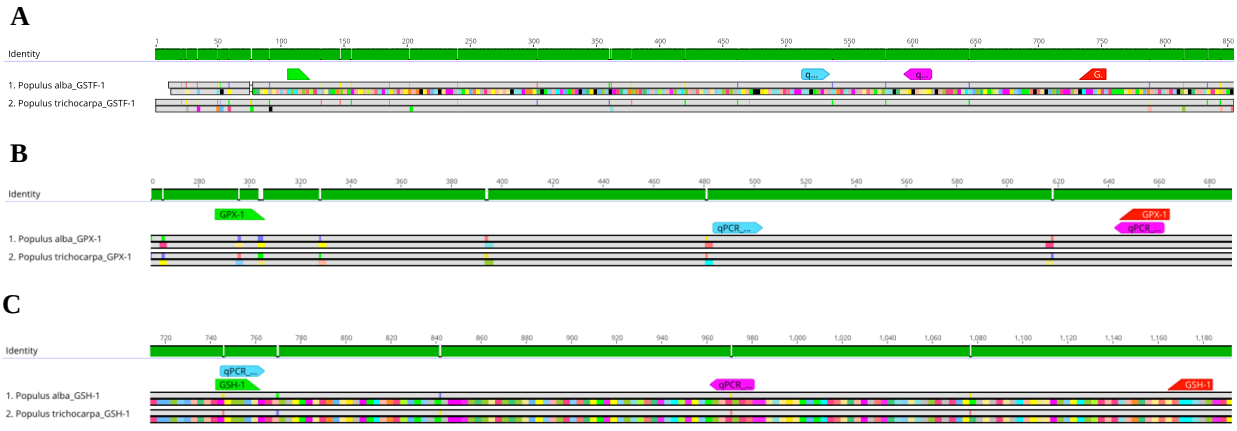

Figure 24. Pairwise Sequence Alignment of coding sequences of (A) *GSTF1*, (B) *GPX1* and (C) *GSH1* of *P. trichocarpa* and *P. alba*.

4.5. Gene amplification by PCR

To verify the presence of the selected genes in the genome of *P. alba* "Villafranca" clone, PCR assays were performed. For each gene, two different pairs of primers were designed to target different regions of the genome. This approach ensured comprehensive amplification coverage, confirming the presence of these sequences in the clone's genome. Different temperatures were also tested during PCR to optimize the specificity of the primers. However, all temperatures tested showed positive results. The presence of the three genes was confirmed in the genome of *P. alba* "Villafranca" clone through the electrophoretic gel (Fig. 25). For *GSTF1* the full length of the sequence (843 bp) and a fragment of 696 bp were amplified. For the *GPX1* gene, the complete sequence (2198 bp) and a smaller fragment of 1613 bp were also amplified. For the GSH1 gene, two distinct fragments were amplified, one of 1560 bp and the other of 1784 bp.

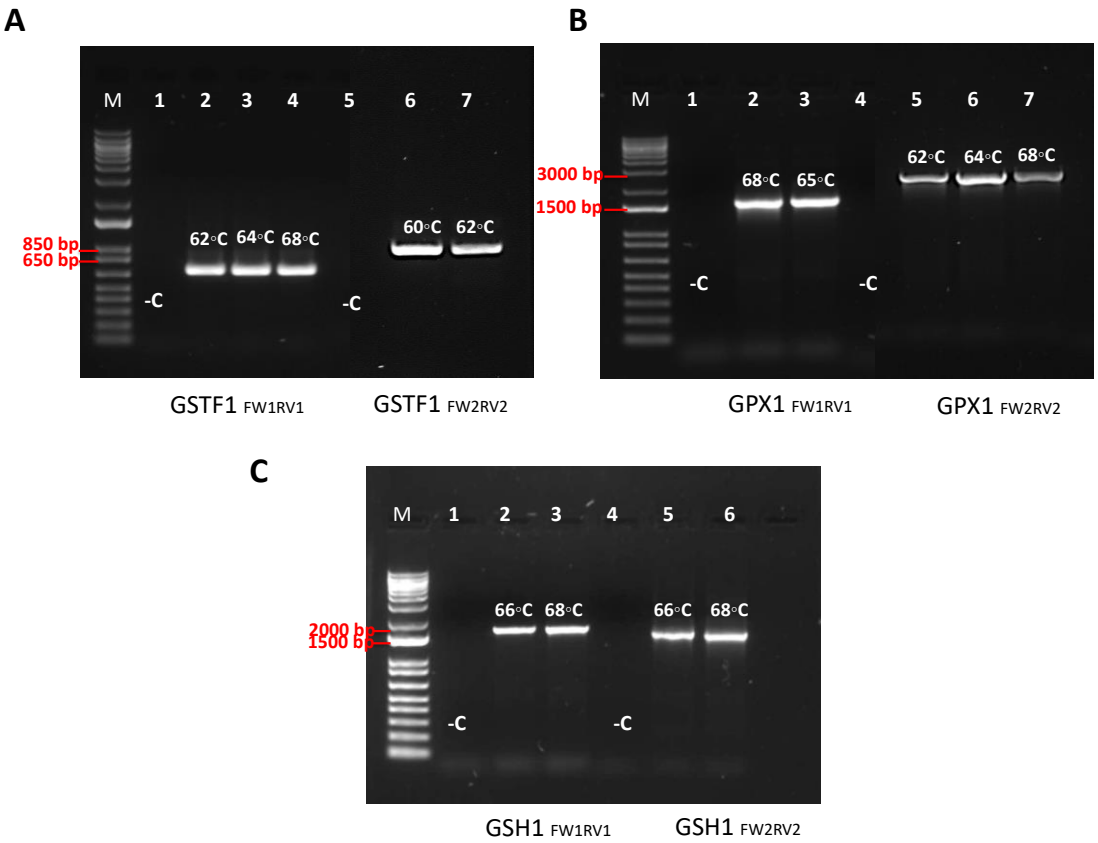

Figure 25. Polymerase Chain Reaction (PCR) amplification of target genes in the genome of the *Populus alba L.* "Villafranca" clone. (A) Amplification of both the full-length (843 bp) and a 696 bp fragment of the *GSTF1* gene. (B) Amplification of both the full-length (2198 bp) and a 1613 bp fragment of the *GPX1* gene. (C) Amplification of two distinct fragments of the *GSH1* gene using different primer sets. M, marker; -C, negative control.

