Response strategies to salinity and hyper-salinity of guayule and castor plants: growth and physiological parameters

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Response strategies to salinity and hyper-salinity of guayule and castor plants: growth and physiological parameters

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Abstract

Salinity is one of the major environmental constraints that affect many regions of the globe, and its rate of expansion is expected to increase. The interest in phytoremediation of salt-rich and contaminated soils and water has been increased in recent years, but several aspects about the plants' responses to salinity still need to be clarified. Guayule (*Parthenium argentatum* Gray) and castor bean (*Ricinus communis* L.) are among the plant species considered for growing in saline environments. This study aims to investigate the responses of guayule and castor to high concentrations of sodium chloride (NaCl), and the recovery capacity of castor plants. To meet these purposes, hydroponically grown guayule and castor plants were exposed to increasing NaCl concentrations. Growth parameters (morphological determinations and biomass production), physiological parameters (chlorophyll fluorescence, gas exchanges and photosynthetic pigments) and chemical analyses (sodium and mineral nutrient tissue-contents) were evaluated. The typical symptoms of salt-stress were observed in the two species, with differences in their responses to osmotic and ionic-specific stress components. Variations in growth and physiological parameters indicate that guayule and castor showed symptoms of ionic stress when exposed to concentrations of NaCl around 15 g L^{-1} and 10 g L^{-1} respectively. Guayule and castor did not survive at hypersaline conditions (above 35 g L^{-1} NaCl), but they survived at high saline conditions (above 5 g L^{-1} NaCl), indicating that the two species are suitable to be grown in saline soils and wetlands.

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List of abbreviations

A: Net CO_2 assimilation rate

A/g_s: Intrinsic water-use efficiency

C_i: Intercellular CO_2 concentration

Chls: Chlorophylls

Chl *a*: Chlorophyll *a*

Chl *b*: Chlorophyll *b*

DW: Dry weight

E: Transpiration rate

EC: Electric conductivity

FAO: Food and Agriculture Organization

F_v/F_m: Potential efficiency of PSII photochemistry

FW: Fresh weight

g_s: Stomatal conductance

HMR: Height to mass ratio

L: Leaf length

LA: Mean leaf area

LAR: Leaf area ratio

LMA: Leaf mass per area

LMR: Leaf mass ratio

LN: Leaves number

NAR: Net assimilation rate

NPQ: Non-photochemical quenching

PAR: Photosynthetic Active Radiation

PGA: Plant growth analysis

PPFD: Photosynthetic photon flux density

PSII: Photosystem II

RGR: Relative growth rate

RL: Root length

RWC: Rate water content

SE: Standard error

SL: Stem length

SMR: Stem to mass ratio

ST: Stem length

TW: Turgid weight

W: Leaf width

Φ_{PSII}: Actual photon yield of PSII photochemistry

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1 Introduction

1.1 Understanding salinity

The term “salinity” refers to the concentration of dissolved salts in water or soil. Dissolved salts are usually sodium and chloride ions (Na^+ and Cl^-), but there can be many others, such as potassium and bicarbonate ions. Salinity ranges from fresh to hyper-saline category (Table1.1).

Salts (g L^{-1})	**Category**
up to 1.00	Fresh
1.00 to 3.00	Fresh to brackish
3.00 to 5.00	Brackish
5.00 to 35.0	Saline
35.0 and above	Hyper-saline

Table 1.1: Salinity range (EPA, 2020)

Soil salinity occurs under all climatic condition and mainly affect arid and semi-arid regions (Zama et al., 2018). The accumulation of salts in soil depends on the balance between water supply and evapotranspiration, which relies on rainfalls and/or irrigation activity, the level of underground water, the characteristics of the vegetation cover and of the soil itself (permeability, depth). Salinity is caused by numerous causes of natural and anthropogenic origin. The natural processes that lead to salt stress are mainly the weathering of rocks and the deposition from rainfalls of salts evaporated from the oceans. The raising of aquifers level, the salt accumulation by irrigation, and seawater intrusion to coastal areas are the main processes from anthropogenic activities which cause the increase of salinity. The first process is due to the thinning and cutting of perennial vegetation, or its replacement with shallow rooted crops and grazing activities. These changes in the land use cause a reduction of water taken up by plants, either rainfall or irrigation water. By capillary rise, groundwater reaches the surface and evaporates, releasing on the soil surface salts that were stored in the deep soil. The second process mainly occurs in clay soils, where the irrigation is associated with high evaporation rates, resulting in limited leaching and ions accumulation on surface. Finally, over-exploitation of groundwater may reduce the levels of the aquifers, with a consequent seawater intrusion in coastal areas (Shahid and Rahman, 2011).

Either from natural causes or from anthropogenic activities, soil and water salinity represents one of the major environmental constraint worldwide, and no country is currently

completely free from the salinity phenomenon and related concerns. Global statistics on salt-affected soil vary according to different data sources (Zaman et al., 2018); however, the FAO has estimated that there are 4 million square kilometres of salinized lands and approximately 50% croplands is salt stressed, threatening the agricultural productivity (Devi et al., 2016). The rate of expansion of salinization is expected to increase due to several factors. The expansion of world population and the spread of agriculture in arid and semi-arid ecosystems, will lead to changes in the hydrologic balance of the soil between water supplied and water used by crops, increasing and exacerbating the soil salinization (Chaves et al., 2009). Different scenarios for climate change will lead to the use of lower-quality water, the increase of salinization induced by irrigation and dryland (caused by the increase of arid and semi-arid areas), and the rise of sea level, with direct salinization of nearby soils or indirect contamination of soils through saline intrusion in aquifers (Jesus et al., 2015).

Salinity can cause a number of environmental damages, including land degradation, reduction of water quality and detrimental effects to vegetation (Shelef et al., 2012). It has severe negative impacts on agricultural productivity and sustainability, causing productive lands to become barren (Devi et al., 2016). Salinity contributes to the land degradation by destabilizing soil aggregation due to slaking, swelling and dispersion (Jesus et al., 2015). In addition, the high water table affects biological activities in the soil (Shahid and Rahman, 2011), with reduction of biodiversity and productivity. Different approaches could be used to reduce the salinity and recover impacted areas. Plant-based technologies (phytoremediation) offer sustainable advantages, but require the selection of appropriate plant species and a suitable strategy to be effective (Barbafieri et al., 2017).

1.2 Phytoremediation

Phytoremediation is a family of technologies that use living plants for *in situ* treatment of contaminants in soils, sludge, sediments, surface water and groundwater, leading to its containment, removal or degradation. In recent decades, phytoremediation has been successfully used for the treatment of soils contaminated with various pollutants, as well as a technological complement for treatment of polluted waters in wetlands (Truu et al., 2015). It is clean, cost-efficient and easy to implement methods that can be applied to a variety of environmental contaminants and does not require extraction and transport of soil (Devi et al., 2016). This technique is publicly acceptable for being in line with the principles of sustainable development, it’s environmentally non-disruptive and gives opportunities of land valorisation. Moreover, phytoremediation technique limits the soil erosion and leaching, and contributes to the

maintenance of microflora. Phytoremediation presents limits, mainly related to the survival of plants in unfavourable environmental conditions (Barbafieri et al., 2018), the extension of the polluted soil that would be colonized by the root system, and the relatively long timing that the application of this phytotechnology requires (generally related to the plant lifespan), thus being often used in long-term projects. In addition, the complete remediation of the pollutant depends on the chemical nature (e. g. organic or inorganic) and its bioavailability for plants, so often only a fraction can be degraded or removed (Bert et al., 2012). Thus, researches are devoted to improving phytoremediation applicability and efficiency (Barbafieri et al., 2017; Barbafieri et al., 2018).

Phytoremediation includes different types of application, the main ones being phytostabilization, rhizodegradation and phytoextraction, in dependence of the main biological processes involved. The first application occurs when plants reduce mobility and bioavailability of a pollutant, without necessarily involving the bioaccumulation in their tissues (Shelef et al., 2012). Rhizodegradation occurs when the presence of plant roots enhances microbial activity in the rhizosphere (also thanks to the production of root exudates and extracellular enzymes), leading to the degradation of the pollutant (Bert et al., 2012). Phytoextraction (or phytoaccumulation) occurs when plants extract, uptake and concentrate contaminants from soil or water to aboveground harvestable parts (Shelef et al., 2012). Ideally, plants for phytoextraction should combine a high accumulation capacity with a high biomass production and an extensive root system (Truu et al., 2015). Earlier researches on phytoremediation focused on the phytoextraction capacity of hyperaccumulator plants, which are plants able to stock in aboveground shoots a concentration of pollutants hundreds or thousands of times higher than that usually stocked by other most plants growing in the same environment (Reeves, 2003; van der Ent et al., 2013). However, these are essentially very specific and endemic plants, with a natural slow growth rate. Consequently, their use in phytoextraction is limited by their low biomass production (Cassina et al., 2011). An alternative approach developed is based on the utilisation of plant species characterized by a higher biomass production, growth rate and plurennial cycles, and hence such plants are able to store a large amount of pollutants during their lifespan (Evlard 2012; Bert et al., 2013).

As regards salt stress and salinity, phytoremediation consists in the cultivation of salt accumulating or salt-tolerant plants for the reduction of salinity in the environment. Plants may also be used to lower the water table and enhance the drainage, while the salt uptake into the shoots prevents their leaching to groundwater (Jesus et al., 2015).

1.3 Phytoremediation in constructed wetlands

Constructed wetlands are man-made systems that utilize natural processes for the elimination of a wide range of contaminants from wastewater, improving water quality for recycling (Shelef et al., 2012). These systems have been used mainly for the treatment of domestic wastewaters, and more recently also for the remediation of industrial effluents and petrochemical wastewater (Ji et al., 2001). Phytoremediation technologies are used to improve the performance of existing wastewater treatments in constructed wetlands (Truu et al., 2015). The role of plants in the purification process of constructed wetlands is mainly based on the increase retention time by reducing water velocity, and on the improvement of hydraulic conductivity by the root growth and activity. Plants may affect the elemental composition of wastewater by the utilisation of elements as nutrients, such as nitrogen and phosphorus; their roots provide surfaces for microbial growth and can be used as bioindicators in the constructed wetlands management. Moreover, plants in wetlands provide a positive impact on the neighbourhood, as they can prevent odour nuisances and enhance the aesthetic appearance of the system (Shelef et al., 2012).

Nevertheless, the mode of action and technological aspects of the plant application for the pollutants removal in treatment wetlands is so far less studied, when compared to the phytoremediation of polluted soils, probably because of the complex and synergistic nature of the ongoing processes (Truu et al., 2015). Moreover, there are still many aspects to be clarified about salt phytoremediation in both soil and wetlands, including the performance of the different plant species. Indeed, plants differ in their salinity tolerance and the mechanism by which they regulate salt content in their tissues (Jesus et al., 2015).

1.4 Salt effects on plants and tolerance mechanisms

Salinity is one of the primary abiotic stresses seriously affecting growth, photosynthetic process, development and survival of plants, and thus the overall productivity of an ecosystem. The nature and impact of damage resulting from salt stress vary considerably according to the plant species and the environmental conditions (Shah et al., 2017). Understanding how plants respond to salt stress can play a pivotal role in both stabilization of crop performance and protection of natural vegetation under saline conditions (Chaves et al., 2009). Morphologically, the most typical symptom of saline injury in plants is the reduction of growth, which results from a combination of physiological responses including the modification of water status, photosynthetic efficiency, ion balance, carbon allocation/utilization, and the induction of antioxidant and defence enzymatic and non-enzymatic systems (Di Baccio et al., 2004;

Janmohammadi et al., 2012). Salt interference with plant growth presents two phases: an early-occurring osmotic stress, and a slower response occurring later due to the accumulation of ionic Na^+ and/or Cl^- within the plant (Deinlein et al., 2014).

In the first osmotic phase, high concentrations of salt at the roots surface create a low water potential zone outside, decreasing the ability of the root system to absorb water, as well as nutrients, which immediately affects the cell growth and associated metabolism (Munns and Tester, 2008). Osmotic stress exposes plants to drought stress and it can be mitigated by the reduction of water loss through stomatal closure, and the maximization of water uptake through loss of cell turgor, which causes a reduction in cell expansion. These protective mechanisms affect the plant growth, mainly shoot dry matter or leaf area, and the photosynthetic process and apparatus (Mugnai, 2014; Munns and Tester, 2008).

A rapid growth inhibition, as well as the decrease of photosynthetic capacity, may be transitory responses to salt stress. The inhibition of leaf expansion aims to preserve carbohydrates for sustained metabolism, prolonged energy supply, and for better recovery after stress relief (Deinlein et al., 2014). A small decline in stomatal conductance limits the entry of CO_2 into leaves, reducing photosynthesis (Stepien and Johnson, 2009), but it may have protective effects against stress leading to water saving and improves plant water-use efficiency (Chaves et al., 2009). However, the degree of growth inhibition and of photosynthetic efficiency reduction caused by osmotic stress mainly depends on the severity of the stress, as well as on the time scale of the response, the particular tissues and species studied, and how the salinity stress occurs (rapidly or gradually) (Deinlein et al., 2014; Chaves et al., 2009).

The second phase of plant response to salinity is ion-specific, and gradually takes over time when toxic concentrations of salt accumulate inside the plant tissues (Munns and Tester, 2008). Salts absorbed by the root system undergo long-distance transport in the transpiration stream, and eventually accumulate in leaves. A high accumulation of ions such as Na^+ in the cytoplasm disrupts the uptake of other cations into plant cells, especially nutrients (as calcium, magnesium or potassium), with adverse effects on many metabolic pathways (Yang and Guo, 2017). This phytotoxic effect appears first in old leaves, that are no longer expanding due to osmotic stress, therefore no longer diluting the salt incoming; this accelerates their senescence till premature fall (Mugnai, 2004). New leaves also reduce their growth rate if the rate at which old leaves die is greater than the rate at which new leaves are produced. The leaf mortality rate is crucial for the survival of plants under salt stress. Having less surface to assimilate CO_2, the photosynthetic capacity of the plant is no longer able to supply the carbohydrates demand (Munns and Tester, 2008). When the salt stress is intense or prolonged, irreversible damages to cellular

structures and biomolecules, and therefore leading to tissue death, are added to the generalized reduction in growth. The primary components of the photosynthetic apparatus, such as membranes, enzymes, chlorophylls and carotenoids are mainly damaged. On a macroscopic level, these damages can be observed as anomalous colouring and necrosis events on the leaf blade.

Mechanisms that plants counteract against ionic stress are Na^+ exclusion from leaf blades and tissue tolerance. The Na^+ exclusion by roots avoids that leaves accumulate toxic concentration of ions. The tissue tolerance mainly refers to the tolerance of tissue in accumulating Na^+. It requires compartmentalization of Na^+ at the cellular and intercellular level to minimize salt concentration in the cytoplasm, where the main metabolic activities take place (Munns and Tester, 2008).

1.5 Physiological parameters

The following physiological parameters are among the key parameters for the study of salt stress in plants.

1.5.1 Chlorophyll fluorescence

The measurement of chlorophyll fluorescence is an *in vivo*, precise and quick technique widely used in investigating photosynthetic responses to the environment, as it can be performed on intact and attached leaves (Chaves et al., 2009).

Light energy absorbed by chlorophylls in leaves can be processed mainly in three ways: photochemical processes, that use solar energy to activate the photosynthetic electron transport, and non-photochemical processes, which include the exclusion of the excessive solar energy through its dissipation as heat or as chlorophyll fluorescence. These three processes are in competition with each other, such that any increase of the efficiency of one of them causes the decrease in the yield of the other ones. Therefore, by measuring the yield of chlorophyll fluorescence, information on variations in the efficiency of photochemistry can be gained (Maxwell and Johnson, 2000). When measuring fluorescence, the two alternative processes of energy dissipation are known as mechanisms of fluorescence quenching (White and Critchley, 1999). Photochemical quenching refers to an increase in the rate at which electrons are transported away from the photosystem II (PSII), promoted by light-induced activation of enzymes involved in CO_2 assimilation and the opening of stomata. Non-photochemical quenching (NPQ) refers to an increase in the efficiency with which energy is converted to heat (Maxwell and Johnson, 2000). In

order to gain useful information about the photosynthetic efficiency of a plant, it is mandatory to be able to distinguish between the photochemical and non-photochemical contributions to quenching (Murchie and Lawson, 2013). Pulse-amplitude-modulated measurement (PAM) is a technique based on the principle of separating photochemical and non-photochemical components of light energy use and dissipation by plants.

In dark-adapted leaves (when all PSII reaction centres are open and the NPQ is null), fluorescence is initially measured by switching on a measuring light, that elicits a minimum value for chlorophyll fluorescence (F_o). When a saturating pulse is applied the maximal values for fluorescence (F_m) is recorded. The difference between F_o and F_m is the variable fluorescence, F_v, and $F_v/F_m = (F_m - F_o) / F_m$ gives a robust indicator of the maximum quantum yield of the PSII photochemistry. The measure of F_v/F_m after an appropriate period of dark adaptation is one of the most common techniques for measuring stress in plants. In a healthy non-stressed plant, F_v/F_m is around 0.83, while any type of stress that results in inactivation or damage to PSII (photoinhibition) causes a decreasing of F_v/F_m (Murchie and Lawson, 2013).

The steady-state level of fluorescence under actinic illumination, is termed F'. Under this condition, the application of a saturating pulse closes all the PSII reaction centres, providing a value of maximal fluorescence in light-adapted leaves (F_m'). The actual photon yield of PSII in the light is calculated as $\Phi_{PSII} = (F_m' - F') / F_m'$ and is the most commonly used light-adapted parameter. It gives a proportion of absorbed light that is actually used in the PSII photochemistry, and therefore, it can be used as an estimator of the rate of electron transport through the PSII (Murchie and Lawson, 2013).

1.5.2 Photosynthetic gas exchanges

The carbon-water balance in plants is controlled by stomata, that are involved in the regulation and control of photosynthetic and transpiration fluxes between leaf and atmosphere. Carbon dioxide (CO_2) diffusion inside the leaf is regulated by two factors: 1) the diffusion gradient, which is the difference between the concentration of CO_2 around the leaf surface, and the one inside it, and 2) the stomatal conductance (g_s), which is proportional to the CO_2 flux through stomata, depending on the leaf stomata density, as well as their opening degree. The stomatal conductance can be defined as the reciprocal of the stomatal resistance to the diffusion of H_2O (to the outside) and CO_2 (to the inside). The stomatal closure causes a lower concentration of CO_2 in the leaf intercellular air space (C_i) (Giuliani et al., 2013). The water use efficiency is commonly defined as the amount of carbon fixed in photosynthesis per unit of water transpired (Lawson and Blatt, 2014) and the balance between CO_2 and H_2O fluxes can be characterized by

intrinsic water use efficiency, i.e. the ratio between the net CO_2 assimilation rate (A) and g_s, A/g_s. The conservation of water via stomatal closure, and thereby low g_s, limits CO_2 uptake into leaves, reducing A. Similarly, a high g_s leads to higher rates of A, as well as to a greater cost of water loss via transpiration (E) (Matthews et al., 2018).

Gas exchange systems allow a sensitive, *in vivo* measurement of leaf transpiration and photosynthetic response to light variation as water vapour fluxes and assimilated CO_2. Such systems are equipped with a gas-exchanges chamber. Leaves are inserted into the chamber and, through a highly sensitive infrared differential analyser, the CO_2 concentrations and water vapour incoming and outgoing the leaf tissues are measured. Through particular algorithms, a series of photosynthetic parameters are obtained, such as the stomatal conductance (g_s), the transpiration rate (E), the net CO_2 assimilation rate (A), and the intercellular concentration of CO_2 (C_i) (Long and Bernacchi, 2003).

1.5.3 Photosynthetic pigments

Harvesting the light energy from the sun to store it as chemical energy, chlorophylls (ChIs) are a dominant factor controlling leaf properties of healthy green vegetation. The most significant plant pigments for the oxygenic conversion of light energy are chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) (Sub et al., 2015). Together with accessory pigments such as carotenoids, they absorb the photosynthetically active radiation (PAR), which then migrates to the reaction centres of photosystem I (PSI) and II (PSII), where the photosynthetic energy conversion process takes place (Jangpromma et al., 2010). Leaf chlorophyll contents can vary significantly in value among different plant kingdoms and growing stages and provides useful information about the photosynthetic potential (Sub et al., 2015). Chlorophyll loss is associated to environmental stress, while the variation in total chlorophyll/carotenoids ratio may be a good indicator of plants stress, as chlorophyll rapidly disappears when the tissue dies, and carotenoids gain in relative importance for the protection of photosystems and their antioxidant activity (Sub et al., 2015; Netto et al., 2004).

Traditionally, the leaf chlorophyll concentration is determined by solvent extraction from leaf samples. Chlorophyll content is subsequently measured by spectrophotometric or chromatographic techniques (Jangpromma et al., 2010), and their amount in leaves is normally expressed on leaf matter (µg Chl / g tissue) or area basis (µg Chl / cm^2 tissue) (Sub et al., 2015). However, such determinations are destructive, expensive and time consuming, and therefore they may not be applicable for all purposes. Alternative and more rapid methods estimate the leaf concentrations of Chl *in vivo*, by exploiting the optical properties of leaves. These non-destructive

methods are based on the reflectance and/or absorbance of radiation by chlorophyll (Uddling et al., 2007). Figure 1.1 shows the absorption spectra of chlorophyll *a* and *b* pigments (Sub et al., 2015).

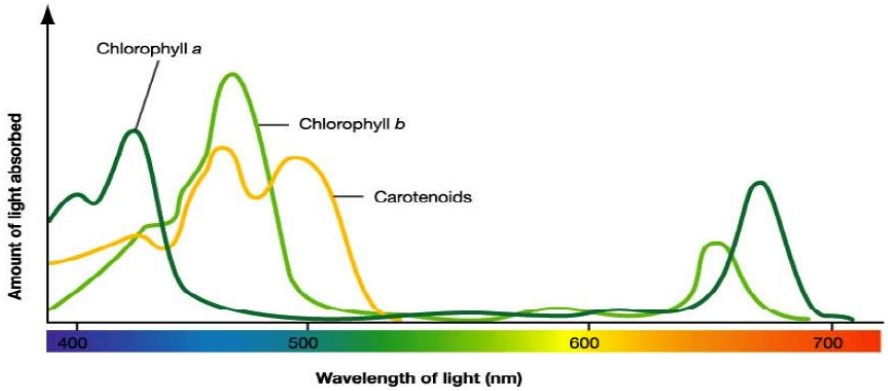

Figure 1.1: Absorption spectra of Chlorophyll a, b and carotenoids (Sub et al., 2015)

The SPAD-502 chlorophyll meter is a hand-held device that makes instantaneous readings of relative chlorophyll concentration of leaves, based on the quantification of light absorbance by the tissue sample at specific wavelengths. Chlorophyll maximum absorption occurs in the red-light domain, and near-infrared light is measured to record differences in the different plant leaf structures. The meter is equipped with a red LED (peak wavelength: approximately 650 nm) and an infra-red LED (peak wavelength: approximately 940 nm). When the measuring head is closed, these two LEDs emit light in sequence from the emitting window to a photodiode detector. Light passes through the leaf sample and a certain amount is transmitted through the tissue, strikes the reception and is converted into electrical signals. As an output, SPAD-502 meter calculates index-values (i.e. SPAD-value, arbitrary units) by division of light transmission intensities at red and infra-red wavelengths. The numerical SPAD value is proportional to the chlorophyll content within the sample (Netto et al., 2004; Sub et al., 2015). Chlorophyll meters provide a substantial saving of time and resources and they have been extensively used in both agricultural and research fields (Ling et al., 2011).

SPAD-values should resemble absolute total chlorophyll concentration (Chl *a* + Chl *b*) expressed in µg / cm^2, but in order to obtain realistic data, calibration curves between meter readings and the biochemical determination of chlorophyll concentration through solvent extraction must be made (Sub et al., 2015). The relationship between SPAD values and chlorophyll concentration has been widely investigated, and it has been found to have considerable inter-specific variations, caused by structural differences between the leaves of different plant species and, consequently, different light reflection or scattering effects. Therefore,

the conversion of relative SPAD-values into units of absolute chlorophyll concentration requires a calibration equation derived specifically for the particular species of interest (Ling et al., 2010). The studies performing such calibration of the SPAD meter usually parameterise linear relationships (Uddling et al., 2007).