4.6. Gene expression

The relationship between salt stress and the expression levels of the genes *GSTF1*, *GPX1*, and *GSH1* was evaluated in control and 180 mM NaCl-treated group, specifically in roots and basal leaves, as these organs were most affected. Among the three genes involved in the glutathione pathway, only *GSTF1* showed a significant change during salt stress (Fig. 26). In both basal leaves and roots, an increase in gene expression was observed when the plant was exposed to stress, indicating that *GSTF1* is up regulated under stress conditions. In basal leaves, there was a 162.5 % increase in expression in the 180 mM NaCl-treated group compared to the control. In roots, the increase was 179.6 %. Thus, *GSTF1* is expressed more in roots than in leaves, and its expression significantly increases during stress.

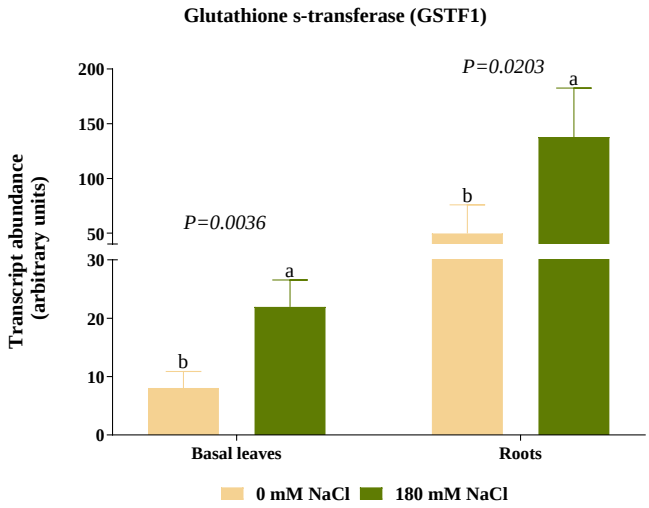

Figure 26. Relative transcript abundance of *GSTF1* in basal leaves and roots of *Populus alba L.* "Villafranca" clone after one month of salt treatment. The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by unpaired two-tailed t-test with Welch's correction. Values followed by different letters are significantly different ($P < 0.05$).

Regarding the *GPX1* and *GSH1* genes, no significant changes were observed (Fig. 27). The *GPX1* gene is more expressed in basal leaves than in roots, with a significant difference, but there was no difference among the groups. In the *GSH1* gene, no differences are observed between organs, leaves and roots show a very similar level of expression. However, among the groups, it is possible to observe a decrease in the group treated with NaCl, but these changes are not significant.

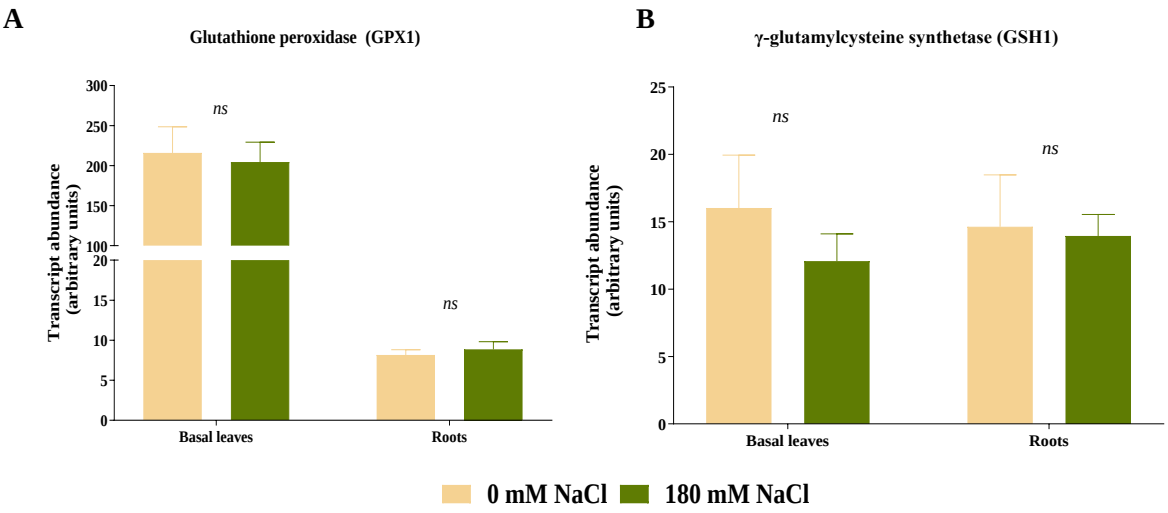

Figure 27. Relative transcript abundance of (A) *GPX1* and (B) *GSH1* in basal leaves and roots of *Populus alba L.* "Villafranca" clone after one month of salt treatment. The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by unpaired two-tailed t-test with Welch's correction. Values followed by different letters are significantly different ($P < 0.05$). ns, non-significant.

5. Discussion

Soil salinity is one of the main limitations to plant growth and development, posing a significant challenge to agriculture and ecology worldwide. Among the many salinity-sensitive species, *Populus alba L.* emerges as a species of particular interest due to its wide geographic distribution and economic and ecological importance. Furthermore, *Populus alba L.'s* ability to adapt to a variety of environmental conditions makes it an excellent candidate for studies on the effects of salinity. However, despite its importance, our understanding of the mechanisms underlying the effects of salinity in this species and different clones is still developing. In this context, this study investigated the effects of salinity on *Populus alba L.* "Villafranca" clone, exploring its morphological, physiological, biochemical, and molecular responses under different levels of salinity.