1.6 Guayule

Guayule (*Parthenium argentatum* A. Gray) is a xerophytic plant belonging to the Asteraceae family. This species is a perennial, woody shrub not exceeding 65 cm height, that grows slowly and in the wild environment it can live forty or fifty years (Suchat, 2012). Guayule is mainly found in the semi-arid mountain slopes of the Big Bend region of Texas and Chihuahua desert of north-central Mexico, where temperature ranges from −18 °C to 49.5 °C (Rasutis et al., 2015). Guayule produces high-quality, hypoallergenic natural rubber, that has been shown to prevent transmission of viruses and other pathogens, making it suitable for medical use (Coffelt and Nakayama, 2007). The widespread occurrence of allergy to proteins in the natural rubber products of *Hevea brasiliensis* (Muell. Arg.), the “rubber tree”, is enhancing the interest in agronomic studies on guayule (Foster and Coffelt, 2004).

Well-adapted to live in arid ecosystems, guayule has an history of natural exposure to several environmental stresses, including soil salinity (Sundar, 2003), and it is considered to have the potential of becoming an agronomic crop in saline areas (Miyamoto et al., 1990). However, the salinity-tolerance level of guayule has to be defined yet, and guayule responses to environmental stresses has been studied mainly in relation to its resin and rubber production. Guayule responses to salinity depend on plant development and age, and its tolerance has been reported to be strongly higher in mature than in emergence and seedling life stages (Foster and Coffelt, 2004; Posher et al., 2005). While seeds germination was reported to be successful in saline solution up to 23 dS m^{-1}, germination did not always coincide with a successful emergence stage (Miyamoto et al., 1985). More recent studies found that lower EC levels (8 dS m^{-1}) affected both the percent seed germination and the mean time of germination, especially when salt-stress was combined with suboptimal temperature conditions (Sanchez et al., 2014). Therefore, guayule establishment by direct seeding in saline areas would be unreliable (Miyamoto et al., 1990).

However, the field guayule planting is more often established through seedlings transplanting, regardless excessive soil salinity. This method involves first the seeding in nurseries or greenhouses for the production of seedlings, that are then transplanted in fields (Foster and Coffelt, 2004). Ten-weeks-old guayule seedlings grown in greenhouse, transplanted in spring and irrigated with water of 4.6 dS m^{-1} can survive with minimal losses, while mortality

increases up to 21% at 7.2 dS m^{-1} (Miyamoto et al., 1984). Apart the transplant mortality, salinity affected guayule mainly by reducing its dry matter production, water-use-efficiency (defined as the amount of rubber produced per unit of water applied), and rubber content (Foster and Coffelt, 2004). Although a moderate environmental stress, including excess soil salinity, was linked to an increasing of rubber production, a soil EC of 3.2 dS m^{-1} didn't increase rubber production, and an increase of salinity beyond the threshold of 7.5 dS m^{-1} caused a reduction of rubber production (Hoffman et al., 1988).

1.7 Castor bean

Ricinus communis (L.), commonly known as castor bean or simply castor, is a fast-growing perennial crop plant, belonging to the *Euphorbiaceae* family. It probably originated from Ethiopia, tropical Africa, and it is mainly cultivated in Asia, especially India. The castor varieties that are currently cultivated reach 60-120 cm of height during the first year, while it can reach several meters in the following years. Castor often grows in waste, degraded and contaminated soils as a wild plant (Kiran and Prasad, 2017), and for its known drought resistance, it is considered an important agricultural source for areas characterized by poor soils and stressed climatic conditions. Castor bean oil is among the most versatile vegetable oils found in nature and of great interest for unique chemicals which can be derived from it. Possible uses of castor bean oil include manufacturing surfactants, coatings, greases, fungistats, pharmaceuticals, cosmetics and many other products. Moreover, castor-bean-derived products are biodegradable and eco-friendly, thus they are considered preferable than petrochemical products (Bajay et al., 2011; Janmohammadi et al., 2012).

Castor is mainly grown in semiarid areas, often affected by salinity (Janmohammadi et al., 2012), and it's among the oilseed crops evaluated for cultivation in salt affected areas (Severino et al., 2014). Experiments under semi-controlled environment showed that NaCl concentration up to 200 mM caused inhibition of plant growth, but this condition was not lethal (Janmohammadi et al., 2012). Castor bean can tolerate saline lands, growing 2-5 m height in one season with full sunlight, heat and adequate moisture (Wu et al., 2011). Experiments conducted under field conditions to identify eventual development stages with higher tolerance to salinity, suggested that responses to salt stress of castor plant were not different according to the growth stage (Costa et al., 2013).

Being both a salt-tolerant plant and an important oilseed crop, castor bean was evaluated as candidate for amelioration of saline soils (Wu et al., 2011), combining phytoremediation with bioenergy production (Olivares et al., 2013). Wu et al. (2011) found that after planting castor bean

in saline soil for two growing seasons, soil salinity was significantly reduced, with an amelioration of the soil density and nutrient condition.

1.8 Study objectives

Guayule and castor bean are considered salt-resistant plants and they spontaneously grow in arid ecosystems, where soil salinity occurs. However, their highest salt tolerance threshold is yet to be defined, as well as their growth, biochemical and physiological variations in high salinity conditions. The aim of this work was to study the effects of high and increasing concentrations of sodium chloride (NaCl) on morphological, biochemical and physiological characteristics of guayule and castor plants. Moreover, the recovery capacity of castor plant under salinity conditions was investigated. In order to meet these goals, experimental tests were conducted in semi-controlled conditions (greenhouse), using hydroponic techniques. Analyses performed includes growth and physiological parameters, and mineral nutrient determinations.

2 Material and methods

2.1 Plant material and experimental design

2.1.1 Plant material and pre-culture conditions

Guayule (*Parthenium argentatum* A. Gray) plants were obtained by commercial seeds and grown inside soil vessels in controlled conditions, as the most common growth system for guayule culture (Dissanayake et al., 2007). When plants were about 50-60 days old, they were transferred to the experimental greenhouse. In order to test their resistance and possible growth capacity in hydroponic conditions, plants were washed from soil using tap water for a week, and then placed in the hydroponics systems, where they were subjected to acclimation (Figure 2.1).

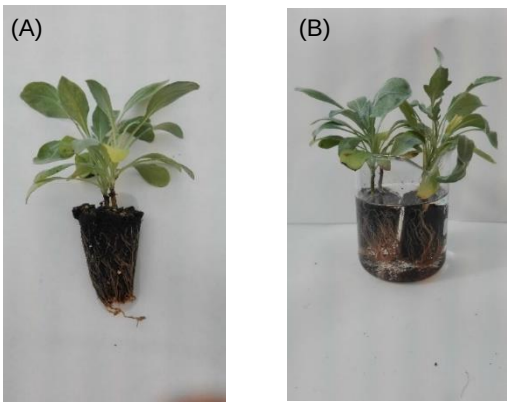

Figure 2.1: A – Guayule plants in soil and B – guayule transferred to solution to be washed from soil

Castor plants (*Ricinus communis* L.) were obtained from seed, supplied by the Department of Agricultural, Food and Agro-environmental Sciences of the University of Pisa. Seeds were surface sterilized with a 75% ethanol solution, decorticated, sown on inert substrate (perlite) and germinated inside plastic containers. Seedlings (16 days of age) were transferred to hydroponic conditions, where they were maintained for a two-weeks-period of acclimation and tested for resistance and growth capacity.

2.1.2 Experimental conditions

Two-factorial experiments (sodium, Na, concentration and treatment duration) were set, with the common objectives of investigating the response of guayule and castor plants to increasing concentrations of salt on a morphological, biochemical and physiological level. The factor “concentration” was the increasing concentration of salt, and the factor “duration”

represented the time of salt exposure. Based on the results of these experiments, a recovery test was set up with the objective of investigating the castor recovery capacity after exposure to high salinity conditions. The study of salt stress in plants, with responses to varying amounts of salt, requires a specific root zone environment; for this reason, all the experiments were conducted under hydroponic conditions.

The experiments occurred in a greenhouse with a controlled temperature system, natural humidity and additional lamps for light exposure during the daytime (from 7.00 a. m. to 22.00 p. m.) in order to reach a mean PAR (Photosynthetic Active Radiation) of 110 ± 40 µmol m^{-2} s^{-1}. The plants were exposed to the ambient conditions shown in Table 2.1. In order to assure equal growing conditions for all plants, optimal arrangement of hydroponic systems in the greenhouse was determined according to preliminary measurement of ecological parameters. Moreover, the position of every system was exchanged randomly twice a week.

Table 2.1: Range of environmental conditions in the greenhouse measured between October 2019 and January 2020. PAR = Photosynthetic Active Radiation

Ecological parameter	**Measurement**
Daytime temperature range	16-30 °C
Relative humidity	60 %
Ambient CO_2 concentration	400 ± 60 ppm
PAR h. 10:00-17:00	60-180 µmol m^{-2} s^{-1}

In all the experiments, hydroponic conditions were achieved with a non-recirculating (“air-gap”) system, where the roots hang directly into a nutrient solution reservoir (water and nutrients) (Rorabaugh et al., 2002). Hydroponic system consisted in a white rectangular polystyrene tank (23 x 16 cm), filled with 3 L of liquid fertilizer (Flora Series ®) diluted in tap water. Each system contained 3 plants receiving the same treatment. The plants were placed inside the system by circular plastics vases mechanically supported by a polystyrene plate, floating above the water surface. In all the experimental tests set, control plants were supplied with basal nutrient solution (fertilizer opportunely diluted with tap water), while treated plants were supplied with basal nutrient solution added with amounts of sodium chloride (NaCl) to reach the concentrations each time analysed. Electric conductivity (EC) and pH of control and nutrient solutions were measured at least twice a week with a portable instrument (2301T conductivity meter and digital pH electrode, XS Instruments, Carpi MO – Italy).

2.1.3 Salinity tolerance tests

A first test was conducted on 3-months-old guayule plants after 2 weeks of adaptation to hydroponics conditions. Twelve plants among those ones resulted the best adapted to hydroponic conditions were selected and randomly distributed to the two test conditions: 6 plants of control and 6 plants treated with NaCl (n = 6). The controls were supplied with tap water + fertilizer, while the treated plants with tap water + fertilizer + NaCl at increasing concentrations. The sodium chloride concentration was set at 1.5, 2.5, 5, 10, 15, 20, 30, 40 g L^{-1}, increasing every 3-4 days. The plants exposure to NaCl started on October the 17th, 2019, and the nutritive solution was changed weekly. Guayule responses to salt stress were evaluated on the basis of growth and physiological parameters as described below. The salt tolerance of guayule was reported to vary according to plant stages of growth, and it increases with plant establishment (Posher, 2005; Miyamoto et al., 1990). Therefore, a subsequent salt-tolerance test on guayule older plants was developed.

The second salinity tolerance test was performed on guayule plant and, contemporary, with the same modalities, the test was applied for the first time on castor plants. Among the guayule plants (about 4-months-old) which best responded to a 4-weeks hydroponic adaptation period, five ones were selected for the control and five for the NaCl treatment (n = 5), in order to assure the best homogeneity of the two populations. When castor reached the first stage of true leaves (30-days-old plants), twelve plants (6 of control and 6 treated with NaCl, n = 6) were randomly selected. The controls were supplied with tap water + fertilizer, while the treated plants with tap water + fertilizer + NaCl at increasing concentration. The tested NaCl concentrations were set at: 2.5, 5, 10, 15, 20, 25, 30, 40 g L^{-1} for guayule and 2.5, 5, 10, 15, 20, 25 g L^{-1} for castor. The treatments started on November the 7th, 2019, and the NaCl concentration was increased every 3-5 days, while the nutrient solution was changed weekly. Tables 2.2 and 2.3 show the time-course and NaCl concentrations (and correspondent conductivity) of solutions used for treatments.

The responses of guayule and castor plants to salt stress were evaluated on the basis of their growth and physiological parameters as described below. The evaluation of the results obtained from castor suggested to develop a subsequent experiment, testing the plant recovery capacity after the exposure to high (visible symptoms of suffering without death) salt concentrations.

Table 2.2: Data of NaCl treatment for test on guayule. EC values are means (n = 2)

Day	NaCl (g L^{-1})	EC (dS m^{-1})
0	0	1.81
1	2.5	6.63
5	5	11.2
8	10	18.2
13	15	28.5
16	20	31.6
19	25	37.3
22	30	50.1

Table 2.3: Data of NaCl treatment for test on castor. EC values are means (n = 2)

Day	NaCl (g L^{-1})	EC (dS m^{-1})
0	0	1.93
1	2.5	6.59
5	5	11.5
8	10	17.7
13	15	28.5
16	20	31.1
19	25	37.4

2.1.4 Recovery test

A recovery test was conducted on twelve castor plants (6 of controls and 6 treated with NaCl, *n* = 6). In order to ensure a better adaptation to hydroponic condition, plants were previously placed for about 4 weeks in a float system as described above, with the addition of oxygen supplied to the roots by an aquarium pump (Rorabaugh et al., 2002). Oxygen supply was removed with the beginning of the test. Castor plants (2 months old) were exposed to increasing doses of NaCl to be gradually adapted to 10 g L^{-1} NaCl, as limit threshold identified during the previous salinity tolerance test. The salt treatment started on January the 10th, 2020, and NaCl concentrations increased every 1-4 days (5, 6, 7.5, 8.5, 10 g L^{-1} NaCl), while the nutrient solution was changed weekly. Salt stress was removed after 3 days of 10 g L^{-1} NaCl treatment. Castor recovery capacity was evaluated on the basis of growth and physiological parameters measured

in correspondence with salt stress removal and one-week later. Growth parameters were also measured 30 days after the stress removal.

2.2 Growth parameters

2.2.1 Non-destructive determinations

In all the experiments set up, before the start (T_0) and during the salt exposure, in correspondence with NaCl solution increase (twice a week), biometric measures were performed on each plant. The growth parameters monitored were:

- root length (the maximum length of the root apparatus),
- number of leaves (green leaves),
- leaf morphological traits (main length and width for surface expansion)

2.2.2 Destructive determinations

At the end of the experiments, total fresh and dry weight (FW and DW) of different plant organs were measured by destructive analyses. In particular, plants were divided into leaves (surface and, eventually, petioles), stem and roots (Figure 2.2), washed free of minerals and NaCl traces with tap and distilled water, dried of any surface moisture with tissue paper and immediately weighed to determine FW. Simultaneously, the stem length was measured and leaf discs (10 mm or 7 mm, diameter) were sampled with a cork borer for the determination of leaf mass per area

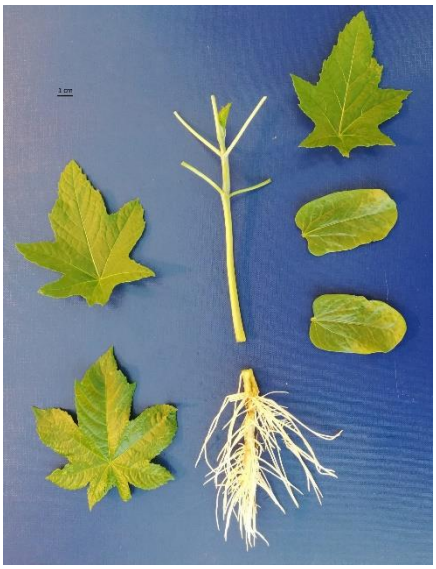

Figure 2.2: Castor bean divided into leaves, stem and roots for destructive analysis

(LMA, g m^{-2}) and leaf relative water content (RWC, %). Then, all the plant material was oven dried at 50 °C to a constant weight.

2.3 Growth analysis

2.3.1 Regression models for leaf area determination through non-destructive measurements

Before the NaCl treatment (T_0), plants were selected (different age and expansion level) to determine leaf area (LA) by a destructive method. The LA of harvested leaves was measured using an imaging analysis software (ImageJ, IJ 1.46r). Leaves were arranged on white sheets and scanned with an acquisition system. The images obtained were graphically elaborated (Figure 2.3) to determine the foliar area (Di Baccio et al., 2009).

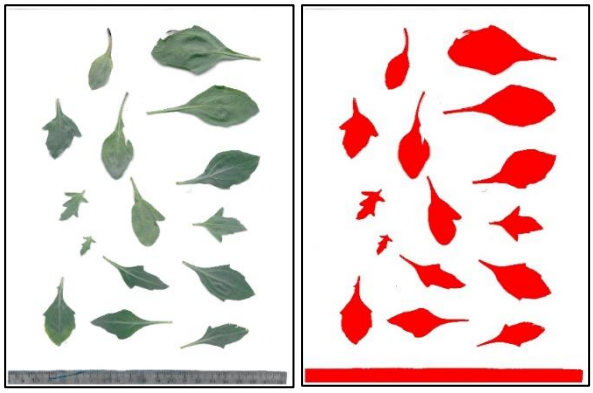

Figure 2.3: Graphical modification for the measure of guayule leaf area

Such measurements of LA were used to create correlations with leaf morphological non-destructive measurements registered during the time-course of experiments, in order to monitor the plants growth during salt treatments without loss of biomass. By relating the morphological traits (for example: maximum and minimum length) of each leaf with the correspondent area, a regression model was achieved using the statistical software R Studio (Onofri and Sacco, 2018). In both guayule and castor plants, the relationship between LA and non-destructive leaf measurements was linear, and the best model was found relating LA with the product of the two main orthogonal leaf diameters (length, L, and width, W) (Figure 2.4). For guayule, the maximum leaf width and the maximum length including the petiole were considered as “width” and “length”, respectively. For castor, the maximum leaf width and the maximum length excluding the petiole were considered as “width” and “length”, respectively. The regression found was significantly high

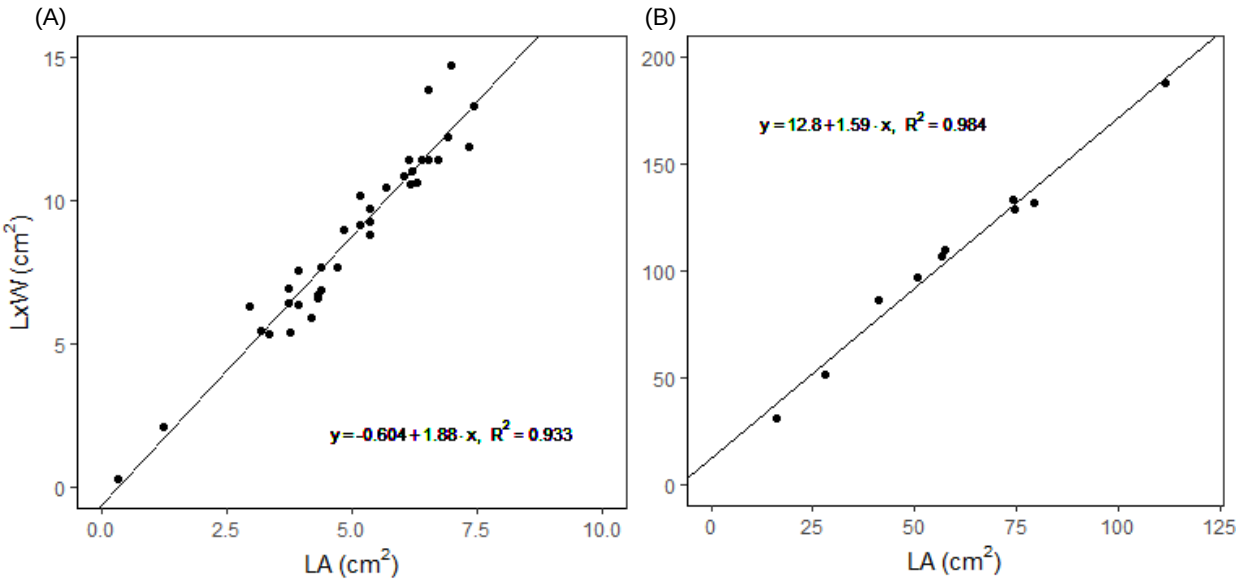

Figure 2.4: Linear regression model between leaf area (LA, cm²) and the product of maximum leaf length and width (L x W, cm²) for A – guayule and B – castor

for both guayule and castor (*P*-value < 0.001) and associated with elevated correlation coefficients (R^2 = 0.93 and 0.98, respectively).

The leaf area was determined throught L x W during the time-course of the tests, in correspondence of the salt solution renewal (twice a week), on every experimental plants. At the end of the tests, after 30 days for guayule and after 22 days for castor, LA was determined by destructive method, together with main orthogonal leaf diameters (L x W). Such measurements were compared with those of T_0 plants and used for verifying the model constructed to explain

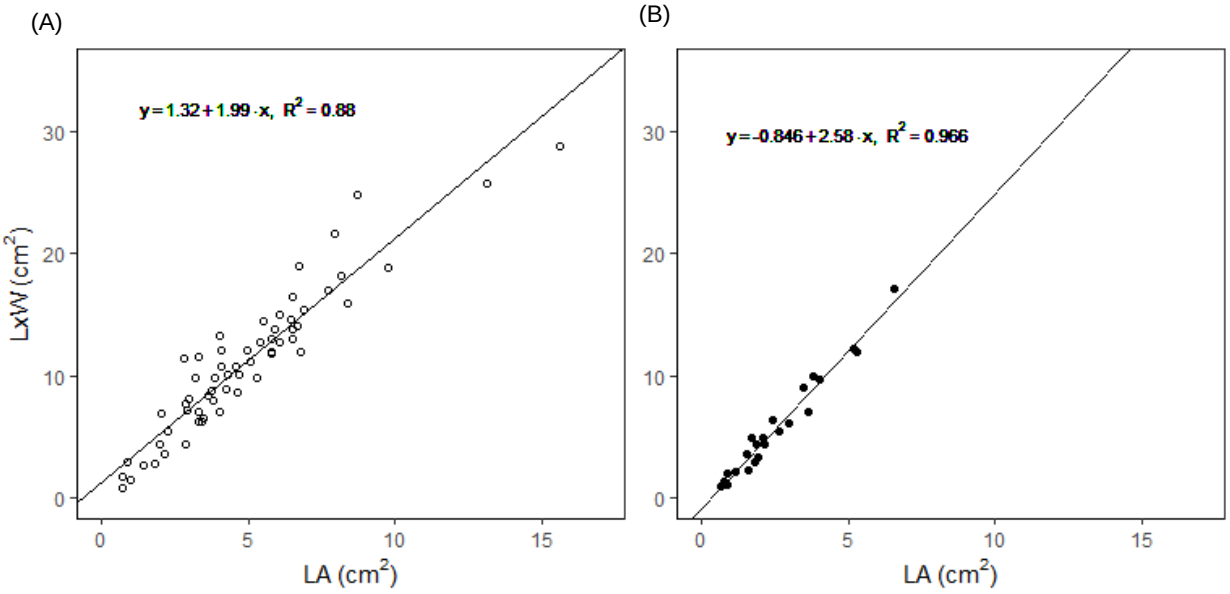

Figure 2.5: Linear regression models between leaf area (LA, cm²) and the product of maximum leaf length and width (L x W, cm²) in guayule for A – control plants and B – plants exposed to 40 g L⁻¹ NaCl

the relation between LA and L x W. As salt stress may cause morphological changes, the analysis of covariance was applied at a significance level (P) ≤ 0.05 in order to verify the influence of NaCl treatment on such relation (Fuchs, 2011). For guayule, comparing the intercepts of regression lines of controls and treated plants, no significative differences were observed (P-value = 0.30). However, a significative interaction between the LA and treatment was found (P-value = 0.02), indicating that the slope of the regression between leaf orthogonal diameters and leaf area was significantly different for controls and treated plants. The results obtained show that the relation between guayule leaf morphological traits and leaf area measured by destructive analysis is influenced by the NaCl treatment. Therefore, two different equations were used for controls and stressed plants (Figure 2.5). The regression model elaborated for LA determination in castor was also tested for covariance. No significative differences were observed in the intercept (P-value = 0.66), as well as in the slope (P-value = 0.51), between the regression lines of controls and treated plants (Annexe 1). This indicate that in castor the relation between leaf orthogonal diameters and LA was not influenced by NaCl treatment. Therefore, for the determination of LA during the NaCl tolerance test in castor, one common equation for controls and treated plants was used (Figure 2.6).

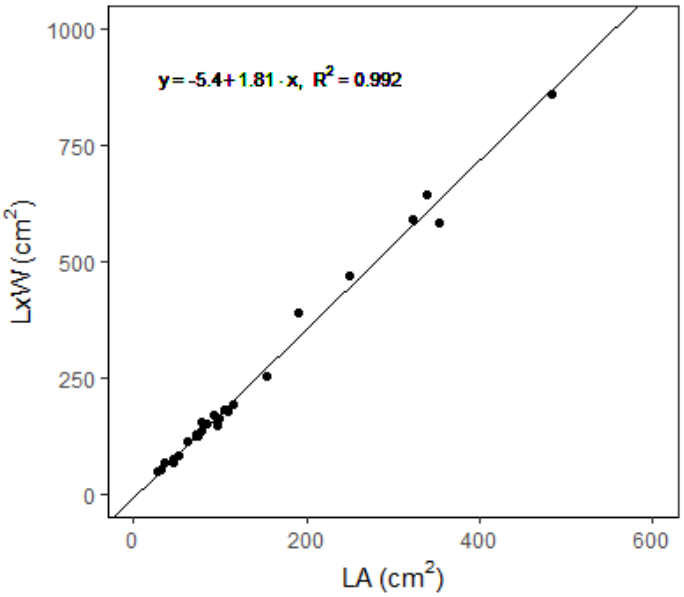

Figure 2.6: Linear regression model between leaf area (LA, cm^2) and the product of maximum leaf length and width (L x W, cm^2) in castor (controls and NaCl treated plants)

2.3.2 RGR and derivative indices

Plant growth analysis (PGA) is an approach that uses primary data (weights, areas, tissue volumes) of plant single organs to investigate processes involving the whole plant (Hunt et al.,

2002). The central parameter of PGA is the Relative Growth Rate (RGR), that represents the relative velocity or rate with which one plant or part of it grows. Mathematically, the RGR is the unit increment of biomass during time or the first derivative function of the biomass growth curve:

1. $RGR = (\ln Mt_f - \ln Mt_0) / t_f - t_0$,

where Mt_f is the biomass produced at time t_f, i.e. the end of the experiments, and
Mt_0 is the biomass produced immediately before NaCl exposition (T_0) (Neto et al., 2004).
The RGR of a plant can also be expressed with the relation:

2. $RGR = NAR \times LAR$,

where NAR (g d^{-1} m^{-2}) is the net assimilation rate, or DW increment per leaf area unit, and
LAR (m^2 g^{-1}) is the leaf area ratio, dependent on the leaf area and thickness (Di Baccio et al., 2009). The LAR can be obtained with the following relation:

3. $LAR = LMA \times LMR$,

where LMA (g m^{-2}) is the leaf mass per area, it is used to describe typical leaf traits of a plant species, and calculated as ratio of the leaf DW (g) to the leaf area (m^2) and
LMR (g g^{-1}) is the leaf mass ratio, or the fraction of the total biomass allocated to the leaves, calculated as the ratio of the leaf biomass (g DW) to the total plant biomass (g DW) (Di Baccio et al., 2009).

The stem length (SL, cm) was also measured and used to calculate the height to mass ratio (HMR, cm g^{-1}), the ratio of SL and stem DW (g). Finally, the stem to mass ratio (SMR), was calculated as the ratio of the stem DW (g) to the whole-plant DW (g) (Di Baccio et al., 2009).

2.3.3 Relative Water Content

The relative water content (RWC), is a major determinant of metabolic activity and leaf survival (Sinclair and Ludlow, 1985). Although it is related to cell volume, RWC reflects the dynamic balance between water supply from root system and the ability to conserve water via stomata regulation (Meher et al., 2017). In this study, RWC was determined to give indication on the water status of guayule and castor plants at physiological conditions and under salt stress. At least 6 completely expanded leaves from 3 different plants (*n* = 3) for both controls and treated plants were used. Leaf discs (10 mm or 7 mm, diameter) were collected with a cork borer, avoiding the mid-rib. Discs were immediately weighted (FW), soaked in vials with 1.5-2.0 mL of distilled

water and stored for at least 24 h. After soaking, discs, completely hydrated, were quickly dried of any surface moisture using a tissue paper and the fully turgid weight (TW) was measured. Leaf discs were then oven dried at 50 °C to obtain their DW. The RWC was calculated from the following formula:

4. $\mathrm{RWC}\ (\%) = \frac{\mathrm{FW-DW}}{\mathrm{TW-DW}}\ \mathrm{x}\ 100$

2.4 Physiological parameters

2.4.1 Chlorophyll fluorescence

Chlorophyll fluorescence measurements were conducted on guayule and castor plants to evaluate NaCl effects on the functionality of photosystem II (PSII). The measure was conducted in correspondence with salt solution renewal (twice a week) on intact, fully expanded and exposed leaves using a pulse-amplitude-modulated fluorometer (Mini-PAM; Heinz Walz GmbH, Effeltrich, Germany) on every experimental plant. As chlorophyll fluorescence may be affected by leaf senescence, both old and new leaves were chosen for each plant when needed.

The potential efficiency of PSII photochemistry (F_v/F_m) was evaluated on plants adapted to dark for at least 30 min. as $F_v / F_m = (F_m – F_o) / F_m$, where F_v is the variable fluorescence in the dark, F_o represents the minimum fluorescence yield in the dark and F_m is the maximum fluorescence yield in the dark after application of saturation flash of light, which completely closes all the PSII reaction centres (Scartazza et al., 2020).

The actual photon yield of PSII in the light (Φ_{PSII}) was measured on long-term light adapted leaves (100 ± 40 µmol m^{-2} s^{-1} of PAR, growing light conditions) and determined as $\Phi_{PSII} = (F_m' - F_s) / F_m'$ at steady state, where F_m' represents the maximum fluorescence yield with all the PSII reaction centres in the reduced state, obtained by superimposing a saturating light flash during exposure to actinic light, and F_s is the fluorescence at the actual state of PSII reaction centres during actinic illumination (Scartazza et al., 2020).

2.4.2 Gas exchanges

On the bases of the results of chlorophyll fluorescence measurements, gas-exchanges were measured during salt tolerance tests in order to evaluate plants response to 15 g L^{-1} NaCl. Measure was performed on each plant, on the same leaves used for chlorophyll fluorescence, using a photosynthetic portable system Li6400 (LiCor, Lincoln, NE, USA) equipped with the leaf

chamber fluorometer. Chamber conditions were set at 20 °C and 400 µmol mol^{-1} of CO_2 concentration. The PPFD (photosynthetic photon flux density) inside the chamber was set at 100 µmol mol^{-2} s^{-1} with a red/blue LED light source. Evaluated parameters are: stomatal conductance (g_s), transpiration rate (E), net CO_2 assimilation rate (A), intercellular CO_2 concentration (C_i), actual photon yield of PSII photochemistry (Φ_{PSII}) and intrinsic water-use efficiency (A/g_s).

2.4.3 Photosynthetic pigments determinations and relations with SPAD measurements

Before the beginning (T_0) and after the end of the experiments, both control and NaCl treated plants of guayule and castor were selected for the determination of photosynthetic pigments (chlorophyll *a*, chlorophyll *b* and total carotenoids) in the leaves. Leaf discs (10 mm or 7 mm diameter: 0.785 cm^2 or 0.385 cm^2 area, respectively) were collected from each plant with a cork borer. Completely expanded leaves were chosen, covering a wide range of vital and senescent leaves, and the disc was cut avoiding the mid-rib. Leaf discs were immediately weighted and then put in vials with 1.5-2.0 mL of 80% (v/v) cold acetone. After stirring for 12 h at 4°C, the samples were exposed to three sonication (Branson 1210, Bransonic Ultrasonic Cleaner) cycles of 5 minutes each (working frequency: 47 kHz ± 6%). At the end of each sonication cycle, the leaf disc samples were transferred on ice for 5 min. to avoid the overheating of cells in the disruption phase. After 15 min. of sonication, each sample was stirred at 4°C until the completely bleaching of the disc. The absorbance of the liquid extracts was measured at 663.2, 646.8, and 470.0 nm using an UV-vis spectrophotometer (UV-1800 Spectrophotometer, Shimadzu). The determination of chlorophyll *a* [Chl *a* = $(12.25A_{646.8}) – (5.1A_{663.2})$], chlorophyll *b* [Chl *b* = $(12.5A_{646.8}) – (5.1A_{663.2})$] and total carotenoids [C_{x+c} =$(1000A_{470}$ – 1.52Chl *a* – 85.02Chl *b*) / 198] contents in the leaves of guayule and castor were performed using the equations indicated by Wellburn (1994) when the extraction of vegetal material was conducted on an acetone solvent basis.

Before the beginning of the salt tolerance tests (T_0), when guayule plants were 3-months-old and castor 30-days-old, the amount of total chlorophylls was determined with a portable chlorophyll meters (SPAD, SPAD 502 Plus Chlorophyll Mater, KONICA Minolta Europe) in correspondence of the leaf area where the disc would be sampled for pigments extraction. Leaves sampling covered a range of SPAD values (relative units) between 24 and 60 for guayule and 21 and 66 for castor. The leaf adaxial side was always placed toward the emitting window of the device. The chemical analyses of total chlorophylls (chlorophyll *a* + chlorophyll *b*, Chl *a* + *b*) expressed as µg cm^{-2} were plotted against the correspondent SPAD values (Figure 2.7), as also

proposed by Sub et al (2015) experimental methods. For both guayule and castor, the regression found (linear) was significantly high (*P*-value < 0.001) and associated to elevated correlation coefficients (R^2 = 0.90 and 0.75 for guayule and castor respectively).

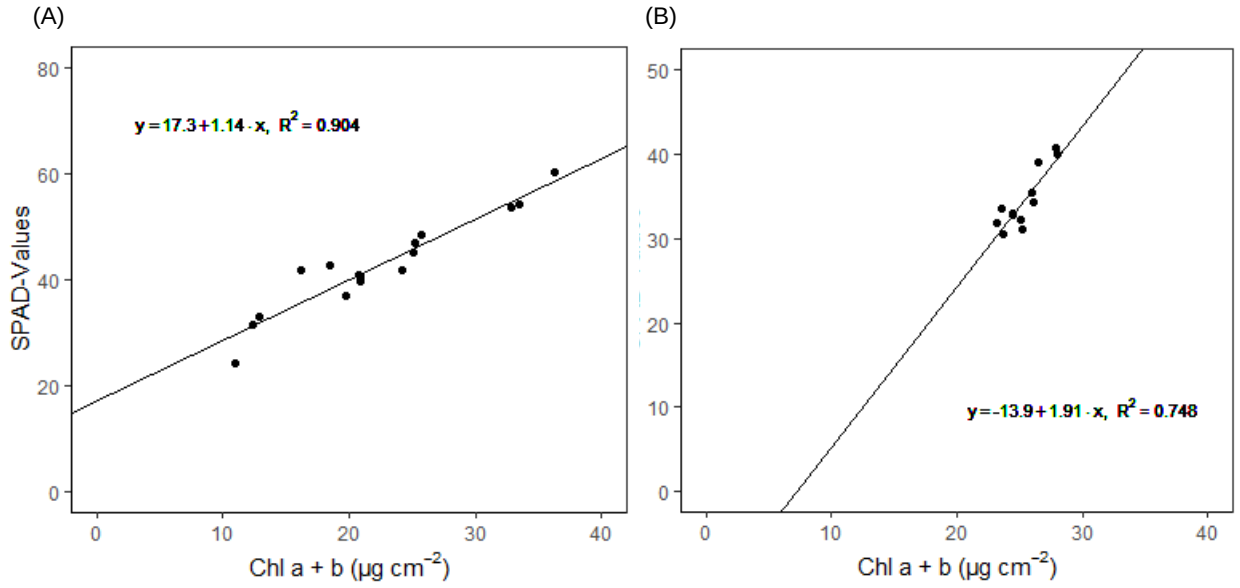

Figure 2.7: Linear regression model between leaf SPAD-values and total chlorophyll concentration (Chl a + b) expressed in µg cm^{-2} for A – guayule and B – castor

The SPAD measurements were performed during the time-course of the salt tolerance tests, in correspondence of the renewal (twice a week) of NaCl solution on at least three leaves of each experimental plant. At the end of the salt tolerance test (after 30 days for guayule and 22 days for castor), together with the SPAD measurements, also the chemical analyses of photosynthetic pigments were carried out on control and treated plants, as described above. They were compared with those of T_0 plants and used for verifying and comparing the relations between SPAD determinations and chlorophyll measurements on control and treated plants. In order to evaluate if treatment influenced the linear regression model elaborated, an analysis of covariance was applied at a significance level (*P*) ≤ 0.05. Results of the comparison between the regression linear curves of guayule controls and NaCl treated plants, showed no significant interaction between the two experimental conditions, indicating that the slope of the regression line is similar for both controls and treated plants. A significative difference in the intercepts between the regression lines of the two groups was observed (*P*-value < 0.001), indicating that the intercept of treated plants was significantly higher than that of controls. The results show that the relation between SPAD values and Chl *a* + *b* in guayule was influenced by salt treatment. However, ANOVA test showed that the model was not affected by removing the interaction between the two conditions (*P*-value = 0.42) (Fuchs, 2011). Thus, the model chosen consisted in two parallel lines,

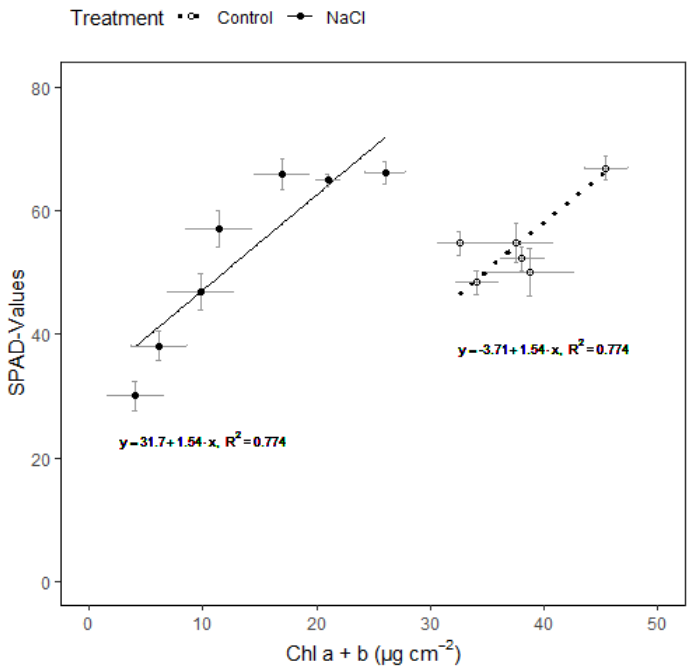

Figure 2.8: Linear regression models between leaf SPAD-values and total chlorophyll concentration (Chl a+b) expressed in µg cm^{-2} for controls and treated guayule plant exposed to increasing concentrations of NaCl up to 40 g L^{-1}.

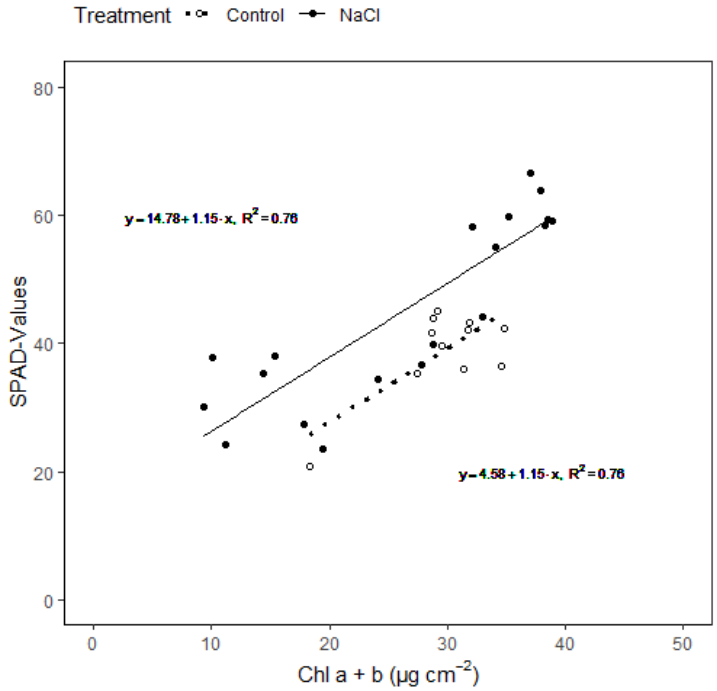

Figure 2.9: Linear regression models between leaf SPAD-values and chlorophyll concentration (Chl a+b) expressed in µg cm^{-2} for controls and treated castor plants exposed to increasing concentrations of NaCl up to 25 g L^{-1}.

one for the controls and one for treated plants, with the same slope but different intercepts (Figure 2.8, Annexe 2). Similarly, the difference between the slopes of regression lines of castor controls and treated plants was not significant (*P*-value = 0.94), but the intercept of treated plants was significantly higher than that of controls (*P*-value < 0.001). Therefore, a model of two parallel lines was chosen for castor controls and treated plants (Figure 2.9) (Ismay and Klim, 2019; Caro, 2016; Mirman, 2014).

2.5 Mineral content determination

The dried leaf, stem and root, samples of guayule and castor from salinity tolerance tests were ground to a fine power using a blender and a ceramic mortar. The elements, including sodium (Na^+), potassium (P), calcium (Ca^{2+}), magnesium (Mg^{2+}) and phosphorus were determined using different methods.

Plant material was digested with microwave digestion. Well-mixed samples were weighed and pre-digested for one night in Teflon vessels with concentrated nitric acid and perchloric acid. Vessels were then treated with two cycles of microwave digestion at 250 Watt for 10 min., followed by 5 min. of ventilation. After this process, samples were filtered through a Whatman paper filter. The filtrate was collected in 25 mL volumetric flasks and diluted with milli-Q water.

Sodium determination was performed using a fast-sequential atomic absorption spectrometer (AA240FS, Agilent), reading samples at 589 nm.

A sequential inductively coupled plasma atomic emission spectrometer (ICP-OES, Liberty AX, Agilent) was used for Ca^{2+}, Mg^{2+} and K^+ determinations. The selected wavelengths were: 766.790 nm (K^+), 315.887 nm (Ca^{2+}) and 279.079 nm (Mg^{2+}).

For P determination, the sample extract was mixed with p-nitrophenol solution and drop by drop NaOH solution was added to turn the indicator colour into yellow. Then, sulfomolybdic reagent was added and, after 10 min. of incubation, the samples were read spectrophotometrically at 720 nm. The phosphorus (mg L^{-1}) was obtained with a previously prepared calibration curve and results were expressed as $\mathrm{P\ mg/Kg} = \frac{\mathrm{P\ mg/L} * \mathrm{V}}{\mathrm{p}}$, where V is the extraction volume and p is the mineralization weight.

2.6 Statistical analysis

The 4 NaCl tolerance tests performed were evaluated separately. Data shown in graphs and tables represent the mean values ± standard error (SE). One-way analysis of variance (ANOVA) was applied on all the parameters in order to evaluate the effect of the increasing

sodium concentrations; *P*-values ≤ 0.05 were considered as significant. For percentage results, before statistical analysis, an arcsine transformation was applied. Statistical analysis was conducted using R Studio (2020) and Microsoft Excel (2019).

3 Results

3.1 Guayule test results

3.1.1 First salt tolerance test

A preliminary test was conducted on 3-months-old guayule plants adapted to hydroponics for 2 weeks.

Figure 3.1 and 3.2 show growth measures performed during the salt tolerance test, while table 3.1 shows destructive measures performed at the end of the test. Plants exposed to 40 g L^{-1} NaCl presented a significative reduction in both fresh weight (FW) and dry weight (DW) of leaves, as well as leaves percentage DW, compared to the control (Table 3.1). In the same condition, the leaf number (LN) became almost 2-fold lower (from 14.2 to 8.2). The LN showed a decreasing trend from the application of the lowest salt treatment (1.5 g L^{-1} NaCl, Figure 3.1), and leaf area (LA) showed a 64.1% reduction at 40 g L^{-1}. At the end of the test (40 g L^{-1} NaCl), the leaf area

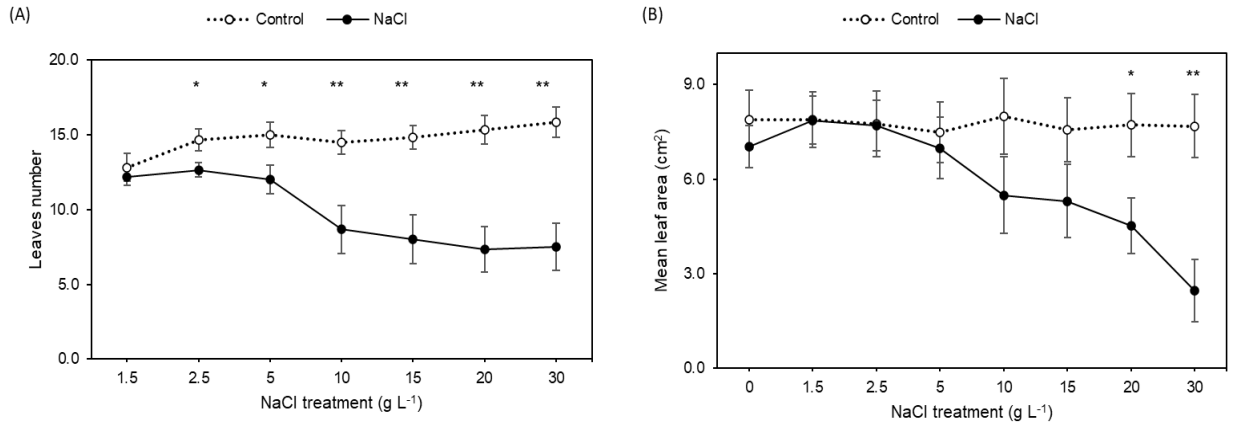

Figure 3.1: A - Number of green leaves and B – leaf area (cm^2) of guayule plant under increasing NaCl concentrations. Values are means ± SE (n = 6). Means accompanied by stars were significantly different from the control at P < 0.05 (*) and P < 0.01 (**) (one-way ANOVA).

ratio (LAR) and leaf mass per area (LMA) significantly varied compared to the control (Table 3.1). The stem of stressed-plants presented a significative reduction of fresh weight and length (SL) compared to the control, but no significative difference where observed in dry weight and DW percentage. The stem height related to its mass (HMR) reduced to the half after NaCl treatments. The roots of treated plants did not show significative differences compared to the control in their length (Figure 3.2), and at the end of the test, the root DW and % DW did not change, as well as the shoot/root ratio. The relative growth rate (RGR) of the whole plants was not affected by the NaCl treatments, while that of the leaves was reduced (Table 3.1). The leaf relative water content (RWC) drastically decreased (-53%) in stressed plants.

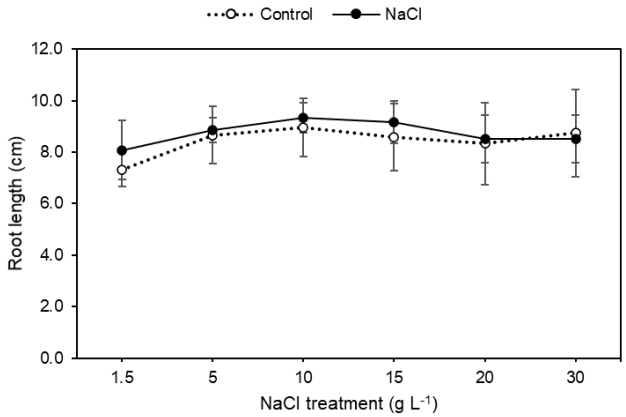

Figure 3.2: Root length of guayule plants under increasing NaCl concentrations. Values are means ± SE (n = 6).