Among all the negative effects of saline stress, the first responses noticed are in the morphology of the plant. In this work, the morphological changes analyzed were in relation to the weight, height, and appearance of the plants. After one month of treatment, a gradual reduction in fresh weight was observed in all organs (leaf, stem, and root) of the plants proportional to the increase in salinity. This can occur because, in the presence of high concentrations of salt ions, an osmotic gradient is created that is unfavorable for water absorption by the roots. This can lead to dehydration of the plants and a decrease in their fresh weight (Zahra *et al*., 2020). Regarding plant growth, the salt concentrations used in this work did not seem to interfere with this parameter in *P. alba* "Villafranca" clone, as no significant changes were observed. The same was reported by Della Maggiora *et al.* (2023), in a study of salinity in *Populus alba L.* "Marte" clone. In terms of appearance, it is possible to notice clear symptoms of stress in the basal leaves of *P. alba* treated with 120 and 180 mM NaCl, which present yellow colors, this is due to excessive concentrations of saline ions that harm the photosynthetically active leaves, leading to chlorosis.

Abiotic stresses such as Na exposure can affect the photosynthetic system and decrease fixation of CO_2 by affecting components such as the aperture of stomata through which CO_2 and H_2O gases are exchanged. Photosynthetic gas exchange of poplar leaves is highly sensitive to salt stress and even low concentrations of NaCl can reduce net assimilation without visibly damaging the leaves. In this work, a clear decrease in g_s and P_n was observed in all groups treated with NaCl. Even in the group treated with the lower concentration of 60 mM NaCl, where no visible changes were observed, there was a significant decrease in G_s and P_n in the second week of treatment. After one month of treatment, the values of G_s and P_n of this group decreased by 60.5 % and 55.1 %

respectively. A similar decrease in these parameters during salt stress was also reported by Della Maggiora *et al*. (2023) in *P. alba* "Marte" treated with 100 mM NaCl and by Abbruzzese *et al*. (2009) for three different genotypes of *P. alba* (6K3, 2AS11 and 14P11) treated with 250 mM NaCl. The same is observed in different plant species such as *Portulaca oleracea* (Hnilickova *et al*., 2021), *Vicia faba* L. (El-Dakak *et al*., 2023) and Olive (Aliniaeifard *et al*., 2016). This decrease in stomatal conductance occurs because salt stress leads to the closure of stomata to optimize water use efficiency and minimize the transpiration rate. However, gas exchange in leaves occurs through stomata, thus causing a reduction in carbon dioxide (CO_2) intake. The reduction in stomata conductance also affects the photosynthetic rate. Although environmental stress may have direct effects on stomatal characteristics that cause a decrease in photosynthesis, non-stomatal factors also limit the photosynthetic performance. Therefore, the reduction in photosynthetic rates could occur due to stomatal and/or non-stomatal factors. Stomatal closure leads to CO_2 shortage. Non-stomatal factors include increased CO_2 diffusive resistance in the mesophyll, PSII reduction, decreased chlorophyll content, and inhibited electron transport (El-Dakak *et al*., 2023). In summary, the decrease in G_s and P_n in plants under saline stress can be seen as an adaptive response, where the plant tries to reduce water losses and optimize the use of resources in the face of unfavorable soil conditions.

Regarding chlorophyll content, the plants were analyzed using a non-destructive measurement, the SPAD meter. Until the 23rd no significant changes were noticed, this can be seen as a period of adaptation of the plant or by the location of the measured leaf. During the analysis, a leaf located in the middle of the plant was chosen, but at the end of the treatment it was possible to notice that the most affected leaves are in the basal part, this may explain the delay for changes in the chlorophyll content. However, in the last week of treatment, from day 25 onwards, it is possible to notice significant changes in the chlorophyll content. Chen *et al.* (2018) also reported an extensive decrease in total chlorophyll content in *Populus* × *euramericana* cv. '74/76' after 25 days of treatment with 6 % NaCl (Vol 150 mL). The study carried out by Lu *et al. (*2020), observed a significant decrease in the chlorophyll content in WT *Populus* treated with 200 mM NaCl for four weeks. The reduction in chlorophyll content in plants under salt stress is a process already observed in many plant species, not just poplar. For example, in *Oryza sativa* leaves, chlorophyll reduction was observed after 14 days of treatment with 200 NaCl (Amirjani, 2011). In *Cucumis sativus* L. the decrease in total chlorophyll content was 30 % at 5 dS m^{-1} after 14 days of treatment (Khan *et al.,* 2013). In general, the decrease in

chlorophyll content under salt stress is a commonly reported phenomenon in several species, this may be due to different reasons, one of them is related to membrane deterioration.

The flavonol content is also a parameter greatly affected by salinity, as it is a secondary metabolite and acts as an antioxidant against ROS produced during stress. Throughout the treatment period, the flavonol content of the groups treated with 60 and 120 mM NaCl remained statistically like the control, which can be interpreted as a response of the antioxidant system against short-term salt stress. However, in the group treated with 180 mM NaCl there was a significant decrease during the treatment period, which demonstrates that *P. alba* "Villafranca" is susceptible to high concentrations of NaCl. This means that under extreme conditions of environmental stress, the production of antioxidants in plants cannot keep up with the magnitude of oxidation, leading to an increase in the ROS content in the cell and a decrease in the antioxidant content (Shomali *et al*., 2022).

Regarding proline concentration, it was affected with increasing salinity. Between the apical leaves and the roots, it is possible to observe a trend, where there is an increase in proline with increasing salinity, and which decreases after treatment with 180 mM NaCl, which may show that beyond this concentration, negative changes are observed in the plant health. However, contrary to this, the study by Kulczyk-Skrzeszewska and Kieliszewska-Rokicka (2021) using *P. nigra* "Italica", reported an increase in proline concentration along with an increase in salinity. Where the highest proline concentration was observed in the group treated with the highest saline concentration (250 mM NaCl). However, proline concentration is a parameter that changes according to the species and clones, which may explain this difference. For example, the study by Khoma *et al.* (2021) used four different *Populus* clones and four drought levels. And the results showed that the concentration of proline did not vary according to stress, but rather according to the clones. Different clones have different responses. In basal leaves, no trend was observed; plants treated with 60 mM NaCl resulted in an increase in proline concentration, while the other groups had lower and statistically equal results.