Table 3.1: Growth parameters of guayule plants exposed for 30 days to increasing concentrations of NaCl up to 40 g L^{-1}. FW = fresh weight; DW = dry weight; RGR = Relative growth rate; LN = leaf number; LA = mean leaf area; LAR = leaf area ratio; LMA = Leaf mass per area; LMR = Leaf mass ratio; NAR = Net assimilation rate; SL = stem length; HMR = Height to mass ratio; SMR = Stem to mass ratio; RL = root length; RWC = relative water content. Values are means ± SE (n = 6). Data were analysed by one-way ANOVA. The significance level (P) is shown

Growth parameter	**NaCl treatment**		***P*-value [a]**
	Control	**Treated**	
Initial total FW (g)	2.01 ± 0.27		
Leaf FW (g)	1.17 ± 0.55	0.13 ± 0.07	0.011
Stem FW (g)	0.11 ± 0.06	0.01 ± 0.01	0.016
Root FW (g)	0.28 ± 0.14	0.13 ± 0.07	0.142
Total FW (g)	1.63 ± 0.77	0.15 ± 0.08	0.024
Initial total DW (g)	0.32 ± 0.04		
Leaf DW (g)	0.16 ± 0.08	0.05 ± 0.03	0.049
Stem DW (g)	0.04 ± 0.02	0.01 ± 0.01	0.227
Root DW (g)	0.03 ± 0.01	0.01 ± 0.01	0.196
Total DW (g)	0.21 ± 0.11	0.06 ± 0.04	0.065
Leaf DW %	7.39 ± 3.95	13.05 ± 7.16	0.006
Stem DW %	4.67 ± 2.19	6.81 ± 3.76	0.121
Root DW %	1.64 ± 0.92	2.23 ± 1.13	0.249
Total DW %	6.63 ± 3.54	5.94 ± 3.16	0.065
Shoot/root (g g^{-1})	2.26 ± 1.08	1.12 ± 0.60	0.252
RGR (d^{-1})	$1.26 \times 10^{-2} \pm 6.45 \times 10^{-3}$	$5.35 \times 10^{-3} \pm 2.83 \times 10^{-3}$	0.098
RGR_L (d^{-1})	$1.26 \times 10^{-2} \pm 6.22 \times 10^{-3}$	$6.51 \times 10^{-3} \pm 3.33 \times 10^{-3}$	0.040
RGR_S (d^{-1})	$1.03 \times 10^{-2} \pm 5.72 \times 10^{-3}$	$6.80 \times 10^{-3} \pm 4.04 \times 10^{-3}$	0.270
RGR_R (d^{-1})	$1.49 \times 10^{-2} \pm 8.55 \times 10^{-3}$	$7.24 \times 10^{-3} \pm 3.73 \times 10^{-3}$	0.335
LN	14.17 ± 0.79	8.00 ± 0.58	< 0.001
LA (cm^2)	4.88 ± 0.35	2.58 ± 0.33	< 0.001
LAR (cm^2 g^{-1})	$1.09 \times 10^{-2} \pm 9.93 \times 10^{-4}$	$4.88 \times 10^{-3} \pm 6.80 \times 10^{-5}$	0.015
LMA (g m^{-2})	68.8 ± 7.53	135.0 ± 0.15	0.003
LMR (g g^{-1})	0.72 ± 0.01	0.65 ± 0.03	0.033
NAR (g d^{-1} m^{-2})	2.13 ± 0.87	3.07 ± 0.55	0.567
SL (cm)	5.50 ± 0.45	4.33 ± 0.11	0.029
HMR (cm g^{-1})	23.2 ± 12.6	12.6 ± 7.30	0.806
SMR	0.02 ± 0.01	0.03 ± 0.02	0.214
RL (cm)	10.2 ± 2.03	10.7 ± 1.62	0.876
RWC %	60.4 ± 2.46	28.5 ± 8.67	0.011

a: Before statistical analysis, percentages values were compared after arcsine transformation.

Chlorophyll fluorescence measurements (Figure 3.3) showed that NaCl treated plants maintained the same levels of PSII functionality of the control until the 5 g L^{-1} treatment, while beyond this threshold we observe a gradual reduction of PSII photochemistry. However, the functionality of PSII significantly decreases only after 15 g L^{-1} (15 days-treatment).

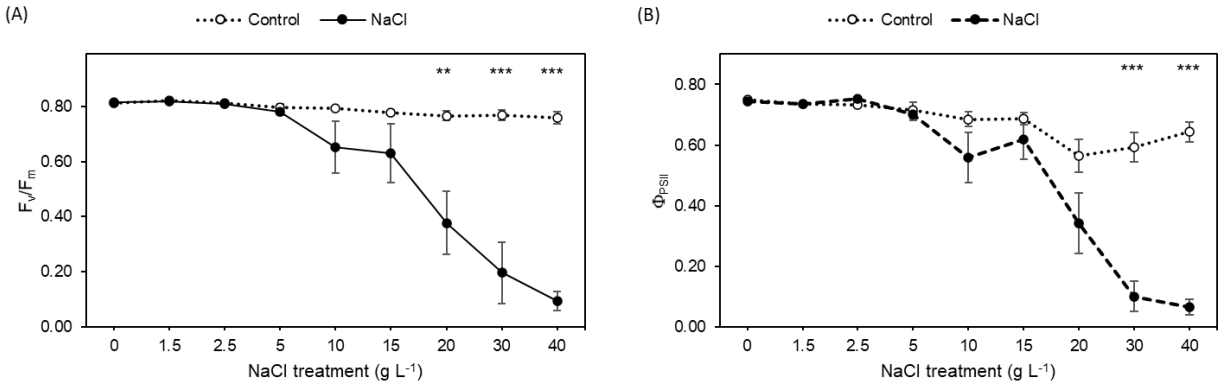

Figure 3.3: A – Potential efficiency of PSII photochemistry (F_v/F_m); B – Actual photon yield of PSII in the light (Φ_{PSII}) measured at 100 ± 40 µmol m^{-2} s^{-1} of PAR, growing light condition. Values are means ± SE (n = 6). Means accompanied by stars were significantly different from the control at P < 0.01 (**) and P < 0.001 (***) (one-way ANOVA).

Figure 3.4 shows the results of gas-exchanges measurements at 15 g L^{-1} NaCl, which represents the salt concentration threshold just before the fall of the photochemical activity. The measure was performed on both “old” and “new” leaves, where for “old” and “new” leaves we considered green leaves that were fully expanded at the beginning of the experiment and green leaves that have expanded during the treatment period, respectively. Salt stress caused a significant reduction of stomatal conductance (g_s) and transpiration rate (E). The partial stomatal closure caused a reduction of CO_2 uptake and, hence, a decrease of net CO_2 assimilation rate (A) and Φ_{PSII}. The intercellular CO_2 concentration (C_i) did not decrease in stressed plants compared to the control and, in contrast, tends to a slight increase. All parameters differed between old and new leaves for both control and treated plants. In particular, the new leaves of both control and treated plants presented a higher A and Φ_{PSII} and a lower C_i compared to the old leaves. Moreover, treated new leaves showed C_i values similar to those of the control. The intrinsic-water use efficiency, i.e. the A/g_s ratio, decreased in the treated old leaves compared to the control ones, while the new leaves maintained values similar to those reached in the control. However, the difference between old and new leaves was not significant (*P*-value > 0.05) according to one-way ANOVA.

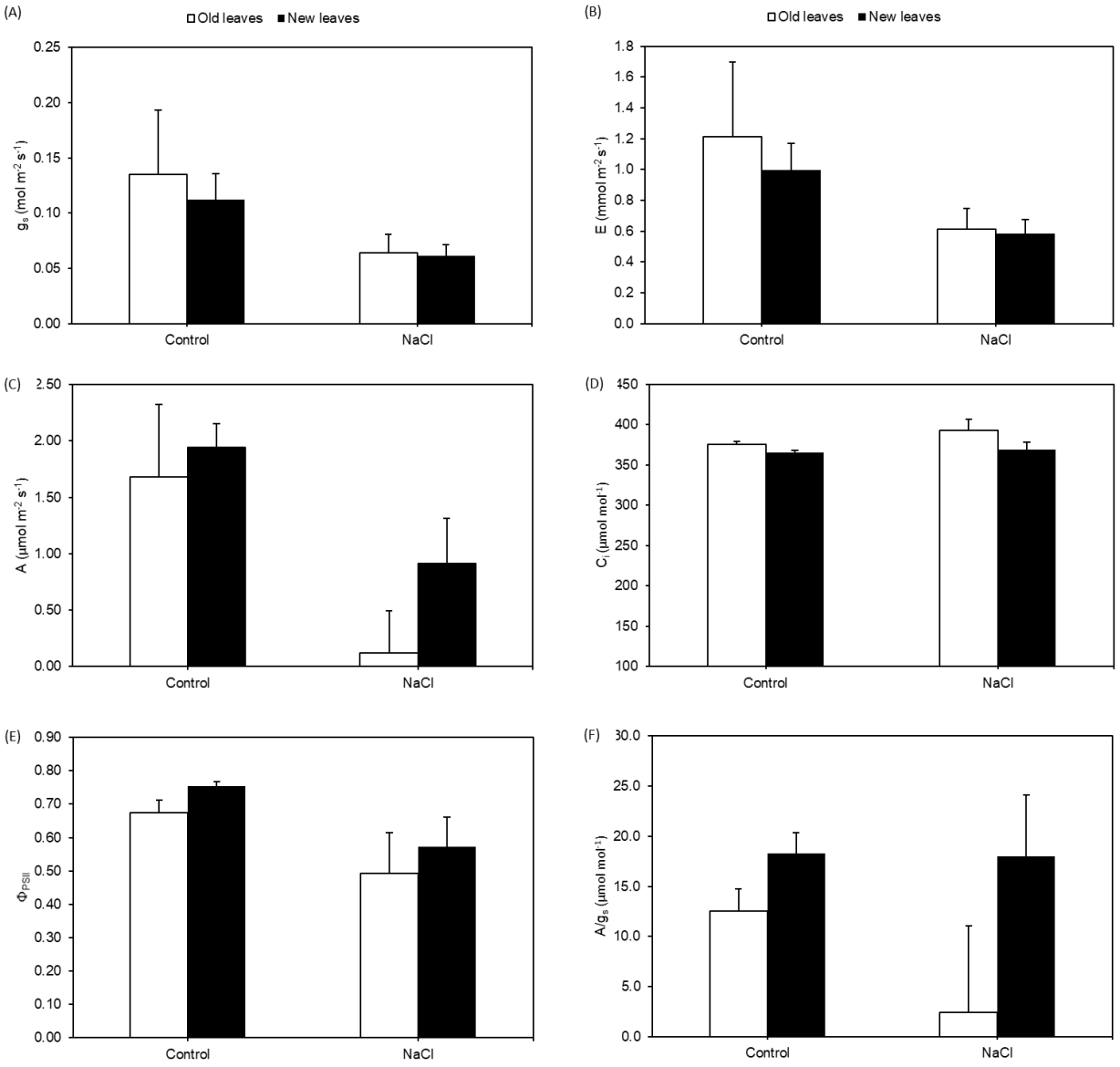

Figure 3.4: A – Stomatal conductance (g_s); B – transpiration rate (E); C – net CO_2 assimilation rate (A); D – intercellular CO_2 concentration (C_i); E – actual photon yield of PSII photochemistry (Φ_{PSII}); F – intrinsic water-use efficiency (A/g_s). Values are mean ± SE (n = 6)

During the salt tolerance test, at any NaCl treatment, the leaf chlorophyll content was sensitively higher in the control plants than in treated (Figure 3.5). The chemical extraction of photosynthetic pigments performed at the end of the test, showed a reduction in Chl *a* + *b* and Chl *a* (2-fold and 2.6-fold lower, respectively) in salt-stressed plants compared to the control (Figure 3.6). The Chl *a* to Chl *b* ratio markedly decreased (almost 5-fold lower) in stressed plants compared to the controls. The leaf content of carotenoids in treated plants decreased (3.6-fold

lower) compared to the control, while the total chlorophyll/carotenoids ratio was significantly higher (1.6-fold).

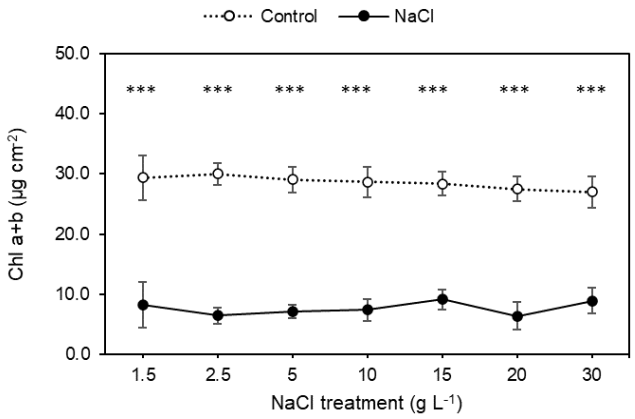

*Figure 3.5: Leaf total chlorophyll concentration (Chl a+b) in guayule plants under increasing concentrations of NaCl. Values are means ± SE (n = 6). ***: significantly different at the P < 0.001 level according to the one-way ANOVA test*

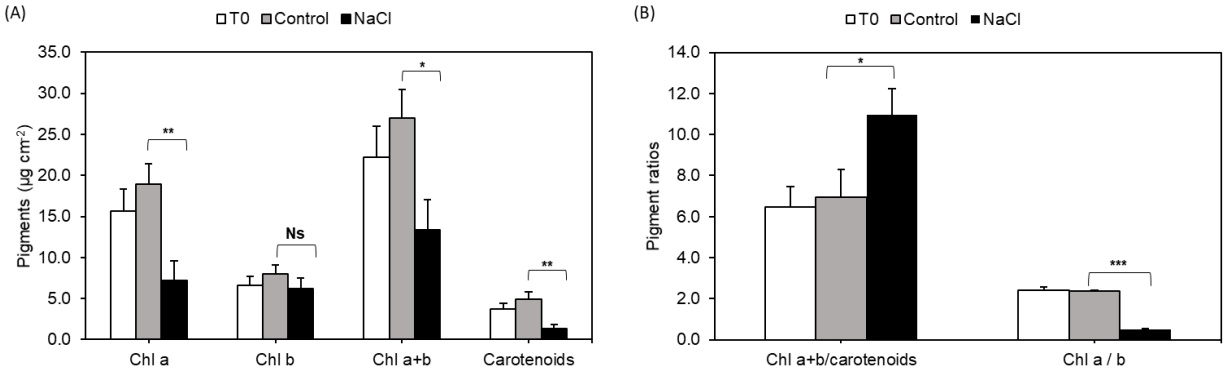

*Figure 3.6: A – Leaf concentration of chlorophylls and carotenoids and B – pigment ratios in guayule plants exposed to 40 g L^{-1} NaCl, distinguished among T_0, controls (control) and treated plants (NaCl). Values are mean ± SE. Ns: not significant; *, ** and ***: significantly different at the P < 0.05, P < 0.01 and P < 0.001 level, respectively, according to the one-way ANOVA test. Chl a: chlorophyll a; Chl b: chlorophyll b; Chl a+b: total chlorophyll*

3.1.2 Second salt tolerance test

As salt tolerance of guayule varies according to the plant growth stages, a second salinity tolerance test was performed on 4-months-old plants, which had been adapted to hydroponics for 4-weeks.

3.1.2.1 Growth parameters

The total fresh weight (FW) and dry weight (DW), biomass partitioning among organs, leaf area and the lengths of stem and root were measured after 40 g L^{-1} NaCl treatment, and used as

basis for the analysis of plant growth. The main results of this analysis are presented in Table 3.2, together with some leaf morphological parameters and the leaf relative water content (RWC). Guayule plants exposed to 40 g L^{-1} NaCl, showed a significative decrease of total FW compared to the control. However, no significative difference was observed in the total DW, although the *P*-value was close to the significant threshold (*P* = 0.08), as well as the stem DW (*P*-values = 0.06). The percentage DW (DW, %) was significantly higher than that of the control, mainly due to a sharp increase of the leaf percentage DW. The leaf number (LN) and leaf area (LA) were also strongly affected by salinity, showing a reduction of 52.2% and 64.8% respectively. The leaf area ratio (LAR) and leaf mass ratio (LMA) varied according to LA and leaf DW variations (Table 3.7). Figure 3.7 shows the gradual decrease of LN in salt-stressed plants during the time-course of the test. Dehydration and premature death of the oldest leaves in stressed-plants was noted after 15 g L^{-1} NaCl (15 days exposition), and gradually enhanced with salt concentration increase. The stem length (SL), the height to mass ratio (HMR) and the stem to mass ratio (SMR) were not affected by salt stress. The root length, DW and %DW of stressed plants were not impacted by salinity, as well as the shoot/root ratio and NAR when compared to the control. The leaf relative water content (RWC) was clearly reduced (2.7-fold lower) by salinity (Table 3.2).

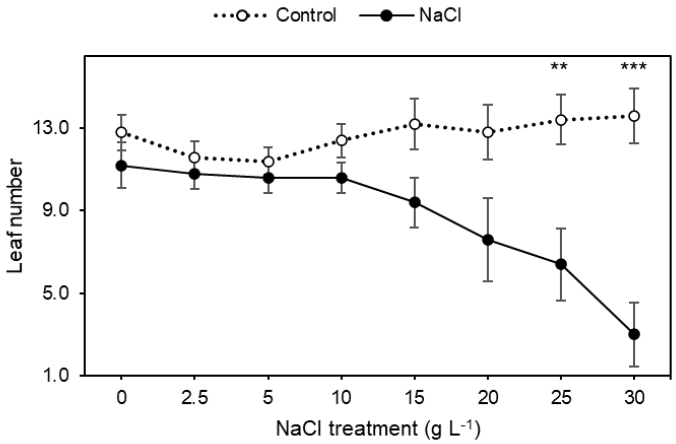

Figure 3.7: Number of green leaves of guayule plant under increasing NaCl concentrations. Values are means ± SE (n = 5). Means accompanied by stars were significantly different from the control at P < 0.01 (**) and P < 0.001 (***) (one-way ANOVA).

Table 3.2: Growth parameters of 4-motnhs-old guayule plants exposed for 30 days to increasing concentrations of NaCl up to 40 g L^{-1}. FW = fresh weight; DW = dry weight; RGR = Relative growth rate; LN = leaf number; LA = mean leaf area; LAR = Leaf area ratio; LMA = Leaf mass per area; LMR = leaf mass ratio; NAR = Net assimilation rate; SL = stem length; HMR = Height to mass ratio; SMR = Stem to mass ratio; RL = root length; RWC = relative water content. Values are means ± SE (n = 5). Data were analysed by one-way ANOVA. The significance level (P) is shown

Growth parameter	**NaCl treatment**		***P*-value [a]**
	Control	**Treated**	
Initial total FW (g)	1.63 ± 0.77		
Leaf FW (g)	2.27 ± 0.64	0.37 ± 0.03	0.017
Stem FW (g)	0.49 ± 0.06	0.25 ± 0.00	0.005
Root FW (g)	0.77 ± 0.28	0.34 ± 0.07	0.170
Total FW (g)	3.71 ± 0.96	0.95 ± 0.09	0.028
Initial total DW (g)	0.21 ± 0.11		
Leaf DW (g)	0.51 ± 0.11	0.30 ± 0.04	0.109
Stem DW(g)	0.16 ± 0.02	0.10 ± 0.02	0.059
Root DW (g)	0.08 ± 0.02	0.05 ± 0.01	0.170
Total DW (g)	0.75 ± 0.14	0.45 ± 0.04	0.080
Leaf DW %	24.4 ± 2.68	79.9 ± 5.60	0.000
Stem DW %	32.9 ± 1.48	41.5 ± 2.86	0.027
Root DW %	12.0 ± 1.04	13.9 ± 1.57	0.358
Total DW %	23.3 ± 2.60	47.2 ± 2.46	< 0.001
Shoot/root (gg^{-1})	10.0 ± 2.94	10.6 ± 3.22	0.889
RGR (d^{-1})	$8.12 \times 10^{-3} \pm 5.75 \times 10^{-3}$	$5.90 \times 10^{-3} \pm 2.84 \times 10^{-3}$	0.045
RGR_L (d^{-1})	$9.91 \times 10^{-3} \pm 6.70 \times 10^{-3}$	$7.87 \times 10^{-3} \pm 4.50 \times 10^{-3}$	0.086
RGR_S (d^{-1})	$6.71 \times 10^{-3} \pm 3.76 \times 10^{-3}$	$9.76 \times 10^{-3} \pm 4.94 \times 10^{-3}$	0.051
RGR_R (d^{-1})	$2.26 \times 10^{-2} \pm 9.53 \times 10^{-3}$	$5.37 \times 10^{-3} \pm 8.37 \times 10^{-3}$	0.211
LN	13.00 ± 1.92	7.20 ± 1.40	0.001
LA (cm^2)	5.93 ± 0.49	2.09 ± 0.25	< 0.001
LAR ($cm^2\ g^{-1}$)	$1.20 \times 10^{-2} \pm 3.66 \times 10^{-4}$	$4.13 \times 10^{-3} \pm 6.08 \times 10^{-4}$	< 0.001
LMA ($g\ m^{-2}$)	56.4 ± 1.00	145.2 ± 15.86	0.002
LMR ($g\ g^{-1}$)	0.67 ± 0.02	0.66 ± 0.05	0.922
NAR ($g\ d^{-1}\ m^2$)	3.39 ± 0.58	3.32 ± 2.01	0.969
SL (cm)	4.88 ± 0.23	4.66 ± 0.45	0.672
HMR ($cm\ g^{-1}$)	4.05 ± 2.21	20.06 ± 10.80	0.145
SMR	0.04 ± 0.03	0.07 ± 0.04	0.835
RL (cm)	9.49 ± 1.32	9.00 ± 1.33	0.800
RWC %	77.5 ± 4.47	28.9 ± 11.03	0.001

a: Before statistical analysis, percentages values were compared after arcsine transformation.

3.1.2.2 Physiological parameters

As shown in Figure 3.8, the functionality of photosystem II (PSII) of guayule gradually decreased starting from 15 g L^{-1} (16 days of treatment), while behind this threshold treated plants maintained the same levels of the control. However, one-way analysis of variance (ANOVA) between F_v/F_m of control and treated plants showed a significative difference (*P*-value < 0.05) only after 25 g L^{-1} NaCl treatment (22 days of treatment), when the average potential PSII

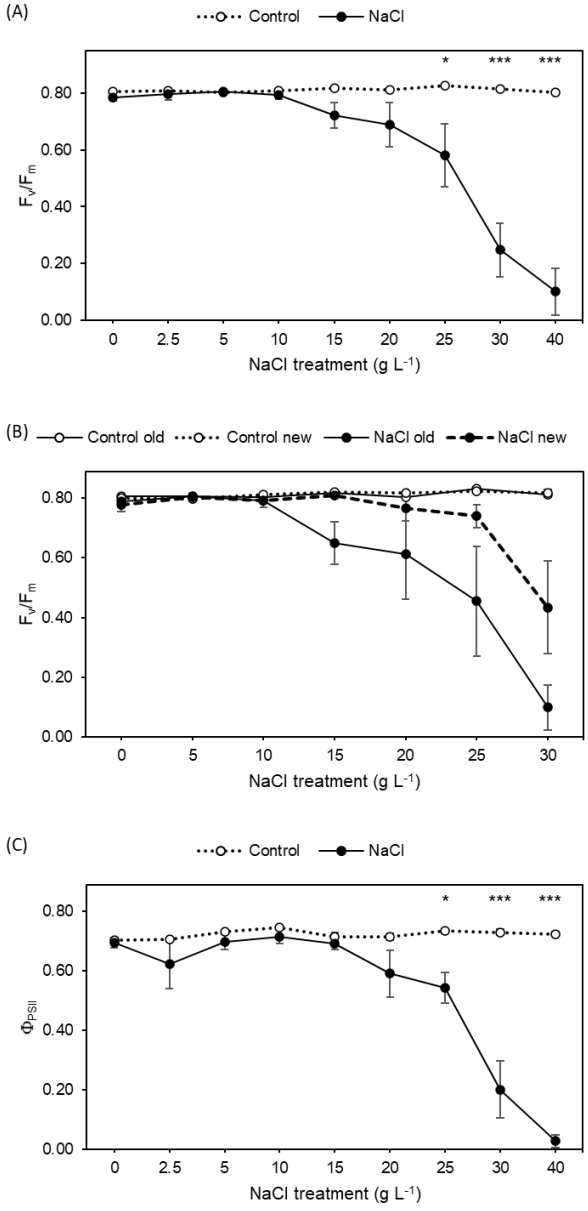

Figure 3.8: A – Potential efficiency of PSII photochemistry (F_v/F_m); B – potential efficiency pf PSII photochemistry distinguished in old and new leaves for controls and NaCl treated plants; C – actual photon yield of PSII in the light (Φ_{PSII}) measured at 100 ± 40 µmol m^{-2} s^{-1} of PAR, growing light conditions (Φ_{PSII}). Values are means ± SE (n = 5). Means accompanied by stars were significantly different from the control at P < 0.05 and P < 0.001 (***) (one-way ANOVA).

efficiency of treated plants was 0.581. Figure 3.8 (B) shows that treated the new leaves maintained a higher PSII functionality than the old ones, although the difference was not statistically significant according to one-way ANOVA (*P*-value > 0.05).

Gas-exchanges (Figure 3.9) were measures at 15 g L^{-1} NaCl (16 days of treatment), being the concentration threshold at which potential efficiency of PSII photochemistry started to decline. Stressed plants showed a reduction in stomatal conductance and transpiration rate, indicating a partial stomatal closure. The latter causes a reduction of the CO_2 uptake and, hence, a significative decrease of actual photon yield of PSII photochemistry and net CO_2 assimilation rate. The average values of the latter are 2.90 µmol m^{-2} s^{-1} in the controls, and 1.08 µmol m^{-2} s^{-1} in treated plants. The intercellular CO_2 concentration did not vary in salt-stressed plants compared

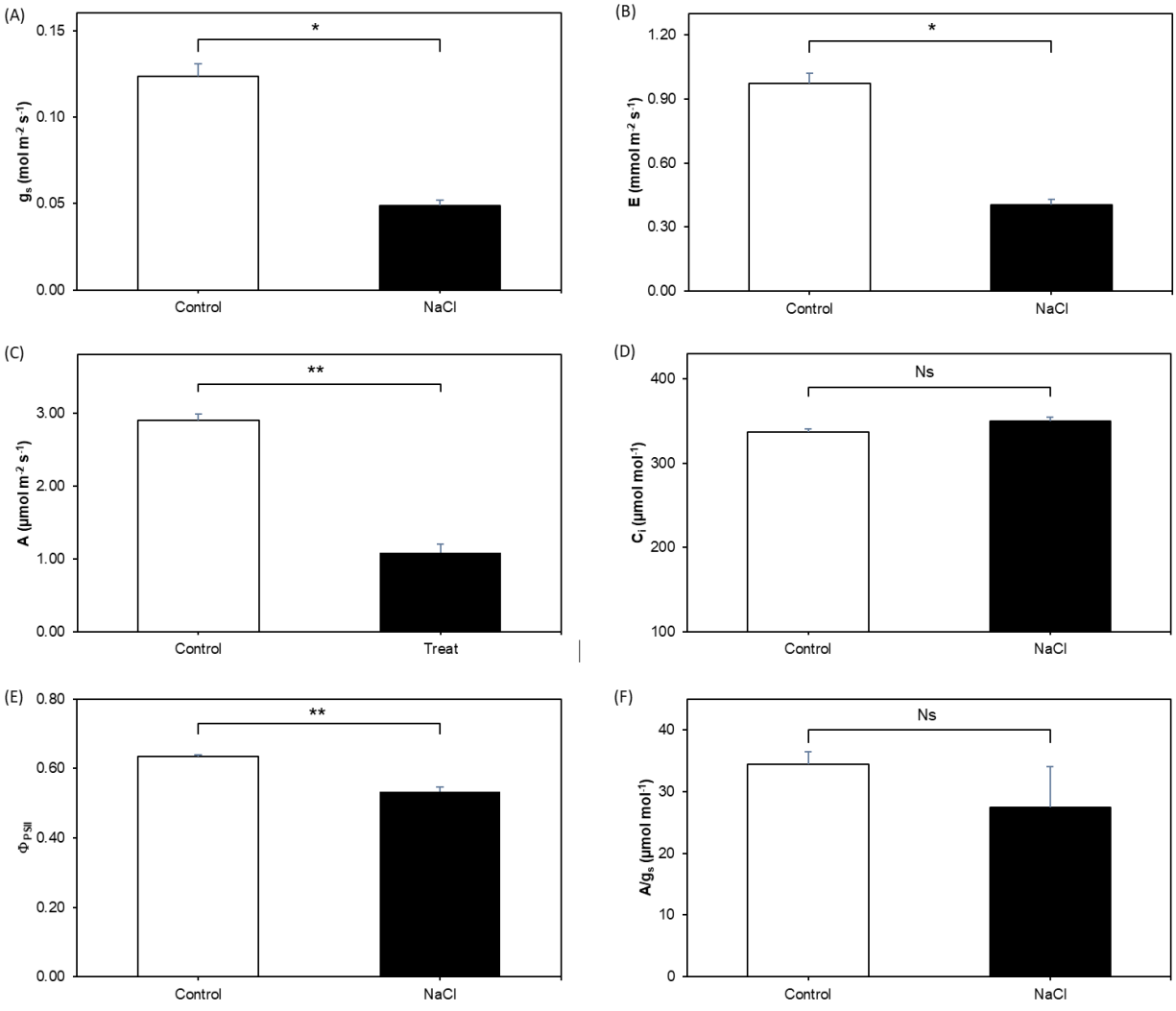

*Figure 3.9: A – Stomatal conductance (g_s); B – transpiration rate (E); C – net CO_2 assimilation rate (A); D – intercellular CO_2 concentration (C_i); E – actual photon yield of PSII photochemistry (Φ_{PSII}); F – intrinsic water-use efficiency (A/g_s). Values are mean ± SE (n = 5). Ns: not significant; * and **: significantly different at the P < 0.05 and P < 0.01 level according to the ANOVA single-factor test, respectively*

to the control, presenting values around 350 µmol mol^{-1} and 337 µmol mol^{-1} respectively. The A/g_s ratio in treated plants did not significantly vary compared to the control.

The SPAD measurements registered during the time-course of the NaCl test (Figure 3.10), showed that in treated plants the total chlorophyll concentration (Chl *a* + *b*) was significantly lower than the controls. At the end of the test, (40 g L^{-1} NaCl), and Chl *a*, Chl *b*, Chl *a* + *b* and carotenoids were significantly lower (3-, 1.4-, 2.4-, 3-fold, respectively) in stressed plants compared to the controls, (Figure 3.11). The relative content of total Chl *a* +*b* to carotenoids increased (1.6-fold higher) in treated plants compared to the control, while the Chl *a* to Chl *b* ratio decreased (almost 5-fold lower).

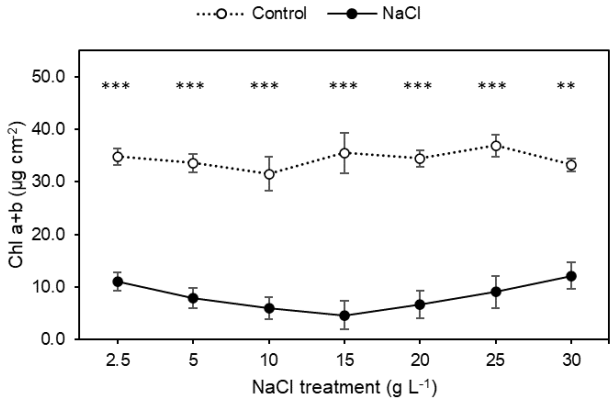

*Figure 3.10: Total chlorophyll concentration in guayule plants under increasing concentrations of NaCl. Values are means ± SE (n = 6). ** and ***: significantly different at the P < 0.01 and P < 0.001 level, respectively, according to the one-way ANOVA test*

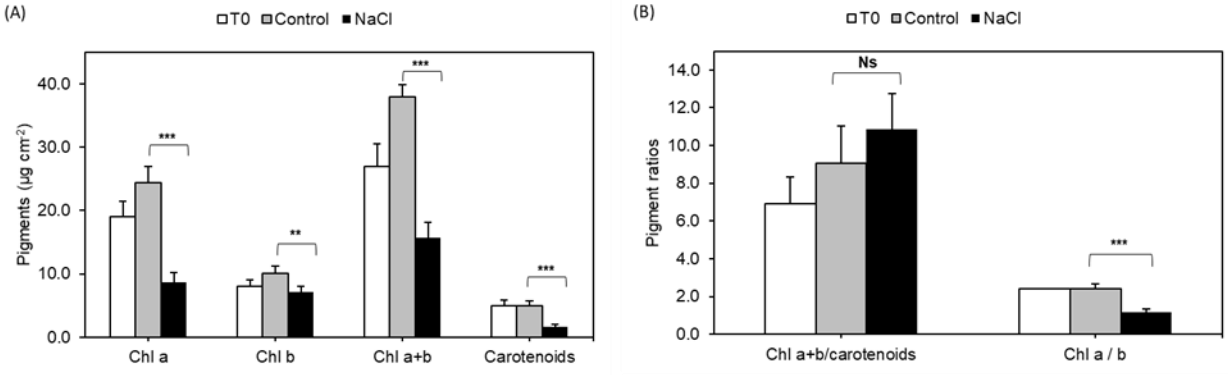

*Figure 3.11: A – Leaf concentration of chlorophylls and carotenoids and B – pigment ratios in guayule plants exposed to 40 g L^{-1} NaCl, distinguished among T_0, controls (control) and treated plants (NaCl). Values are mean ± SE. Ns: not significant; ** and ***: significantly different at the P < 0.05 and P < 0.001 level according to the one-way ANOVA test, respectively. Chl a: chlorophyll a; Chl b: chlorophyll b; Chl a+b: total chlorophyll*

3.1.2.3 Mineral composition

After 40 g L^{-1} NaCl treatment, Na^+ concentrations and contents in the tissue of the different organs analysed in guayule plants significantly increased compared to the control (Table 3.3 and Figure 3.12). The Na^+ concentration in organs of treated plants was relatively higher in stems than in leaves and roots, where this cation reached almost the same level (stem > leaves ≈ roots). The average Na^+ concentration in salt stressed plants was 38.1 mg g^{-1}, while it was 1.82 mg g^{-1} in the controls, and differences in the distribution within organs were observed. The shoot/root Na^+ content ratio (calculated by dividing the sum of leaf and stem Na^+ content by the root Na^+ content) was 3.27 in controls and 8.14 in treated plants, showing a significant difference (*P*-value < 0.001).

Table 3.3: Sodium (Na^+) concentration (mg g^{-1}) in organs, total Na^+ uptake (mg $plant^{-1}$) and shoot to root Na^+ ratio in guayule control and treated plants exposed for 30 days to increasing concentrations of NaCl up to 40 g L^{-1}. Values are means ± SE (n = 4). Data were analysed by one-way ANOVA. The significance level (P) is shown

Tissue	NaCl treatment		P-value
	Control	Treated	
Leaves (mg g^{-1})	1.33 ± 0.29	37.18 ± 3.03	< 0.001
Stem (mg g^{-1})	2.33 ± 0.42	42.18 ± 2.72	< 0.001
Roots (mg g^{-1})	3.43 ± 0.46	36.11 ± 4.39	< 0.001
Whole plant (mg $plant^{-1}$)	1.21 ± 0.02	17.51 ± 1.98	< 0.001
Shoot Na^+ / root Na^+	3.27 ± 0.85	8.14 ± 0.77	0.002

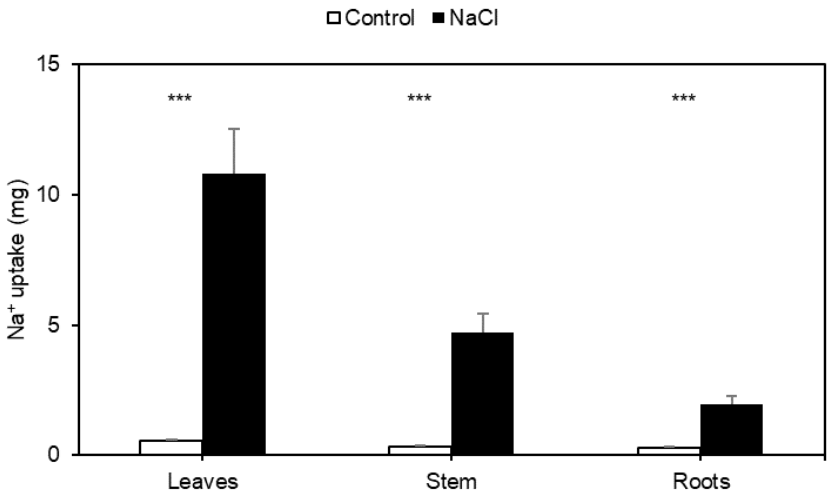

*Figure 3.12: Sodium (Na^+) uptake (mg) in different organs of guayule control and treated plants exposed for 30 days to increasing concentrations of NaCl up to 40 g L^{-1}. Values are means ± SE (n = 4). ***: significantly different at P ≤ 0.001 level according to the one-way ANOVA test*

In both groups, leaves were the organ presenting the highest Na^+ content, corresponding to 47.8% of the whole-plant-content in controls and 61.8% in treated plants, while in roots it was only 24.7% in controls and 11.2% in treated plants. One-way ANOVA separately applied to the two groups of plants (control and treated) revealed that in treated plants the Na^+ stem-content was significantly higher than the roots-content (*P*-value = 0.01), while the same phenomenon did not occur in controls (*P*-value = 0.50).

The effect of 40 g L^{-1} NaCl were investigated on the organ concentration and uptake of mineral elements (Table 3.4 and Figure 3.13). The potassium (K^+) concentration in roots presented a 67.9% reduction compared to the control, while the phosphorus (P) concentration was not affected by salinity condition, as no significant variations were observed compared to the control. Calcium (Ca^{2+}) and magnesium (Mg^{2+}) concentrations showed a significant difference compared to the control only in stem, where Ca^{2+} decreased by 51.5% and Mg^{2+} increased by 133%. Potassium and calcium contents (Figure 3.12) significantly reduced in stem of NaCl treated plants compared to the control (*P*-value = 0.03 and 0.04, respectively), while no significant difference was observed in P and Mg^{2+} stem-content (P-value = 0.81 and 0.16, respectively). The contents of all elements evaluated (K^+, P, Ca^{2+} and Mg^{2+}) tended to decrease in leaves of guayule treated plants, although the *P*-values resulted not significant. This is probably due to the

Table 3.4: Concentration (mg g^{-1} DW) of potassium (K^+), phosphorous (P), calcium (Ca^{2+}) and magnesium (Mg^{2+}) in organs of guayule control and treated plants exposed for 30 days to increasing concentrations of NaCl up to 40 g L^{-1}. Values are means ± SE (n = 4). Mineral content was analysed by one-way ANOVA (P ≤ 0.05). The significance level (P) is shown

Mineral (mg g^{-1})	**Tissue**	**NaCl Treatment**		**P-value**
		Control	**Treat**	
K^+	Leaf	36.51 ± 4.69	30.22 ± 2.89	0.297
	Stem	16.46 ± 2.24	12.06 ± 1.19	0.209
	Root	31.82 ± 5.04	10.21 ± 1.31	0.006
P	Leaf	1.84 ± 0.58	1.61 ± 0.06	0.705
	Stem	4.10 ± 1.17	5.77 ± 0.61	0.251
	Root	7.47 ± 0.91	9.67 ± 2.71	0.471
Ca^{2+}	Leaf	17.32 ± 0.73	18.20 ± 2.35	0.734
	Stem	11.12 ± 1.30	5.39 ± 1.84	0.044
	Root	7.81 ± 2.39	9.13 ± 1.59	0.662
Mg^{2+}	Leaf	4.05 ± 0.16	4.58 ± 0.68	0.297
	Stem	0.49 ± 0.08	1.14 ± 0.04	< 0.001
	Root	1.44 ± 0.23	1.30 ± 0.08	0.588

replication variability of leaf biomass production, as shown by the relatively higher standard errors than in stem and roots (Figure 3.13). No significant reduction of element content was observed in roots of guayule stressed plants in comparison with the control. The K^+: Na^+ ratio of the whole plant was 21.55 in controls and 0.61 in treated plants, showing a high significant decrease (*P*-value = 0.033). This ratio was significantly reduced in treated plants compared to the control in both shoot and roots (*P*-value = 0.034 and 0.031, respectively), data not shown. The Ca^{2+}:Na^+ ratio of the whole plant also significantly decreased in salt-stressed plants in comparison with the control (*P*-value = 0.007), data not shown.

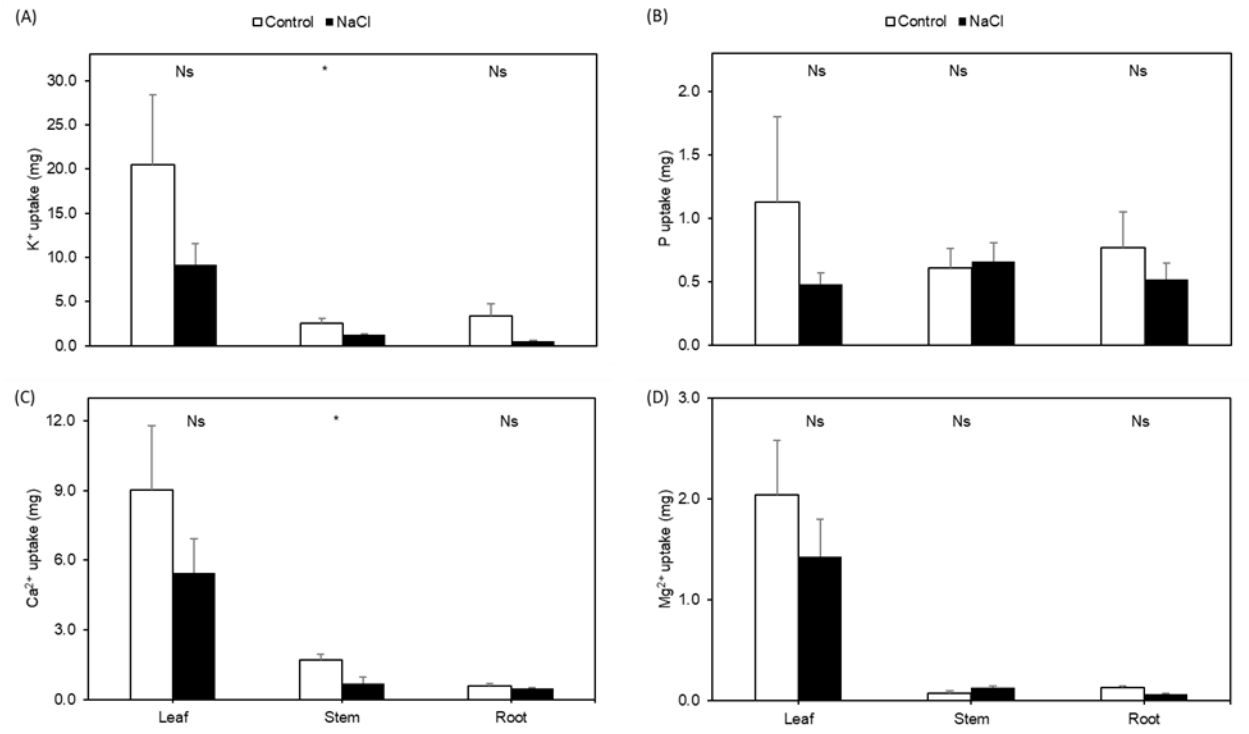

*Figure 3.13: A – potassium (K^+), B – phosphorus (P), C – calcium (Ca^{2+}) and D – magnesium (Mg^{2+}) uptake (mg) in organs of guayule control and treated plants exposed for 30 days to increasing concentrations of NaCl up to 40 g L^{-1}. Values are means ± SE (n = 4). Ns: not significant; *: significantly different at the P ≤ 0.05 level according to the one-way ANOVA test*

3.2 Castor tests results

3.2.1 Salt tolerance test

The salinity tolerance test on castor (*Ricinus communis* L.) was performed on 1-month-old plants adapted to hydroponics for 2 weeks.

3.2.1.1 Growth parameters

Figure 3.14 and 3.15 show growth measures performed during the salt tolerance test, while table 3.5 shows destructive measures performed at the end of the test. Castor growth was clearly affected by salinity (Table 3.5): the fresh weight (FW) showed a significant decrease in each organ compared to the control. The biomass production (shoot and root dry weight, DW) of stressed-plant was reduced by the salinity condition. The shoot/root ratio (calculated by dividing

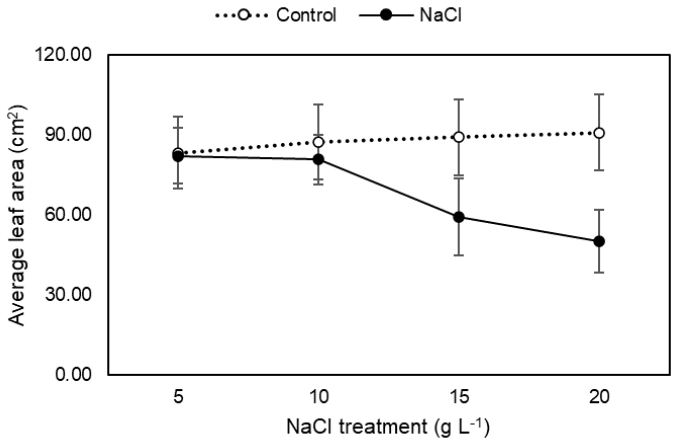

Figure 3.14: Leaf area (cm^2) of castor plants under increasing NaCl concentrations. Values are mean ± SE (n = 6)

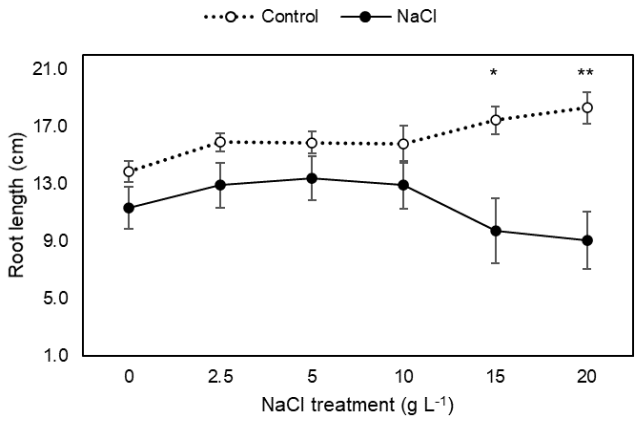

*Figure 3.15: Root length (cm) of castor plant under different NaCl concentrations. Values are mean ± SE (n = 6). * and **: significantly different at the $p < 0.05$ and $p < 0.01$ level according to the ANOVA single-factor test, respectively*

the sum of leaf and stem DW by the root DW) significatively differed from the control, and the dry matter production of the leaves, stem and roots presented differences in their response to salt stress. The leaf and root DW was averagely more affected by NaCl treatment than the stem, that, anyway, showed a sensitive reduction (about -45%), although not significantly different compared to the control according to a significant level (P = 0.085) very close to the defined threshold of the Fisher test ($P \leq 0.05$). The leaves and roots DW decrease was reflected in a remarkably reduction of their relative growth rate (RGR), as opposite as the RGR of stem (RGR_S), which did not vary compared to the control. However, the stem percentage DW significantly increased in comparison with the control, and salt stress induced a reduction (-27%) in stem length (SL) and an increase (+19%) in the stem to mass ratio (SMR). The leaf DW % was also impacted by salinity (4-fold higher), while the mean leaf area (LA) of treated plants was significantly lower (about -62%) than the control. Figure 4.1 shows the progressive reduction of the mean LA during the treatment, estimated using leaf morphological traits. ANOVA single factor between control and treated plants did not show a significative difference at each NaCl treatments, although in correspondence of 20 g L^{-1} NaCl the mean LA of castor plants became about 1.5-fold lower than in controls. However, LA was significantly different compared to the control at 25 g L^{-1} NaCl, presenting a 63.1% reduction (Table 3.5). The leaf area ratio (LAR) and leaf mass per area (LMA) also varied according to leaf DW and LA, with a decrease (-39.4%) in the first case and an increase (+79%) in the second one. No significant variation of root percentage DW was observed (Table 4.2), but the salinity reduced the root elongation just after 15 and 20 g L^{-1} NaCl (Figure 4.2), causing a 44 and 50.3% decrease of RL, respectively. At the end of the tolerance test (25 g L^{-1} NaCl) the RL of treated plants showed an average reduction of 27% compared to the controls (Table 3.15). The leaf relative water content (RWC) clearly decreased (about -61%) in stressed plants compared to the controls.