As expected, mineral analysis showed that Na^+ levels increased in all organs with increasing salinity. In leaves, sodium accumulation was only significant in the group treated with the highest concentration (180 mM NaCl). While in the roots there was a considerable increase in all treatments, as they all showed a difference in relation to the control. This accumulation of ions in the root shows the exclusion capacity of this clone,

which prevented the translocation of Na^+ to the aerial part of the plant. However, it was only efficient until treatment with 120 mM NaCl, as with higher concentrations, such as 180 mM NaCl, it was unable to prevent translocation. Regarding potassium concentration, there was no significance in the leaves, only in the roots. In the roots, it is possible to observe that with an increase in salinity there is a decrease in the concentration of K^+. This happens because Na^+ can compete with K^+ for the binding sites of the uptake system, resulting in an imbalance of the K^+/Na^+ ratio that eventually causes ion toxicity (Zhang *et al.*, 2019). For this reason, it is essential that the plant can maintain a balance between K^+/Na^+ ratio, otherwise it will result in stress. The same trade was reported in the work of Della maggiora *et al*. (2023) in *P. alba* "Marte" clone treated with 100 mM NaCl. Concerning calcium concentration, only in the leaves was a significant difference observed in relation to the control. It is possible to observe that the calcium concentration increases proportionally to the increase in salinity. Ca^{2+} is an important secondary messenger in plants and mediates poplar salt tolerance by increasing Na^+ exclusion, restricting K^+ efflux, and sustaining cell membrane selectivity. During NaCl exposure, the resulting elevated cytosolic Ca^{2+} levels are detected by SOS3, which activates SOS2 and stimulates the membrane-localized Na^+/H^+ antiporter SOS1, resulting in Na^+ efflux into the root apoplast (Zhang *et al.*, 2019). Therefore, an increase in Ca^{2+} concentration is a signal to salt transporters that the plant is under stress. In summary, a strong relationship is observed between the apical leaves and roots of the control group with potassium, basal leaves with calcium and treated roots with sodium. And there is also a negative relationship between sodium and potassium and a positive relationship between sodium and calcium.

Regarding gene expression, three genes from the glutathione pathway were analyzed: *GSTF1*, *GPX1*, and *GSH1*. However, only *GSTF1* showed changes after salt stress. Following RT-PCR analyses of different organs- basal leaves and roots - a predominant expression in the roots was observed. The work by Gao *et al.* (2022), which analyzed the expression of the same gene, *PtGSTF1*, in *Shanxin yang* plants treated with 150 mM NaCl, demonstrated that the highest level of expression was observed in the leaves and not in the roots, as found in the present study. Furthermore, they observed the highest level of expression during a short period of stress exposure, only 4 hours. This indicates that the expression of this gene can vary significantly according to different factors such as species, organs, and exposure time. Song *et al*. (2024), also reported an increase in the expression of the *GSTF* gene in poplar (*P. alba*×*P. glandulosa*) during salt stress. The

highest level of expression was presented in the treatment with 150 mM NaCl, which suggests a potential involvement of this gene in the poplar response. The work also reported that in the group treated with 200 mM NaCl, the gene expression decreased, indicating that beyond this concentration, the accumulation of ROS is already greater than the antioxidant response. Other studies have also shown an increase in the expression of this gene family during stress exposure, suggesting that it is an important gene in the plant's defense system (Gao *et al*., 2022; Niu *et al*., 2024).

Concerning *GPX1* and *GSH1*, no differences in expression were observed after NaCl treatment. Similar results were found by Meng and Wu (2017), who analyzed the expression levels of different genes in the *GPX* family in poplar under both salt stress and normal conditions. Their study demonstrated that among the various *GPX* genes, *GPX1* exhibited a low level of expression in both conditions. In contrast, several studies demonstrate that in other species both genes are up-regulated during salt stress (Rubio *et al*., 2009; Shavrukov, 2013; Cheng *et al*., 2015). Therefore, as they are genes with lower expression in *P. alba*, but which demonstrate positive effects on the response to stress, a promising solution would be their overexpression in the plant genome.

6. Conclusion and future perspectives

This study investigated the responses of *Populus alba L.* "Villafranca" clone to salt stress at multiple levels, from morphological characteristics to gene expression. Based on these results, we can conclude that at a moderate salinity level of 60 mM NaCl, no negative effects on the plant's morphology were observed. However, at concentrations above this, chlorosis and a decrease in plant weight were noted. Analyzing the physiology, all measured values decreased across all treatments with sodium chloride, indicating that these parameters are sensitive to salinity. Regarding proline content, it increased up to the 120 mM NaCl treatment, indicating a plant response to stress, but above this concentration, the proline levels decreased. The results for mineral elements showed that salinity stress increased the levels of sodium and calcium while decreasing potassium. Sodium mainly accumulated in the roots, with only a small amount moving to the aerial parts of the plant, which is an important adaptive response. At the molecular level, there was a significant increase in the expression of the *GSTF1* gene in the group treated with 180 mM NaCl, indicating its role in the plant's defense mechanisms. In summary, at moderate salinity levels, such as 60 mM NaCl, *P. alba* “Villafranca” can grow normally, but at concentrations above this, more intense negative effects are observed.

Different types of responses were observed in plants subjected to stress, highlighting the importance of understanding these mechanisms for sustainable agriculture. The identification of genes such as *GSTF1*, paves the way for future research to develop strategies to increase plant tolerance to salinity. Controlled overexpression of this specific gene could potentially lead to the development of genetically modified plants more tolerant to salt stress. Furthermore, this study highlights the potential of *Populus alba L.* as a valuable model for salt stress tolerance studies, providing valuable information that can also be applied to fruit trees. In this way, conclusions presented here contribute not only to the understanding of plant defense mechanisms but also to the development of more efficient and sustainable agricultural practices, essential to face future challenges.

7. References

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8. Appendix

Appendix 1. Genomic sequences of *GSTF1*, *GSH1* and *GPX1* genes, with exonic regions highlighted in grey.

GSTF1: 1206 bp genomic sequence.