Table 3.5: Growth parameters of castor plants exposed for 22 days at increasing concentrations of NaCl up to 25 g L^{-1}. FW = fresh weight; DW = dry weight; RGR = Relative growth rate; LN = leaf number; LA = mean leaf area; LAR = Leaf area ratio; LMA = Leaf mass per area; NAR = Net assimilation rate; SL = stem length; HMR = Height to mass ratio; SMR = Stem to mass ratio; RL = root length; RWC = relative water content. Values are means ± SE (n = 6). Data were analysed by one-way ANOVA (P ≤ 0.05). The significance level (P) is shown

Growth parameter	**NaCl treatment**		***P*-value [a]**
	Control	**Treated**	
Initial total FW	9.97 ± 0.71		
Leaf FW (g)	19.83 ± 3.72	2.45 ± 0.14	0.001
Stem FW (g)	11.72 ± 2.28	4.78 ± 0.42	0.013
Root FW (g)	15.36 ± 2.56	5.22 ± 0.26	0.003
Total FW (g)	46.92 ± 8.44	12.45 ± 0.66	0.002
Initial total DW	0.91 ± 0.07		
Leaf DW (g)	2.65 ± 0.49	1.30 ± 0.06	0.021
Stem DW(g)	0.85 ± 0.19	0.47 ± 0.04	0.085
Root DW (g)	0.56 ± 0.08	0.19 ± 0.02	0.002
Total DW (g)	4.06 ± 0.75	1.96 ± 0.10	0.020
Leaf DW %	13.5 ± 0.25	53.8 ± 4.29	< 0.001
Stem DW %	7.07 ± 0.23	9.92 ± 0.28	< 0.001
Root DW %	3.73 ± 0.13	3.78 ± 0.47	0.991
Total DW %	8.68 ± 0.22	15.8 ± 0.57	< 0.001
RGR (d^{-1})	$6.09 \times 10^{-2} \pm 8.79 \times 10^{-3}$	$3.17 \times 10^{-2} \pm 2.37 \times 10^{-3}$	0.009
RGR_L (d^{-1})	$6.40 \times 10^{-2} \pm 8.55 \times 10^{-3}$	$3.52 \times 10^{-2} \pm 2.26 \times 10^{-3}$	0.009
RGR_S (d^{-1})	$5.74 \times 10^{-2} \pm 1.11 \times 10^{-2}$	$3.64 \times 10^{-2} \pm 3.60 \times 10^{-3}$	0.102
RGR_R (d^{-1})	$5.16 \times 10^{-2} \pm 3.49 \times 10^{-3}$	$5.24 \times 10^{-3} \pm 4.03 \times 10^{-3}$	< 0.001
Shoot/root (g g^{-1})	6.11 ± 0.41	9.48 ± 0.83	0.004
LN	5.0 ± 0.0	4.0 ± 0.0	
LA (cm^2)	176 ± 33.23	65.2 ± 7.76	0.008
LAR (cm^2 g^{-1})	$3.31 \times 10^{-2} \pm 3.06 \times 10^{-3}$	$2.00 \times 10^{-2} \pm 3.95 \times 10^{-3}$	0.030
LMA (g cm^{-2})	20.52 ± 1.53	36.69 ± 5.45	0.009
LMR (g g^{-1})	0.656 ± 0.011	0.662 ± 0.008	0.669
NAR (g m^{-2} d^{-1})	2.06 ± 0.39	1.87 ± 0.34	0.748
SL (cm)	20.7 ± 1.6	15.1 ± 0.8	0.009
HMR (cm g^{-1})	29.77 ± 4.96	32.53 ± 1.85	0.614
SMR	0.201 ± 0.014	0.239 ± 0.008	0.039
RL (cm)	18.9 ± 1.56	13.8 ± 1.55	0.048
RWC %	46.83 ± 2.26	18.42 ± 4.46	< 0.001

a: Before statistical analysis, percentages values were compared after arcsine transformation.

3.2.1.2 Physiological parameters

Chlorophyll fluorescence measurements (Figure 3.16) showed that salt-stressed castor plants maintained the same levels of PSII functionality as the control until 5 g L^{-1} (8 days of treatment), while over this threshold the PSII photochemistry gradually decreases. However, the one-way ANOVA applied to F_v/F_m of control and treated plants showed a significative difference (*P*-value < 0.05) only after 20 g L^{-1} NaCl treatment (19 days of treatment), when the death of 5 out of 6 salt-stressed plants occurred.

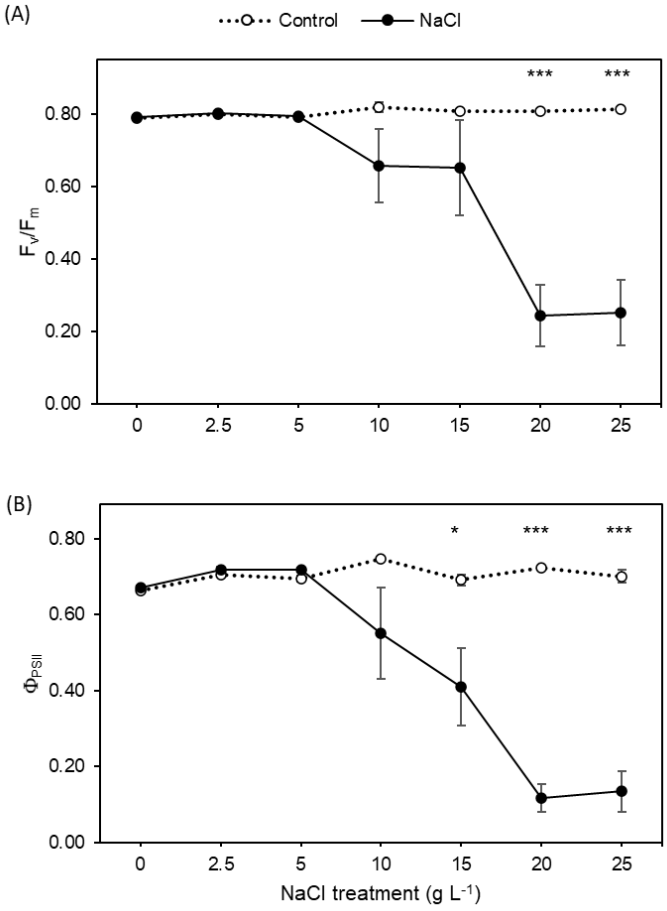

Figure 3.16: Potential efficiency of PSII photochemistry (F_v/F_m); B – actual photon yield of PSII in the light (Φ_{PSII}) measured at 100 ± 40 µmol m^{-2} s^{-1} of PAR, growing light conditions (Φ_{PSII}). Values are means ± SE (n = 6). Means accompanied by stars were significantly different from the control at P < 0.05 (*) and P < 0.001 (***) (one-way ANOVA).

Figure 3.17 shows gas-exchanges measured at 15 g L^{-1} NaCl on every castor plant (*n* = 6) under tolerance test. Salt stressed plants showed a significant reduction in stomatal conductance (g_s, 0.57 and 0.06 mol m^{-2} s^{-1} in controls and treated plants, respectively) and transpiration rate (E, 3.18 and 0.46 mmol m^{-2} s^{-1} in controls and treated plants, respectively). The

partial stomatal closure caused a reduction in net CO_2 assimilation rate (A), with average values of 5.88 and 0.43 µmol m^{-2} s^{-1} in controls and treated plants, respectively. Intercellular CO_2 concentration (C_i), as well as the intrinsic water-use efficiency, i.e. A/g_s ratio, did not vary in treated plants compared to the control (*P*-value = 0.925 and 0.926, respectively).

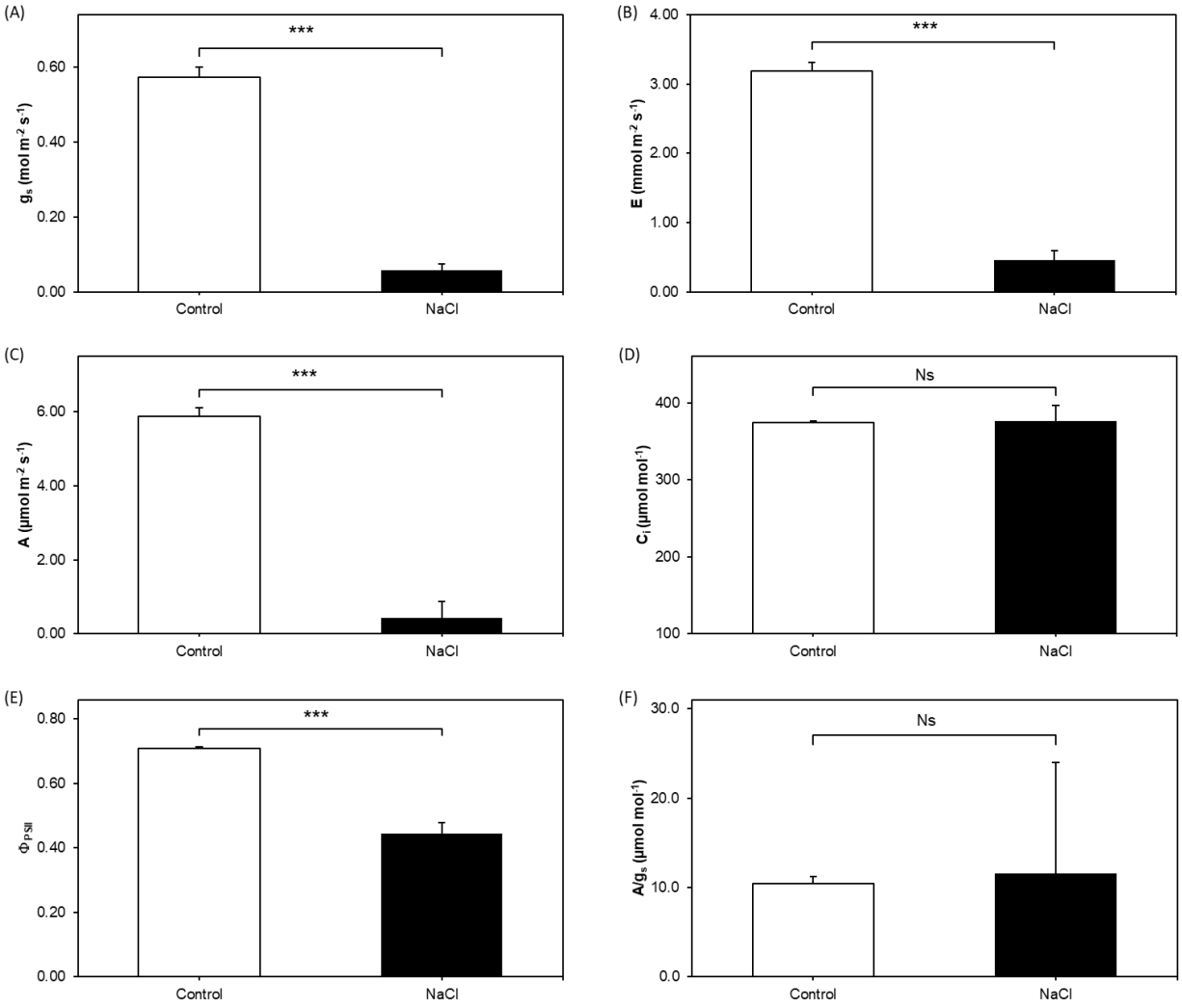

*Figure 3.17: A – Stomatal conductance (g_s); transpiration rate (E); net CO_2 assimilation rate (A); intercellular CO_2 concentration (C_i); actual photon yield of PSII photochemistry (Φ_{PSII}); intrinsic water-use efficiency (A/g_s). Values are mean ± SE (n = 6). Ns: not significant; * and **: significantly different at the p < 0.05 and p < 0.01 level according to the ANOVA single-factor test, respectively*

During the time-course of the NaCl tolerance test, the leaf total chlorophyll concentration (Chl *a* + *b*) estimated from SPAD values using a regression model, showed a relative increase between 15 and 25 g L^{-1} (Figure 3.18). This is consistent with the progressively decrease of LA and the remarkable increase of LMA (+79%) registered in response of the highest NaCl treatment (Table 3.5 and Figure 3.14). At the end of salinity test (25 g L^{-1} NaCl), Chl *a* + *b* concentration did not vary in stressed plants compared to the control, as well as chlorophyll *b* (Chl *b*), while

chlorophyll *a* (Chl *a*) and Chl *a*/*b* ratio were significantly lower (-17.6 and -32%, respectively) in stressed plants (Figure 3.19). The leaf total carotenoids concentration was significantly reduced (-17.6%) in the salinity condition compared to the control, and the Chl *a* + *b* / carotenoids ratio resulted significantly higher (+31.4%) in stressed plants.

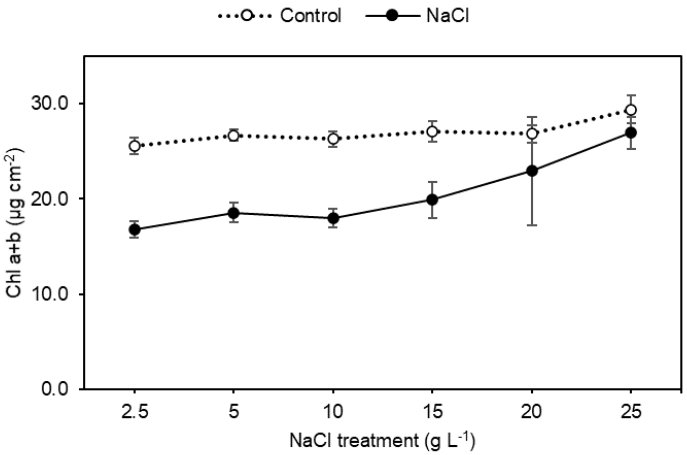

Figure 3.18: Total chlorophyll concentration (a + b) estimated from SPAD-values in castor plants under increasing concentrations of NaCl. Values are means ± SE (n = 6)

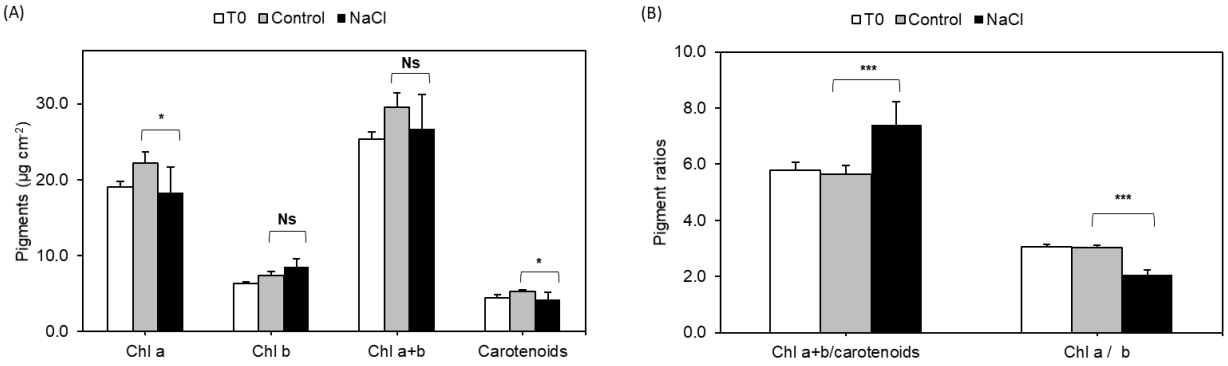

*Figure 3.19: A – Leaf concentration of chlorophylls and carotenoids and B – pigment ratios in castor plants exposed to 25 g L^{-1} NaCl, distinguished among T_0, controls (control) and treated plants (NaCl). Values are mean ± SE. Ns: not significant; ** and ***: significantly different at the P < 0.05 and P < 0.001 level according to the one-way ANOVA test, respectively. Chl a: chlorophyll a; Chl b: chlorophyll b; Chl a+b: total chlorophyll*

3.2.1.3 Mineral composition

Castor salt-treated plants presented a significative increase of Na^+ concentration in all the tissues leaves, stem and roots analysed in comparison with the control (Table 3.6). In control plants Na^+ content was higher in stem than leaves, but not different from the Na^+ content of roots (stem ≈ roots > leaves), while in treated plants the Na^+ content progressively increased from roots to the stem and leaves (leaves > stem > roots) (Figure 3.20). However, shoot/root Na^+ content

ratio (calculated by dividing the sum of leaf and stem Na^+ content by the root Na^+ content) was 2.01 in controls and 7.09 in treated plants (Table 3.6). The Na^+ content in castor leaves was 24 and 56.8% of the total Na amount in controls and treated plants, respectively.

Table 3.6: Sodium (Na^+) concentration (mg g^{-1}) in organs, total Na^+ uptake (mg $plant^{-1}$) and shoot to root Na^+ ratio in castor control and treated plants exposed for 22 days to increasing concentrations of NaCl up to 25 g L^{-1}. Values are means ± SE (n = 4). Data were analysed by one-way ANOVA. The significance level (P) is shown

Tissue	NaCl treatment		P-value
	Control	Treat	
Leaves (mg g^{-1})	0.40 ± 0.06	24.86 ± 0.17	< 0.001
Stem (mg g^{-1})	2.32 ± 0.36	36.59 ± 3.43	< 0.001
Roots (mg g^{-1})	2.36 ± 0.17	36.75 ± 3.39	< 0.001
Whole plant (mg $plant^{-1}$)	3.13 ± 0.33	52.74 ± 1.58	< 0.001
Shoot Na^+ / root Na^+	2.01 ± 0.24	7.09 ± 1.69	0.025

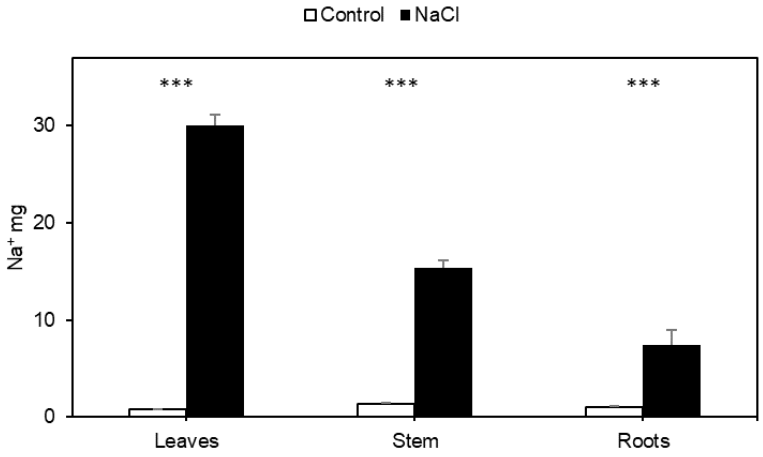

*Figure 3.20: Sodium (Na^+) uptake (mg) in different organs of castor control and treated plants exposed for 22 days to increasing concentrations of NaCl up to 25 g L^{-1}. Values are means ± SE (n = 4). ***: significantly different at P ≤ 0.001 level according to the one-way ANOVA test*

The effect of 25 g L^{-1} NaCl were investigated on the organ concentration and uptake of mineral elements (Table 3.7 and Figure 3.21). The concentration of K^+, Ca^{2+} and Mg^{2+} remarkably decreased as a consequence of NaCl treatment in all the organs analysed, with the exception of Ca^{2+} in roots and Mg^{2+} in stem (Table 3.7). The concentration of P did not show significant variations in any organs analysed. Salinity altered the tissue-contents of K^+, Ca^{2+} and Mg^{2+} (Figure 3.21). The K^+ content of treated plants presented a significant difference compared to the control in each organ, with a reduction of about 63% in leaves, 75% in stem and 89% in roots. The K^+: Na^+ ratio significantly decreased in stressed plants compared to the control (*P*-value < 0.001) in

every organ. The Ca^{2+} content of treated plants significantly decreased by about 63, 54 and 58% in leaves, stem and roots, respectively, compared to the control. The Mg^{2+} content was mainly reduced in leaves and roots (61.7 and 77.2%, respectively) of castor treated plants, while it significantly decreased by 11.4% in stem compared to the control. As for P concentration, also the P content of salt stressed plants was not affected by NaCl treatments in any tissue-organ analysed.

Table 3.7: Concentration (mg g^{-1} DW) of potassium (K^+), phosphorous (P), calcium (Ca^{2+}) and magnesium (Mg^{2+}) in organs of castor plants exposed for 22 days to increasing concentrations of NaCl up to 25 g L^{-1}. Values are means ± SE (n = 4). Mineral content was analysed by one-way ANOVA (P ≤ 0.05). The significance level (P) is shown

Mineral (mg g^{-1})	**Tissue**	**NaCl Treatment**		**P-value**
		Control	**Treat**	
K^+	Leaf	27.6 ± 2.44	17.4 ± 1.19	0.010
	Stem	47.1 ± 2.72	17.7 ± 0.78	< 0.001
	Root	64.9 ± 8.36	17.5 ± 1.11	0.001
P	Leaf	1.58 ± 0.40	2.37 ± 0.62	0.322
	Stem	3.22 ± 0.64	4.61 ± 0.70	0.192
	Root	3.71 ± 1.34	4.79 ± 0.71	0.506
Ca^{2+}	Leaf	22.7 ± 1.27	14.1 ± 1.18	0.003
	Stem	18.6 ± 1.78	12.4 ± 1.19	0.027
	Root	10.8 ± 0.72	10.6 ± 1.73	0.897
Mg^{2+}	Leaf	3.91 ± 0.12	2.51 ± 0.20	0.001
	Stem	2.14 ± 0.12	2.84 ± 0.27	0.058
	Root	8.28 ± 0.54	4.53 ± 0.37	0.001

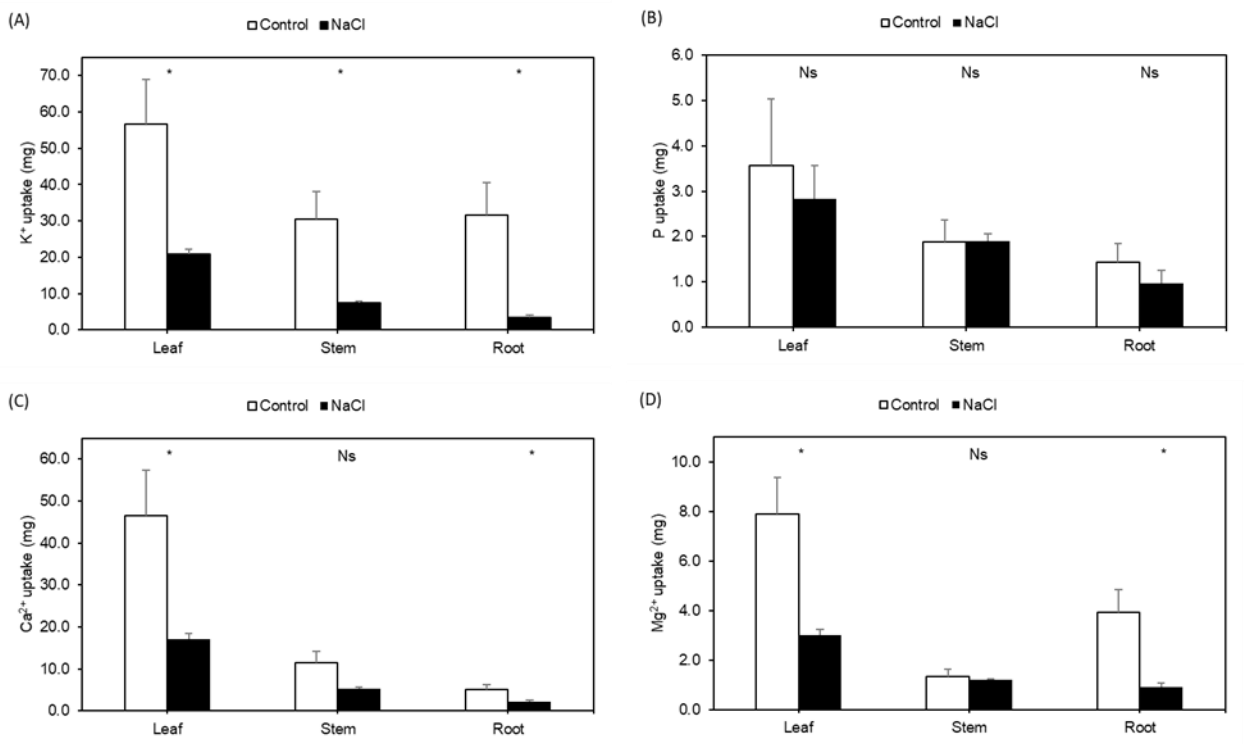

*Figure 3.21: A – potassium (K^+), B – phosphorus (P), C – calcium (Ca^{2+}) and D – magnesium (Mg^{2+}) uptake (mg) in organs of castor plants exposed for 22 days to increasing concentrations of NaCl up to 25 g L^{-1}. Values are means ± SE (n = 4). Ns: not significative; *: significantly different at the P < 0.05 level according to the one-way ANOVA test*

3.2.2 Recovery test

Castor plants (2-month-old) were monitored for recovery test, using the concentration of 10 g L^{-1} NaCl as initial dose.

After 30 days of recovering, the FW of castor plant's leaves and roots significantly decreased compared to the control, while the stem FW was unaltered (Table 3.8). Leaves were smaller (-64% reduced LA) and their number was significantly lower, as the salinity caused the premature death of all leaves that were expanded when salt-stressed was applied. However, new leaves emerged during recovery time. Both stem FW and length did not show differences from the control.

Table 3.8: Growth parameters of castor plants after 30 day of recovery from 10 g L^{-1} NaCl treatment. FW = fresh weight; LN = leaf number; LA = mean leaf area; SL = stem length. Values are means ± SE (n = 6). For each line, data were analysed by one-way ANOVA test, the significance level (P) is shown.

Growth parameter	NaCl treatment		P-value
	Control	Treat	
Leaf FW (g)	10.25 ± 0.98	2.84 ± 0.92	< 0.001
Stem FW (g)	13.9 ± 1.56	12.7 ± 1.92	0.659
Root FW (g)	13.68 ± 2.10	6.42 ± 0.96	0.009
Total FW (g)	39.3 ± 2.98	22.4 ± 3.09	0.004
LN	3.60 ± 0.24	1.67 ± 0.56	0.016
LA (cm^2)	181.3 ± 20.9	65.0 ± 25.1	0.007
SL (cm)	28.4 ± 1.12	28.5 ± 1.45	0.959

Figure 3.22 shows chlorophyll fluorescence measured at the time of salt stress removal (day 0) and one-week later (day 7). Under salt stress, the photosynthetic efficiency drastically decreased in treated plants compared to the control, but it increased after 7-days of recovering, when measured on the new developing leaves. Indeed, 10 g L^{-1} NaCl caused dehydration and premature fall of all expanded leaves, that were permanently damaged. Therefore, a photosynthetic efficiency as high as the control was observed only in the apical leaves produced after the salt-stress removal and not yet expanded after 7-days of recovery.

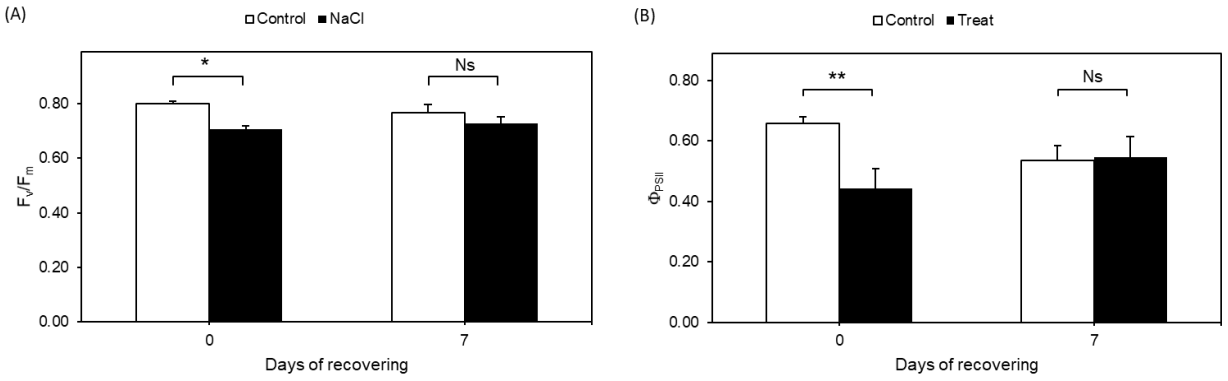

*Figure 3.22: A – Potential efficiency of PSII photochemistry (F_v/F_m) and B – actual photon yield of PSII in the light (Φ_{PSII}) measured at 100 ± 40 µmol m^{-2} s^{-1} of PAR, growing light conditions. Values are means ± SE (n = 6). Ns: not significant; * and **: significantly different at the P < 0.05 and P < 0.01 level, respectively, according to the one-way ANOVA test*

4 Discussion

4.1 Guayule responses to salinity

In this study guayule (*Pathenium argentatum* A. Gray) was exposed to increasing salt concentrations with the main objective of evaluating the growth and physiological responses and the salt resistance threshold of such perennial plant species native to Mexico. Two tests were conducted in order to investigate salinity responses of guayule at two different development ages (3-months-old and 4-months-old plants). Guayule tested in hydroponics survived at saline concentrations closed to hypersaline condition (above 35 g L^{-1}). However, results obtained on growth and physiological parameters, suggested that plants underwent ionic-stress phase at much lower concentrations.

Salt stress is known to alter the plant biomass production and allocation to different organs (dry weight of leaves and stem, shoot, roots) (Munns, 2002). The stem and roots DW of stressed guayule plants were not significantly different than those of the controls in both NaCl tolerance tests, while the stem length was reduced only in the first test (Table 3.1 and 3.2). However, the primary and main effect of salt stress on plants is the reduction in the size and number of leaves (Munns and Tester, 2008), and in this study leaves were the most impacted organs by salinity. Premature death of old leaves, a typical signal of ionic stress, was particularly evident on 3-months-old guayule plants, that presented evident signs of early senescence when exposed to 2.5 g L^{-1} NaCl (Figure 3.1), such as leaf turgor loss and colour change, while it appeared later in 4-months-old plants (Figure 3.7). However, at the end of both tests, treated plants presented a significant decrease of leaves number and area, and an increase in DW % (Table 3.1 and 3.2), consistent with leaf dehydration and fall. The leaf mass per area (LMA) and leaf area ratio (LAR) also varied according to salt treatment in both tests, indicating physiological and metabolic adjustment such as reduction of surface expansion of leaves and alterations in their thickness, biomass concentration and volume (Munné-Bosch and Alegre, 2004). LMA is a biological parameter associated with the thickness of the leaf, such that the leaves are thinner when LMA is higher (Poorter, 1989). A low LMA enables plants to increase leaf area and maximize light interception, while high values of LMA are often associated with relative low concentrations of nutrient and a reduced photosynthetic capacity of leaves (Sun and Frelich, 2011). Variations in LMA commonly results in the variation of LAR (Medek et al., 2007). LAR is the ratio of leaf area to the total plant weight, and, consequently, an index of photosynthetic machinery per unit of plant biomass (Amanullah et al., 2007). In both guayule tests, LAR decreased and LMA increased

Figure 4.1: Aerial part of guayule plants under increasing NaCl concentration

accordingly with a reduction of leaf area and an enhancement of leaf thickness, typical response of plants subjected to salt and/or drought stress, which reduce their size and total surface with decrease of transpiration rate and concentration of energy products and biomass to survive and adapt to adverse environments (Yang and Guo, 2017). The slowdown growth and the reduction in size adopted by guayule plants under salt stress conditions is also consistent with the general reduction of the relative growth rate (RGR), of the whole plant or related to leaves (RGR_L) or stem (RGR_s) (Tables 3.1 and 3.2). The leaf relative water content (RWC), that was determined to obtain information on the plant water status under salt condition, was clearly reduced by salinity. This sharp decline indicates that under severe salt condition, guayule presented a reduced ability to variate the absorption of water via the root system and/or to control water loss through stomata. It may also indicate a limited ability of osmotic adjustment to maintain tissue turgor, and hence physiologically activities, under severe salt stress.

Premature senescence of older leaves was confirmed by physiological parameters, that revealed that guayule reacted to salinity by speeding-up the senescence process. In NaCl treated plants, the new leaves maintained a photosynthetic functionality higher than the old ones (Figure 3.8), and all the gas-exchange parameters analysed differed between old and new leaves (Figure 3.4). In particular, the new leaves of both control and treated plants presented a higher A and Φ_{PSII} and a lower C_i compared to old leaves, suggesting a higher photosynthetic capacity. Moreover, treated new leaves showed C_i values similar to the control, suggesting that these leaves suffered less damage to the photosynthetic apparatus than the old leaves. This was reflected on the intrinsic-water use efficiency, i.e. the A/g_s ratio. Indeed, this ratio decreases in treated old leaves compared to the control ones, indicating a reduction in the intrinsic water-use efficiency, while new leaves maintain values similar to those of the control.

Analyses related to the main parameters of chlorophyll fluorescence, revealed a diversification of the behaviour within the two tests proportional to the saline concentration tested (Figure 3.3 and 3.8). The parameter F_v/F_m indicates the potential efficiency of capturing the energy of excitation by the PSII, and in non-stressed plants has values around 0.83. Three out of six 3-months-old plants did not survive at 20 g L^{-1} NaCl exposition, with F_v/F_m values close to 0.00, while at the same concentration F_v/F_m values of 4-months-old stressed plant were not significantly different from the control. This may confirm results of previous studies, testing lower salinity concentrations in soil systems, that found that guayule resistance increased with plant development (Posher et al., 2005).

However, gas-exchanges measures suggested that guayule suffered non-stomatal limitation to photosynthesis starting from the salt threshold of 15 g L^{-1} NaCl in both tests (Figure

3.4 and 3.9). Guayule reacted to saline condition by closing the stomata in order to prevent dehydration. Consequently, osmotic stress induced by 15 g L^{-1} NaCl caused a reduction of stomatal conductance and transpiration rate. The partial stomatal closure caused a reduction of the CO_2 uptake and, hence, a significative decrease of actual photon yield of PSII photochemistry and net CO_2 assimilation rate. Despite a higher stomatal closure than the control, intercellular [CO_2] did not significantly decrease in salt-stressed plants. The decrease in incoming CO_2 was not counterbalances by an efficient use of the intercellular CO_2 in chemical reactions responsible for the conversion of CO_2 into glucose. In addition, the A/g_s ratio did not increase in salt-stressed plants compared to the control, indicating a reduction of intrinsic water-use efficiency, that was reflected on the plant water status. This indicates that, in addition to a clear stomata response, stressed plants presented also a reduced photosynthetic capacity (non-stomatal effect), which contributed to decrease the CO_2 assimilation rate in salt-stressed plants of guayule. The presence of possible photoinhibition and/or photodamages to PSII are confirmed by the lower values of F_v/F_m in stressed plants compared to the control. This suggests that the reduction of stomatal conductance observed at 15 g L^{-1} NaCl was not a mechanism of osmotic-adjustment, and plants were already undergoing an ionic-specific phase of salt stress. Even if plants survived to higher salt concentrations, with average photosynthetic capacity similar to that of the control, it may be speculated that mechanisms against ionic stress of guayule may not be effective at NaCl concentrations higher than 15 g L^{-1}.

The observed reduction of guayule growth rate, biomass production and photosynthetic efficiency in response to high NaCl doses are primary and general effects of an osmotic response induced by the decrease in the environment water potential (hydroponic system with high electrical conductivity), which reduces the plant ability to uptake water and nutrients and induces a decrease in leaf expansion. With the persistence or increase of the salt stress, more specific effects occur due to ion toxicity (Na^+ and/or Cl^-) in the old leaves because of the rise in salt concentrations in cell walls or cytoplasm (Munns, 2002; Munns and Tester, 2008). The excess presence of the ion Na^+ in the plant cell can cause damages to several structure and biomolecules, by disruption or substitution with other cations such as Mg^{2+} in chlorophylls or Ca^{2+} involved in cell membrane stability (Pak et al., 2009; Kronzucker et al., 2013). Indeed, one of the major responses to salt stress in plants is the degradation of photosynthetic pigments (Jogawat et al., 2013), which is one of the first and typical symptom of oxidative stress (Taibi et al., 2016). Chlorophylls (Chl) and carotenoids play a major role in photosynthesis and plant photoprotection mechanisms, and the extent of reduction of their contents depends on the salt tolerance level of the plant species. In case of some species, the Chl content is a potential biochemical indicator of

(A)

(B)

Figure 4.2: A – 3-months-old and B – 4-months-old guayule control plants and plants treated with increasing concentrations of NaCl up to 40 g L^{-1} for 30 days

salt tolerance, although this is not true for all species (Jogawat et al., 2013). In both our NaCl tolerance tests, the leaf total chlorophyll concentration (Chl *a* + *b*) of treated plants decreased in comparison with controls just from the lowest NaCl treatments, and then it maintained almost the same level during the time-course of the tests (Figure 3.5 and 3.10). In the older guayule plants (4-months) of the second test the highest reduction of Chl *a* + *b* was observed in correspondence of the 15 g L^{-1} NaCl treatment (Figure 3.10), when also the measurements of chlorophyll fluorescence and gas exchange parameters indicated impairments in photosynthetic processes (Figures 3.8 and 3.9). However, at the end of experimental tests (40 g L^{-1} NaCl) Chl *a* + *b* was reduced due to the degradation or inhibition of synthesis of Chl *a* or of both Chl *a* and Chl *b*. In any case, these variations are associated with a decrease of Chl *a*/*b* ratio, more relevant in the younger plants (-78.8%) than in the older ones (-51.5%), indicating that Chl *b* gained in relative importance (Figure 3.6 and 3.11). In both tests leaf total carotenoids significantly decreased

compared to the control, but in 3-months-old plants the total chlorophyll/carotenoids ratio augmented, while in 4-months-old plants this ratio did not vary compared to the control. Beside a sharp reduction in chlorophyll concentration, in older plants the relative rate between the two classes of pigments was maintained, confirming that these plants were less impacted by salt stress than younger ones. In fact, variations in the relative amounts of chlorophylls and carotenoids in plant leaves indicate changes or impairments in size and/or compositions of the two photosystems (PSs) and related antenna complexes (Light harvesting complexes, LHCs), with consequent inefficient functionality of the photosynthetic apparatus (Anderson et al., 2008; Ruban, 2015).

Mineral nutrients play a major role in determining plant resistance to salinity (Hu and Schmidhalter, 2005), and it is shown that NaCl stress significantly increased Na^+ concentration, while decreased P, K^+, Mg^{2+} and Ca^{2+} (Asik et al., 2009; Amiriani et al., 2010). Potassium is a competitor of Na^+ under saline conditions, and when sodium ion reaches toxic accumulate in root membranes and, it interferes with K^+ selective ion channels, altering the availability of nutrients and affecting plant growth (Yetisir and Uygur, 2009). The maintenance of high K^+ concentrations and a higher K^+: Na^+ ratio may be a mechanism underlying plants salt tolerance capacity (Tavakkoli et al., 2010 A; Hu and Schmidhalter, 2005). The K^+: Na^+ ratio significantly reduced in treated plants compared to the controls, but in the same condition the K^+ concentration was not significantly affected in leaves and roots (Figure 3.13 A). In treated guayule plants, leaves represented the organ with the highest Na^+ content, impacting the K^+: Na^+ ratio of shoot. As well as potassium, calcium concentration commonly decreases in sensitive plants under salinity condition, and the ability of plants to retain Ca^{2+} is associated with their salt resistance (Hu and Schmidhalter, 2005). Calcium content significantly decreased in salt-stressed guayule plants in stem, while the variation in leaves and roots was not significantly different from the control (Figure 3.13 C). The Ca^{2+}: Na^+ ratio significantly decreased in both shoot and roots of salt-stressed plants compared to the control. The decrease of Ca^{2+}: Na^+ ratio under salinity condition causes significant changes on morphological levels and plays a major role in the inhibition of plant growth (Hu and Schmidhalter, 2005). The decrease in Mg^{2+} uptake under salt stress conditions might be due to the suppressive effect of Na^+ on this cation or due to reduced transport of Ca^{2+} and Mg^{2+} ions (Varshenv et al., 1998). Magnesium is a component of chlorophyll molecules and the reduction or impairments in its concentration, as observed in guayule plants under salt stress, can explain the inhibition of its synthesis or degradation due to the interaction and/or substitution of Mg^{2+} with excess of Na^+ cation (Tavakkoli et al., 2010 A; Taibi et al., 2016)

Mineral nutrients were analysed in guayule tissue at the end of the NaCl tolerance test, after exposing the plants to very NaCl concentrations (up to 40 g L^{-1} NaCl), which are lethal to most other non-halophyte plants. However, the results obtained here suggest that guayule, exposed to high salinity stress, can adopt physiological (modulation of stomatal conductance and transpirations rate) and biochemical (relative biomolecule content and ion regulation) mechanisms, which make the plants able to withstand salt stress and survive, contrasting growth inhibition trough the reduction of growth rate and biomass production.

In general, the plant responses to the osmotic phase of salt stress can be observed within hours or few days after salt stress imposition, while the ionic specific phase of the stress impacts the plant much later (Munns and Tester, 2008). However, in dependence of the intensity and duration of the salt stress and of the plant species and development stage, the two phases of the salt stress can overlap or occur close together in time (Yang and Guo, 2017). While hydroponically grown guayule survived to a 30 g L^{-1} NaCl short-term exposition, physiological and growth parameters indicated that ionic stress may be occurred at lower concentrations (15 g L^{-1} for 4-months-old plants). This has to be confirmed by further studies testing the long-term capacity of guayule to adapt at such high concentrations, and investigating mechanisms such as Na^+ exclusion and tissues-tolerance to Na^+ accumulation.

4.2 Castor responses to salinity

In this study castor (*Ricinus communis* L.) plants were exposed to increasing salt concentrations and survived at concentrations up to 20 g L^{-1} NaCl. However, like guayule, castor presented variations in growth, morphophysiological and chemical parameters, suggesting that ionic-stress was induced by lower salt concentrations, when symptoms of osmotic stress occurred.

The effects of salt treatments are visible at the whole-plant level as the biomass was sensitively reduced (Table 3.5), with differences among the organs (leaves, stem and roots). The shoot/root ratio (calculated by dividing the sum of leaf and stem DW by the root DW) of treated plants significatively differed (1.6 fold higher) from the control, indicating that these in the two groups of plants the above and below-ground components didn't reduce in the same proportion and differently allocated the dry matter. Moreover, the shoot to root ratio is a sensitive indicator of plant stress by chemical or physical agents, and can be used as a tool for estimating the plant carbon storage and modelling the plant response at various levels (Agathokleous et al., 2017). Indeed, the treatment caused a reduction of the stem length and enhanced the stem to mass ratio compared to the control (Table 3.5). However, the height to mass ratio (HMR) did not vary

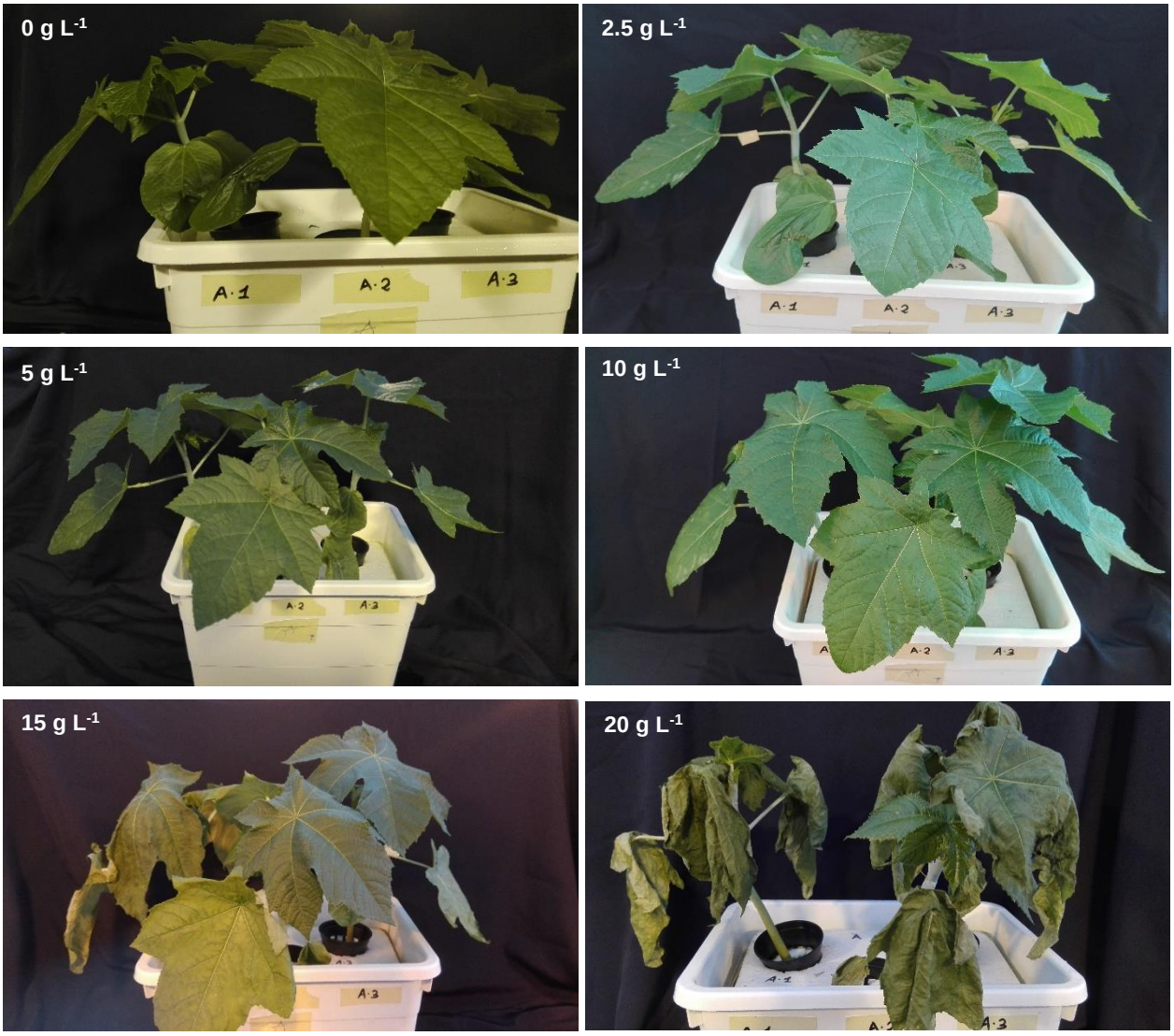

Figure 4.3: Aerial part (stem and leaves with long petioles) of castor plants under different NaCl concentrations

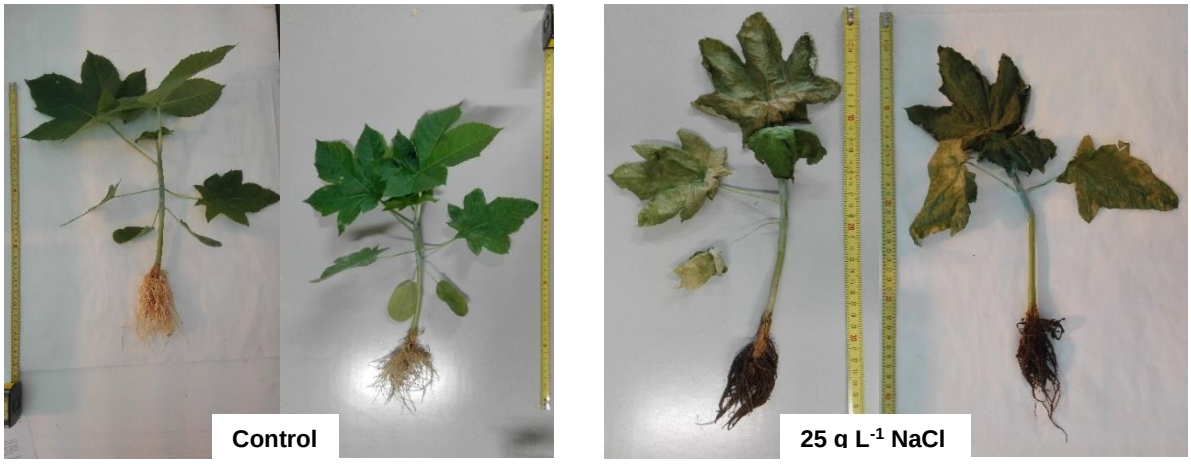

Figure 4.4: Castor plants exposed to increasing concentrations of NaCl up to 25 g L^{-1} for 22 days

according to salt treatment. The HMR is related to stem density, so salinity impacted castor stem on its length, but its density did not vary, causing the stem DW of treated plants not to decline (*P*-value = 0.085, one-way ANOVA). The stem resulted to be the organ less impacted by salinity when compared to leaves and roots. Salt stress caused the premature fall of cotyledons, and both leaf DW and leaf area were reduced in treated plants, causing opposite variations in leaf area ratio (LAR), which decreased (-39.4%) and leaf mass per area (LMA), which increased (+78.8%). Salinity induces physiological drought, leading to an increase in LMA that can also be observed under drought stress. However, mechanisms of salt exclusion cause increase of the LMA in some tolerant plants to increase only in higher salinity conditions. On the other hand, species that accumulate NaCl in the vacuole, show a higher enhancement in LMA due to the increase in mesophyll cell size or number of cell mesophyll layers (Poorter et al., 2009). In general, the plant root growth tends to be less sensitive to salt stress than the shoot growth. In salinity condition, the latter presents an immediate reduction due to osmotic stress, while roots are impacted later, when high accumulation of salt takes over time (Munns and Tester, 2008). The reduction of total DW in castor stressed plants compared to the control was caused by the reduction of both leaves and roots DW. In these conditions, also the growth rate (RGR) of plants decreased, due to the reduction of leaf and root RGR (Table 3.5). Salinity inhibited roots length: a root growth trend similar to that of the control was observed in salt stressed castor plant until 15 g L^{-1} NaCl, when root length significantly decreased (Figure 3.15).