>NW_023271512.1 *Populus alba L.* glutathione S-transferase F11-like (LOC118052639)
CTTCCTGCTGTGTGACTATAAGTAAAGTGACCAGAGCACGCTAAAATCCACTCCAGCAGCAAGACGTAAT
CTTTCCTGCAACTAGCAAATCTAATGGCAACTCCGGTGACTATTTACGGGCCACCATTGTCCACGGCAGT
GTCGAGAGTCCTAGCTACTCTGATCGAGAAAGATGTGCCCTTTCACCTCATTCCCATTGACCTCTCCAAA
GGTGAACAGAAGAAGCCCGAGTACCTCAAGATCCAGGTACTGATCAGTATCTCTATCTTGGTGACTGCCA
TTTTCTTTCCTCCATTCACATCCTCACCGCCTCCTTTGACTCTTAACAGCCCTTTGGCCAAGTACCAGCT
TTTAAAGACGAGAGCATCACCCTATTTGGTAAACTACACCAAATTCTAAATTATGATCATCATTCTCCAT
TCACTTAATCCTCTTCATAATCCCTCGTTAATTGTTCTTTTTACATGTGTTTTTTGATTGTTTGTTGCAG
AGTCAAGAGCAATATGCAGGTACATATGTGACAAATATGCTGACAAGGGAAATAAGAGCTTATACGGCAC
GGACATCTTATCAAAAGCAAATATAGATCAATGGGTAGAAACGGATGGGCAGACTTTCGGTCCACCAAGC
GGGGACTTGGTGCACGACCTTCTGTTTAGTAGTGTCCCAGTAGACGAAGCCTTGATAAAGAAGAACGTAG
ATAAGTTGGCGAAAGTGCTCGACATTTACGAGCAGAAGCTTGGACAGACTCGGTTTTTGGCCGGAGATGA
ATTTTCTTTTGCTGATCTTTCACACCTGCCTAATGGGGACTACTTGGTGAATTCAACCGACAAGGGATAC
CTGTTTACTTCCAGGAAGAATGTAAACAGGTGGTGGACTGAGATTTCTAACAGGGAATCCTGGAAGAAGG
TTCTTGAAATGAGGAAAAATGCTTGAAATTGGTGGTCGTTTTTTGCTCGGTCTGCCAGCACTCTGCACTC
CGCAGTCTCAAGTGAGGTTTTTAAATAAGTTGGCGGCCTCTACCTTGCTCGGTGTGATTTGTAATTTTGT
AATGCAATTATCTATGTTTCCAACCTTATCCATGTGTCTCAGCTGATGTTGTCTGTAGCATGTGTGCTTT
AGACATCGAGGCGATAGGTTGCCTGTTTCTTGTTGATCCAATAAAAACTGCCATGTCTTGGGCTTCATGA
CCCATTCTTCCTTTTT

GSH1: 7347 bp genomic sequence.

>NW_023272025.1 *Populus alba L.* glutamate-cysteine ligase, chloroplastic-like (LOC118033691)
TGTTTGTAGGTCCGCACTCATTTGATTTTTTTCCTTCCTTTCCTCACTCTCTAGTCTCTTCGCTTTACTA
CAAAAGCCAACCAGCCCAACCAAAAGATCTTTCCTTTCTTAGCTGCCACCTCTCTCTCAAAAATATATTT
CTCCTCTCGCAAATTCTTCAATCCATAAGAACCTGTTACTATTCAATCCCTACAATTGTTTACTTTTCTC
TTCTTCTTAAAGGTAAAAACTTCGTCCTTTTTTTTCCTTCAAGTTTTTGGTTCATTTGATGCTTTCGTTG
CGTTACTGGTCTTTTTATATTATATGTTTTTACCTAATGGTAGAAATGAATGTCGCGTTGTTAAATGGTG
CATTGGGTATTGTGAAGGTAAGGAGAAGTGGAGTATTTTAATTTCTTTAGCATTTTTTTTTTCGCAAATT
TTTGTGAAGCGACCCTTTGTTGATTCTTGATTTGATAAGCTAATGTGGGTACTGTTTATCCTGATATGAT
AGGCAGCGTAGCACATGCTGGAGTTTTTTTTTTTTTTTTTTTTAAGAAATGGATATCATGTGAAATTATT
GGAAGTATCTCTTTAACCTTTTTTTTTTTTTTTTTTTTTGAGTTTTGGTCACTGAAAGTTGAAAAGGGGC
ATTATGTGAAATTGTTGAAAGCTGAATAAGTTCCGTATAGCTAACTTTTCCGACGTTTTACCACTGGCTG
GAATATAATTTGTTTGTGTTTATGGTTTTCTCGCTGGATGCTTGGTTCCTGTTGATTTTGACTTTGTAAG
TTGTAATTGAAAGGATGGGTGGGTTGTTGCAGGCATGACGCTGGTTGGCCAGGCTGGTCCATCATCCTCT
ATACTTACGGAGATAAGCCGCTGTAAAGCTGCACAAAATGTGGTTATTGCTATGGCTAGAAGCACCGATG
CATCTAAGCTAAAGGAAACACCTCCCAGATTCTCTTCATTGTCATGTAATTCTAGCAAGACACCTCAGAT
TCTGAGTTCAGAGAGTCTGTCAGTAGGAATGAAGAGAGGGCATCGAGTTGTTGTTGCCGCAAGCCCTCCT
ACAGAAGATGCTGTCATTGCCACAGAACCACTAACAAAAGAGGATCTTATCGGATACCTTGCCTCTGGTT
GCAAACCTAAGGAAAATTGGAGGTAAATTCTCAATCCGTGATTCTAGTTGGATTAGAGTGGTATCTTCTT
GTCAAAATTTTGCATAGATAAAAGGCTCTTCTTTTGCCCATTCCACCAATCCTAAATTCTGCAAATCGTA
GAGAGTCTTGAATTTTTGCAATTCAAATACTCTGTCTCAAGACATGTACATATCCATCGAAGGTCAATTA
CGATCAAACATATATATCCATAAGGGTTTGTTGTACCCTTCTGACTAGTCTATCTCAAACTGTAGTGCTT
TGCATGTTGTAAATCCAAGTACCTTCTGTTAAGGAAAAGGGTTCTGTTCCTTGTCCTTGGTAACAAACTT
GTTATTATCCCATTTCATTTTGTTTTGGTTGATACTCAGTACTATTGTGTTTTCATACTTGATTTCTGGT
TTTACCTATACCCTAGGTATTGCTCATTTACCGTGTTGCTTTTGCAGAATAGGTACTGAACACGAGAAGT
TTGGTTTTGAGATTGGAACTTTACGTCCTATGAAATATGAACAAATTGCAGAGTTGCTTCATGGTATTGC
TGAGAGGTTTGACTGGGACAGAATAATGGAAGGTGACAACATCATAGGACTTCAACAGGTAAAATGGTCA
TCATGGTTATCTTTTTCATAGCTAACTGCATCTCATACTTATGCATATCTTATACTCTGCTCTGGACTTA
TTTTCTACTGTTTATATACAAACTACTTGCATATCTTACATAGGAAATTTTGTGCTGTTTTCTCAAATGG
CCTGGGTTGTGGATTATGTGGTTTTAATTGCTGTGGCCAGGGGAAGCAAAGCATATCATTAGAGCCTGGT
GGTCAGTTTGAGCTCAGTGGTGCTCCTGTAGAAACTTTGCATCAAACGTGTGCTGAGGTTAATTCACACC
TTTATCAGGTAAGTTGAGATTACTACACATCAACATGACTTTTTCTCAGGTTAATTCAACATTTGGATTT

ATTTCTGGCAGTAATGACTTTTGCTCAGGTTAATTCAACATTTGGATTTATTTCTGGTAGTAGGACATTT
TTTTTTTGCAAACATCATCTGGATGAATATGAGATTGCGTGATGGTGCTGTTTTCTTTTTCTCAAATTTT
TTATGAAACACACATTCACTATGTCCTTGTGATCTTGCTGTCTTCATATGTTAATTTTACCAGGTGAAAG
CTGTCGCAGAGGAAATGGGAATTGGATTCTTAGGAATTGGCTTCCAGCCCAAATGGGGACTTAAAGACAT
ACCTGTTATGCCCAAGGTATAATAATCCAATCAATTTATTCTCCTTCTCATGCAATTAGCTTGGGTGGAA
AATATACTAAGACATGAAGACATGGATTATTAAGGAAAGTAAAAGGTAATAGTTGTCATGGAATGAGCTT
GAACATGCTTTCCATGCAAGACAAATGTGCCCTTGTAAACTTTTATGCAAGTTTTTGTGACATTTAACAG
AAGGGATAGAATAAATAAAATTATGATCATACACGTTTGTATTCTCAGTTTTTATATTGTTTCATGGGTT
TCATGTTTGGCCTTTTCTTGTCAAACCTTCATAGTATGCCATGAATGCTTCCTTCTTTGTTAAATATGGC
AGATTGTGTCTCTGAGCTTTCACATATCTTATGCCAATCTCTTGAGAACTGTTTAGTTTTGTGGCTCAAA
GCTGATTTGTCCAGCCAACTATACTCCCTAGTATATTCTTTCTATAAGCCATCTAAAGTTGCATGTGTAG
ACTGATTTAAGTGTTCATGCCTAGAGGAAAAGCCTAGAAATACAGGGGCTACTTTTAAGTATTGGAATAT
TTCATAGAGGTTCCTTTCCAATCTATCCTACAACATACCATCAGTGATATTTAATTTATGTTTGTTATCC
CAGCGATACTAGGCTTCTCATTTTAATCTCTTTTTTCAATGCCTCTTTTCTTTTCCTTTTGTTTTTACTG
CTGTTTATAATCATGCTTTCCCTTGGAAATTTGATTGCTTGTCTACTGACATGTATGTTGTCCAGGGAAG
ATATGATATCATGAGGAATTACATGCCTAAAGTTGGCTCGCTGGGACTAGATATGATGTTTAGGACTTGC
ACCGTTCAGGTTAGATGAATTTTGCTTTATTCACTGGTTTGGTCATTTTGAAGCAATTTCTTGCCTGGTG
GCTAATTTTCTAGTATAAACTTCCTTTTCTGTATTTGATGATTTTTTGTCTAATGTATATTGATAACTTT
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TTTTTCTGATTCTGTGATATCTTTTTATTGGAAAATAAACAGGTAGCAACAGCTCTATTTGCAAACTCAC
CTTTCACCGAAGGAAAACCAAATGGCTATCTCAGCCTAAGAAGGTTTCTAAACGTAATTGCTCTAGTGTA
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GATACTGACAACAATCGTGCTGGCATGCTTCCATTTGTTTTCAATGACACCTTTGGGTAAGTTATTAAGT
TGTTATTTGAGAGGAAAATAACATTTAAGCTTTTAATTTTTGGGTTGGGGGTTGTGGAGGATGAGCAGGT
TTGGGAATCCATCTACAGAATCTGTATTTCTGAGTTAATTTTTATTTTGAATACTGTAATGTACAGAAAT
CTAAGTTGGCATCGACTATGAGCACAGATGATCACTATCTGTGTGAGTATATATCTAAGGATATGTATAG
TTTAGCTCCTTGCTATTTTGTTTGCGATTAAGAGGAGTCATCATCACCCCTGACTTCTGAGTTAAAAAGG
ATTTAACATTGCCAGCATCAATCCCCTTTTTTATCACTTCTCAGATCAGGGGTAAATAAATGTGGACACA
TGCCCATTCCTGCATACAAATACATGTTACATGTATGTTTGCAAGGTTAAGTTCATAATGTATGCATGAA
TACTCGCATAAACAAATGCGCATGCACATGGTGCATGAAGTTGATTGTATGATAGTAGTTCTGCTTTATT
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GAGTGAGAGTCATAGTTGTACGATGTTTTCTTTTAGGATGTCATGCAGTAAACTGAATATAAGCAAAAGA
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GAAAGAGACTTTCATTTACTTTTTCTCAACATTCCTGATGCAAGTTCATGAAACCTGACATTGCTAGAGG
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TGATTCTGATATAAAATTGGTTTTTAGGTTCCAAAGACAGGTCTGAAGACACCATTTCGTGATGGACTGT
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GAGTTATGAACTATTGGATTAAATTTTATTTTATTATATGGTAAGAATTATGAAATTGATTCTTAATTAG
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GAGAAAAAATAATCAAATGCCATTAAAAAAAGCCATGGGTTTGTTGTAAAAAAGCCTAGTTGCATGGACT