Salinity not only affects the growth of plants, but almost every aspect of plant physiology and biochemistry (Babu et al., 2012) and in many physiological studies on salt stress, the inhibition of plants growth has been primary related to a reduction in photosynthesis (Ruiz et al., 1997), the main metabolic pathway of vegetation. The potential photochemical efficiency of PSII (F_v/F_m) of castor bean presented values around 0.80 at salt concentrations up to 5 g L^{-1}, while beyond this threshold, F_v/F_m started to decline (Figure 3.16). The potential photochemical efficiency of treated plants was significantly different from the control only after 20 g L^{-1} NaCl treatment, when stressed castor plants showed average value of F_v/F_m lower than 0.40. However, gas-exchange measures suggested that castor manifested the first signals of non-stomatic limitation to photosynthesis at 15 g L^{-1} NaCl (Figure 3.17). Plants perceived the osmotic stress and reacted by closing stomata to prevent dehydration. Consequently, the stomatal conductance and transpiration rate, as well as the net CO_2 assimilation rate, drastically decreased. However, partial stomatal closure did not lead to a reduction of intercellular CO_2 concentration in salt-stressed plants compared to the controls. This evidence, together with the reduction of both Φ_{PSII} and F_v/F_m in treated castor plants, supports the hypothesis that this salt threshold induced non-stomatal limitations to photosynthetic

CO_2 uptake, causing photoinhibition and/or photodamages to photosystem II. According to this hypothesis, both growth and physiological parameters, indicate that castor plants treated with 15 g L^{-1} NaCl manifested symptoms of ionic-stress.

One of the main indices reflecting the leaf photosynthesis ability and general plant health condition is the leaf chlorophyll concentration and its changes under salt stress can be used as parameter for selection of tolerant and sensitive plant species (Doganlar et al., 2010). The total chlorophyll concentration (Chl *a* + *b*) of castor treated plants, estimated through SPAD values, were lower than those of control plants from the beginning of the NaCl treatment (2.5 g L^{-1} NaCl) to 10 g L^{-1} NaCl, when it apparently increased until the end of treatments (25 g L^{-1}), reaching levels of control values (Figure 3.18). SPAD measurements are based on the light transmission though an intact leaf at 650 nm and 940 nm (Sub et al., 2015), so they are determined and influenced not only by pigments concentration, but also by a series of leaf morphological factors, including spatial pigments distribution in the leaf. Therefore, the relationship between *in vivo* and *in vitro* chlorophyll determination is largely influenced by environmental conditions affecting leaf morphology (Monje and Bugdee, 1992), so that at a certain value of total chlorophyll, the corresponding SPAD value may differ due to alterations in the internal structure and general architecture of the leaf caused by the salinity. Such ultrastructure and structural modifications (e.g.: cell dehydration, number of cell layer, thickening of cell wall, increase of leaf thickness, etc.)

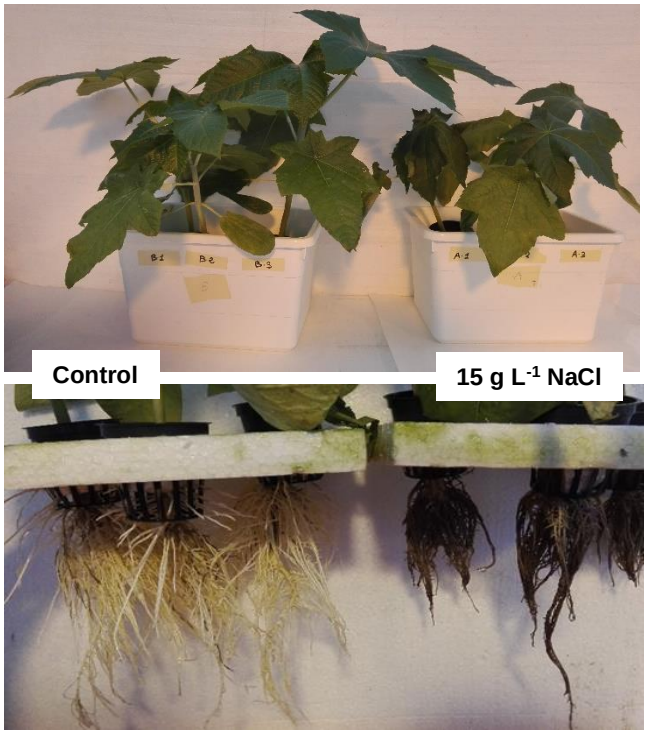

Figure 4.5: Aerial part and roots of castor plants under 15 g L^{-1} NaCl

can alter the leaf optical properties, with consequent changing of its absorption and transmission response. Even when the pigment content remains unchanged, alterations in leaf morphology due to salt stress may translate into variation of the spectral response in the near-infrared region. Therefore, under stressed conditions, it is possible to register different SPAD values for the same extractable chlorophyll content (Shah et al., 2017). Several morphological changes were observed in leaves of castor salt stressed plants, such as a progressive reduction in leaf area and the strong increase of LMA (Figure 3.14 and Table 3.5). These modifications may explain the apparent increase of leaf total chlorophyll concentration measured by the SPAD meter at the highest NaCl treatment (15-25 g L^{-1} NaCl). However, the extraction of photosynthetic pigments (chemical method) at the end of the test showed not significant variation of total chlorophyll in salt-stressed plants compared to the control (Figure 3.19 A). On the other hand, a significant decrease in Chl *a* (-18%) was observed, while Chl *b* did not vary, with a consequent reduction of the Chl *a*/*b* ratio. In the same NaCl stress condition, the total carotenoids content was reduced of similar amount (-21%) as Chl *b*. Moreover, the total chlorophylls/carotenoids ratio significantly augmented in stressed plants compared to the control, indicating that carotenoids gained in relative importance (Figure 3.19 B). Carotenoids are accessory photosynthetic pigments increasing leaf capacity for light absorption in the photosynthesis process, but they are also involved in the defence mechanism against oxidative stress, and have the function of dissipating excess energy, providing protection to reaction centres. As a photo-protection mechanism, carotenoids are retained during the process of chlorophyll degradation at leaf senescence (Shah et al., 2017). For these reasons the chlorophylls/carotenoids ratio is a good indicator of plant stress (Netto et al., 2004), and in our study, we can hypothesise that salinity caused leaf tissues death or early senescence in castor, with consequent chlorophylls loss and a relative increase in carotenoids. However, it is true that in the leaves of castor treated plants, the salt stress induced a certain decrease of carotenoids content. Anyway, we have to consider that the salt treatments imposed here, are in the range of high and hyper salinity stress, and in such conditions the protection by carotenoids is not one of the most important defence mechanisms, being involved other antioxidant non-enzymatic and enzymatic systems like as flavonoids, phenolic compounds and ascorbate-glutathione cycle (Di Baccio et al., 2004; Taibi et al., 2016).

Salt stress disturbs the mineral-nutrient composition and relations in plants through their effects on nutrient availability, transport and partitioning in the plant (Hu and Schmidhalter, 2005). Plants acquire essential nutrients from their root system environment, and under NaCl conditions, the nutritional balance results altered, with lower ratios of K^+: Na^+, Ca^{2+}: Na^+ and Mg^{2+}: Na^+, which may cause plant growth reduction (Ruiz et al., 1997). Chemical analyses of castor exposed to

salt stress, revealed a significant increase of Na^+ concentration in all the plant organs (Table 3.6), while the K^+ concentration and the K^+: Na^+ ratio significantly decreased in NaCl treated plants compared to the control (Table 3.7). The toxicity of excess Na^+ ion may induce ions deficiency or imbalance in plants, through competitive interaction with nutrients such as K^+ and Ca^{2+}, or by affecting the ion selectivity of membranes. The root system plays a major role in the maintenance of a high K^+: Na^+ ratio, through the antagonist uptake of Na^+ and K^+, presenting a selectivity of K^+ over Na^+ and a preferential loading of K^+ rather than of Na^+ into the xylem (Hu and Schmidhalter, 2005). Under high salt stress, castor plant did not maintain a high K^+ concentration in both shoot and roots. The reduction in K^+ uptake and accumulation under salt-stress condition, is likely an important growth limiting factor, as this element plays a major role in many plant processes (Ruiz et al., 1997). In addition, when Na^+ accumulates in root membranes, interfering with K^+ selective ion channels, it alternates the availability of many other nutrients, causing further adverse effects on plant growth and development (Babu et al., 2012). The concentrations and contents of Ca^{2+} and Mg^{2+} in leaves significantly reduced in castor salt stressed plants, as well as Ca^+ and Mg^+ content in roots (Table 3.7 and Figure 3.21). The reduction of Ca^{2+} content under saline conditions in related to plant growth inhibition and morphological changes (Hu and Schmidhalter, 2005). Magnesium deficiency may contribute to the reduction of plant photosynthetic activity and growth, as this element is the central atom of the chlorophyll molecule, and it influences on the size, structure and function of chloroplasts (Ruiz et al., 1997). So, the Mg^{2+} decline in castor treated plants at the end of the NaCl tolerance test can partially explain the observed impairments of photosynthetic processes and the synthesis inhibition or degradation of chlorophyll *a*. Phosphate was the only mineral element that did not vary in treated plants compared to the control. Therefore, inhibition of plant growth observed in salt-stressed castor plants may be related to K^+, Ca^{2+} and Mg^{2+} deficiencies, while the P availability seems not to impose limitations in plant growth.

The presence of non-stomatal limitation to photosynthesis at 15 g L^{-1} NaCl led to the decision of testing the castor recovery capacity at 10 g L^{-1} NaCl, the immediately previous NaCl dose tested. The main macroscopic effects of salt stress on plants, such as the reduction in the size and number of leaves, and in roots growth, were observed (Table 3.8). In general, salt firstly accumulates in older leaves, and in this test, it caused the dehydration of almost all the leaves that were fully expanded during the time of salt stress. The old leaves of castor were permanently damaged and fell during the recovery test, indicating that plants underwent a phase of specific-ion toxicity. The drastic reduction of leaf surface assimilating CO_2, reduced plant ability to respond to carbohydrates demand, with impacts on growth capacity. The fresh weight of leaves and roots was significantly reduced in stressed plants. At day 7 of recovery, 2 out of 6 stressed-plants

presented dried apical leaves, and over a longer period of time (1 month) the salt, probably accumulated at toxic levels, together with reduction of leaf surface, caused their death. However, salinity did not lead to death 4 out of 6 stressed-plants. The photosynthetic efficiency drastically decreased in stressed plants compared to the control, but it increased again after 7-days of recovering (Figure 3.22). Salt stress caused dehydration and premature death of the all expanded leaves, which were permanently damaged during the test-time. However, in this condition, castor maintained a vital vegetative apex, able to produce new leaves that, even if not yet expanded during fluorescence measures, presented a photochemical efficiency similar to that of the control. This effect on the photosynthetic performance, associated with a slight increasing trend in leaves number and surface during 1-month recovery, suggests that castor plants were able to recover from salt-stress, although with a high loss of biomass. Further studies on castor recovery capacity are of particular interest considering that the salinity stress in the field may present large ranges of spatial and temporal variations (Tavakkoli et al., 2012).

4.3 Phytoremediation of saline soil and wetlands

This study aimed to investigate growth, biochemical and physiological responses of guayule and castor to high salinity, in order to help in understanding the possibility of using these plants for phytoremediation in both soil and wetland ecosystems. For the characteristics of hydroponic tests, phytoextraction is the only phytoremediation application that can be evaluated. Ideally, plants suitable for phytoextraction are able to extract from the environment and store in their tissues large amount of pollutants (Truu et al., 2015). The analysis of plant mineral composition performed shows that both guayule and castor grown in hydroponics presented a Na^+ shoot/root content ratio significantly higher in stressed plant compared to the control (8.14 and 7.09, respectively), indicating that they stored much higher concentrations of Na^+ in leaves and stems than roots (Table 3.3 and 3.6). However, such analyses were performed after exposing the plants to very high salt concentrations, with the aim to find their survival Na^+ thresholds in extreme environments. Therefore, it would be interesting and useful a further investigation on the accumulation capacity of these plant species when subjected to lower NaCl concentrations.

Few considerations can still be done about the destructive analyses performed at the end of salt treatment, including the mineral content and biomass production. Guayule survived at higher NaCl concentrations and for longer time than castor bean. Moreover, plant growth and mineral nutrients content were less impacted by salinity in guayule than in castor. However, although the Na^+ concentrations in different plant organs of guayule and castor were comparable, the Na^+ tissue-contents were significantly higher in castor than in guayule, and results suggest

that one of the salt-tolerance mechanisms adopted by guayule may be relative low uptake of Na^+. This confirms that guayule should be considered as an agronomic crop in saline area. In the test conditions analysed, castor showed a higher capacity of salt accumulation than guayule. In addition, the shoot dry weight of castor was significantly higher than guayule, roots were longer and castor is a fast-growing plant (Kiran and Prasad, 2017), while guayule grows slower (Suchat, 2012). These factors may indicate that castor bean combines a relevant salt accumulation capacity in the aboveground shoots with a high biomass production and an extensive root system, which are all features characterizing the phytoextraction capacity of plants employed in phytoremediation plants (Truu et al., 2015). Therefore, while castor may be more sensitive than guayule at high concentrations of salt, it may present more advantages than guayule for phytoremediation in lower salinity conditions.

4.4 Differences between hydroponic and soil growth systems

Many studies on salt stress assume that plant responses in hydroponics mirror those observed in soil. However, Tavakkoli et al. (2012) suggested that there may be differences in responses to salt stress between plants grown in hydroponic and soil systems. Experimental studies compared responses to salinity in hydroponic and soil growing conditions of different cultivars of barley, imposing ionic stress to approximately the same degree, based on the EC of the respective solutions in hydroponics and soil cultures. Results show that barley plants growing in hydroponic systems presented different salt-sensitivity levels than those growing in soil. In addition, the concentrations of Na^+, as well as of Cl^-, in leaves of plants grown in hydroponics were much higher than those in soil, suggesting differences in plant uptake and exclusion mechanisms. The different sensitivity of plant to salt tolerance when grown in soil or hydroponics suggested that there are fundamental differences in the nature of these two growth systems, influencing plants responses to salt stress (Tavakkoli et al., 2012).

Differences between hydroponic and soil growing conditions may be mainly related to the effect of solid matric on the uptake of ions and on plant-water relations. Soil differs from hydroponics because the cation exchange complex affects the relative activities of cations and anions in the soil solution and, consequently, the availability of nutrients for plants. Moreover, the plant responses to salinity are influenced by differences in specific plant-water relations. In soil, the water potential and water uptake by plants are determined not only by the characteristics of the saline soil solution, but also by the physical properties of the soil. While water uptake in hydroponic is only affected by the osmotic potential of the nutrient solution, plants growing in saline soils have to cope with the effects of the saline soil solution and of the soil matric potential,

which is affected by the pore size distribution. The effect may be exacerbated when high concentrations of Na^+ can alter the physical structure of the soil, limiting the availability of soil moisture and restricting the plant growth (Tavakkoli et al., 2010 A).

Relationships that may arise in soils may not appear in hydroponic systems. While in this study the reduction of phosphorous content in plant tissues induced by the salinity stress was not observed, this phenomenon is frequently found in studies conducted in soil. The low solubility of Ca-P minerals may reduce the P availability under saline conditions in soils (Hu and Schmidhalter, 2005). Moreover, the rate of change of salt stress may also differ between hydroponic and soil systems. Because of the soil buffering capacity associated with the cation exchange complex, sudden changes in salt concentrations in the soil solution are unlikely to occur. On the other hand, plants grown in hydroponics immediately perceive variations in the salt medium concentrations, and they have less time to adapt to such variations than plants grown in soil (Tavakkoli et al., 2010 A).

5 Conclusion and future perspective

Salinity is a major environmental concern and a primary abiotic stress threatening plants survival and productivity. However, several plant species have the ability to adapt to such environmental stresses and understanding how plants respond to high concentrations of salt opens up future opportunities for the selection of plants suitable for growing in lands or wetlands with different salinity levels.

Four experimental tests were developed in semi-controlled conditions (green-house) in order to evaluate guayule and castor bean responses to high NaCl concentrations distributed in a hydroponic growth system, other that the castor recovery capacity after the removal of salt stress. The use of hydroponics technology permitted to monitor the sodium concentration in the media without interferences of the soil matric, with an easier understanding of the complex plant responses to salinity at morphophysiological and biochemical levels. This knowledge is also important when evaluating the opportunity of integrating phytoremediation techniques in the wastewater treatments of constructed wetlands or in the remediation of salt-rich or contaminated soils, although plants responses in hydroponic system do not necessary mirror those in soils.

The typical symptoms of salt-stress were observed in the two plant species investigated in this study, with differences in their responses to both osmotic and ionic-specific components of such stress. Guayule and castor did not survive to hypersaline conditions (above 35 g L^{-1}), but they survived to saline conditions (above 5 g L^{-1}). Growth and physiological parameters indicated that guayule and castor mainly underwent the ionic-specific stress phase after the application of 15 and 10 g L^{-1} NaCl, respectively. However, it is very difficult to distinguish the effects of osmotic phase from those of ionic-specific phase in the plant responses to salt stress, and so to identify the exact time of their occurring at physiological and metabolic levels. Growth analysis and mineral content determination suggest that guayule may adopt resistance mechanisms of salt exclusion, while castor may present a higher Na^+ accumulation capacity.

This study identifies a series of morphological, physiological and biochemical responses to high salt stress (acute exposure in short-term), confirming that guayule and castor are suitable to be grown in salinity conditions and allowing a better evaluation of this possibility. The application of destructive and non-destructive measurements experienced here showed that for the evaluation of plant tolerance and/or adaptability to environmental stresses and the identification of physiological threshold, the combination of different techniques and instruments (portable and analytical tools) are necessary. The knowledge generated with this work should be

integrated with further analyses on guayule and castor responses to long-term exposition to high salinity, in order to better identify plant defence mechanisms against osmotic and ionic stress.

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Annexes

Annexe 1: Analysis of covariance verifying the influence of NaCl treatment on the regression model constructed to explain the relation between leaf area (LA) and morphological measures (LxW), in castor. Analysis performed on R Studio. A similar R code was used for guayule plants

```
> Data1 = read.csv("Area Castor 1.csv", sep =";", header = T, stringsAsFactors = F)
> # Intercept
> mod1 <- lm(LxW~LA + Treatment, data = Data1)
> summary(mod1)

Call:
lm(formula = LxW ~ LA + Treatment, data = Data1)

Residuals:
    Min      1Q  Median      3Q     Max
-48.663 -11.654   0.921   6.875  48.081

Coefficients:
              Estimate Std. Error t value Pr(>|t|)
(Intercept)    -2.74646    8.03131  -0.342    0.735
LA              1.80529    0.03653  49.420   <2e-16 ***
TreatmentTreat -3.76379    8.35133  -0.451    0.656
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 19.08 on 25 degrees of freedom
Multiple R-squared:  0.9923,    Adjusted R-squared:  0.9917
F-statistic:  1618 on 2 and 25 DF,  p-value: < 2.2e-16

> #Slope
> mod2 <- lm(LxW~LA * Treatment, data = Data1)
> summary(mod2)

Call:
lm(formula = LxW ~ LA * Treatment, data = Data1)

Residuals:
    Min      1Q  Median      3Q     Max
-49.422 -12.566   0.262   6.454  48.020

Coefficients:
                 Estimate Std. Error t value Pr(>|t|)
(Intercept)      -3.51161    8.19850  -0.428    0.672
LA                1.80961    0.03748  48.278   <2e-16 ***
TreatmentTreat    6.39694   17.24071   0.371    0.714
LA:TreatmentTreat -0.14839    0.21952  -0.676    0.506
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 19.29 on 24 degrees of freedom
Multiple R-squared:  0.9925,    Adjusted R-squared:  0.9915
F-statistic:  1055 on 3 and 24 DF,  p-value: < 2.2e-16
```

Annexe 2: Some parts of R code (R Studio) used for construction of parallel slope model explaining the relation between SPAD-values (here, "SPAD") and total chlorophyll concentration expressed as µg cm^{-2} (here, "Chlab") in guayule, including the list of used R packages. A similar R code was used for castor plants

```
Data = read.csv("Pigment Guayule2.csv", sep =";", header = T, stringsAsFactors = F)
# Intercept
mod1 <- lm(SPAD~Chlab + Treatment, data = Data)
#Slope
mod2 <- lm(SPAD~Chlab * Treatment, data = Data)
#Comparing mod1 and mod2 with ANOVA
anova(mod1, mod2)
```

```
#Plot
library(ggplot2)
library(broom)
library(dplyr)
library(moderndive)

Control <- subset(Data, Treatment=="Control")
Treat <- Data[Data$Treatment=="Treat",]

lm_eqC <- function(){
  mod<- lm(SPAD ~ Chlab + Treatment, Data);
  eq <- substitute(y == a + b %.% x*","~~R^2~"="~r2,
                   list(a = format(unname(coef(mod)[1]), digits = 3),
                        b = format(unname(coef(mod)[2]), digits = 3),
                        r2 = format(summary(mod)$r.squared, digits = 3)))
  as.character(as.expression(eq));
}
```

```
ggplot(Data, aes(x = Chlab, y = SPAD, linetype = Treatment,
                 color= Treatment, size=Treatment)) +
  geom_parallel_slopes(se = FALSE) +
  coord_cartesian(xlim = c(0, 50), ylim = c(0, 80)) +
  xlab(expression(paste('Chl a + b (µg ',cm^-2,')',sep=''))) +
  ylab(expression(paste('SPAD-Values',sep=''))) +
  geom_errorbar(aes(ymin=SPAD-SE, ymax=SPAD+SE), size = .1, color = '#999999',
                linetype = 'solid') +
  geom_errorbar(aes(xmin=Chlab-SE, xmax=Chlab+SE), size = .1, color = '#999999',
                linetype = 'solid') +
  geom_point(aes(x =Chlab, y = SPAD, shape = Treatment), size = 1.5) +
  scale_shape_manual(values=c(21, 19)) +
  scale_linetype_manual(values=c("dotted", "solid"))+
  scale_color_manual(values=c('Black', 'Black'))+
  scale_size_manual(values=c(1.3, .7)) +
  geom_text(x = 10, y = 23, label = lm_eqT(), parse = TRUE, size=2.5, color="black")
  geom_text(x = 40, y = 38, label = lm_eqC(), parse = TRUE, size=2.5, color="black")
  theme_bw() +
  theme(legend.justification = c(0,1), legend.position = 'top',
        legend.background = element_rect(fill = 'white', color = 'white', size = 2.5)
  cleanup
```