TGAGGCAGGTTAATGTGTTAGATAATTTGTTTTTTCTTTTTACCTTGTTCTTTGAAAAAAAAAGTTCTGT
GACAGAGGACTGTCAACAACAAAAACTAATAGCCATCCTAGTGCTTGAACCTACAACCTTGCTTGATTAA
GAGTCTGTTTAGTATTATGGTAGTGATTATTTTTTAAAGTGTTTTTTACTTGGAAATATATCAAGATAAT
ATATATTTTTTTTTTTTAATTTATTTTTGACATCAGTCAACACAAGTTAAAAACATAAAAAACTTAATTT
GAAGCAAATAAAAAAAAAAATAAGTTTTTTTTAAAAATGCTTTTGAAATGTAAAAACAAAAAGGCACCAA
GGATACATGTTAAACCAAGTGAGTTACAGAACAAATATGTCATCAAACAATATATTAACTTGGTTAAACC
GACCCTGGTCTTTTTAGTTGTTTTGATAATTGTTCTCTAAACACGCTTATTCTACTGCAGGATGGCTTAG
AAAGGAGAGGCTTCAAGGAAGTTGGTTTCTTGAATGCAGTGTCTGATGTGGTTAGCACAGGTAATTTAGA
AGTAATTATGCATTTATCTTGCATGTTTCCCCAGTAATTTGGCTATGTTAACCTGCATAAGTTGGTTTCT
TAATGGTCATGATGATTGATAATTTTATGGCAGGAGTAACACCGGCAGAAAAGCTTTTGGAACTTTACCA
TGGAAAGTGGGGGCGGTCTATAGATCCTGTGTTTGAAGAGCTACTTTACTAAGGTGAATTAGATTTTTGT
AGCACTGCAGCAGTGTCCTTGTGTTTAATTCTCTCTCTCTCTCTCTGGGTTTAAACCTTGTTTATGTTCT
GAACTACTTTCTTCAACTGATACTGATAGTTTGTCTTTTGCCGAATAAATTTCAATGTAAATCCTTGGTA
TGAGGATTCTCACAAAACTCTCATCGAATAACACCCACATAGTTTGGAGCCAGTGAACAATTTTTTA

GPX1: 2613 bp genomic sequence.

>NW_023271433.1 *Populus alba L.* probable phospholipid hydroperoxide glutathione peroxidase (LOC118050184)
GGGTCAACTTTCAGACAATAGACTCATCCATGTAAGAAAATCAAGCAAGCAGACAAAAGGCAGCCTCGTG
GAGCCTTCACCTCCACCACAGACTTCACCTCAGAAACCCCAACATCGAGTTCCAACTTCTAGCTCCAGTT
CACATGCTATTAGGACACAAACATCAGCTTCCATCTCCCTAGAACCTCTCACCAACTCCCAGAATCCGCT
GATCAAACAGTAGTACCACATGGCTTCCTTACCTTTCTCCTGTTCATCTGTACTGGGTTTGAAAATCCAT
CCAAACCCTACAACAATGGCTCCCTTTGTCACTTCCATCAAGTCCCCACTTGGGCCTTCCAAATCTGCTT
TTTTGCAACGTGGGTTTTCTTTACAGTCTCCAAATCTTCCTAGGTTTGCCTCTAAAGCCCGGTCCTTTGG
TGTTTTTGCTAGAGCTGCTACAGAGAAATCTGTTCATGACTTCACTGTTAAGGTAGTAGCTCTTTTGATA
AAGCTTTTTGTTTTTTTACAGTACTGGTTCAACTGTTCAGTTTATCACCTTTCTGAGTCATGGAAGCTGG
GATTTGTTTTGATTTCTCTTGCTTGATTTAGGAGTTTGGACTTGATTGTTCATGAGGTTGTTAGGTTTCG
ACTTAGTAGATGCAAAGATAAATCTTTGCTGTTTGAATTGGTAAAAGTTTTGAACTTGAAATGTTTTTGC
GCTTCTTGGACTGGTAGAGCGCATAAGAATCAAGAGCTATGCACTCTGGAATATAGTTTTTGAGGCTGTA
AGATTTGAAGATCCTAGATATTGATTCATAATTAGCCCTGAAAATATGAAGATGTAAAGTGAGGAATAAC
CTTTTTTCCATTTACTTGATGCAGGATATTGATGGGAAGGATGTTGCTCTTAGCAAATTTAAGGGGAAAG
CTCTCTTGATTGTTAATGTCGCTTCGAAATGGTACTCTCTCCTTTGAGAATGCTTTATTCTGTGATCTGT
TTTGTTCTCTCATAGTTGGTTGATTAGCTTAGTTTGTTACGTGTCTTGTATATGAAGAATATATATTTCG
AAGTTGTACCAAATTTTTATTTATCTACTTTTTTCACGACACTTGAGATCTTATTGGAAGTTTTTGCTCT
GACTCCAGTGGTTTGACATCATCAAATTATTCGGAACTCACACACATATACGAGAAGTACAAAACTCAAG
GTAAGCTAAATTTTTGTGAGGAAAGCATTTTGATTTTTGGCTTTTTAGTGCCTACAACCTGATTGTTTTG
GAAAGAAGTTCTGCTTTTATAAGCTTAATACCTTTACGACGAGGCTGTCGATGGAATTGGTGTGATCTTT
CGCTCTATTTGATATCTTCTTGCTTTCTTTTGGCACCCAAAGCCTTTACACATCTAAAATAGGTGGCTCA
ATTTGTATGTGGGAGTGATAGGGGCGTGGTATTTGAACTACTATTTGAGTAGGGAGTTTGTGCCCGTCTC
ATACATGTTATATAGATTTTAATTTCTGCTAGCCCTAGTTAATGAGCAATCTAATGTTTTGTCATGCACC
TGAAGCTCTTCTGACATTGTATTCGTGTGGTGTCCTGCAACTAGTGGACATTGAAAATCTCCAGGATCAA
AGCAGGCTTCTTTGTTAAGATTGTAAGAGTAACAATATTGTCAGATCAATAAATTGATGGACTCCATATG
AATATAAGGAAGAGAACATCCCTGGGGATATTTCGATATACACATCGTTAAGTTGCCTATTGGCATCATC
TCTTTACTTGATTTGAGAAATTTAAAGCAATAGTGAAAAGATCTCTCTTTTATTAAATTGATGAAGCTAA
TCTCTATTATGTAACTTTCATTGCAGGCTTAGAAATTCTGGCTTTTCCTTGCAATCAGTTTGGTGGGCAA
GAACCTGGATCAAACCCTGAGATTAAACAATTTGCTTGTACCAGGTACAAAGCAGAATTTCCAATATTTG
ATAAGGTAAGCGGAATTATTCTCTCCTATTTTTGTAAAATGTCTCTTGGAAAAAGAACAATTTAATTACT
CAAGGTGTTGCATGACAGGTTGATGTGAATGGACCAAGTACAGCTCCAGTCTATCAGTTTCTGAAATCAA
GTGCTGGAGGATTTTTAGGTGATTTGATCAAGTGGAACTTTGAGAAGTTCTTGGTGGACAAGAATGGCAA
GGTTGTGGAGAGATATCAACCAACAACATCACCTTTCCAAATCGAGGTACACTTTTCTTCTATTTTCAAG
CATTTGGATGTTCTTTGAATTCTTCTATTTGTCATGACTGATAGGAAGTGGCAACATCTGCTCTTATAAA
CCGTGCCATCTGCATACCAATCCTGTCTTCACATTTCAAGTATCAACCGTGCTTTCTTAAATTTTTTCTT
CTCTTTTCCTGTTACAGAAGGACATTCAGAAGCTCCTCGCCGCATGAATGATTGCTGGCAGCAGTGACAT
GTATGAGATGTATCATCAGTACCAATAGAAGAAACACAAATCAATACATGTAAATTTGTGCTCCAATCTT
TACAGTTTGTTTATATACAAATGTTTTCTGTAATAGGAATCTAATATAATAGTGTTTCAATTGCCAGAAA
TTTTTGAAGTTTCTTTTGTTTGT

Appendix 2. Dilution factor of the samples, used in the mineral element analysis.

Groups	Dilution factor		
	Na	Ca	K
0 mM NaCl	9	60	60
60 mM NaCl	13	65	65
120 mM NaCl	13	65	65
180 mM NaCl	13	65	65

Appendix 3. Instrumental parameters (wavelength, nebulizer flow and viewing position) for each element analysed.

Element	Wavelength (nm)	Nebulizer flow (L/min)	Viewing position
Na	588.995	0.95	0
K	766.491	0.75	0
Ca	393.366	0.6	0

Appendix 4. Instrumental common conditions for the analysis of mineral elements.

Common conditions	
Replicates	3
Pump speed (rpm)	20
Uptake time (s)	90
Rinse time (s)	30
Stabilization time (s)	20

Appendix 5. PCR conditions for the amplification of *GSTF1*, GSH1 and *GPX1* genes.

Step	Temperature (ºC)	Time (min)	Cycles
Initial denaturation	98	0.30	
Denaturation	98	0.05	
Primer's annealing	*	0.30	35
Extension	72	**	
Final extension	72	5	
Hold	4	∞	

*: variable depends on the Tm of the primers (Table 3) **: variable depends on the size of the fragment. NB: Phusion DNA polymerase amplifies 1Kb/30s

Appendix 6. PCR reaction for the amplification of *GSTF1*, GSH1 and *GPX1*.

Component	Quantity (µL)
Nuclease-free water	7
Primer FW (10 µM)	1
Primer RV (10 µM)	1
Phusion DNA Polymerase (2 U/µL)	10
DNA (10 ng/µL)	1
Total volume	20

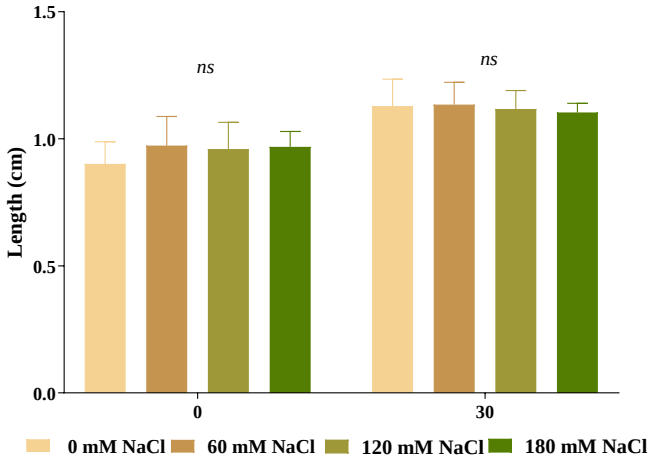
Appendix 7. Temperatures tested to optimize the primers used in PCR, for the *GSTF1*, *GPX1* and *GSH1* genes.

Primer	Temperature (ºC)
GSTF1 FW1/RV1	60 and 62
GSTF1 FW2/RV2	62, 64 and 68
GPX1 FW1/RV1	62, 64 and 68
GPX1 FW2/RV2	65 and 68
GSH1 FW1/RV1	66 and 68
GSH1 FW2/RV2	66 and 68

Appendix 8. RNA extraction buffer preparation (Vennapusa *et al.*, 2020).

Reagent	Concentration	Volume
Tris pH8	1 M	2.5 ml
NaCl	3 M	5 ml
EDTA pH8	0.5 M	500 µl
SDS	10 %	10 ml
DEPC water		32 ml

Add before use 10 µl of 2-mercaptoethanol per sample.



Appendix 9. Length (cm) of *Populus alba L.*"Villafranca" clone at the day 0 and 30 under salt treatment (0, 60, 120 and 180 mM NaCl). The values are expressed as mean ± standard deviation (n = 4). Results were analyzed by one-way analysis of variance (ANOVA). Values followed by different letters are significantly different according to Tukey's Multiple Comparisons Test ($P < 0.05$). ns, non-significant.